TUESDAY—MORNING SESSION

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*

In the development of chemical indices of decomposition and filth in foods the ever present problem of interferences is not easily solved. In this discussion an "interference" may be defined as a substance not identical with the one you want to measure, but which acts as if it were, and which will measure as the same compound to a greater or lesser degree. Theoretically, interferences can be eliminated by suitable separation techniques. In practice, to eliminate an interference may be very difficult; in fact, to remove the interference may be more trouble than the gain is worth, particularly in view of the blank which is often encountered. "Blank" may be defined as that amount of the substance being measured which occurs naturally even though no decomposition is present. In the ideal situation the blank is zero; in practice this is seldom true. An example where it is true is succinic acid in eggs. So far we have never found a measurable quantity of this acid in passable eggs. The situation is different with water-insoluble acids in butter. Even in butter made from sweet cream the value may run to more than 100 mg per 100 g of fat. In butter made from rotten cream the value will be several hundred mg. There's nothing you can do about eliminating a positive blank; it's with you always and makes regulatory application of that particular index more difficult. Where the blank is an appreciable proportion of the value for decomposed material then one must analyze a large number of authentic samples to get a reasonable idea of the maximum blank that will be encountered in practice.

The quantities with which we are dealing in our studies on decomposition range from a few micrograms per 100 grams of food, as with indole in shrimp, histamine in fish, or uric acid as an index of filth, to the several hundred milligrams of water insoluble acids in butter made from decomposed cream. With this last mentioned amount possible interferences are unimportant.

A few ways to eliminate or to get around the difficulties of interferences may be mentioned. Chromatography is our most useful tool in separating interferences. For succinic acid in eggs two separations are necessary. The first one is a standard chemical procedure to separate lactic from succinic acid by precipitation of the succinic acid as its barium salt in 80 per cent ethyl alcohol. This is necessary because lactic and succinic acids do not separate completely on the column we now use.

Histamine is an organic compound which has a pronounced physiological activity in more ways than one. Its antagonists, the antihistamines, are known in name by almost everyone. In microgram quantities histamine affects the blood pressure of mammals, and we believe that we can use this property to measure the amount of histamine in fish. When a fish decomposes, histamine may be conjectured to form by the decarboxylation of the amino acid histidine. Perhaps other amino acids are also decarboxylated; however these do not have any appreciable effect on blood pressure. Histamine is a poisonous substance—a feature to be considered in regulatory application to decomposed foods; most of our other indices are not poisons per se.

Our newest tool is paper chromatography, a powerful means of separating complex mixtures when only microgram amounts of each component are present; in fact, as ordinarily used, it is limited to microgram amounts. This extreme sensitivity has its disadvantage too; you must have a color reagent which will give a visible color with these quantities and not react with the paper to give an interfering color. As yet we have not had enough experience in applying paper chromatography to decomposition problems to predict where it will be particularly useful.

The method **16.34** to **16.39** for succinic acid in eggs is in a first action status. The collaborative results reported by Hillig (*This Journal*, **33**, 722 (1950)) supporting that action are also adequate support for adoption of the method as official at this time.

RECOMMENDATIONS*

It is recommended that the first action method for succinic acid, **16.34** to **16.39**, be adopted as official.

Further, the Referee concurs in the recommendations of the Associate Referees for Decomposition and Filth in Foods (Chemical Indices) as follows:

A. **Fruits and Fruit Products.**

(1) That the study of galacturonic acid as a possible measure of decomposition in apples and strawberries be continued.

(2) That collaborative studies be conducted on the napthoresorcinol and carbazole methods for galacturonic acid.

(3) That further studies be made of the method for intermediate polygalacturonides as an index of decomposition in fruits.

(4) That search be made for other indices of decomposition in fruits.

B. **Tomatoes.**

(1) That studies to determine the correlation of rot and succinic acid in tomatoes be made.

C. **Shellfish.**

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* For report of Subcommittee C and action of the Association, see *This Journal*, **35**, 50 (1952).

(1) That the search for suitable chemical indices of decomposition in shellfish be continued.

D. **Filth in Foods—Chemical Indices.**

(1) That the study of uric acid as a possible criterion of insect filth in cereal and fruit products be continued.

(2) That the search for indicators of animal fecal matter in foods be pursued.

REPORT ON DECOMPOSITION IN FRUITS AND FRUIT PRODUCTS

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

A. DETERMINATION OF GALACTURONIC ACID

In last year's report a method for the determination of galacturonic acid in fruits was proposed. This acid is as good an index of fruit decomposition as anything known at the present time. The method proposed is based on the reaction with naphthoresorcinol in hydrochloric acid solution at 50°C. The method has some disadvantages; the principle ones are the instability of the reagent and the difficulty in reproducing readings with different solutions of the reagent, particularly when it is from different batches. Because of these difficulties the writer endeavored to find a more suitable reagent and method for the determination.

Some years ago Z. Dische[1] found that carbazole in the presence of concentrated sulfuric acid produced a violet colored complex with uronic acids which was fairly specific. A modified version of this method was proposed by S. M. Stark, Jr.,[2] for determination of pectin in cotton fiber. This reaction appeared to offer some promise and, as proposed by the authors, was carried out, first in a cold and then in a boiling water bath. In attempting to apply the test to the acids of fruit samples, it was found that the high concentration of sulfuric acid decomposed the normal fruit acids and rendered the test void at the higher temperature. Further experimentation showed that if the acid was added gradually at ice bath temperature, and the reaction completed afterward at 50°C., there was little or no interference from the normal fruit acids (malic or citric).

The carbazole method developed and reported below offers several advantages over the naphthoresorcinol method. The reagent itself is stable and its solutions are also comparatively stable and can be kept for several weeks without deterioration. The reagent is inexpensive as compared to naphthoresorcinol, is readily obtainable, and produces a reproducible straight-line standard curve. It has two disadvantages, how-

[1] *J. Biol. Chem.*, **167**, 189 (1947).
[2] *Anal. Chem.*, **22**, 1158 (1950).

ever; it is somewhat less specific than naphthoresorcinol and gives a higher blank on sound fruits than that obtained by the latter reagent. The method follows:

METHOD

APPARATUS

(a) *Spectrophotometer and cells*—(A neutral wedge photometer with filter centered at 540 mμ, and a 1-inch cell can be used in place of the spectrophotometer.)

(b) *Two glass tubes* 25 mm by 200 mm with a 5 to 6 mm O.D. tube sealed to one end are prepared (Figure 1). Inside each tube at the bottom, place a 15 mm layer of glass wool, and on this place a 10 mm layer of coarse white sand (20–30 mesh). In a graduate, measure 28–30 ml of wet cation exchange resin, Duolite C-3. After settling well, place the resin in one tube. Measure a like quantity of anion exchange resin, Duolite A-4, in the same manner and place it in the other tube.

(c) *A thermostated bath* maintained at 50°C. and controlled to ±0.3°C.

REAGENTS

(1) *Carbazole soln.*—Dissolve 0.100 g of carbazole (Eastman grade was used) in alcohol and dilute to 100 ml with the same solvent. Make alcohol aldehyde free by U.S.P. method.

(2) *Anion exchange resin.*—Duolite A-4.* (Some other resins tried were not satisfactory because of blank.)

(3) *Cation exchange resin.*—Duolite C-3.*

(4) *Malic or citric acid solns.*—Prepare a soln containing 0.7% malic acid and 0.4% potassium malate, or 0.7% citric acid and 0.4% potassium citrate, in water. Soln used will depend on which is the principal acid in the fruit. Add a few drops of $CHCl_3$ or CCl_4 as a preservative.

(5) *Standard galacturonic acid stock soln.*—Dissolve 250 mg of pure galacturonic acid and 0.7 g of malic (or citric) acid in water and dilute to 100 ml with H_2O. Add a few drops of $CHCl_3$ or CCl_4 to preserve. (*Note*—If pure galacturonic acid is not available, the crude acid can be purified by precipitation as the calcium salt, removal of the calcium, and liberation of the acid on a cation exchange column.) The effluent (filtrate) is concentrated under vacuum to a syrup, the syrup is taken up in hot methyl alcohol and cooled to 5 to 10°C. The acid is filtered off, washed with a little cold methyl alcohol, and dried in a vacuum desiccator at room temperature over H_2SO_4.

(6) *Activated charcoal, washed.*—Place 20 g of good activated charcoal (Nuchar C 190-N was used) in a 600 ml beaker, add 300 ml H_2O, 100 ml HCl (1+9), and digest on steam bath for 45–60 minutes. Filter on a Büchner funnel and wash with hot H_2O. Return carbon to beaker, add 300 ml alcohol, 25 ml HCl (1+9), and digest 1 hour on steam bath. Filter on Büchner funnel, wash with hot alcohol and, finally, with about 150 ml of acetone. Transfer to dish, heat on steam bath until odor of acetone is gone, grind gently with pestle, and dry in oven. Preserve in closed bottle.

PREPARATION AND CONDITIONING OF ION EXCHANGE RESINS

Place the resin tubes containing the charge of resin above the sand and glass wool in an exactly vertical position. Wash each column by counterflow (backwash) with a small amount of water to classify the particles in the column. To do this, attach a reservoir of distilled water to each column and control the backflow with the screw clamp. If the resin has not been used, condition it before use by putting it through a cycle of regeneration and exhaustion.

* Obtainable from Chemical Process Co., Redwood City, Calif.

Activate (regenerate) the cation resin column by passing 100 ml of HCl (1+5), introduced from a separatory funnel, through the column by down flow at the rate of about 7–8 ml per minute. Follow this with distilled water (about 200 ml) at about the same rate until the effluent shows no acidity (*p*H 6–7) with Universal indicator

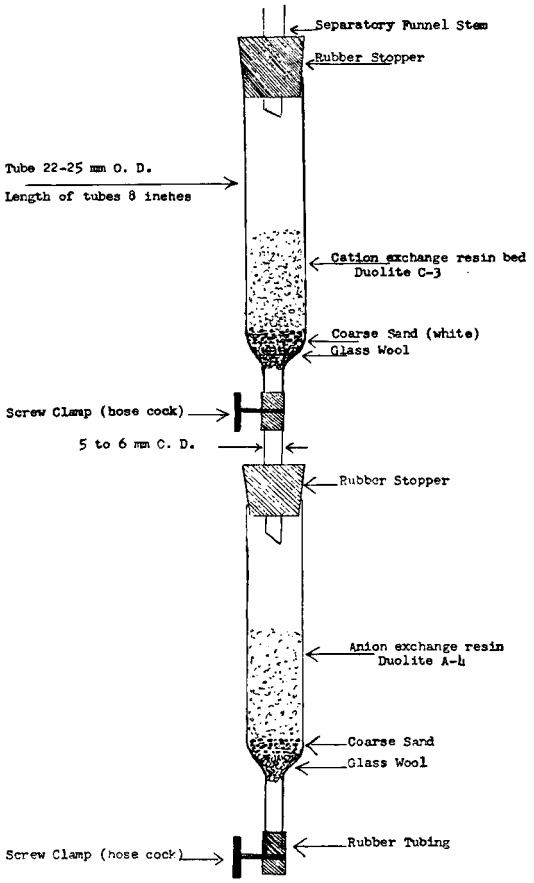

FIG. 1. Ion Exchange Tubes, Assembly B. (For Assembly A see *This Journal*, **34**, 509 (1951).)

paper. Now pass about 30 ml of 5% NaOH through the column to exhaust it and follow with water until the *p*H drops to 6–7. Repeat cycle once, then regenerate with HCl (1+5), wash with H_2O until free of acid (*p*H 6–7), and the column is ready for use.

Activate and condition the anion exchange resin in a similar manner by washing by counterflow with about 100 ml of H_2O to classify particles. Regenerate with 120 ml of 5% NaOH, wash with water until the effluent *p*H drops to about 7, then exhaust with 40 ml HCl (1+9) and wash again with water. Use rates given under the cation resin. Repeat the cycle once and finally regenerate with 120 ml of 5% NaOH, wash with H_2O until the effluent is neutral, and the column is ready for use.

PREPARATION OF SAMPLE

Fruits.—Grind in a food chopper and strain through cheesecloth, using pressure, if necessary, or comminute in a blendor. Juice citrus fruits by bisecting and reaming. Quickly heat the juice or blended mixture of fresh fruits in a H_2O bath to about 90°C. in a covered flask to destroy enzymes. Cool, place 100 ml of the prepared mixture in a 200 ml volumetric flask, and add acetone gradually with continuous shaking to the 200 ml mark. Stopper, mix well, and let stand several minutes in a bath at about 20°C. Then fill to mark again with acetone, remix well, and let stand for precipitate to flocculate. Add a gram or two of "filteraid" (mix equal amounts of Filtercel and Celite 545), mix again, and filter on a rapid fluted filter (18.5 cm is suitable). Cover filter during filtration. The filtrate should be clear; if it is not, pour back until clear. Transfer 50 and 100 ml aliquots (equal to 25 and 50 ml sample) to beakers and evaporate off the acetone on the steam bath, using glass beads or boiling tube to aid evaporation. Remove when acetone odor is nearly gone and cool. If insoluble matter separates after evaporation of acetone, add some filteraid mixture, stir and filter on a Büchner funnel (about 7 cm) with suction. Wash beaker and filter twice with 8–10 ml of water. Keep 50 and 100 ml aliquots separate. If fruit sample is only moderately acid (apples) use the larger aliquot for acid adsorption. If of high acid content (grapefruit) use the smaller portion for acid adsorption, and reserve the other portion in either case.

ADSORPTION OF ACIDS ON RESINS

Transfer the filtrate of the selected portion to a separatory funnel. Arrange the ion exchange tubes so that the effluent from the cation tube discharges into the anion tube (Fig. 1). Fit the separatory funnel (containing sample) into the mouth of the cation tube with a perforated stopper. The rubber tubing attached to the bottom of the tubes containing the resins should be provided with screw-type hose cocks to adjust the flow of liquid through them. (If set-up B (Fig. 1) is used, the liquid in the tubes should always be $\frac{1}{4}$ to $\frac{1}{2}$ inch above the resin.)

With the hose cocks on the resin tubes closed, open the stopcock on the separatory funnel and adjust the hose cocks so that the sample soln flows through the tubes at a rate of 5–6 ml per minute. When the last liquid in the funnel reaches the stem, wash down the sides of funnel with 15–20 ml of H_2O from a wash bottle. Allow this liquid to pass into stem of funnel, then add 170 ml of water to funnel and allow it to pass through the tubes to carry the acids through the cation tube into the anion tube and to wash out the non-acid materials. Discard the effluent and washings. Separate the anion column from the cation column. Place a fine screen (stainless steel or monel, about 40 mesh) in the mouth of the anion tube with a pad of glass wool on top of the screen to catch any fine particles of resin. Backwash the column with water at the rate of 50 ml per minute until 200 ml has passed through. Then drain off excess liquid to $\frac{1}{4}$ to $\frac{1}{2}$ inch above resin. Fit a rubber stopper on the stem of a separatory funnel (125 to 250 ml) and fit it to the open top of the anion tube. Place 40 ml of HCl (1+9) in the separatory funnel, open the stopcock and adjust the hose cock so that the acid flows through the tube at about 7–8 ml per minute. When acid reaches funnel stem, follow with distilled H_2O at the same rate. Receive the effluent in a 50 ml graduate at first. When nearly 30 ml of effluent has collected, test effluent for acidity (blue litmus), and if neutral, pour that collected into a beaker. Continue collecting effluent in the graduate in 5 ml portions and test each time for acidity. If not acid, pour the portion into the beaker. When the emerging effluent becomes acid (after about 45 ml have collected) substitute a 200 ml volumetric flask as a receiver and pour the last 5 ml portion collected in graduate into flask. Rinse graduate once with about 2 ml H_2O. (*Note*—The progress of the acid

down column can be followed fairly well by the color change of the resin.) Continue the flow of H_2O through column until about 180 ml effluent has collected in flask. Discard water in beaker collected at first.

By the same procedure adsorb the ions from a 50 ml aliquot of reagent 4 and wash and elute in the same way. Then add 300 ± 5 mg of the washed carbon (reagent 6) to each volumetric flask, shake to disperse it, and place the flask on the steam bath (over a 50–60 mm opening) for about 12 minutes, or in any suitable bath so that the temperature rises to about 80°C. After heating 5 minutes, shake at minute intervals until 12 minutes, in all, have elapsed. Remove, cool to 25°C., make to 200 ml, and filter on rapid filter. Pour back until bright. (The filtrates should be practically water white.) Use the effluent obtained from reagent 4 as a blank soln.

DETERMINATION

Place 2.0 ml of reagent 5 in a 100 ml volumetric flask, dilute to the mark with blank soln, and mix well. (This standard soln contains 5.0 mg in 100 ml.) Prepare standards by pipetting into a series of test tubes (25 × 150 mm) 0.5, 1.0, 1.5, and 2 ml respectively of this standard soln and make each tube to exactly 2 ml with the blank soln. In two similar test tubes place, respectively, 2 ml of sample effluent and 2 ml of blank soln. From a 1 ml graduated pipet, add to each tube 0.5 ml carbazole soln (Reagent 1). Place the tubes in an ice bath until cold. Prepare two other ice baths in pans or in large beakers.

Fill a 50 ml burette with concentrated H_2SO_4 and place one of the ice baths beneath it. Add 3 ml of conc. H_2SO_4 dropwise to each tube at the rate of 2–2.5 ml per minute while shaking the tube constantly in the ice baths. Place each tube in turn in the third ice bath after adding the acid. (*Note*—A simple way to control the rate of acid flow is to attach a snug-fitting rubber tube fitted with a screw-type hose cock to the top of the burette. A piece of wire of small diameter inserted in the top of the rubber tube and extending through the hose cock will aid in the adjustment. If preferred, the rubber tube on the burette can be connected to a smaller rubber tube by means of a short glass tube and the hose cock attached to this smaller tube. With this flowmeter it is necessary to fill the burette 2 or 3 cm above the top graduation so that the flow rate will have become steady when the acid reaches the zero mark. The tubes must be changed at each 3 ml without stopping the flow, and the burette should be refilled after 12 or 15 ml have been run out.) When 3 ml of acid have been added to each tube, remove the attached "flowmeter" and add 9 ml more acid at a rapid dropwise rate. Shake in the ice bath during this addition and stop the flow between tubes. Keep the tubes in the ice bath until the total of 12 ml has been added to all tubes, then remove them to a bath, held at about 28°C., for 6

TABLE 1.—*Galacturonic acid found in various samples by the Carbazole Method*

SAMPLE	GALACTURONIC ACID		
	ADDED	FOUND	RECOVERY
	mmg	*mmg*	*per cent*
Synthetic mixture of fruit acid and salts	50	47	94
Synthetic mixture of fruit acid and salts	50	47.5	95
Synthetic mixture of fruit acid and salts	100	94	94
Frozen sound strawberries	0	42	—
Apples, No. 1	0	7.5	—
Apples, No. 1	50	55.5	96
Rotten pear	0	1149	—
Apple juice	0	25	—

minutes. Finally, place the tubes in the thermostated bath maintained at 50°C. ±0.3° for a period of 70 minutes. Remove tubes and place in a water bath at about 22°C. for 5 or 6 minutes. Read the optical densities of the solns in a spectrophotometer at a wave length of 540 millimicrons and use the blank determination as a reference of 0 density (100% transmission). A neutral wedge photometer with a filter centered at 540 millimicrons may be used.

From the reading obtained on the standards construct a curve (straight line) plotting optical densities or photometer readings against micrograms of galacturonic acid. Obtain the galacturonic acid in the sample aliquot from the curve and calculate the sample content in p.p.m.

Some results obtained by the method are given in Table 1. These data show that the method gives reasonable recovery of galacturonic acid when added to fruit samples.

B. Determination of Intermediate Poly-Galacturonide Decomposition Products of Pectin

In a former report[3] the author called attention to the presence, in substantial amounts, of a mucilaginous substance, apparently a polysaccharide, in rotten apple juice which was present only in minute amounts in sound apple juice. It was reported that the material was not a starch or a pentosan, although insoluble in alcohol. It was found that it did not precipitate in the pectic acid determination, either by the A.O.A.C. method or the calcium pectate method. Further investigation has led the author to the conclusion that the material is a polygalacturonide, intermediate between pectin and galacturonic acid which results from a partial breakdown of the pectin molecule. The following carbazole method, in which the material is hydrolyzed to galacturonic acid, is offered for the determination of this material. Results are reported in terms of galacturonic acid. A few results by the method are given in Table 2.

CARBAZOLE METHOD

With the exception of the following, apparatus and reagents are as given in Part A.

(1) *Galacturonic acid stock soln.*—Dissolve 200 mg of pure galacturonic acid in water and dilute to 100 ml. Add several drops $CHCl_3$ or CCl_4 and store in refrigerator.

(2) *Standard galacturonic acid soln (dilute).*—Make 5 ml soln (1) (stock) to 100 ml with H_2O. Add several drops $CHCl_3$ or CCl_4 and preserve in refrigerator.

PREPARATION OF SAMPLE

Fruits.—Peel and core such fruits as apples and pears. Clean berries of the calyx, stems, etc., and separate a suitable sample (200 g or more). Crush and chop the fruit in a food chopper and quickly weigh 100 grams into a tared 600 ml beaker. Add about 200 ml of hot 95% alcohol, digest a short time on the steam bath, then pour the contents of the beaker into a blendor (Waring, or similar). Rinse beaker with a little alcohol and comminute the sample for about 4 minutes. Pour the

[3] *This Journal*, **32**, 513 (1949).

TABLE 2.—*Determination of galacturonides (as galacturonic acid)*

SAMPLE	MMG/G
Sound apple	28
Rotten apple (1)	1540
Rotten pear	476
Rotten apple (2)	2144

mixture back into the beaker, rinse blendor with alcohol (about 50–75 ml), cover beaker, and digest the mixture on steam bath about one hour.

Fruit Juices.—Measure a 100 ml portion into a 600 ml beaker. Add, with continuous stirring, 325 ml of warm alcohol and digest the mixture on steam bath about one hour.

Place a rod in the beaker and stir all sample mixtures thoroughly at 5–10 minute intervals during the digestion. Remove from bath, cool to room temperature, and transfer quantitatively to a 500 ml volumetric flask, using alcohol to rinse beaker. Make to 500 ml with 95% alcohol, and mix thoroughly. Add a few grams of filteraid (equal weights of filtercel and Celite 545) to the flask and mix again. Filter the mixture on a 11 cm Büchner funnel with suction, using a Whatman 41 H paper. (Remove filtrate from flask and, if desired, use an aliquot for determination of galacturonic acid as in Part A.) Rinse flask and wash the residue on the filter with 200–300 ml of hot 80% alcohol. The last washings should be quite clear and nearly colorless. To wash effectively, smooth down the edges of filter pad and residue with a spoon or smooth object to keep filter tight.

After washing, remove the filter pad with residue from funnel, strip filter from pad of residue and Celite, and place this material from the filter pad in a 600 ml beaker. Wash off the filter paper with hot water into the beaker and add a total of 400 ml of water to the beaker. Stir well, cover beaker, and digest on steam bath for 50–60 minutes, stirring well at intervals. Remove beaker, cool to 25°C., and transfer contents quantitatively to a 500 ml volumetric flask with the aid of water from wash bottle. Make to mark with water, mix, and pour into a dry beaker. Add several grams of Celite to contents of beaker, stir well, and filter on a 11 cm Büchner funnel with suction, using Whatman 41 H filter paper.

REMOVAL OF PECTIC ACID

Measure a 125 ml aliquot of the filtrate into a 400 ml beaker and reserve the remainder of filtrate. Place 125 ml of water in a similar beaker as a blank. Cool the beaker to 15 to 18°C. in a bath, and add 3.0 ml of 15% NaOH with stirring. Let beakers stand in bath at about 15–18°C. for 20 minutes. Remove from bath and acidify contents of beakers with 0.95 ml of acetic acid (glacial). Dilute the contents to 200 ml and add to each beaker 3 ml of 11% $CaCl_2$ soln with stirring.

Allow to stand a few minutes, then heat the liquid to boiling and continue boiling for 5 minutes. Remove and filter the hot soln through a 15 cm Whatman 41 H paper on a fluted or ribbed funnel. Wash beaker and filter several times with hot H_2O from wash bottle. Discard the filter and precipitate. Add 450 mg of anhydrous Na_2SO_4 to the filtrate and concentrate to about 75 ml by boiling or by evaporation on steam bath. Transfer the liquid quantitatively to a 100 ml volumetric flask and dilute to about 90 ml. Add 300 mg of activated charcoal to the contents of flask, and mix. Place the flask on the steam bath over the 50–60 mm opening and heat for 11 minutes. Shake at frequent intervals after heating 5 minutes. Remove, cool to 25°C., make to 100 ml with H_2O and filter on a quantitative filter paper. If necessary pour back until bright.

DETERMINATION

Place 2 ml of the sample filtrate and 2 ml of the blank filtrate respectively in two 25×150 mm test tubes. Prepare standards by placing 0.5, 1.0, 1.5, and 2.0 ml of reagent (2) in similar test tubes. Make the volume to 2 ml in these standards with the liquid from the blank soln.

Proceed with the determination as in the carbazole method for galacturonic acid beginning, "From a 1 ml graduated pipet add to each tube 0.5 ml carbazole soln (Reagent 1)." However, heat the tubes only 1 hour at 50°C.

Report results in terms of micrograms galacturonic acid per ml or g of sample. (The final soln represents 25 ml or 25 g of original sample in 100 ml of soln.)

The results are encouraging as an additional index of decomposition. Other criteria of decomposition are being investigated. By the use of paper chromatography, the presence of various compounds in rotten tissue are being detected and a number of leads have been found. These will be investigated further, but the writer is not at present ready to report on this work.

RECOMMENDATIONS*

It is recommended—

1. That collaborative studies be conducted on the naphthoresorcinol method and on the carbazole method for galacturonic acid.
2. That further studies be made of the method for intermediate polygalacturonides as an index of decomposition in fruits.
3. That studies of other criteria of decomposition be continued.

REPORT ON GALACTURONIC ACID IN STRAWBERRY JUICE

By Paul A. Mills (Food and Drug Administration, Federal Security Agency, San Francisco 2, California), *Associate Referee*

Last year's report concluded with the recommendation that the color reaction be further studied and that authentic data on the galacturonic acid content of strawberries be developed. Difficulties encountered in obtaining reproducible results and the elimination of interferences consumed nearly all the available time, and few data on the galacturonic acid content of strawberries were obtained.

In an attempt to eliminate uncertainties due to impure naphthoresorcinol as purchased, a quantity was synthesized according to the procedure in *Organic Synthesis*, Vol. 25. This material was pale yellow in color, melted at 122°C., and was thought to be very pure. However, its use produced weak colors and variable results.

Some work done near the beginning of the investigation showed that

* For report of Subcommittee C and action of the Association, see *This Journal*, 35, 50 (1952).

reducing agents such as sodium hydrosulfite destroyed the color of the galacturonic acid-naphthoresorcinol compound and that hydrogen peroxide restored it, at least in large measure. In the synthesis, the last step involved crystallization in the presence of sodium hydrosulfite. Some reducing substances were probably carried over. Experiments showed that the addition of small amounts of hydrogen peroxide to the reaction mixture produced strong colors.

The initial experiment was conducted by adding 1% hydrogen peroxide solution dropwise, to tubes of reaction mixture. These consisted of 1 ml of a solution containing 100 mmg of galacturonic acid, 2 ml of 1+3 acetone solution containing 20 mg naphthoresorcinol, 2 ml hydrochloric acid (conc.) and varying numbers of drops of 1% hydrogen peroxide solution. The tubes were heated 75 minutes at 65°C. then each was shaken with ether and the colors determined as described in last year's report.[1] The experiment indicated that from 1 to 3 drops of 1% hydrogen peroxide solution produced maximum colors (0.1 ml to 0.2 ml per tube).

In order to place it on a more quantitative basis the following experiments were conducted. In practice a series of 6 tubes was developed at one time. For this series, naphthoresorcinol solution was prepared by dissolving 130 mg in 13 ml 1+3 acetone. Each 2 ml of this solution contained 20 mg of naphthoresorcinol.

In the following experiment, 1% peroxide solution was added to the 1+3 acetone solution, so that the final volume was 13 ml ($12\frac{1}{2}$ ml acetone +0.5 ml peroxide, 12 ml acetone +1 ml peroxide, and so on). In order to be sure that the galacturonic acid content was not a factor, varying quantities of galacturonic acid were used. Results are listed in Table 1.

TABLE 1.—*Effect of concentration of hydrogen peroxide on depth of color*

GALACTURONIC ACID	OPTICAL DENSITIES				
	0.5 ML H_2O_2	0.75 ML H_2O_2	1 ML H_2O_2	1.5 ML H_2O_2	2 ML H_2O_2
mmg					
10	.055	.082	.082	.079	.066
30	.200	.247	.248	.241	.194
50	.365	.461	.425	.405	.325
70	.520	.590	.590	.532	.460
100	.730	.850	.850	.810	.640

These 5 trials, made under as nearly the same conditions as possible, indicate that from 0.75 to possibly 1.25 ml of 1% peroxide solution, in a total volume of 13 ml of 1+3 acetone solution of naphthoresorcinol, produced maximum colors. 1 ml in this volume was arbitrarily selected as the optimum quantity. In some later work, 8 tubes were used simultaneously

[1] *This Journal*, 34, 513 (1951).

and the strength of the peroxide solution adjusted so that the proper quantity for a total volume of 17 ml could be added in one ml.

The peroxide solution was originally prepared by diluting 3 ml of Superoxol to 100 ml. (Strength by titration was found to be 1.1% for the particular bottle of Superoxol used.) By calculation it was found that 3.9 ml of this Superoxol diluted to 100 ml would contain enough hydrogen peroxide in 1 ml to furnish the required quantity for 17 ml of naphtho-resorcinol-acetone solution which contained 170 mg naphthoresorcinol.

All of the commercial naphthorescorcinol purchased had been used before this phenomenon was noted, so it has not as yet been possible to test the effect of hydrogen peroxide on commercially available naphthoresorcinol.

Since peroxide apparently cleared up the difficulty with the color reaction, and since the proposed method recommended the isolation of acids by the use of ion exchange resins, work was continued on pure solutions passed through ion exchange resins. Solutions of 1% citric acid containing varying quantities of galacturonic acid were used. Usually 25 ml of 1% citric acid and the indicated quantity of galacturonic acid were passed first through the cation column (in all cases Duolite C-3) and then through the anion column. The column was washed with water until the total volume through the resins was about 250 ml. The anion columns were then stripped with dilute HCl and a total volume of 100 ml of eluate was collected. In stripping the anion column 20 ml of 1+3 hydrochloric acid was added drop by drop followed by water to 100 ml (flow rate for the columns used was 2–3 ml per minute). Resin particle size for the anion column was 20 to 40 mesh. This tended to eliminate troublesome channeling.

TABLE 2.—*Recovery through the resins and reproducibility of the color reaction*

GALACTURONIC ACID PER 100 ML ELUATE	OPTICAL DENSITY		
	INITIAL DEVELOPMENT	REPEAT	REPEAT
mg			
3.0	.249		.238
5.0	.432	.434	.425
7.5	.620	.624	.612
10.0	.805		.807
3.0	.235	.246	
5.0	.398	.393	
7.0	.529	.532	
10.0	.795	.785	.783
3.0	.234		.240
5.0	.397		.385
7.0	.548		.552
10.0	.755	.755	.765

Three sets of four levels of galacturonic acid were used as shown in Table 2. Additional color reactions were developed on each set on subsequent days to test the reproducibility of the color reaction.

The color reactions for the same solution on subsequent development is reproducible as shown in columns headed "Repeat." Recoveries through the resins leave something to be desired. However, on these 3 runs the maximum variation is only 6.3%.

Other experiments were conducted to test the effect of passing pure solutions through the resins. In all cases eluates from the resins produced less color than the pure solutions used. Colors developed from eluates, when graphed, produced straight line curves differing only in slope from that obtained for pure solutions. Two anion resins were used. Optical densities are shown in Table 3. It was concluded that standard solutions

TABLE 3.—*Galacturonic acid colors before and after elution*

GALACTURONIC ACID	OPTICAL DENSITY		
	PURE SOLUTIONS	THROUGH IRA 400	THROUGH DUOLITE A-4
mg/100 ml			
1	.085		
3	.252	.24	.248
5	.445	.40	.393
7	.603	.54	.579
10	.846	.78	.818

should be passed through the resins and the acids stripped from the anion column in the same manner as with samples.

The data in Table 3 indicate that either IRA 400 or Duolite A-4 could be used equally well on pure solutions of galacturonic acid, but when samples of strawberry juice were passed through IRA 400 resin some interference was picked up which caused the formation of off colors in the color reaction, and optical densities were much too high. Duolite A-4 did not produce this effect. This behavior of IRA 400 may be related to its strongly basic character. Table 4 shows optical densities obtained on eluates from both resins using the same sample of juice. In view of the results obtained with IRA 400 resin, work with this material was discontinued in favor of Duolite A-4.

TABLE 4.—*Comparison of optical densities obtained from eluates from 2 anion resins*

SAMPLE	IRA 400	DUOLITE A-4
25 g juice	.235	.083
50 g juice		.175
25 g juice+3 mg Galact. Acid	.417	
25 g juice+5 mg Galact. Acid	.523	.313
25 g juice+7 mg Galact. Acid	.720	.630

Results reported here are sketchy and it is planned to study these phenomena further during the analysis of authentic samples. So far only two samples of authentic fresh fruit have been examined. These represent two varieties grown commercially in California. Galacturonic acid was added to portions of each to test recovery and results are shown in Table 5. These varieties are given by symbol rather than by name.

TABLE 5.—*Galacturonic acid content of fresh strawberry juice based on various sample weights. Recovery of galacturonic acid when added to strawberry juice*

SAMPLE	GALACTURONIC ACID ADDED	GALACTURONIC ACID FOUND		
		PER ML ELUATE	PER GRAM JUICE	RECOVERY
g juice	*mg*	*mmg*	*mmg*	*per cent*
Z2 variety				
25	0	10	40	
50	0	22	44	
25	3	38		93
25	7	78		97
H1 variety				
25	0	13	52	
50	0	26	52	
100	0	47	47	
25	10	111		98

Data in Table 5 show good recovery and reasonable accuracy. The resin columns used, containing a resin bed about 17 mm in diameter and 150 mm deep, have an apparent capacity of up to 100 g of strawberry juice. The acids may be eluted in a volume of 100 ml. The few figures shown here indicate some loss with the 100 g sample. Fifty grams may prove to be the practical maximum.

Larger resin columns have been used but no advantage resulted because larger volumes were required to remove the acids from the larger quantity of anion resin.

RECOMMENDATION*

It is recommended that work be continued.

REPORT ON CHEMICAL INDICES TO DECOMPOSITION IN SHELLFISH

By GEORGE MCCLELLAN (Food & Drug Administration, Federal Security Agency, New Orleans 6, Louisiana), *Associate Referee*

The studies described here were limited to canned shrimp.

The best chemical index at present available for confirming decomposi-

* For report of Subcommittee C and action of the Association, see *This Journal*, **35**, 50 (1952).

tion in shrimp is probably the indole content. Its single defect is that it fails to detect some types of decomposition, notably that known as "ammoniacal."

In the search for some index of more universal application than indole, several "feeling-out" experiments were performed on canned fresh and canned decomposed shrimp. The purpose was to make brief tests of several possible indices in the hope of finding one or more that would show a wide difference in results on the two samples. Among the criteria tested were volatile bases, ether-soluble volatile bases, bases adsorbed on permutit from an acetone extract of the shrimp, volatile and permutit-absorbed bases precipitated by phosphotungstic acid, and bases volatile at room temperature in the presence of formaldehyde. In all of the tests, the results for canned decomposed shrimp were higher than those for canned fresh shrimp, but the differences were not considered sufficient to warrant further investigation of these indices.

Work was begun on methods for isolating and determining free tryptophane, arginine, and cystine in shrimp. Recovery experiments on canned shrimp, using an adaptation of Duggan's method for tryptophane in cream[1] showed recoveries from 75 to 95 per cent of added tryptophane in canned shrimp and of from 52 to 70 per cent on raw shrimp.

RECOMMENDATIONS*

It is recommended that the work be continued.

REPORT ON FISH (INDOLE IN CRAB)

INDOLE AS AN INDEX OF DECOMPOSITION IN DUNGENESS CRAB

By DAVID W. WILLIAMS (Food and Drug Administration, Federal Security Agency, San Francisco 2, California), *Associate Referee*

The presence of indole in decomposed shrimp was found by Duggan and Strasburger (1) to be a good index of decomposition. There was also some indication that decomposed crabmeat prepared from the blue crab (*Callinectes sapidus*) contained indole (2).

This year's project included the analysis by the Associate Referee of two experimental packs of canned Dungeness crab (*Cancer magister*) for indole (a total of 28 determinations) using the A.O.A.C. method (3), and some studies on the applicability of the method to this species.

One experimental pack was prepared from crabs decomposed in the shell under various conditions of temperature and time, in the shell after killing the crabs, and crabs decomposed after picking. Each type of spoilage was

[1] Duggan, R. E., *This Journal*, **31**, 151 (1948).
* For the report of Subcommittee C and action of the Association, see *This Journal*, **35**, 51 (1952).

canned separately. Separate packs of good, decomposed, advanced decomposed, and putrid crabmeat from each type of spoilage were prepared, with no citric acid dip and with citric or acetic acid dips of different durations.

The other experimental pack was prepared from crabmeat progressively spoiled as picked meat or as butchered uncooked crabs. Packs were made from the four stages of resulting decomposition with no acid dip, with a citric acid dip, or with an acetic acid dip of two different concentrations.

RESULTS

In the first pack no indole was found in Stage 0 crabmeat and the indole did not exceed 5 mmg/100 g in Stage 3 crabmeat (advanced decomposed). In the second pack analyzed, no indole was found in Stage 1 crabmeat, and amounts of indole in the range of 0–21 mmg/100 g. were found in Stage 3 crabmeat.

DISCUSSION OF THE METHOD

Difficulty was experienced with foaming in the distillation and this greatly extended the time of analysis. This difficulty was corrected by using a silicone antifoam (D.C. Antifoam A). Standards and blanks were run using the antifoam and it was found to have no effect on the results.

LITERATURE CITED

(1) *This Journal*, **29**, 177 (1946).
(2) Private Communication.
(3) *Methods of Analysis*, 7th Ed., p. 302 (1950).

REPORT ON URIC ACID IN FRUIT PRODUCTS (CHROMATOGRAPHIC ISOLATION)

By DORIS H. TILDEN (Food and Drug Administration, Federal Security Agency, San Francisco 2, Calif.), *Associate Referee*

In the 1950 report of the Associate Referee on "Uric Acid in Fruit Products (4) it was recommended that the study of the isolation and determination of minute amounts of uric acid in fruit products by means of paper partition chromotography be continued. The difficulty encountered at that time consisted chiefly of the lack of an effective means of concentrating the very small amounts of uric acid that might be expected in fruit syrups made from insect infested raw stock. A sufficient quantity must, of course, be present when aliquot portions of syrup in the magnitude of 0.01–0.03 ml are spotted on filter paper for final separation. A second difficulty, which is closely associated with the first, is caused by the accompanying build-up of sugars and caramel with any type of concentration which involves evaporation.

The procedure of Geren *et al.* (2), involving the adsorption of uric acid on charcoal, was tried on fruit syrups with soluble solids content of about 10 per cent. It was found that brown substances of the syrup, whether originating from the fruit or from degradation of sugars, migrate with and adversely affect the partitioning of small amounts of uric acid. When treated with alkali this material strips from the carbon along with any uric acid present. Experiments with fruit syrup also indicate that the adsorption of uric acid on charcoal and its subsequent removal is not quantitative.

Many types of solvent systems were tried in unsuccessful attempts to separate uric acid from caramel. Paper treated with buffer solution, talc, and starch were tried, but without success. The solvent systems and developing reagents used by Berry *et al.* (1) in connection with their studies of the behavior of uric acid on paper strips, were also investigated. Their ammoniacal silver nitrate reagent for developing a black spot of uric acid against a white background has advantages in manipulation over the formerly reported (4) procedure of producing a mercuric nitrate-nitric acid complex, but obviously it cannot be used when the phenol-acetic acid-water solvent system is employed unless all phenol is removed from the paper. A butyl alcohol-ammonium hydroxide solvent system affects a fairly satisfactory migration of uric acid and allows for the use of ammoniacal silver nitrate. Here again, however, closely associated caramel-like material reacts with the developing reagent and tends to obscure definition of the area occupied by small amounts of uric acid.

Current experiments with Duolite resins C-3 and A-4 in tandem columns (3) have given very promising results in concentrating uric acid added to fruit syrups, although there is still more caramel-like material accompanying the eluate than is desirable. Uric acid was retained on the A-4 column and eluted with 4 per cent hydrochloric acid. The solution was then made alkaline with lithium carbonate, evaporated to dryness, taken up in a small amount of water and subjected to paper chromatography (4).

Experimentation with other resins is contemplated, as the present work indicates that a procedure based on a combination of column and paper chromatography may be made relatively rapid, capable of determining very small amounts of uric acid, and probably quantitative.

RECOMMENDATION*

It is recommended—

That the use of column chromatography followed by paper chromatography as a means of concentrating and isolating minute amounts of uric acid in fruit products be further studied.

* For the report of Subcommittee C and action of the Association, see *This Journal*, **35**, 51 (1952).

REFERENCES

(1) BERRY, H. K., *et al.*, *University of Texas Bulletin*, 5109 (1951).
(2) GEREN, WM., *et al.*, *J. Biol. Chem.*, **183**, 21 (1950).
(3) MILLS, P. A., *This Journal*, **34**, 513 (1951).
(4) TILDEN, D. H., *This Journal*, **34**, 498 (1951).

REPORT ON URIC ACID IN CEREAL PRODUCTS

By HELEN C. BARRY (Food & Drug Administration, Federal Security Agency, New Orleans 16, La.), *Associate Referee*

Experiments using 2–6 dichloroquinone-chloroimide (CQC) as color reagent and quinoline as a solvent for uric acid were continued. Blank or weakly positive tests for uric acid were obtained on flours containing little or no insect excreta; strongly positive tests were obtained as insect excreta pellets became numerous.

As used by the Associate Referee, the CQC-quinoline method has semiquantitative status only. The tendency of CQC to darken or discolor within a short time after color development in flour extracts presents a serious difficulty. This reagent is apparently sensitive to traces of the usual solvents for uric acid. Experiments are being continued on flour, and various solvents and methods of extraction are being investigated.

It is recommended that the study of uric acid in cereal products be continued.*

REPORT ON SUCCINIC ACID IN TOMATOES

By HALVER C. VAN DAME (Food and Drug Administration, Federal Security Agency, Cincinnati 2, Ohio), *Associate Referee*

In last year's preliminary report on succinic acid as an index of decomposition in tomatoes† it was recommended that the problem be studied further. This year the Associate Referee has collected more data to show that succinic acid is formed when tomatoes rot. The method as used last year has been modified to allow for the addition of 50 ml of 20 per cent phosphotungstic acid before the centrifuging procedure. This helps the filtration step considerably. All analyses this year have been made with this modification.

EXPERIMENTAL

In order to test whether or not canned tomatoes would break down after a year's storage to give greater amounts of succinic acid than freshly canned tomatoes, succinic acid was determined on a sample of tomatoes canned last year. Last year this sample of tomatoes showed 2.6 milli-

* For report of Subcommittee C and action of the Association, see *This Journal*, **35**, 51 (1952).
† *This Journal*, 34, 522 (1951).

grams of succinic acid per 100 grams. The result of the present analysis is shown in Table 1.

It was decided to investigate the effect of allowing tomatoes to rot after inoculation with some of the molds commonly found to produce rot in tomatoes. Pure cultures of *Altenaria Sp.*, *Muccor Sp.*, and *Fusarium Sp.*, obtained from the Division of Microbiology of the Food and Drug Laboratories in Washington, were used for this purpose.

After thorough washing, followed by dipping in boiling water for one minute, tomatoes were placed in wide-mouth sterile jars. Each tomato

TABLE 1.—*Correlation of succinic acid and mold decomposition of tomato products*

PRODUCT	MOLD COUNT	SUCCINIC ACID (MG/100 G)		
	PER CENT	ADDED	FOUND	PER CENT RECOVERY
Commercial Puree #1	64	0.0	7.3	—
		20.0	26.3	95.0
Commercial Puree #2	44	0.0	6.0	—
		20.0	25.6	98.0
Fresh Tomatoes	0	0.0	2.1	
Tomatoes Canned 1 Yr. Ago	0	0.0	2.5	
Tomatoes Inoculated with *Allernarium*	100	0.0	20.0	
Tomatoes Inoculated with *Muccor*	100	0.0	86.0	
Tomatoes Inoculated with *Fusarium*	100	0.0	93.8	

was then cut with a sterile scapel and inoculated with one of the above molds. The jars were allowed to incubate at 37°C. for 48 hours. After this time the tomatoes were comminuted in a Waring blendor and succinic acid was determined on a portion. The results are listed in the table.

The succinic acid isolated from the rotten tomatoes was identified from the melting point of its anilide derivative prepared in the following manner:

The butanol-chloroform mixture and the water layer remaining after the titration of the succinic acid with sodium hydroxide is evaporated to 2 or 3 ml, poured into a small test tube, and evaporated to dryness. 0.4 ml aniline and 0.1 ml HCl are then added to the test tube, and the mixture is heated in an oil bath for one hour at a temperature of 150°–160°C. 2 ml of alcohol are added, and the mixture brought to a boil. The contents of the tube are then poured into a small beaker containing 5 ml of H_2O. The mixture is heated on the steam bath and evaporated to about 2 or 3 ml. It is next cooled in an ice bath, the crystals are collected on a filter paper, washed and recrystallized from 50% alcohol. The melting point of the anilide derivative is 226°C.

CONCLUSIONS

(1) Canning and storage of tomatoes for periods of a year do not appear to cause formation of succinic acid.

(2) A relatively large amount of succinic acid is produced in tomatoes by molds which produce rot.

RECOMMENDATIONS*

It is recommended—

(1) That the problem be studied further to determine the correlation of rot and succinic acid. This should be done on authentic purees containing known amounts of rotten tomatoes.

(2) That the method be given collaborative study.

No reports were given on acids in fish products, animal fecal matter, carbohydrate decomposition in pineapple, pigments in strawberries, uric acid in nuts, lactic acid, gallacturonic and succinic acids in spinach, or on gallacturonic acid in tomatoes.

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*

This report will be divided into two parts, viz. (A) concerning inorganic metals, and (B) the newer organic toxic substances and residues.

A. INORGANIC SUBSTANCES IN FOOD

COPPER AND ZINC

The topics of copper and zinc can be discussed together. They are mildly toxic metals but have importance as trace metals in important foods. The Associate Referee on copper did not conduct any collaborative studies but checked the results of a previous collaboration that had never been reported and cleared the decks for further collaborative study next year. The results of the first study by three of the laboratories most experienced in the determination of copper were not highly satisfactory. If three such experienced collaborators cannot agree on their copper results there must be something lacking in the chemistry of the two methods themselves or in their wording. If different meanings are given to the same wording by different analysts they probably need revision. The Referee had some conversation with the Associate Referee on Copper in Foods about ways and means for conducting the work next year.

No report was made by the Associate Referee on the determination

* For the report of Subcommittee C and action of the Association, see *This Journal*, **35**, 51 (1952).

of zinc. The Referee has pointed out a number of times in previous years that the present method could be revised to bring it more in conformity with other dithizone methods for metals now in the official Methods of Analysis. He repeats his observation that the extraction of zinc from glassware by alkaline fluids disturbs the blank determination so important in the case of small quantities and that this could be minimized by making the extraction and determination of zinc at a mild acid *p*H in an acetate buffer solution, according to the directions of Hellmut Fischer.[1] Your Referee hopes that some report on this idea will be made next year. He therefore recommends that work on copper and zinc be continued.

FLUORINE

The Referee's attention was recalled to the fluorine determination during the year. The method for fluorine as adopted mentions gelatinous silica, aluminum, and boron as interferences in the fluorine determination. It describes how the fluorine may be isolated from aluminum and boron compounds previous to its determination, but it is silent with respect to silica. The chemists responsible for the development of the fluorine method had had no experience with silica compounds and thought that perhaps this interference was of more theoretical than practical interest. Several of the Western States have had a fluorine problem thrust upon them since the last war. New aluminum plants have at first emitted fumes containing fluorine compounds which were carried over the countryside by the winds. Before scrubbing operations on the emitted gases were undertaken the fumes damaged forest and fruit trees and ornamental plants and caused fluorosis in cattle eating grasses from pasture thus contaminated. Similar situations existed earlier in certain sections in the East. The Food and Drug Administration was interested in the situation but could find little evidence of excess fluorine in interstate foods coming from the areas and little or none in milk from fluorine intoxicated cows.

State chemists soon found that fluorine results by the A.O.A.C. method on certain grasses and hays and on pine needles were always low. On the other hand, clover hay caused no difficulties. Sudan grass and pine needles contain considerable silica, while clover contains little. Apparently it is this silica in the ash that caused the retention of fluorine in the distillation. Collaborative samples were exchanged between the state officials and our laboratory and the results were always about the same—low recoveries in the silica-containing plants and satisfactory ones from the others. The incident forces this Association to reopen the fluorine question, at least as far as the silica interference is concerned. It is our belief that the ashing of the grasses or pine needles with excess lime in the sample preparation causes the formation of a very refractory calcium-fluorine-silica complex that does not give up its fluorine readily to either a sulfuric or

[1] *Z. Anal. Chem.*, 107, 241 (1936).

perchloric acid distillation. Larger volumes of distillate and higher temperatures of distillation may counteract the effect of reasonable amounts of gelatinous silica but they do not seem to be the answer to complex formation. Fusion with alkali before distillation has helped some, but the Referee believes that fusion followed by silica removal by zinc perchlorate (rather than zinc nitrate), similar to the macro methods for fluorine determination in enamels pioneered by the Bureau of Standards, is the answer to this particular problem. He therefore recommends that the Associate Refereeship on fluorine determinations be reactivated with respect to this interference of excess silica.

MERCURY

The Associate Referee on the determination of mercury has been conducting collaborative studies for a number of years, with success by most collaborators but failure by a few others. It has been found that a complete destruction of organic matter before extraction of the mercury was necessary in some instances, as, for example, on apples, and was apparently unnecessary in the case of biological material. Destruction of organic matter without loss of mercury required a special apparatus. Last year's low recoveries in the case of one collaborator were traced to the use of silicone grease in the all-glass apparatus. Precipitated silica retained the mercury and prevented quantitative extraction of the metal. This required that a specific prohibition against the use of silicone grease be incorporated into the method. Another change was the partial neutralization of the digest with ammonia instead of alkali, or lowering of the *p*H by dilution with water. Separation of mercury from copper, the most serious metal interference, seems to be best made with sodium thiosulfate, the excess of which is destroyed by sodium hypochlorite and this in turn by hydroxylamine hydrochloride. This method of oxidizing thiosulfate avoids the use of potassium permanganate, which was found to have a variable mercury blank. The collaborative results this year, together with the majority of the results last year, justify the adoption of a mercury method suitable for the determination of small amounts of mercury in almost any kind of organic material. The Referee seconds the recommendation of the Associate Referee for the adoption, first action, of the mercury method and the discontinuance of further work on mercury for the present.

B. ORGANIC RESIDUES IN FOOD

DDT is the first and only one of the numerous chlorinated hydrocarbon insecticides that has arrived at the dignity of having a method for its determination in spray residue adopted as first action[1] by the Association. The Referee believes that some discussion of the present stature of the methodology of benzenehexachloride and methoxychlor is of interest as an index of things to come.

[1] *Methods of Analysis*, 7th Ed., p. 379.

BENZENEHEXACHLORIDE

Two colorimetric methods for the determination of benzenehexachloride (BHC) have been proposed and presented at the 1951 Boston meeting of the American Chemical Society. One (Schechter-Hornstein)[2] is based on the dechlorination of BHC by zinc and acetic acid to benzene, nitration to meta dinitrobenzene (85%), and formation of a magenta color with alkaline methyl-ethyl ketone. The other method (Fairing) rests on the reaction of the BHC with aniline and formation of dichlorodiphenylamine, which in turn produces a magenta color when oxidized with vanadic oxide. The Division of Food of the Food and Drug Administration has subjected both methods to trial. The latter method requires the complete elimination of fats, waxes, and other organic matter by several chromatographic separations and a rigid purification of the aniline and organic solvents. Benzenehexachloride can be determined in the presence of fats and waxes from fruits by the former method. Its critical point seems to be the previous removal of proteins and free amino acids as far as possible. Proteins must be removed because certain amino acids of proteins contain phenyl rings and probably produce high blanks by complete or partial conversion to benzene and hence to dinitrobenzene. This method is currently used in the determination of BHC in peanuts and peanut butters. Benzenehexachloride in the soil, no matter how it got there, has a very disagreeable effect on the taste and flavor of peanuts, somewhat analogous to its effect on potatoes. The chemical can be actually detected in contaminated peanuts but has never been identified in potatoes. The problem of the determination of BHC in peanuts and peanut butter, has not been completely solved, particularly with respect to the matter of sample preparation and elimination of blanks. However, the Referee believes that the colorimetric determination of BHC in foods and biological materials has advanced to the point where collaborative studies can begin, and therefore recommends the appointment of an Associate Referee to conduct studies, collaborative if possible, of these two competitive methods.

METHOXYCHLOR

No report was made this year on the determination of methoxychlor or on its differentiation from DDT. From the report last year and personal discussion this year, it is the Referee's understanding that DDT can be successfully determined by the Schechter colorimetric method in the presence of methoxychlor if the insecticides are first separated on a chromatographic column as in the removal of fats.[3] The methoxychlor is held on the column with the fat and the DDT comes through. The Fairing method for the determination of methoxychlor is specific, but the sample needs a careful "clean up" before applying the test because of the discoloration produced by the strong sulfuric acid used in the method on organic matter in general. This matter of clean-up is becoming of greater

[2] *Anal. Chem.*, **24**, 544 (1952).
[3] Davidow, B., *This Journal*, **33**, 130 (1950).

importance in other methods as well. The Referee believes that more work will have to be done on the isolation of methoxychlor from accompanying organic matter, particularly plant extractives, before its determination can be placed on a firm footing. When this has been done, we shall be in a position to carry out necessary collaboration. The Referee therefore recommends further investigation, including collaboration if possible, on the subject of the determination of methoxychlor in food products.

OTHER CHLORINATED HYDROCARBONS

Chlordane, heptachlor, aldrin, dieldrin, and toxaphene may be included in this group. Progress has been made in the colorimetric determination of the first four but no progress whatever can be reported on toxaphene. Commercial chlordane is such a variable mixture of isomers and related products that the stumbling block in its determination is not the production of a qualitative color complex but of what to use for a standard. A number of colored derivatives of some of the compounds in commercial chlordane have been reported in the literature, but no one will produce a color with all of them or to the same degree of sensitivity in the case of all of those compounds that do produce colors. This presents us with a dilemna on what to do right now. Chlordane is a very toxic substance, and its continued use as a spray material will depend largely on its absence or practical removal from food products at harvest time or before consumption. That means a low tolerance, if any, and the lower the quantity tolerated the more difficult becomes the problem of supplying a sensitive and accurate method. Perhaps a reference batch or batches of chlordane can be made with the help of the manufacturer which can be used as the standard of comparison for the insecticide itself or formulations thereof, but will it do for the determination of weathered spray residues? The Referee has heard reports that the manufacturer has colorimetric micro methods for both chlordane and heptachlor that are said to be both sensitive and accurate. It is to be hoped that they will "crystallize" before the next meeting. Heptachlor is one of the compounds in commercial chlordane, but the Referee understands it is not produced from chlordane but by another process. If it should become the predominant commercial article, methodology would be greatly simplified. At least we would be dealing with one definite chemical compound instead of half a dozen, and no doubt pure heptachlor can be obtained for use as a standard.

Aldrin is another chlorinated hydrocarbon that is believed to need an extra sensitive and accurate method to determine it as spray residue because of its high degree of chronic toxicity. The manufacturer's chemists declare that they have developed a colorimetric method to the "rough draft" stage that will detect 0.1 p.p.m. of this insecticide as spray residue. All that remains to be done, they say, is to make certain improvements in the clean-up step. When the method is finally released, it can be determined if performance measures up to promise. The Referee hopes that it

will determine amounts of aldrin of at least this magnitude with a satisfactory degree of accuracy. The difference between the words *detect* and *determine* may be of considerable significance in future thought on spray residue.

PARATHION AND OTHER PHOSPHORUS-CONTAINING INSECTICIDES

No report was made on the determination of parathion. This method also depends on a clean-up of the sample before the parathion in it can be determined in the lower ranges with a high degree of accuracy. The "sample blank" (a device not in favor by regulatory officials) on some agricultural products, particularly tobacco (1–2 p.p.m.), containing no parathion, is too high. The Referee hopes that the clean up methods found useful in the determination of other insecticides may in turn be applied to parathion. He therefore recommends that the study of the determinations of parathion be continued.

There are a number of phosphorus-containing insecticides, other than parathion and some of the simpler organic phosphates, that are coming into use here and in other parts of the world. They are systemic poisons and can be translocated in plant as well as animal tissue. Like parathion they do not possess a high degree of volatility and linger around for quite a while. Unlike tetraethylpyrophosphate they are not easily and quickly hydrolyzed into innocuous compounds. Their high degree of toxicity renders then dangerous products to handle, and not much is known at present about their residues, either in or on plants. Methods of analysis for such residues, so far suggested, are based on the determination of organic phosphorus (after transformation into inorganic phosphates) by some variety of the colorimetric molybdenum methods. The Referee has often thought of this idea as the basis for a check method for the determination of parathion or other organic phosphorus compounds, analogous to the determination of organic chlorine in chlorinated hydrocarbons. The critical point is the complete separation of the organic phosphorus compounds from inorganic phosphates plentiful in foods. Some form of vacuum distillation can, perhaps, make the greater part of the separation, but there is always the problem of small blanks due to a spray carry-over in the distillation, or the presence of volatile natural organic phosphorus compounds in the plant material. In the absence of specific color reactions, the size of the blank is the critical part of this determination. If the chronic toxicity of the insecticide is high, the tolerance for it in foods must naturally be low. Where the blank approximates the tolerance or the level of insecticide to be determined, results are liable to be not very significant or accurate. This illustrates and emphasizes the difficulties analytical chemists have had and will continue to have in devising methods for determining smaller and smaller quantities of the more toxic insecticides existing as spray or translocated residues.

MONOFLUOROACETIC ACID

The Associate Referee has refined the qualitative thioindigo test for monofluoroacetates (1080) to the point where 0.5 mg can be detected with certainty when extracted from foods or biological material. If it can be obtained in pure condition, even smaller quantities can be identified. This compound is so toxic to certain species of animals that often only very small quantities can be isolated from carcasses at autopsy. Here the qualitative test will be extremely useful. About the only interferences in the qualitative test are the monochloracetates sometimes used as preservatives. Therefore in the event that a qualitative test is positive, a quantitative fluorine determination will serve to pin the identification on to the fluoroacetate and determine how much is present as well. The Referee recommends that this investigation be continued particularly with respect to collaborative studies.

DECOMPOSITION OF INSECTICIDES

The Referee does not intend to discuss the metabolism of insecticides in the animal body in this section but only the breakdown of insecticides that may occur in weathering or processing of agricultural products. In the early days of DDT, one of the most stable of the chlorinated hydrocarbons, some work was done showing that this insecticide could, under certain conditions, undergo complicated decompositions under the influence of ultra violet light. Fortunately it was found that the breakdown products of DDT were more volatile than the insecticide itself and never seemed to accumulate in the open. Therefore the problem of their composition seemed of no great importance. It was shown later that certain canning processes do break down partially, or almost completely, DDT and some of the other chlorinated hydrocarbons. Nothing is known about the composition of such breakdown products, whether they are toxic or not or whether they can interfere in the determination of the residual insecticides. Certainly they cannot escape from the interior of a processed can. We have not had any reports for the last two years. The great increase in the number of organic insecticides expands the problems of what happens to these products in the canning or other processes that agricultural products are subjected to. The Referee hopes that this subject will not be forgotten and that investigation will be resumed.

Finally, the Referee wishes to leave the following thought behind as his last contribution to the years of work on the development of methods for the determination of insecticides as spray residues. He does not know what the outcome of the spray residue hearings is going to be, but he firmly believes that the analytical methods adopted by this Association must have an accuracy that can be correlated with the tolerances set up as legal limits. It can be taken as almost axiomatic that the greater the degree of chronic toxicity, the lower must be the limit of tolerance. An error of 0.1 p.p.m. due to any cause whatever in the determination of any insecticide as spray residue is quite acceptable if the tolerance is set at

1 p.p.m. or over, but it cannot be ignored if the tolerance is 0.1 p.p.m. or less. In other words, sensitivity and accuracy of spray residue methods must be geared to any future tolerances officially set up, after due hearing, and based on degree of toxicity and amounts of insecticide present on or in foods at time of shipment or consumption. Therefore the present Referee suggests that future Referees keep this doctrine in mind in developing acceptable methods, and that they reject those that do not measure up to the current tolerances and replace them with others that can.

RECOMMENDATIONS*

It is recommended that—

(1) Work on methods for the determination of copper and zinc be continued.

(2) The subject of the determination of fluorine be reopened with respect to efforts to restrict the interference of silica, particularly in plant products containing excessive amounts of silica.

(3) The method for the determination of micro amounts of mercury developed by the Associate Referee be adopted, first action, and that further work be discontinued subject to the call of the Referee.

(4) An Associate Referee be appointed to determine which of two proposed colorimetric methods for the determination of benzenehexachloride is superior and to subject the selected one to collaboration.

(5) The work on the determination of methoxychlor be continued.

(6) The investigations on the determination of parathion and related phosphorus-containing compounds be continued.

(7) The qualitative and quantitative methods for the determination of the monofluoroacetates be subjected to collaboration.

(8) The investigation of the decomposition products of insecticides in the weathering, canning, or other processing of agricultural products and their effect on analytical methods be continued.

REPORT ON MERCURY

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

In last year's report, *This Journal*, **34**, 529 (1951), it was recommended that both Laug's method of evaluating mercury, *This Journal*, **25**, 399 (1942), and the one proposed by Klein be submitted to further collaborative study. This was considered necessary because one of seven collaborators submitted very poor results by both procedures. Most of last year's collaborators expressed a preference for Klein's procedure, stating that removal of interfering metals from the reagents specified in the Laug procedure is quite difficult. The method is as follows:

* For report of Subcommittee C and action of the Association, see *This Journal*, **35**, 51 (1952).

PRINCIPLES

The procedure consists of wet digestion of the sample with nitric and sulfuric acids under reflux in a special apparatus; isolation of mercury by dithizone extraction; removal of copper; estimation of mercury by photometric measurement of mercuric dithizonate.

PRECAUTIONS

The critical part of procedure is digestion of sample. Unless sample digestion is almost complete, residual organic matter may combine with mercury and prevent or hinder extraction with dithizone. Oxidizing material in digest must also have been destroyed or dithizone reagent is decomposed and mercury is not quantitatively extracted. Because of volatility of mercury compounds, careful heating of digest during sample preparation is required. The acidity of final sample solution (after partial neutralization with ammonium hydroxide) prior to extraction should be ca 1 *N* and no higher than 1.2 *N*.

APPARATUS

(1) Digestion apparatus—see Figure 1.

(a) A 500 ml two neck standard taper round bottom flask.

(b) Standard taper modified Soxhlet unit devoid of syphon overflow, but equipped with stopcock on tube leading to digestion flask.

(c) Standard taper condenser affixed to top of Soxhlet unit. When stopcock of latter is open, assembly is in reflux position. When closed, unit serves as trap for condensed water and acids.

(d) Standard taper dropping funnel connected to outer vent of digestion flask.

Unit A, modified Soxhlet, 5 cm O.D., 200 ml capacity before overflow. Unit B, dropping funnel, ⅋ 24/40, capacity 75 ml. C, Friedrichs condenser, 35 cm total length. D, 500 ml digestion flask with two ⅋ 24/40 holes, 3 cm apart, for clearance of A and B.

(2) Separators (preferably Pyrex) of 250 and 500 ml or liter volume.

NOTE: As mercury compounds tend to adsorb on glassware, the apparatus, particularly separators, should be rinsed with dilute nitric acid and then distilled water before an analysis.

REAGENTS

All reagents must be of analytical quality.

(1) *Mercury standard.*—Prepare from dry, recrystallized mercuric chloride. A convenient stock

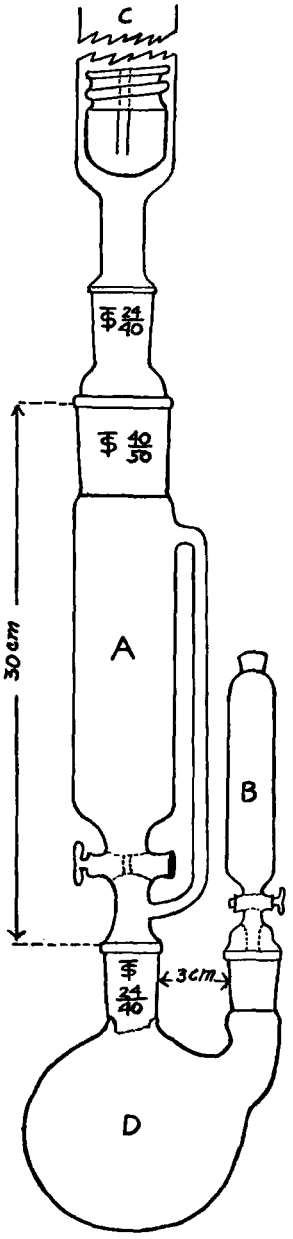

FIG. 1.—Special Digestion Apparatus

soln is 1 mg mercury per ml. Prepare dilute standard solns from stock soln and store in Pyrex glass. Add conc. hydrochloric acid in the proportion of 8 ml per liter to all standards before diluting to final volume. A convenient dilute standard is 2 mmg per ml.

(2) *Chloroform.*—Distil from hot water bath and collect distillate in brown bottle containing 10 ml absolute ethyl alcohol per liter distillate. Swirl receiver intermittently during the distillation.

(3) *Dithizone.*—The reagent as now distributed needs no further purification. A convenient stock soln is 100 mg/liter in reagent (2). Store in refrigerator when not using. As dilute solns of dithizone are somewhat unstable, prepare dilutions from the stock as needed.

(4) *Sodium thiosulfate 1.5% w/v.*—Prepare daily.

(5) *Sodium hypochlorite.*—C. P. Preferably 5% available chlorine reagent. As distributed, reagent varies in available chlorine content. Determine strength by analysis. Store in refrigerator when not using and determine titer at monthly intervals. (Certain preparations of hypochlorite intended for household use contain traces of mercury. If these preparations are used, run careful blank. A reagent with more than 0.1 mmg Hg per ml should not be used.)

(6) *Acetic acid,* 30% by volume.

(7) *Hydroxylamine hydrochloride 20% w/v.*—Extract with dilute dithizone until chloroform layer remains green, remove excess dithizone with chloroform, and filter.

SAMPLE PREPARATION

(Conduct Acid Digestion in Hood)

In all determinations use amount of sample equivalent to no more than 10 g, dry weight.

(a) *Fresh fruits or vegetables and beverages.*

Place weighed sample into digestion flask along with 6 glass beads, connect assembly, and add thru dropping funnel 20 ml of conc. nitric acid. Send rapid stream of water thru condenser, adjust stopcock of Soxhlet unit to reflux position, and apply small flame to flask. Use an asbestos board with 1–2″ diameter hole between flask and flame. (The original reaction must not proceed violently or the evolved nitrogen dioxide will carry vapors of the digest mechanically thru condenser and cause loss of mercury.) After initial reaction is complete, apply heat so that digest just refluxes. Should the mixture darken, add nitric acid dropwise thru funnel as needed. Continue refluxing for 0.5 hr. or until digest does not change consistency. Allow to cool, add slowly 20[1] ml of cold nitric-sulfuric acids (1+1). Heat with small flame, adding subsequently nitric acid dropwise as needed to dispel darkening of the digest. Continue heating until fibrous material (fruit skin, cellulose, etc.) is apparently digested. Turn stopcock of Soxhlet unit to trap water and acids and continue heating. Allow digest to become dark brown (not black) before adding further increments of nitric acid. (Fats and waxes can not be totally digested by the hot acids under reflux. No attempt should therefore be made to effect a complete digestion in this step.) When all but fat and wax is in solution, allow digest to cool and drain water and acids cautiously into the main digest. Cool and pour two 25 ml portions of water thru condenser and intermediate unit. Remove reaction flask, chill under cold water or by surrounding with ice to solidify fats and waxes, and filter off insoluble matter on a small pledget of glass wool. Rinse reaction flask and filter pad successively with two 10 ml portions of water. Remove Soxhlet unit and wash it and the flask with hot water to remove insoluble material. Pour hot water thru condenser to remove volatile fats and oils. Discard all washings. Connect flask contain-

[1] Use 10 ml acid mixture for 5 g or less (dry weight) of sample.

ing filtered sample soln to assembled apparatus, heat, and collect water and acids in the trap. Complete the digestion, using small additions of nitric acid as needed. In final stage of digestion, adjust flame until digest reaches incipient boiling (soln simmers) and acid vapors do not rise beyond lower half of condenser. Continue heating for 15 min. after last addition of nitric acid. Digest should now be colorless or pale yellow. Allow digest to cool, drain trapped liquids carefully into reaction flask, and add two 50 ml portions of water thru condenser. Reflux soln until all nitrogen dioxide is dispelled from apparatus. Add 5 ml urea reagent 40% w/v and reflux 15 min. (Digest should be colorless or a pale yellow soln.)

(b) *For dried fruit, cereal, seeds, and grains.*

Dilute sample with 50 ml of water before adding nitric acid, and proceed with sample preparation as described in **(a)**.

(c) *For meats, fish, and biological material.*

Because of high fat and protein content of these materials, initial digestion must be conducted carefully to avoid foaming of digest into condenser. Add 20 ml of nitric acid to sample, swirl flask, and let stand 0.5 hr. in digestion assembly before heating. Add 25 ml of water and heat cautiously with small rotating flame until initial vigorous reaction is over and foaming has ceased. Proceed therefrom as described in **(a)**.

Titrate 1 ml of sample soln thus prepared with standard alkali. Add the calculated amount of concentrated ammonium hydroxide to reduce the acidity to 1.0 *N*; swirl the flask during the addition of the ammonia in order to avoid local excess. (Soln should never be ammoniacal to avoid formation of mercury complexes.)

ISOLATION OF MERCURY

The following table is useful in preparing standard curve and for establishing approximate mercury range in sample solution.

Mercury Range	*Dithizone Conc.*	*Volume Dithizone*	*Cell Length*
mmg	*mg/l*	*ml*	*cm*
0–5	4	10	5
0–10	5.5	10	2.5
0–50	10	25	2.5
0–100	15	25	1

Transfer sample soln to 500 ml separator. Add 10 ml of 4 mg/l dithizone and shake vigorously for 1 min. (If green color of dithizone is apparent in chloroform layer, indicating excess of dithizone, the amount of mercury is within 0–5 mmg.) Allow layers to separate and transfer chloroform layer quickly to a second separator containing 25 ml of 0.1 *N* HCl and 5 ml of reagent **(7)**. (A small amount of oxidizing material may still be present. On long contact with dithizone soln, oxidizing substances may destroy dithizone reagent and prevent extraction of mercury.) Repeat extraction of sample soln with two additional 5 ml of dithizone, transferring chloroform layer successively to the second separator. If the first extraction indicates mercury in excess of 5 mmg add stronger concentrations of dithizone, as indicated by the table, until, after 1 min. vigorous shaking, the chloroform layer contains dithizone in marked excess. Drain chloroform layer into second separator containing the 0.1 *N* HCl and repeat extraction of the sample soln with two 10 ml portions of 4 mg Dz/l concentration of dithizone, draining each successive extract into the second separator. Shake contents of second separator vigorously for 1 min., and drain chloroform layer into third funnel containing 50 ml of 0.1 *N* HCl. (Shaking dithizone extract with dilute acid in second funnel removes entrained organic matter which may be present. With biological materials or those of high protein content,

aqueous layer is usually light yellow because of nitrated organic compounds. Small amounts are carried into third funnel where they are destroyed by chlorine.) Extract soln in second funnel with 1–2 ml chloroform and transfer organic layer to third separator. To contents of third separator add 2 ml thiosulfate reagent, shake vigorously for 1 min., allow layers to separate, drain off chloroform as completely as possible, and discard. (Copper if present is thus removed as the dithizonate.) Extract again with 1–2 ml of chloroform, drain carefully, and discard. Add 3.5 ml of hypochlorite reagent (or amount of soln of different titer sufficient to furnish 175 mg of available chlorine) to decompose mercury thiosulfate complex and to oxidize excess thiosulfate, and shake vigorously 1 min. Add 5 ml of hydroxylamine hydrochloride reagent (7) from a pipette, taking care to wet both stopper and neck of separator and shake vigorously 1 min. Hold mouth of separator in front of air vent and blow out any remaining gaseous chlorine. Stopper funnel and shake again vigorously for 1 min. (It is imperative that all hypochlorite be reduced. Traces of the reagent remaining would oxidize dithizone, subsequently added, to yellow oxidized form which would be measured in the photometer as mercury.) Extract soln with 2–3 ml of chloroform, drain off organic layer carefully, and discard. The final aqueous soln should now be colorless. Proceed as directed under Determination.

PREPARATION OF STANDARD CURVE

Prepare working curve of required range, starting with blank and extending to final standard of range, with four intermediate increasing increments. Add appropriate amounts of mercury to 50 ml of 0.1 *N* HCl in separator. Add 5 ml of hydroxylamine hydrochloride reagent, and 5 ml of chloroform, and shake vigorously 1 min. Allow layers to separate, drain off chloroform and discard, being careful to remove as completely as possible all droplets of chloroform. Add 3 ml of acetic acid reagent, the appropriate volume of dithizone soln, shake vigorously 1 min. and allow layers to separate. (The acetic acid aids in stabilizing mercuric dithizonate.) Insert pledget of cotton into stem of separator and collect dithizone extract (discarding first ml) in a test-tube for transfer to appropriate transmission cell. Make photometer readings at 490 mμ. (Since both dilute dithizone and mercuric dithizonate are somewhat unstable, the photometric readings should be taken immediately.)

DETERMINATION

To contents of third separator add 3 ml of acetic acid reagent and appropriate volume and concentration of dithizone soln, as indicated by chart, and proceed with colorimetric evaluation of mercury as described under Preparation of Standard Curve, converting optical density, measured at 490 mμ, to mmg Hg from the working curve.

The method submitted to the collaborators in the present collaborative study differed from last year's in only one respect. After completion of the sample digestion, adjustment of the acidity corresponding to 1.0 *N* was effected by the addition of a calculated amount of concentrated ammonium hydroxide instead of by dilution with water, as formerly. Each collaborator was supplied with a digestion apparatus, an "unknown" in duplicate, and a practice sample. The practice sample, weighing about 100 g, was made from a batch of finely comminuted fresh tomatoes, tested and found to be free of mercury. The "unknown," weighing about 25 g (part of the same batch) contained 16.5 micrograms of added mercury as the

dichloride. To both unknown and practice samples the following metals were added: Cu, Pb, Bi, Zn, Co, Ni, and Cd, in the respective amounts of 2000, 500, 100, 1000, 100, and 100 micrograms per 25 g of sample.

In order to rule out variables, collaborators were requested to divide the final digests of the unknowns into two equal parts, one part for each of the two procedures. Obviously, the amount of added mercury in each was 8.25 micrograms. Collaborators were asked to comment on each procedure and to state their preference. Their results are as follows:

MERCURY RESULTS IN MICROGRAMS PER ALIQUOT

Collaborator	*Laug Procedure*	*Klein Procedure*
A	8.8, 8.6	8.3, 7.5
B	9.5, 9.6	9.5, 9.6
C	7.0, 7.5	8.6, 8.0
D	9.5*, 8.0	8.3, 8.3

* The final solution evaluated for mercury contained zinc as contaminant. This result is therefore excluded from the statistical evaluation of results.

The average value reported by the Laug procedure is 8.42; the average deviation is 0.82 micrograms. The average value reported by the Klein procedure is 8.5 micrograms with an average deviation of 0.52 micrograms. The statistical evaluation of the results obtained by the two procedures does not indicate a marked superiority of either method as to accuracy.

Since the Klein procedure does not require a rigid purification of any of the reagents, and, further, since collaborators in the present study, as well as in the past, expressed a preference for the Klein method, it is recommended† that this procedure be given first action status.

REPORT ON COPPER IN FOODS

DETERMINATION BY MEANS OF DITHIZONE

By ARTHUR L. BRANDON (Anheuser-Busch Co., Inc., St. Louis, Mo.), *Associate Referee*

The last report to the Association was made by Stammer (3) in 1949, at which time he reported on an incompleted study of the Bendix-Grabenstetter Method (1) and the Greenleaf Method (2). Final report on this study was not made because only three of seven collaborators reported results; also a change in Mr. Stammer's work made it impossible for him to complete the report. However he has turned over the entire file on this study to the present Associate Referee.

In reviewing the contents of the file, poor agreement between collaborators was noted and no explanation is given for the erratic results.

Collaborator A had no particular comments to offer. Collaborator B

† For report of Subcommittee C and action of the Association, see *This Journal*, **35**, 51 (1952).

spoke of difficulties in the *p*H adjustment, prior to the original isolation of copper, in the Greenleaf method. He also pointed out a possible source of error in this method; at one stage of the isolation step the copper-dithizonate complex in carbon tetrachloride is broken up by the addition of saturated bromine-water to the appearance of a yellow color. Liberated copper is then transferred by shaking with 0.01 *N* hydrochloric acid. However, upon washing the HCl phase with fresh CCl_4 this collaborator noted that "a pink color (formed) in the solvent layer indicating that the dithizone had not been completely destroyed, and therefore some copper was probably discarded in the original separation." Collaborator C had no explanation for the erratic results. In his opinion, both methods should be capable of yielding reliable data.

COLLABORATORS

O. R. ALEXANDER, American Can Company, Maywood, Illinois.
R. D. COLEMAN, Quartermaster Food and Container Institute, Chicago, Illinois.
W. C. STAMMER, Continental Can Company, Inc., Chicago, Illinois.

The Associate Referee is indebted to Mr. W. C. Stammer for his cooperation in furnishing the records of previous studies.

RECOMMENDATION*

It is recommended—

That collaborative study of the Bendix-Grabenstetter and Greenleaf methods be continued.

REFERENCES

(1) BENDIX, G. H., and GRABENSTETTER, D., *Ind. Eng. Chem.*, Anal. Ed. **15**, 649 (1943).
(2) GREENLEAF, C. A., *This Journal*, **30**, 144 (1947).
(3) STAMMER, W. C., *Ibid.*, **33**, 607 (1950).

Two contributed papers, "The In Vivo Inhibition of Fly Cholinesterase as a Measure of Microgram Quantities of Organic Phosphate Insecticides" and "A Procedure for the Biological Assay of Insecticides by Oral Administration to Flies," both by John P. Frawley, E. P. Laug, and O. G. Fitzhugh, appear on pages 745 and 741, respectively.

The contributed paper "A New Qualitative Test for Monochloracetic Acid," by L. L. Ramsey and W. I. Patterson, was published in *This Journal*, **34**, 827 (1951).

No reports were given on zinc, DDT as spray residue in foods, insecticides in canned foods, parathion, sodium fluoroacetate, or on methoxychlor.

* For report of Subcommittee C and action of the Association, see *This Journal*, **35**, 51 (1952).

REPORT ON ENZYMES
(TESTS FOR UREA WITH UREASE)

By J. W. Cook (Food and Drug Administration, Federal Security Agency, San Francisco 2, Calif.), *Referee*

A test paper for urea which used urease and an acid-base indicator was reported by the referee in 1948 (1). This method, like some other urease-urea methods, depends upon the detection or determination of ammonia liberated by the enzymatic hydrolysis of urea. In this method both the enzyme which liberates ammonia from urea and the acid-base indicator (bromothymol blue), which indicates the ammonia, are incorporated into one test paper. When the paper is wetted and placed in contact with urea a blue spot develops after a short incubation period.

When the method was first developed very little work was done to determine sensitivity, versatility, interference limitations, etc. It was designed as a rapid method for the identification of urea in particles of food products suspected of being urine contaminated. The method stated that "The absence of alkaline substances may be established through the use of an auxiliary test paper impregnated with the indicator alone . . . alkaline salts produce color in both the urease and blank preparations . . . but (urea) does not produce color in the blank preparation (indicator alone)." This difference in action between urea and alkaline salts is easy to establish when a few micrograms of material are available or when two particles of identical material are available. But when the particles are extremely small the interpretation is more difficult. The original report also stated that "When alkaline salts are added to the moistened papers the blue color appears immediately after the alkaline salts are wetted, whereas urea crystals dissolve and seem to disappear; then after a period of 10 to 30 seconds the color appears (on the urease indicator paper)." This latent period of color appearance can be used to differentiate the action of urea from alkaline salts.

The referee has received one report that difficulty was experienced in applying the method to self rising flour, which, of course has finely powdered particles of alkaline carbonates. Here the presence of the alkaline particles can be established by use of test paper containing indicator alone. However, the presence of very small urea contaminated particles incorporated into a self rising flour is more difficult.

This year the referee has studied the method with particular reference to differentiation of the action of sub-microgram quantities of urea and alkaline salts. Some modifications of the technique were investigated and a collaborative study was carried out. All are reported in this paper.

EXPERIMENTAL

It was thought that a diffusion-through-air procedure could be de-

veloped to distinguish the action of alkali from urea. Method **35.103**(2) depends on the liberation of ammonia from urea and its diffusion through air to a drop of chloroplatinic acid solution. Feigl (3) describes a spot test for ammonia in which a piece of litmus paper is hung inside a closed vessel over an ammonia solution made alkaline with sodium hydroxide. Here the liberated ammonia diffuses onto the litmus paper. Conway (4) developed a quantitative micro-diffusion chamber. Neither of these was practical for the detection of sub-micro quantities of urea resulting from urine contamination of cereal products et cetera, so a modification was introduced.

A $\frac{1}{8}$ inch thick piece of clear plastic was perforated as follows: First a $\frac{1}{4}$ inch drill was used to make as many cups as possible in one side of the plastic, being careful not to run one cup into the other. This was done by drilling the plastic on 9/32 inch centers and by not going through the plate. Then the deepest part of each cup was used as a center to drill 3/32 inch holes through the plastic, thus making a large number of funnel shaped holes. When this perforated plate was put onto a piece of enzyme paper, with the large cups down, and covered with a piece of indicator paper, a series of small diffusion chambers was formed. This was placed between two pieces of glass to keep the paper tight on each side of the diffusion plate. Particles of urea placed in the cups are attacked by the urease, and the liberated ammonia diffuses to the indicator paper covering the chamber. Since the indicator paper is separated from the product and enzyme paper by the perforated disc, an indicator paper which has a *p*H change between 4 and 5 can be used. Thus the test is more sensitive to ammonia and interference from nonvolatile alkalis is prevented.

So far this procedure has not been satisfactory because of interference from extraneous ammonia. All of the filter papers that have been tested contain so much ammonium salts that alkaline particles put into the chambers liberated enough ammonia from the paper to give the same diffusion test as that from urea. It is possible to remove most of the ammonium salts from paper, but there is still the possibility that the product which is being tested has both ammonium salts and alkali particles. In this case the product would give a false positive test for urea. However, the possibilities have not been investigated fully.

Other indicators such as hemotoxylin, and manganous sulfate-silver nitrate (3) have been tried. The latter is not as sensitive as an acid-base indicator. Hemotoxylin is very sensitive, but is not as stable as desired and does not distinguish between very small amounts of alkali and urea. Larger quantities of urea are distinguishable with it because alkaline salts produce a blue color and ammonia produces a red. However submicro quantities of alkali also produce a red.

A technique that was given some study was that of heating the developed test paper, which contains blue spots, over a steam bath. When

the action is from a microgram or more of alkaline material the blue spot remains after the paper is dried, but that from urea disappears. When the alkali spot is from a fraction of a microgram the color from it also disappears.

Therefore the best procedure so far found for distinguishing between very small quantities of alkali and urea seems to be that of measuring the latent period of color appearance. That is, when a particle of alkali is put onto the paper the blue color appears immediately, whereas urea does not show action for a short while. These times vary with the amount of chemical involved. For instance, a single small crystal of sodium carbonate will show blue immediately and will make a large spot in a short time, whereas a single piece of finely powdered sodium bicarbonate, as may be found in self rising flour, will make a very small spot and will not appear for a few seconds. A crystal of urea (about 1 microgram) will develop a color spot faster than a fraction of microgram, but there is always a definite delayed action. Therefore the distinguishing feature is the time required for the color to appear, taking into consideration the size of the spot at the end of 5–15 minutes time.

COLLABORATIVE STUDIES

Since distinguishing the action of urea from alkali when large quantities are present is not difficult, this study was designed to test the limits of the technique when very small quantities are present. It was desired to test description of the technique and mechanical manipulations, as well as the theoretical possibilities of the method, in unfamiliar hands.

PREPARATION OF COLLABORATIVE SAMPLE

Farina was sieved, and pieces which passed through a 30 mesh sieve and not through a 40 mesh sieve were accumulated. A portion of this was wetted with a 0.1 *N* NaOH solution and allowed to dry. Another portion was treated with urea solution as follows: To 5 g of 30 mesh farina was added 3 ml of urea solution containing 5 mg per ml. This was allowed to dry. Both the alkali and urea wetted particles were stirred continuously while drying, to keep the particles more evenly wetted, thus bringing about more uniform distribution of alkali or urea.

Four samples of farina were submitted to collaborators for analysis. Sample 1 consisted of farina alone. Sample 2 had 5% (by weight) of farina treated with urea. Sample 4 had 5% farina with sodium hydroxide and 5% farina with urea. Sample 5 had 5% farina with sodium hydroxide. The urea and the sodium hydroxide treated farina particles produced spots of approximately equal size.

Three samples of wheat flour were also submitted to the collaborators. Sample 6 was wheat flour alone. Sample 7 was made by adding 0.01 g urine contaminated flour to 10 g of wheat flour. The urine contaminated flour was prepared by grinding a chunk of caked flour from a sample of

rodent urine-contaminated material. The chunk was ground in a mortar with frequent sieving. The portion that passed thru a 60 mesh sieve and stayed on a 90 mesh sieve was used. Sample 8 was prepared by mixing 0.5 g of self-rising wheat flour with 10 g of wheat flour.

A discussion of the procedure and directions for carrying it out were sent to each collaborator. Each was also furnished with a supply of urease-indicator paper (designated "A") and was furnished with reagents and filter paper to prepare another supply of urease indicator paper (designated "B").

The method and instructions were as follows:*

REAGENTS

(a) *Urease soln.*—0.2 g urease powder per 10 ml H_2O. Wet powder with small amount of H_2O and stir into a paste before diluting to volume.

(b) *Bromothymol blue soln.*—0.15 g bromothymol blue per 50 ml. Rub 0.15 g indicator powder in a mortar with 2.4 ml of 0.1 *N* NaOH soln. After indicator has dissolved wash the mortar and pestle and dilute soln to 50 ml. The soln should be green, which indicates ca *p*H 7.0.

(c) *Test paper A.*—Mix 10 ml of indicator soln **(b)** with 10 ml of urease soln **(a)**. Pour mixture into a watch glass. Using clean tweezers, dip pieces of heavy filter paper (Whatman No. 5, CS&S No. 598, or 589 green ribbon found to be satisfactory) in the mixed urease-indicator soln. (To avoid uneven distribution of indicator and enzyme wet the whole piece of paper at one time by laying it onto the surface of the mixed solns.) Hang the paper to dry in a place free from ammonia fumes, strong air currents, or heat. The paper should be orange colored when dry. Store the dry paper in a well stoppered, dark glass bottle in a cool place.

(d) *Test paper B.*—Dilute indicator soln (b) with an equal portion of water. Dip pieces of filter paper (same kind as used for test paper A) in the indicator soln and hang to dry similarly to test paper A.

DETERMINATION

Neutral solutions can be tested for urea by placing a drop on dry test paper A **(c)**. The appearance of a blue or green color after a few min. incubation at room temp. indicates urea.

For the detection of urea in very small dry particles, dip pieces of both test papers A **(c)** and B **(d)** of appropriate size into water using clean tweezers. Wet each whole paper at one time by laying it onto the surface of the water (the indicator flows unevenly if the paper is wet with drops). Shake each paper to remove excess water and lay it onto a clean flat piece of glass. (If paper on glass has shiny appearance too much water has been added. Allow to dry slightly before using.) Place sample on the papers, cover with another clean, flat piece of glass and press down gently.

Immediate development of blue color on both papers A and B is indicative of alkaline particles. If the alkaline particles are extremely small, color development is delayed 10–30 seconds, but will develop on both papers. Blue spots which develop on test paper A alone are indicative of urea. Urea in the sample is hydrolyzed by the urease in the paper to yield ammonia which changes the bromothymol blue to a blue spot. The reaction under conditions of the test requires 30–60 seconds to give detectable color, the time varying inversely with the urea concentration. The spots

* *This Journal*, **35**, 100 (1952).

TABLE 1.—*Collaborative results using urease-indicator test paper*

SAMPLE	DESCRIPTION	PAPER USED	CONTAMINENT REPORTED	PER CENT OF CONTAMINATING PRODUCT BY LABORATORY						
				1	2	3	4	5	6	7
1	Farina—Blank	A	NaOH	0	0	0	0	0	0	0
		A	Urea	0	0	0	0	0	0	0
		B	NaOH	0	0	0	0	0	0	0
		B	Urea	0	0	0	0	0	0	0
2	Farina—5% with Urea	A	NaOH	0	0	0	0	0	0	0
		A	Urea	2.7	2.6	4.3	7.4	4.5	6.7	3.0
		B	NaOH	0	0	0	0	0	0	0
		B	Urea	1.8	2.0	1.4	3.5	4.5	0	5.5
4	Farina—5% with NaOH+5% with Urea	A	NaOH	0	0	3.6	5.2	4.0	3.4	4.0
		A	Urea	5.7	6.3	1.7	4.1	4.5	3.7	5.0
		B	NaOH	0	0	3.2	4.8	3.5	3.5	5.0
		B	Urea	2.3	6.6	0	3.1	4.0	0.9	2.5
5	Farina—5% with NaOH	A	NaOH	1.0	0	2.0	4.5	5.0	6.1	5.0
		A	Urea	1.0	4.9	0	0	0	0	0
		B	NaOH	0	0	2.7	6.0	6.0	3.8	3.0
		B	Urea	0	6.0	1.5	0	0	0	0
6	Flour—Blank	A	NaOH	0	0	0	0	0	0	0
		A	Urea	0	0	0	0	0	0	0.5
		B	NaOH	0	0	0	0	0	0	0
		B	Urea	0	0	0	0	0	0	1.0
7	Flour—0.1% Urine Contaminated particles	A	NaOH	0	0	13	0	0	0	0
		A	Urea	0	0	0	0	2.5	0	0
		B	NaOH	0	0	0	0	0	0	0
		B	Urea	0	0	0	0	1.5	0	0
8	Flour—5% self-rising wheat flour	A	NaOH	4.5	12.0	5.0	+	12	16	
		A	Urea	0	0	0	0	0	0	
		B	NaOH	3.0	11	22	+	16	11	
		B	Urea	0.5	0.5	0	0	0	0	

continue to develop and enlarge for 10–20 minutes and then fade gradually.

Larger particles may be tested similarly to the very small ones. However, depending on shape and size of particles it may not be practical to cover them with a glass plate. The papers must be protected from NH_3 fumes and from drafts that would remove the NH_3 liberated from the urea. Interpretation is the same as for small particles.

DISCUSSION

Table 1 is a compilation of the results received from the collaborators. Each figure is the average of two determinations. The results obtained by the two different papers, "A" and "B," are shown separately.

All collaborators obtained essentially the same results on samples 1 and 2 and these agree with the composition of the samples. Five of the seven collaborators recognized the presence of both urea and sodium hydroxide in sample 4, whereas 2 collaborators failed to identify the sodium hydroxide. On sample 5 four laboratories obtained nearly exact proportion of sodium hydroxide present but two laboratories showed both sodium hydroxide and urea, and one laboratory failed to recognize sodium hydroxide and called all particles urea.

On the flour samples, the blank, number 6, was entirely satisfactory. However, only one collaborator identified the presence of the small urine-contaminated particles in flour sample number 7. All collaborators identified the alkaline particles from self-rising flour in sample 8.

COMMENTS OF COLLABORATORS

Atlanta.—"Test paper furnished to collaborator developed color much quicker than paper prepared by collaborator. There is a possibility that some of the color developed in Sub 8 may have been due to urea; however, the time was so close to 45 seconds that all color was considered as coming from NaOH. (Note: Bromothymol blue solution, prepared according to direction, turned red on standing. A drop or two of 0.1 *N* NaOH was added before paper was impregnated.)"

Kansas City.—"The sample reagents were made up and the first sub or two checked when it was necessary to drop the work. When resumed after three weeks, the paper seemed less sensitive. The papers you sent later did not seem as sensitive as some I prepared using some urease on hand (containing potassium phosphate). In particular the results on sample 7 were not conclusive with the papers on hand. A buffered system may be helpful. Also, it should be noted that the Referee has some paper prepared in 1948 which is still very sensitive.)"

New Orleans.—"Doubtless due to poor technique the spots formed were very obscure. From my results I would conclude that urea may be present in Nos. 2, 4, and 5 and an alkaline salt in No. 8. All negative papers were checked for spots by other chemists. Many positive papers were recounted by others who agreed within 1 count of the number reported. All spots in No. 8 appeared within 1 minute but by the end of 10 minutes they had faded considerably."

Boston.—"The tests for urea in the absence of an alkali seemed to be definite, but in the presence of an alkali, the results were not sufficiently conclusive. Additional tests with papers used immediately after wetting with solutions were tried to determine whether they would be more sensitive than types A and B. They proved more sensitive, in that stains developed almost instantaneously, but the A and B type papers would be more convenient to use. Test is excellent as to the size of sample

required, and with the use of both type papers it may be the answer to the difficult determination of small amounts of urea on flour."

Los Angeles.—"Upon hanging papers to dry the lower edge of the paper became more concentrated in urease-indicator solution due to downward drainage. This collaborator deviated from the instruction to the extent of removing this excess by touching it with a clean piece of filter paper. It was also found when running Flour Sample No. 8 that some of the spots due to basic contamination appeared rapidly (within the 45 second interval) but faded away entirely before the 10–15 min. incubation period had elapsed. This could cause failure to detect urea if the sample were not observed closely throughout the incubation period, and the relative position of the NaOH spots noted. This precaution was taken on the last three runs of Flour Sample 8. An extra run was made on Farina Sample 5 using test paper "A," but there was no indication of either basic materials or of urea."

Minneapolis.—With the farina samples it was noted that the color produced by the alkaline particles was on or within the particle itself, whereas the urea stain was found only on the paper. In the flour samples the alkaline stains varied in intensity, and the less intense stains were found to disappear before the 15 minute interval elapsed. (All readings were made at 15 minutes.) Although I did not observe any flour samples with both alkaline and urea particles, it would appear that some confusion would result if such were the case.

LIST OF COLLABORATORS

Angus J. Shingler, Atlanta District, Food & Drug Adm., Atlanta, Georgia
H. W. Conroy, Kansas City District, Food & Drug Adm., Kansas City, Mo.
Robert A. Baxter, Los Angeles District, Food & Drug Adm., Los Angeles, Calif.
Helen C. Barry, New Orleans District, Food & Drug Adm., New Orleans, La.
Helen T. Hyde, San Francisco District, Food & Drug Adm., San Francisco, Calif.
Mary C. Harrigan, Boston District, Food & Drug Adm., Boston, Mass.
William A. Bosin, Minneapolis District, Food & Drug Adm., Minneapolis, Minn.

CONCLUSIONS

The results that have been received from these seven collaborators are very satisfactory for this type of experiment. It is the opinion of the Referee that the collaborative study establishes the method as practical even from very small amounts of material. Since the technique is somewhat different from the usual chemical analysis, some experience by the analyst is necessary in order to perform the test properly on all samples. The Referee has been able to readily recognize the presence of the small urine specks (60–90 mesh particles) in flour sample Number 8. One collaborator also identified them, and in all probability others would identify them after some experience with the technique.

All collaborators obtained essentially the same results on the test paper made by themselves and the one made by the referee.

RECOMMENDATIONS

It is recommended* that—

1. The method be adopted as a qualitative method for urea.
2. Further use of enzyme procedures be investigated.

* For report of Subcommittee C and action of the Association, see *This Journal*, **35**, 54 (1952).

REFERENCES

(1) Cook, J. W., *This Journal*, **31**, 797 (1948).
(2) *Official Methods of Analysis, A.O.A.C.*, Seventh Ed. (1950).
(3) Feigl, Fritz, Qualitative Analysis by Spot Tests. Nordeman Publishing Co., New York. (1939).
(4) Conway, E. J. and Byrne, Alfred, *Biochem J.*, **27**, 419 (1933).

No report was given on hydrocyanic glucosides.

REPORT ON FEEDING STUFFS

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

Because there has been an apparent lack of interest in some phases of the Feeding Stuffs work for the past two years and an increased interest in some general areas, it is deemed wise to make several changes in the Feeding Stuffs program. With this explanation in mind, the following suggestions are made:

It is recommended*—

(1) That the following Associate Refereeships be discontinued:
  (a) Mineral Mixed Feeds (Calcium and Iodine).
  (b) Lactose in Mixed Feed.
  (c) Protein Evaluation in Fish and Animal Products.

(2) That the following Associate Refereeships be set up:
  (a) Crude Protein in Feeding Stuffs.
  (b) Ash in Feeding Stuffs.
  (c) Milk by-Products in Mixed Feeds.

It is further recommended—

(1) That work on the following be continued:
  (a) Fat in Fish Meal.
  (b) Crude Fat or Ether Extract.
  (c) Microscopic Examination.
  (d) Mineral Constituents in Mixed Feed.
  (e) Tankage (Hide, Hoof, Horn, and Hair Content).
  (f) Drugs in Feeds.

(2) That the method for sulfaguanidine as adopted, first action, last year, be made official.

(3) That the study of specific methods for nitrophenide (m,m'-dinitro-

* For report of Subcommittee A and action of the Association, see *This Journal*, **35**, 43 (1952).

diphenyldisulfide) and enheptin (2-amino-5-nitrothiazole) be continued as recommended by the Associate Referee.

(4) That the method for the determination of cobalt in mineral feeds as outlined by the Associate Referee, be made first action.

REPORT ON SULFA DRUGS IN FEEDS

DETERMINATION OF NITROPHENIDE AND ENHEPTIN

By RICHARD T. MERWIN (Agricultural Experiment Station, New Haven, Conn.), *Associate Referee*

This year's collaborative study was undertaken to develop methods for nitrophenide (1) and for Enheptin ® (2). Nitrophenide (m,m'-dinitrodiphenyldisulfide) is used for the control of caecal and intestinal coccidiosis in poultry, and enheptin (2-amino-5-nitrothiazole) is used for the prevention of blackhead in turkeys. Both are used at low concentrations in poultry feed, the first usually at 0.0125 per cent and the second at 0.05 per cent and 0.10 per cent.

Since their introduction to feed processors, both drugs have generally been assayed by the methods of the manufacturer. Two other methods, devised by the Associate Referee for comparison, were offered with the current methods for collaborative study.

The assay of nitrophenide involves a preliminary reduction of the drug to a compound containing two amino groups which can be diazotized and coupled with N'-(1-naphthyl)-ethylenediamine dihydrochloride. The ensuing purplish-red color can then be measured at its maximum absorption (545 mμ) in a suitable spectrophotometer. Both methods submitted for study follow this general procedure, but differ in the means used for reduction. A reduction by sodium hydrosulfite in alkaline solution is used in the current method, whereas the second method makes use of a reduction by zinc in hydrochloric acid solution.

The Associate Referee departed from the general procedure in devising a method for enheptin. The current method depends on the loss of density of a spectrophotometric reading after the drug is treated in alkaline solution with a reducing agent. The drug is yellowish in acetone-water solution, but is colorless after reduction. The second enheptin method depends on diazotization and coupling in an oxidative medium. N'-(1-naphthyl)-ethylenediamine dihydrochloride is again used for color formation.

The newer methods have usually produced good results for the Associate Referee, and it was expected that similar results would be obtained by the collaborators. Not all of the collaborators were able to complete the work in time for this report, but most of those who did were unable to attain the expected accuracy.

TABLE 1.—*Analyses of feeds containing nitrophenide*

COLLABORATOR	METHOD NO. 1, PER CENT			METHOD NO. 2, PER CENT		
	PRESENT	FOUND	AVERAGES	PRESENT	FOUND	AVERAGES
No. 1	0.0125	0.0122 0.0115 0.0116 0.0115	0.0117	0.0125	0.0125 0.0110 0.0115 0.0090	0.0110
No. 2		0.0128 0.0135	0.0132		0.0112 0.0112 0.0102	0.0109
No. 3		0.0128 0.0134 0.0166 0.0166 0.0165	0.0152		0.0078 0.0078 0.0073	0.0076
No. 4		0.0153 0.0147 0.0153	0.0151		0.0098 0.0089	0.0094
No. 5		0.0140 0.0110 0.0125 0.0114 0.0135 0.0140 0.0130 0.0125	0.0127		0.0101 0.0100 0.0090 0.0090	0.0095
No. 6		0.0119 0.0120 0.0122 0.0125	0.0122		0.0110 0.0111 0.0111 0.0114	0.0112
No. 7		0.0111 0.0125 0.0124 0.0114	0.0119		0.0117 0.0133 0.0127 0.0129	0.0127
No. 8		0.0139 0.0144 0.0135	0.0139		0.0135 0.0130 0.0135	0.0133
No. 9		0.0143 0.0168 0.0168 0.0198	0.0169		0.0108 0.0108 0.0122 0.0102	0.0110
R.T.M.		0.0137 0.0147 0.0143 0.0153	0.0145		0.0129 0.0129 0.0132 0.0118	0.0127
Average of all			0.0137			0.0109

Two samples, one containing 0.0125 per cent nitrophenide, the other 0.10 per cent enheptin, were sent to each collaborator with recrystallized portions of both drugs for use in preparing standards. Copies of all four methods also accompanied instructions to report four results by each method. No special instructions regarding technique were offered other than those appearing in the methods. Results of the collaborative study on nitrophenide are presented in Table 1, and on enheptin in Table 2.

COLLABORATIVE METHOD NO. 1 FOR NITROPHENIDE

REAGENTS

(1) *Freshly prepared soln of 1% $Na_2S_2O_4$ in 4% NaOH.*
(2) *Freshly prepared 0.50% soln of $NaNO_2$ in water.*
(3) *2.5% soln of ammonium sulfamate in water.*
(4) *0.1% soln of N'-(1-naphthyl)ethylenediamine dihydrochloride in water.*—(Keep in dark bottle.)

Weigh 2 g of ground feed into 100 ml vol flask. Wet with 5 ml of water and add 50 ml of the $Na_2S_2O_4$ soln. Heat in boiling water bath for 20 min. Remove and carefully add 10 ml of conc. HCl to the hot soln. Remove the excess SO_2 by passing in a vigorous stream of air for 20 min., or by adding 3% H_2O_2 until a faint test is obtained for H_2O_2 with starch-iodide test paper. Cool, dil to 100 ml with water and filter through a Whatman No. 42 paper, discarding first 10 ml of filtrate.

To 4 ml of clear filtrate in 25 ml vol flask add 1 ml of the $NaNO_2$ soln, mix and let stand five min. Add 1 ml of the ammonium sulfamate soln, mix and wait two min. Then add 2 ml of the coupling soln. Wait ten min for color development before making to vol of 25 ml. Then read the absorption at 545 mμ against a water blank and refer to standard curve.

Prepare a standard soln by dissolving .0125 g of recrystallized nitrophenide in acetone and make to vol of 250 ml with acetone. Transfer aliquots of 2.5, 5.0, 7.5 and 10 ml to 100 ml flasks and evaporate. Add to each flask 2 g of unmedicated ground feed and proceed as in regular assay, i.e., read absorption of color developed in 4 ml aliquots diluted to 25 ml. Plot readings to obtain standard curve.

COLLABORATIVE METHOD NO. 2 FOR NITROPHENIDE

REAGENTS

(1) *zinc powder, purified grade.*
(2) *1+1 HCl.*
(3) *95% ethyl alcohol.*
(4) *0.10% $NaNO_2$ freshly prepared.*
(5) *0.50% ammonium sulfamate.*
(6) *0.10% N'-(1-naphthyl)-ethylenediamine dihydrochloride.*—(Keep in dark bottle.)

Weigh one g of ground feed and 0.50 g of zinc powder into a 100 ml vol flask. Add 20 ml of alcohol, 20 ml of water and boil on water bath two min., swirling occasionally. Remove flask, and while swirling add 5 ml of 1+1 HCl to the hot soln. Allow to stand exactly five min., with frequent swirling, then add 40 ml of water, cool, and make to vol. Filter through Whatman No. 42 paper, discarding first 5 ml of filtrate.

To 10 ml aliquot of clear filtrate in 25 ml vol flask add 1 ml of 1+1 HCl, 2 ml of the $NaNO_2$ soln and wait 5 min. Add 2 ml of the sulfamate soln and wait 2 min.;

then 2 ml of the N'-(1-naphthyl)-ethylenediamine dihydrochloride and wait 10 min. Swirl contents after addition of each reagent. Finally make to vol with water, using drop or two of alcohol, if necessary, to break foam. Read absorbancy at 545 mμ against water blank in spectrophotometer and refer to standard curve.

Prepare standard soln by dissolving 0.0125 g of recrystallized m,m'-dinitrodiphenyldisulfide in acetone and make to vol of 250 ml with acetone. Take 1, 2, 3, and 4 ml aliquots in 100 ml vol flasks (corresponding to 5, 10, 15, and 20 micrograms) and evaporate. To each flask add 0.50 g of zinc powder, 20 ml of alcohol, etc., and proceed as in assay. Plot resulting absorbancies to obtain standard curve.

COLLABORATIVE METHOD NO. 1 FOR ENHEPTIN

REAGENTS

(1) *75% V/V acetone soln in water.*

(2) *5% W/V NH_4Cl soln in water.*

(3) *Boric acid buffer, pH 9.0, prepared by mixing two solns.*:—(A) 6.203 grams of boric acid and 7.456 grams of potassium chloride made to 500 ml with water; and (B) 0.2 molar sodium hydroxide. Take 50 ml of Solution A and 21.40 ml of Solution B and make to 200 ml with water. (Use C.P. grades).

(4) *1% sodium hydrosulfite in boric acid buffer pH 9.0.*—This soln must be used within 10 minutes after preparation.

Transfer one g of ground feed to 100 ml beaker and add 10 ml of the acetone solution. Heat gently on water bath just to boiling, remove, and allow feed to settle. Decant through funnel containing small plug of cotton into 250 ml beaker. Extract four more times with 10 ml portions. Evaporate acetone on water bath until only faint odor remains. Remove beaker and add 15 ml of the ammonium chloride soln, mix and transfer to 50 ml vol flask, and make to mark with water. After mixing, filter through Whatman No. 42 paper, discarding first 10 ml.

Add a 4 ml aliquot to each of two small test tubes. To the first tube add 0.5 ml of the 1% sodium hydrosulfite in boric acid buffer. Make both tubes to 10 ml and read within 5 min. on a spectrophotometer against water at 388.5 mμ. Subtract the reading of the reduced solution from that of the unreduced and compare resulting absorbancy to standard curve.

Prepare standard soln by dissolving 100 mg of recrystallized 2-amino-5-nitrothiazole in 50 ml of acetone and make to volume of 1000 ml with water. Transfer aliquots of 1, 2, 4, 6, 8, 10, 12, and 14 ml to 100 ml volumetric flasks and dilute to volume with water. Treat 5 ml aliquots of each dilution as in assay procedure, and read the absorbancies of the unreduced solns against the reduced solns as a blank to obtain standard curve.

COLLABORATIVE METHOD NO. 2 FOR ENHEPTIN

REAGENTS

(1) *Acetone.*

(2) *5% soln of NH_4Cl.*

(3) *saturated soln of KIO_4 (cold).*

(4) *soln of H_3PO_4.*—(20 ml made to 100 ml with water.)

(5) *0.10% $NaNO_2$ soln.*

(6) *0.50% ammonium sulfamate soln.*

(7) *0.10% soln of N'-(1-naphthyl)-ethylenediamine dihydrochloride.*—(Keep in dark bottle.)

Weigh 2 g of ground feed into a 50 ml vol flask, add 10 ml of acetone and let stand two min. Swirl, make to vol with water, mix and filter quickly through coarse

TABLE 2.—*Analyses of feeds containing enheptin*

COLLABORATOR	METHOD NO. 1, PER CENT			METHOD NO. 2, PER CENT		
	PRESENT	FOUND	AVERAGES	PRESENT	FOUND	AVERAGES
No. 1	0.100	0.073 0.073 0.074 0.073	0.073	0.100	0.073 0.070 0.063 0.065	0.068
No. 2		0.088 0.080 0.084	0.084			
No. 3		0.088 0.089	0.089			
No. 4		0.084 0.083 0.084 0.083	0.084		0.137 0.134	0.136
No. 5		0.090 0.091 0.091 0.090	0.091		0.099 0.104 0.104 0.106	0.103
No. 6		0.096 0.110 0.105 0.096	0.102		0.092 0.105 0.097 0.092	0.097
No. 7					0.076 0.067 0.069 0.069	0.070
No. 8		0.075 0.075 0.075	0.075			
No. 9		0.080 0.081 0.080 0.081	0.081		0.124 0.126 0.126 0.131	0.127
R.T.M.		0.089 0.099 0.079 0.072	0.085		0.094 0.094 0.092 0.092	0.093
Average of all			0.085			0.099

paper. Transfer 25 ml aliquot to another 50 ml flask, add 15 ml of the NH_4Cl soln and mix. Make to vol with water and filter through Whatman No. 42 paper, discarding first 5 ml of filtrate.

To 5 ml aliquot in 25 ml vol flask, add 1 ml of the H_3PO_4, swirl, then 2 ml of the $NaNO_2$ soln and wait 5 min. Add 2 ml of the sulfamate soln and wait 2 min. Add 2 ml of the N'-(1-naphthyl)-ethylenediamine dihydrochloride soln and after one min add 2 ml of the saturated KIO_4 soln. Let stand *exactly* 5 min before making to vol with alcohol. Read absorbancy *after 30 minutes* at 625 mμ in spectrophotometer against a water blank.

Prepare standard soln by dissolving 0.10 g of recrystallized 2-amino-5-nitrothiazole in 50 ml of acetone and make to 1000 ml with water. Transfer 25 ml to 100 ml vol flask and make to vol with water. To aliquots of 1, 2, 3, 4 and 5 ml, each made to vol of 5 ml with water, in 25 ml vol flasks, add 1 ml of the H_3PO_4 and proceed as in assay, following each step exactly. Plot resulting absorbancies on graph paper.

COMMENTS OF COLLABORATORS

No. 1.—Preference was shown for both No. 2 methods. "It was the feeling of the analyst that with a little practice and familiarity with the methods, greater accuracy could be obtained." "We used our own feed for a blank in establishing a standard on Method No. 1 for nitrophenide and do not feel that this is correct, as the blank should be run on unmedicated feed of the same formulation as that under test."

No. 2 and No. 3.—"We were unable to get a standard curve with Method No. 2, therefore, we are unable to report any values for enheptin."—"Although each chemist duplicated his results on nitrophenide with both methods, agreement between chemists is bad." "The standard curves for nitrophenide could not be duplicated and had to be prepared for each run."

No. 4.—"Both No. 2 methods gave results so different from those of the first procedures, that we frankly have hesitated about reporting them without further confirmatory work. However, time is too short to permit this. While the color of the standards for the enheptin method No. 2 was a clear blue, the sample extracts gave a brownish-purple color. Method 1 for enheptin is still preferable to No. 2 except that the extraction technique of the latter is quicker and more satisfactory. The standard curve was remarkably straight on nitrophenide method 2 but failed to pass through zero."

No. 5.—"In nitrophenide method No. 1, foam in the 25 ml volumetrics caused difficulty in adjusting to volume. We carried out the color reaction in 50 ml beakers and then transferred to volumetrics after the 10 minute standing period and made to volume."

No. 7.—"Enheptin unknown solutions developed a very black-blue color in contrast to the real blue of the standards in the oxidation coupling method. Several attempts using new reagents failed to eliminate the black color."

No. 8.—"The time limit on reading the reduced solutions of enheptin in Method 1 makes it somewhat undesirable when handling large numbers of samples." "The use of a random sample of unmedicated feed as a feed blank in the standards for Method 1 on nitrophenide seems undesirable."

DISCUSSION

There are many reasons for the disparities shown in results between the two methods for each drug and between individual analysts. However, the differences can hardly be attributed to sample variations because of

the great care taken to assure uniformity and correct theoretical percentages during preparation. Lack of familiarity with the second group of methods is probably the major cause of the discrepancies, since no one had had experience with them except the Associate Referee. This is not true of the first methods because they have been used by most feed analysts for more than a year.

The alkaline-sodium hydrosulfite reduction method for nitrophenide produces highly colored feed extracts which vary from feed to feed and contribute varying density readings. If it were possible to prepare standards using the same feed, without medication, as that being assayed, more consistent and accurate results would be obtained. This has been proved experimentally, but such a refinement is obviously impossible in control work. Another cause of variation by this method is the need for removing excess SO_2. If H_2O_2 is used to destroy it, care must be taken to avoid an excess of oxygen. This seems to be a variable factor of significance.

The hydrochloric acid-zinc reduction method for nitrophenide gave most of the analysts lower results than the first method. However, one of the group figures was very good. Correct timing of the reduction period is necessary, and improper timing may largely explain the poorer results. Complete reduction usually takes place within the 5-minute period specified, but over or under reduction produces erratic recoveries.

The low results reported for the first enheptin method are in closer agreement among the collaborators than the figures for the other methods. This consistency, at least, is gratifying, but the low recoveries are not so pleasing. The results are merely proof of the uniformity of the samples and the consistency of the method in yielding low recovery from the sample. Because the method depends on reading loss of density after reduction, it is believed that there is also some loss of feed color, and that the degree of this feed-blank color loss is the major cause of the variation among collaborators.

Most of the collaborators had greater difficulty with the second enheptin method, some finding they could not obtain results worth reporting. The Associate Referee has found that exact timing of all the various reactions is extremely important and believes that there may have been some unintentional variation in time among the analysts. Deviations of more than 30 seconds are to be avoided. The brownish-purple color obtained by one collaborator, and the black-blue color by another, result from waiting too long before making to volume with alcohol. The figures submitted by two collaborators indicate that the method can produce results that are consistent and close to theoretical.

The reports this year indicate the need for continuing studies of assay methods for the two drugs. The collaborators have helped considerably in furthering the work and the Associate Referee expresses his gratitude to the following:

COLLABORATORS

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A. F. Spelman, Agricultural Experiment Station, Amherst, Mass.

RECOMMENDATION*

It is recommended that collaborative studies of methods for nitrophenide and Enheptin ® be continued.

REFERENCES

(1) "Nitrophenide Determination in Chicken Mash," Lederle Laboratories Div., American Cyanamid Co., New York, N. Y.
(2) "Method of Assaying Feeds for Enheptin," ®. Lederle Laboratories Div., American Cyanamid Co., New York, N. Y.

REPORT ON MINERAL CONSTITUENTS OF MIXED FEEDS

COBALT DETERMINATION IN MINERAL FEEDS

By J. C. Edwards (Florida Department of Agriculture, Chemical Division, Tallahassee, Florida), *Associate Referee*

This study was initiated with the aim of establishing a reliable official method for the determination of cobalt in mineral feeds.†

Early in January, 1951, requests for collaborators were mailed to 65 control officials and industry representatives. As a result of this request, 39 analysts offered their cooperation in the program. A mineral feed sample containing a representative amount of cobalt and approximate percentages of the following: calcium 16.1, phosphorus 4.5, sodium chloride 28.6, copper 0.4, iron 5.7, manganese 0.15, was specially prepared and a portion of this sample was sent to each participating laboratory with the detailed procedure of analysis. Of the number who volunteered to take part in the program, 29 reported results as follows:

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* For report of Subcommittee A and action of the Association, see *This Journal*, 35, 43 (1952).
† For the report of Subcommittee A and action of the Association, see *This Journal*, 35, 43 (1952).

COLLABORATOR NUMBER	COBALT FOUND	COLLABORATOR NUMBER	COBALT FOUND	COLLABORATOR NUMBER	COBALT FOUND
	per cent		*per cent*		*per cent*
1.....	.023	12.....	.030	23.....	.027
	.023		.030		.028
2.....	.028		.030		.028
	.027	13.....	.027		.028
3.....	.027		.029		.028
	.027		.028		.029
	.029		.024	24.....	.029
	.029		.028		.025
4.....	.025		.029		.029
	.026		.028		.027
	.027		.028		.027
	.026	14.....	.030		.029
	.026		.030	25.....	.030
	.028		.030		.030
5.....	.026	15.....	.028		.030
	.026		.028		.031
6.....	.022	16.....	.031		.028
	.022		.031		.030
	.021		.032	26.....	.023
	.022	17.....	.031		.024
	.021		.028		.027
	.021		.030		.024
	.021		.030	27.....	.028
	.021		.028		.028
	.021		.030		.027
7.....	.029		.031		.028
	.029	18.....	.028	28.....	.030
	.029		.028		.030
	.029		.028		.030
	.029	19.....	.026		.030
8.....	.031		.026		.031
	.031	20.....	.022	29.....	.030
	.030		.022		.029
9.....	.031		.022		.029
	.032		.022		.029
	.033		.022		.029
10.....	.027	21.....	.026		.030
	.026		.026		
11.....	.029	22.....	.027		
	.028		.027		
			.027		

The mean is .0275% cobalt.

The mean deviation is .0022%.

The high result is .033%.

The low result is .021%.

The high deviation from mean is .0055% and the low deviation from mean is .0065%.

The results are gratifying. They comprise 118 separate determinations by 29 analysts in various sections of the country. Some of the analysts had used the procedure before and were quite familiar with it, while for others it was an entirely new method. Still, all results are so close to the mean that none could be disregarded. The perfect mixing of a sample of this size and composition is difficult, and imperfect mixing could account for the range shown in the results, but even if we assume that the sample was uniformly mixed, the results are still good. Individual error in setting up standards, or differences in standard reagents could cause variation. The method is shown to be reliable and adaptable for the determination of cobalt in mineral feeds.

The procedure and a discussion follow, also pertinent comments of several collaborators:

COBALT DETERMINATION IN MINERAL FEEDS

REAGENTS

Cobalt sulphate ($CoSO_4 \cdot 7H_2O$).—Do not dry, use as received. Dissolve 0.2385 g in water and make up to 1 liter. 1 ml = 0.05 mg Co. This solution may be diluted to suitable concentration in making standard curve.

Nitroso-R-salt ($C_{10}H_4OH \cdot NO(SO_3Na)_2$).—(Eastman) Dissolve 1 g in water and make up to 500 ml.

Spekker acid.—Mix 150 ml 85% H_3PO_4 and 150 ml H_2SO_4 and make up to 1 liter with water.

Sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$).—Dissolve 500 g in water and make up to 1 liter.

STANDARDS

Place 1, 2, etc. up to 11 ml of standard cobalt sulfate soln in 100 ml vol flasks and add 2 ml of Spekker acid, 10 ml of nitroso-R-salt soln, and 10 ml of sodium acetate soln. Prepare a blank by using 2 ml of Spekker acid and 10 ml of sodium acetate soln, but *omit* the nitroso-R-salt. Bring the blank and standard solns to a boil on the hot plate, add five ml of HNO_3 and boil for at least 1 min. and not more than 2 min. Cool the standards and blank and dilute to 100 ml with water.

PROCEDURE

Ash 2 g of sample 2 hrs. at 600°C., transfer to a 200 ml vol flask with 20 ml HCl and 50 ml H_2O, boil 5 min. and make to vol. Allow soln to settle then pipet a suitable aliquot into a small flask. (For samples containing 0.01% to 0.2% cobalt use a .25 g sample. Use more or less according to cobalt expected. The max quantity of cobalt permissible in a sample is 0.5 mg, as above this conc. the soln no longer appears to follow Beer's law.) Pass a brisk current of H_2S thru the soln for 10 min. Filter directly into a 100 ml pyrex vol flask thru a No. 40 Whatman filter paper. Wash with approx 50 ml acidulated H_2S water (1% H_2SO_4). Add 2 small glass beads to flask and boil off H_2S. (Flasks must be given individual attention as violent bumping may occur; therefore shake flask often. Add 5 ml HNO_3 and boil until nitrous fumes no longer appear. Care must be taken at this point as the soln will be low, and spattering and bumping may occur. At first indication of bumping or spattering, remove immediately from hot plate. The small amount of nitric acid remaining will not affect the result. Cool. Add 2 drops phenolpthalein and take to first faint pink with NaOH (approx. 30% soln). Immediately add 2 ml Spekker acid

followed by 10 ml nitroso-R-salt soln and 10 ml sodium acetate soln. Bring to vigorous boil, carefully add 5 ml HNO_3 and then boil at least one min. but not over 2 min. Cool and make to volume. Compare color with standard cobalt solns in colorimeter using a green or No. 54 filter. If a spectrophotometer is used, a wave length of 540 millimicrons is recommended. Color should be read within 2 hours. Report percentage of cobalt to the third place to right of decimal.

The determination is based on the fact that nitroso-R-salt forms color complexes with most of the common elements and that boiling with nitric acid will destroy all the color complexes formed except the cobalt complex. Copper must be removed, and if H_2S is used, all Group 2 elements will be eliminated. Manganese over 10%, nickel over 3%, and chromium over 5% must be removed prior to the color comparison. The following are without effect on the determination, even in quantities over 100 times that of cobalt: Fe, Al, Zn, Ti, Th, U, Zr, W, Ba, Sr, Ca, Mg. (1).

Varying quantities of Spekker acid were added to known ores and to synthetic solutions. It was found that the acid could be varied from 0.5 to 3.0 ml without affecting the color development. Any addition above 3 ml causes a lessening of the depth of color.[2]

The measurement of nitroso-R-salt should be accurate, but no appreciable difference was observed when the quantities of nitroso-R-salt were varied between 10 and 15 ml. The result was low with less than 10 and high with more than 15 ml of nitroso-R-salt solution (2). If kept in glass stoppered containers in the dark, the solution is stable for months.

Sodium acetate is necessary for the full development of the cobalt color and 10 ml is a sufficient amount for most products. It is essential to add sufficient nitric acid to dissolve any precipitate formed with sodium acetate and to destroy color complexes formed with metals other than cobalt. It was found when 10 ml of sodium acetate was used the nitric acid could be increased to 15 ml without affecting the color development (2).

The final boiling must be carefully controlled. The solution must be brought to a vigorous boil, nitric acid added, and the boiling continued from 1 to 2 minutes. The extension of boiling time to three minutes causes little change, but any further extension results in a definite bleaching of the cobalt color. On the other hand, any decrease in boiling time prevents the full development of the red color (2).

There is considerable contradiction in the literature concerning the best wave-length for the measurement of the optical density of the cobalt complex. The question has lately been considered by Claassen and Westerveld (3). In agreement with others they find the transmission minimum lies at about 415 millimicrons. However, they recommend measuring at 550 millimicrons since at this wave length the reagent absorbs hardly at all and any slight variation in the color intensity of the blank will then not be a source of error.

Some suggestions of the various analysts who participated in the study

were incorporated in the procedure. No one objected to the basic method or the chemistry involved. The only questions were matters of technique and differences of opinion about the best way to handle the bumping at the time the H_2S and nitric acid are boiled off. The genuine cooperation and helpful comments of all collaborators have been of great assistance and are deeply appreciated.

COMMENTS OF COLLABORATORS

Collaborator 27.—These results were obtained with a Beckman DU spectrophotometer at 540 mμ *after filtering the final solution.* The solutions were slightly turbid and gave very slightly higher results before filtration (average 0.029%).

Collaborator 26.—Our Coleman Universal Spectrophotometer was not accurate at the high concentrations of standard recommended in the method. We also had considerable difficulty with bumping during the digestions. It was necessary for us to shake the flasks constantly after the nitrous fumes began to appear after addition of nitric acid to the sulfide filtrate. The most serious bumping occurred when we were bringing the final solution to a vigorous boil. Therefore, we found it necessary to shake the flask constantly during the pre-heating stage before the addition of 5 ml. of nitric acid.

Collaborator 25.—The results being submitted to you were obtained with the Beckman DU Quartz Spectrophotometer. It was found that the Fisher Electrophotometer did not have sufficient range to cover more than one-half of the standards using the green filter as directed. The samples require individual attention at the point of evaporation—"until nitrous fumes no longer appear." Constant agitation has to supplement the action of the glass beads at this point or spattering will completely ruin the sample. No other precaution was found to be successful. Also, it was found impossible to perform this evaporation in the 100 ml volumetric flasks. Instead, 125 ml Erlenmeyers were used and a quantitative transfer later made to the volumetric flasks.

Collaborator 22.—A Beckman DU Spectrophotometer was used. At the point in the analysis "boil until nitrous fumes no longer appear" bumping was very violent on either a hot-plate or sand-bath. One set of determinations was allowed to stand about 36 hours when bumping commenced and before boiling down was complete. At this time the fine white precipitate had become coarse and boiling down proceeded smoothly. The precipitate persisted until about 30 minutes after the solutions were finally made up to volume for readings.

Collaborator 20.—After going through the procedure more than once, it was found better to filter into a 150 ml beaker instead of a 100 ml volumetric flask. This gives more surface for rapid evaporation, prevents loss of liquid from bubbling, and makes it easier to take the solution to a faint pink with NaOH in presence of phenolphthalein. A speedyvap watch glass is recommended to cover the beaker to prevent spattering. A Hengar granule gives smoother boiling than glass beads.

Collaborator 15.—The following suggestions may improve the procedure: The use of small Kjeldahl flasks for boiling sample to remove H_2S and concentrating to remove excess HNO_3. When volumetric flasks were used considerable difficulty was encountered from bumping and spattering. Also, some breakage of the ordinary volumetric flasks occurred during the boiling off of the oxides of nitrogen. With pyrex volumetric flasks this difficulty did not occur. After driving off the nitric oxide fumes the solution should be partially neutralized with the 30 per cent NaOH before adding the phenolphthalein, since the phenolphthalein is destroyed when added to the concentrated acid. Using a Coleman Model 11 Spectrophotometer

or Evelyn photoelectric colorimeter, we found the range for the standard curve to be from 0.01 to 0.20 mg Cobalt per 100 ml.

Collaborator 14.—There is a very pronounced tendency for solution to bump when boiling of H_2S and this becomes worse later on when solution is low. To obviate this difficulty we passed air through the solution at the rate of 120 bubbles per minute, employing a 4.5 mm I.D. glass tube. The air was passed through a H_2SO_4 bubbler equipped with a glass wool mist collector before being bubbled through the solution.

Collaborator 13.—Our instrument is a Coleman Model 14 Universal Spectrophotometer. We prepared the standard solution as described, then diluted a portion of it with water in the ratio of 1 to 1, giving us a concentration of 0.025 mg Cobalt per ml. We prefer not to boil solutions in volumetric flasks on hot plates. It may be advisable to substitute beakers for this step and transfer the solutions after they have cooled. Some difficulty was encountered with cloud formation in the sample solution after final addition of the 5 ml of HNO_3 just prior to color measurement. Filtering this solution through Whatman No. 42 filter paper gave us results which were reproducible.

Collaborator 12.—Three separate determinations were made using a Coleman 11 Spectrophotometer. In boiling "until nitrous fumes no longer appear" I found it necessary to boil the solution nearly to dryness. At no time were the fumes strong but they were detectable until all but about 5 ml of the solution had boiled away.

Collaborator 8.—It was found that maximum absorption took place at a wave length of 505 mμ. This was the wave length used. The method is basically good except for the difficulty with bumping when solutions are boiled to eliminate H_2S. Some samples were lost due to violent bumping and spattering. It was impossible to boil the solutions in 100 ml volumetric flasks without loss of the solutions. Boiling was finally done in 250 ml Phillips beakers in which the color was also developed. The solution was then transferred to volumetric flasks. Further work seems desirable to control bumping. Perhaps it might be possible to heat the solutions on a steam bath. It might be possible to develop the final color by immersing the flask for a given period in live steam followed by the addition of 5 ml HNO_3 and a further immersion in live steam for another period.

Collaborator 3.—We have never found it necessary to employ hydrogen sulfide. A reagent blank should include the nitroso-R-salt, otherwise the blank possesses practically the same light absorption characteristics as distilled water.

REFERENCES

(1) YOUNG, ROLAND S., "Cobalt," Reinhold Publishing Corp., 1948.
(2) PINKNEY, E. T., and DICK, R., *Ind. Eng. Chem.*, Anal. Ed., **18**, 474–476 (1946)
(3) SANDELL, E. B., "Colorimetric Determinations of Trace Metals," Interscience Publishers, Inc., New York, 1950.

COLLABORATORS

The order of listing of the collaborators has no bearing on the numerical order of results:

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J. C. Edwards, Assistant State Chemist, Florida Department of Agriculture, Chemical Division, Tallahassee, Florida

Van P. Entwistle, Supervising Feed Chemist, Feed laboratory, Department of Agriculture, Sacramento 14, California

The contributed papers, "The Determination of Fat in Baked Biscuit Type of Dog Foods," by E. F. Budde, "The Urea and Ammoniacal Nitrogen Content of Miscellaneous Feeding Stuffs and Proposed Method for the Determination of Urea," by A. T. Perkins, and "Acidity and Total Solids Measurement in Condensed Milk By-Product Feeds," by Ara O. Call and R. F. Van Poucke, appear on pages 799, 781, and 785, respectively.

No reports were given on mineral mixed feeds (calcium and iodine), lactose in mixed feeds, fat in fish meal, crude fat or ether extract, microscopic examination, protein evaluation in fish and animal products, or on tankage (hide, hoof, horn, and hair content.)

REPORT ON VEGETABLE DRUGS AND THEIR DERIVATIVES

By PAUL S. JORGENSEN (Food and Drug Administration, Federal Security Agency, San Francisco 2, Calif.), *Referee*

RECOMMENDATIONS*

(1) *Aminopyrine, ephedrine and phenobarbital*

No report was received. The Referee recommends that the subject be continued.

(2) *Quinine and strychnine*

No report was received. The Referee recommends that the subject be continued.

(3) *Rutin in tablets*

The Referee recommends that the subject be continued.

REPORT ON RUTIN IN TABLETS

By ARTHUR TURNER, JR., (Eastern Regional Research Laboratory,† Philadelphia 18, Pennsylvania), *Associate Referee*

Forty-three samples of rutin tablets were received by the Associate Referee in response to his request to tablet manufacturers. Since requests were sent to every listed manufacturer, it is felt that the samples received represent, almost entirely, the available supply. These tablets varied from the relatively simple, uncompounded rutin tablet to tablets containing, in addition to rutin, as many as four other active ingredients. The other active ingredients encountered were ascorbic acid, aminophylline, barbiturates, mannitol hexanitrate, nitroglycerin, sodium nitrite, theobromine, and tincture cratageus. Most of the tablets were uncoated, but a few were

* For report of Subcommittee B and action of the Association, see *This Journal*, 35, 47 (1952).

† One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.

covered with a colored sugar coating; two samples were dyed throughout. The weight of rutin per tablet varied from 10 mg. to 100 mg. The following method, a slight modification of the method of Porter, *et al.*[1], has been applied in the analysis of all the available samples.

METHOD

Determine the average weight per tablet by weighing not less than 20 tablets. Thoroughly powder the weighed tablets and weigh the equivalent of 40 mg of rutin into a 50 ml centrifuge tube. Add 0.1 ml of glacial acetic acid and approximately 15–20 ml of 95% ethanol. Suspend the powder in the solvent by stirring, and place in a water bath (70°C.) for 10 min. Stir occasionally during this extraction period. After heating, remove the stirring rod (wash with 95% ethanol) and centrifuge at approximately 2000 rpm for 10 min. After centrifugation, carefully decant the supernatant into a 100 ml vol flask (use a funnel). While the tube is still inverted in the funnel, wash off the lip with 95% ethanol. Repeat this extraction, starting at "Add 0.1 ml of glacial acetic acid," twice more. When the contents of the volumetric flask are at room temperature, dilute to 100 ml with 95% ethanol. Remove any precipitate that may form on cooling or standing by filtration. Transfer 10 ml of this extract to a 250 ml vol flask and dilute to vol with distilled water. Remove by filtration any precipitate that forms. Determine the absorbance of this aqueous dilution at 338.5, 352.5, and 366.5 millimicrons by means of a spectrophotometer. Use 1 cm absorption cells and employ a distilled water blank.

CALCULATIONS

Using the data obtained, the following calulations are made:

$$a_{352.5} = \frac{A_{352.5}}{bc}$$

where a = absorptivity; A = absorbance = Log (I_0/I); b = cell length in cm; and c = concentration of original sample in the final dilution in grams per liter.

$$R_1 = \frac{A_{338.5}}{A_{352.5}} = \text{ratio of absorbance at 338.5 and 352.5 m}\mu.$$

$$R_2 = \frac{A_{366.5}}{A_{352.5}} = \text{ratio of absorbance at 366.5 and 352.5 m}\mu.$$

If R_1 equals $0.909 \pm .009$ and R_2 equals $0.846 \pm .009$, the extracted material can be considered pure rutin and the weight (in mg) per tablet can be calculated by means of the following equation:

$$\text{mg Rutin} \cdot 3H_2O/\text{tablet} = \frac{a_{352.5}}{26.3} \times \text{av. wt./tablet (mg)}$$

Since pharmaceutical rutin[2] may contain up to 5% quercetin an "accept-

[1] Porter, W. L., Brice, B. A., Copley, M. J., and Couch, J. F., U. S. Department of Agriculture, AIC-159, July 1947. (Processed) Eastern Regional Research Laboratory.
[2] National Formulary, 9th Edition, 1950, p. 440. American Pharmaceutical Association, Washington 7, D. C.

able" range is suggested for the above ratios: for R_1 a range of 0.890 to 0.918; for R_2 a range of 0.837 to 0.878. If the sample falls within these ranges the above equation may be used for calculating the rutin content. An increase in R_2 above this limit with a simultaneous decrease below the R_1 limit indicates that the sample contains more than 5% quercetin. In such cases the amount of rutin may be calculated from the following equation:

$$\text{mg Rutin3} \cdot \text{H}_2\text{O/tablet} = (0.1475a_{352.5} - 0.1292a_{366.5}) \times \text{av. wt./tablet (mg)}$$

An increase or decrease beyond the limits of both ratios indicates an interfering absorption which invalidates the analysis.

An increase of R_1 beyond its limit while R_2 remains within its range indicates an interfering absorption at 338.5 millimicrons which diminishes so as to be ineffective at the R_2 wavelength. Under these conditions the correctness of the observed value at 352.5 millimicrons is accepted because any elevation of the 352.5 millimicron reading would lower R_2.

DISCUSSION

Of the 43 samples analyzed, difficulty was encountered with only one sample. This contained a possibly incompatible ingredient and the interence is being studied further. Of the other 42 samples analyzed, 38 gave ratios within the above limits and the rutin content was within the pharmaceutical limit of $\pm 7.5\%$. Of the four that did not meet this requirement, three samples had acceptable ratios and their quantities were just outside the pharmaceutical limits. The fourth sample showed signs of decomposition prior to the analysis and is being studied further.

Under the conditions described in the method the sample weights involved were between 0.150 g and 2.0 g. The absorbance observed was between 0.350 and 0.460.

The Associate Referee intends to submit the method to collaborators.*

No reports were given on aminopyrine, ephedrine, and phenobarbitol, or on quinine and strychnine.

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* For report of Subcommittee B and action of the Association, see *This Journal*, **35**, 47 (1952).

REPORT ON SYNTHETIC DRUGS

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

RECOMMENDATIONS*

Propylthiouracil.—The Associate Referee has submitted a report and recommends that the method which was studied collaboratively be made first action and the subject closed. The Referee concurs.

Sulfanilamide Derivatives.—The Associate Referee has reported on some preliminary tests which indicate the feasibility of chromatographic adsorption methods for the separation of sulfathiazole, sulfamerazine and sulfadiazine in mixtures. He has recommended that this study be continued and that the electrometric titration of sulfonamides be studied. The Referee concurs.

Propadrine Hydrochloride.—No formal report was submitted but the Associate Referee states that work is in progress. It is recommended that the subject be continued.

No reports were received on the following topics: Methylene Blue, Spectrophotometric Methods, and Pyribenzamine and Benadryl. The Referee recommends that these topics be continued.

REPORT ON SYNTHETIC ESTROGENS

DETERMINATION OF DIETHYLSTILBESTROL

By DANIEL BANES (Division of Pharmaceutical Chemistry, Food and Drug Administration, Federal Security Agency, Washington 25, D.C.), *Associate Referee*

The phenol nitrosation method of Lykken, Treseder, and Zahn (1) has been shown (2, 3) to be accurate and reliable for the determination of the synthetic estrogens in simple pharmaceuticals; however, when relatively large amounts of other acidic substances, such as barbiturates, are incorporated in such preparations, the results obtained are less satisfactory. Non-estrogenic phenols, including salicylates and *p*-hydroxybenzoates which are readily nitrosated, also constitute an interference. It therefore became necessary either to find a more specific color test for synthetic estrogens, or to devise a procedure for their separation from the interfering substances present in commercial mixtures. This report deals with experiments on the chromogenic properties of the synthetic estrogens.

The reaction of diethylstilbestrol with metallic compounds in anhydrous media has been noted by several investigators. When treated with antimony pentachloride in ethylene dichloride or a similar organic solvent, all of the synthetic estrogens develop intense red colors which are suitable

* For report of Subcommittee B and action of the Association, see *This Journal*, 35, 47 (1952).

for their quantitative estimation (4). However, the colors are unstable, fading rapidly, and becoming cloudy. Devis (5) has reported that solutions of various metallic oxy-compounds in concentrated sulfuric acid yield distinctive colors when treated with chloroformic solutions of synthetic estrogens. In particular, he found that a solution of vanadyl chloride in sulfuric acid yields red to violet colors with diethylstilbestrol and related compounds, but not with other phenols or diphenols.

Since concentrated sulfuric acid yields colored products with a variety of organic substances, the effect of acetic acid as diluent was tested. Diethylstilbestrol treated with a .01 per cent solution of vanadyl chloride in sulfuric acid, rapidly developed a violet-red color. In a 30 per cent v/v solution of sulfuric acid in acetic acid, 0.1 per cent with respect to vanadyl chloride, color development was slower but could be hastened by warming; in 10 per cent sulfuric acid, a somewhat less intense color was obtained. Very little color was developed, even on prolonged heating, with sulfuric acid solutions less than 8 per cent in strength. In the interest of convenience and specificity, a 10 per cent v/v solution of sulfuric acid in acetic acid was adopted as a standard concentration. In such a solvent, a solution prepared to contain 0.05 per cent vanadyl chloride appeared to yield the optimum quantity of colored product. The yellow-green vanadyl-sulfuric-acetic acid solution turned pale blue on standing, but yielded reproducible violet-red colors with given quantities of diethylstilbestrol. The colors developed rapidly within 30 minutes and increased very slowly thereafter at 100°C. There was no loss in absorbancy after cooling; but at concentrations of diethylstilbestrol greater than 100 micrograms per ml. of reagent, a dark precipitate separated slowly. The colors produced by 10–200 micrograms of diethylstilbestrol heated in 5 ml of reagent solution exhibited maximum absorption of 520 mμ and obeyed Beer's law at that wavelength.

On the basis of these findings, the following method was devised for the determination of diethylstilbestrol.

METHOD

REAGENT

Dissolve 250 mg of dry c.p. vanadyl chloride in 50 ml concentrated sulfuric acid, and warm for 20 min. on the steam bath to expel hydrogen chloride. Cool, add cautiously to about 200 ml of acetic acid, and make to 500 ml with acetic acid.

STANDARD SOLUTION

Dissolve 10.00 mg U.S.P. Reference Standard diethylstilbestrol in alcohol and make to exactly 250 ml with alcohol.

DETERMINATION

Evaporate duplicate aliquots of sample solution containing 100–200 micrograms diethylstilbestrol to a volume of 2–5 ml, and transfer quantitatively with alcohol to test tubes fitted with glass stoppers. Add carborundum chips and evaporate to dry-

ness in a steam bath. Treat similarly duplicate 5.0 portions of standard solution, and a 5.0 ml alcohol blank. Cool 30 min. in a vacuum desiccator. By means of a burette, add 5.0 ml of reagent solution to each tube, stopper loosely, and heat at 100° for one hour. Cool, shake, and let stand 10 min. Transfer to cuvettes provided with lids, and measure the absorbancies relative to the blank at 520 mμ. Compute the diethylstilbestrol content of the sample.

TABLE 1.—*Recoveries of diethylstilbestrol*

DIETHYLSTILBESTROL TAKEN	EXTRANEOUS SUBSTANCES ADDED	DIETHYLSTILBESTROL RECOVERED	RECOVERY
micrograms		*micrograms*	*per cent*
100	None	100.3	100.3
100	Barbital 10 mg	98.7	98.7
100	Salicylic Acid 10 mg	90.0	90.0
150	None	151.9	101.2
150	Barbital 20 mg	147.4	98.2
150	Barbital 40 mg	146.6	97.6
150	Butyl p-Hydroxybenzoate 10 mg	132.3	88.2
200	None	198	99.0
200	Barbital 150 mg	175.3	87.6

Recoveries of diethylstilbestrol from known alcoholic solutions ranged from 97.6–101.2 per cent, even in the presence of 300-fold excess of barbiturates. A powdered sample containing 0.353 per cent diethylstilbestrol, and one hundred times that quantity of barbiturates, was extracted (2) and analyzed according to the method. Recoveries ranged from 100.6 to 101.8 per cent of the put-in quantity on replicate samples. However, very large excesses of barbiturates lead to a loss of diethylstilbestrol (see Table 1). Furthermore, salicylic acid and p-hydroxybenzoates, although they are non-chromogenic under the conditions of the reaction, tend to bleach the color due to diethylstilbestrol. It is proposed to subject the method to collaborative study for diethylstilbestrol in the presence of barbiturates, and to attempt to eliminate the interference of other phenols.*

BIBLIOGRAPHY

(1) LYKKEN, L., TRESEDER, R. S., and ZAHN, V., *Ind. Eng. Chem.*, Anal. Ed., **18**, 103 (1946)
(2) GOTTLIEB, S., *J. Am. Pharm. Assn.*, **36**, 379 (1947); *ibid.*, **37**, 147 (1948).
(3) BANES, D., *This Journal*, **34**, 568 (1951).
(4) DINGEMANSE, E., *Act. Brevia Neerland. Physiol. Pharmacol. Microbiol.*, **10**, 118 (1940).
(5) DEVIS, R., *Compt. rend. soc. biol.*, **140**, 1071 (1946).

* For report of Subcommittee B and action of the Association, see *This Journal*, **35**, 47 (1952).

REPORT ON PROPYLTHIOURACIL

By GORDON SMITH (Food and Drug Administration, Federal Security Agency, New York 14, N.Y.), *Associate Referee*

The spectrophotometric method studied collaboratively this year is essentially the same as that tried out last year.* It consists in comparing the absorbancies of a sample and a standard, both in ammoniacal solution, at 234 mμ. Such a method is too simple to harbor many sources of error, and this year's work was mostly directed toward eliminating such sources, or discovering them if wide deviations in results recurred. A larger weight of sample was used, dilutions were made to larger volumes, instructions were made as specific as possible, and collaborators were requested to run duplicates. They were requested to report absorbancy of the standard, and the slit width used. Two alternative procedures were tried; in one the first solution of sample was pipetted after letting the undissolved excipients settle; in the other it was filtered before pipetting.

To prepare the standard, propylthiouracil powder, obtained through the courtesy of the manufacturer, was dissolved in dilute NH_4OH, reprecipitated with dilute HCl, filtered, washed and dried. The resulting powder melted at 219.5°–220.5° and showed no loss on drying. Part of it was used as a standard; part used in making the tablet mixture used as a sample. The latter was 50.00% propylthiouracil; the other ingredients were starch, talc, lactose and magnesium stearate.

The absorption curve of a dilute ammoniacal solution of the pure propylthiouracil (.0075 mg./ml) shows two peaks, one at 234 mμ and one at 260 mμ; the first is higher. The absorption curve of a dilute ethanol solution of the substance (.00375 mg./ml) shows one peak at 273 mμ. The top portion of this curve is much flatter than either of those in the curve of the ammoniacal solution. For these reasons an ammoniacal solution and a wave length setting of 234 mμ were used.†

Details of the method are given in *This Journal* **35**, 89 (1952).

COLLABORATIVE RESULTS AND CONCLUSIONS

Results are presented in the table below. Those obtained on both the filtered and unfiltered solutions are deemed satisfactory. Filtration evidently does not introduce any error due to adsorption, and this step is included in the method.

One collaborator called attention to the fact that absorbancy readings change when solutions stand overnight. When drawing up the method the writer had assumed that any overnight change would be uniform for standard and sample. Subsequent investigation indicates that this cannot be relied on if the first or more concentrated solutions stand overnight,

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* *This Journal*, **34**, 576 (1951).
† For report of Subcommittee and action of the Association, see *This Journal*, **35**, 47 (1952).

TABLE 1.—*Collaborative results*

COLLABORATOR	WITHOUT FILTRATION	WITH FILTRATION	SLIT WIDTH USED
	per cent	*per cent*	*mm*
1	49.5	48.2	0.8
	49.2	49.2	
2	50.33	50.06	1.0
3	50.19	49.75	1.0
4	49.0	49.2	0.49
	49.3	49.2	
5	48.3	48.1	0.70
	48.6	48.8	
6	50.0	50.1	1.2
	50.1	49.9	
Assoc. Ref.	49.9	49.4	1.0
	49.6	50.4	
Average	49.5	49.4	
Maximum	50.33	50.4	
Minimum	48.3	48.1	

though it does appear to hold true for the final dilute solutions. An instruction has been added to the method to complete the determination in one day.

Because of this time factor, the absorbancies for the standard, which collaborators reported as requested, cannot be relied on to show variation between instruments as intended, and they have not been tabulated. They range from .500 to .5575.

Satisfactory results were obtained with different slit widths. There is an apparent correlation between slit width and precision, in favor of the larger slit widths. This is contrary to expectations, and probably no such conclusion should be drawn from this number of results.

It is recommended* that the method be adopted, first action, and that the subject be closed.

ACKNOWLEDGEMENT

The cooperation of the collaborators, whose names follow, is gratefully acknowledged. All are members of either the Canadian Department of National Health and Welfare, Food and Drug Divisions, or the U. S. Federal Security Agency, Food and Drug Administration.

Jacques Ouellet, Montreal; R. Crisafio and L. G. Chatten, Ottawa; J. H. Cannon, St. Louis; Daniel Banes, Washington; Evelyn Sarnoff, New York.

* For report of Subcommittee B and action of the Association see *This Journal*, 35, 47 (1952).

REPORT ON SULFANILAMIDE DERIVATIVES

By HARRY W. CONROY (Food and Drug Administration, Federal Security Agency, Kansas City 6, Mo.), *Associate Referee*

The investigational work on the separation of mixtures of sulfonamides was continued. It was recommended that a method be developed for the separation of sulfathiazole, sulfamerazine and sulfadiazine in mixtures. While a satisfactory technique was not devised for the above mixture, some preliminary tests indicate the feasibility of chromatographic adsorption methods.

The separation of sulfadiazine and sulfathiazole was first tried by adsorption on commercially prepared alumina. About 0.1 gram of a mixture of the above sulfonamides, dissolved in a minimum amount of acetone, was put on a column of adsorption alumina 80–200 mesh which had been prewashed with 10 ml of acetone. The column of alumina was 7 mm in diameter and 2 g of the adsorbant gave it a height of ca 62 mm. Elution of sulfadiazine was accomplished by 25 ml of a reagent composed of 98 ml acetone, 2 ml 5% ammonium acetate and .25 ml ammonium hydroxide. A test for complete extraction was made by putting an additional 5 ml of the eluant through the column and evaporating the solvent. The sulfathiazole remaining on the column was removed by elution with 60 ml acetone containing .2 ml acetic acid.

	ADDED	RECOVERED	M.P.
	mg	*mg*	°C.
Sulfadiazine	44.8	45.2	252–255
Sulfathiazole	46.8	47.0	196–198*

* After recrystallization.

A separation of sulfamerazine from sulfathiazole was tried by the above procedure, since it was found that sulfamerazine reacts similarly to sulfadiazine toward the eluting reagent. Somewhat lower recovery of sulfamerazine was obtained, but its melting point corresponded fairly closely to the pure substance.

	ADDED	RECOVERED	M.P.
	mg	*mg*	°C.
Sulfamerazine	34.8	33.2	234–237
Sulfathiazole	40.2	41.4	196–197

The separation of sulfadiazine from sulfamerazine was tried by adsorption on alumina and elution of sulfamerazine with 2% methanol in acetone. Preliminary tests on the individual components had shown that

sulfadiazine was not eluted and that the sulfamerazine was rapidly removed. However in the mixture it was found that while sulfamerazine was eluted first, the elution was slow and large volumes of 2% methanol-acetone were required. The above work was done using adsorption alumina as purchased commercially.

Since it has been observed that freshly dried alumina is more adsorptive than the hydrated form, the alumina used in the above determinations was dried at 400°F. for four hours. The loss on drying was 3%. This product was more reactive than when originally received. The elution volumes were increased greatly and separations were not as satisfactory. In view of this difference it will be advisable to specify that anhydrous alumina be used and to redetermine the effects described above.

In the previous report on this subject,* it was recommended that the work on internal indicators for the nitrite titration of sulfonamides be discontinued. It has been suggested by Horwitz† that the electrometric titration be used to determine the end point. The Associate Referee agrees that this method should be investigated.

RECOMMENDATIONS‡

It is recommended—

(1) That the study of methods for the separation of sulfamerazine, sulfadiazine, and sulfathiazole be continued.

(2) That the electrometric titration of sulfonamides be studied.

No report was given on methylene blue, propadrine hydrochloride, spectrophotometric methods, or on di- and triphenhydramine hydrochloride.

REPORT ON MISCELLANEOUS DRUGS

By I. SCHURMAN (Food and Drug Administration, Federal Security Agency, Chicago 7, Illinois), *Referee*

RECOMMENDATIONS§

Mercury Compounds—The Associate Referee has submitted a report which includes a collaborative study on four mercurical compounds. In view of the relatively wide variations in some of the results the Associate Referee has recommended that the subject be continued. The Referee concurs in this recommendation.

Organic Iodides—No report. The Referee recommends that the subject be continued.

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* *This Journal*, **34**, 578 (1951).
† Horwitz, W. A., Private communication.
‡ For report of Subcommittee B and action of the Association, see *This Journal*, **35**, 47 (1952).
§ For report of Subcommittee B and action of the Association, see *This Journal*, **35**, 48 (1952).

Alkali Metals.—No report. The Associate Referee has been transferred and was unable to carry out this assignment. The Referee recommends that the subject be reassigned.

Glycols and Related Compounds—The Associate Referee has submitted a report but because of limited collaborative study he recommends that the subject be continued. The Referee concurs in this recommendation.

Preservatives and Bacteriostetic Agents in Ampul Solutions—No report. The Referee recommends that the subject be continued.

Methanol.—Report to be made by the Referee on Alcoholic Beverages.

Microscopic Tests for Alkaloids and Synthetics.—The Referee recommends that the microscopic tests described by the Associate Referee be adopted, first action, and the subject be continued.

REPORT ON MICROSCOPIC TESTS FOR ALKALOIDS AND SYNTHETICS

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

The substances chosen for study this year were benadryl hydrochloride, pyribenzamine hydrochloride, and pyranisamine maleate. As a departure from the type of microscopical data reported in previous years, the tests conducted this year include other optical-crystallographic data in addition to the characteristic microscopic habit or appearance of the crystalline precipitate in those cases where the precipitate is suitable for further microscopic study.

DIPHENHYDRAMINE HYDROCHLORIDE (BENADRYL HYDROCHLORIDE ®)

Reagents.—5% aqueous solution of platinic chloride ($H_2PtCl_6 \cdot 6H_2O$); glycerin-alcohol soln (1+1).

Preparation of sample.—Add a small amount of the pure substance or tablet material (≈0.2 mg.) to 1 drop of glycerin-alcohol on a microscopical slide and stir into the drop; or prepare an aqueous soln of the substance.

Identification.—To a drop of the test soln, draw in 1 drop of platinic chloride reagent. Aggregates of platy crystals form readily in glycerin-alcohol medium and gradually in the aqueous test drop if Benadryl is present. The plates show jagged edges and have a tendency to twin, forming X-shaped aggregates, hour-glass forms, and dendritic structures. The crystals show first order gray polarization colors and exhibit symmetrical or parallel extinction with respect to the various forms of crystals produced. The plates show positive elongation.

Sensitivity.—1:20,000.

PYRANISAMINE MALEATE

Reagents.—5% aqueous soln of platinic chloride ($H_2PtCl_6 \cdot 6H_2O$); glycerin-alcohol solution (1+1).

Preparation of sample.—Add a very minute amount (ca. 0.1 mg) of the substance

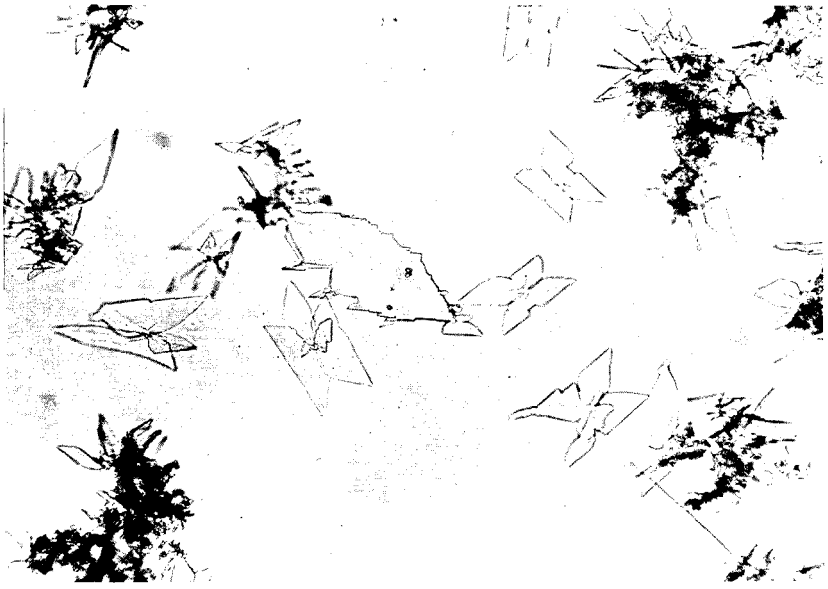

FIG. 1.—Benadryl hydrochloride ® with platinic chloride (×200).

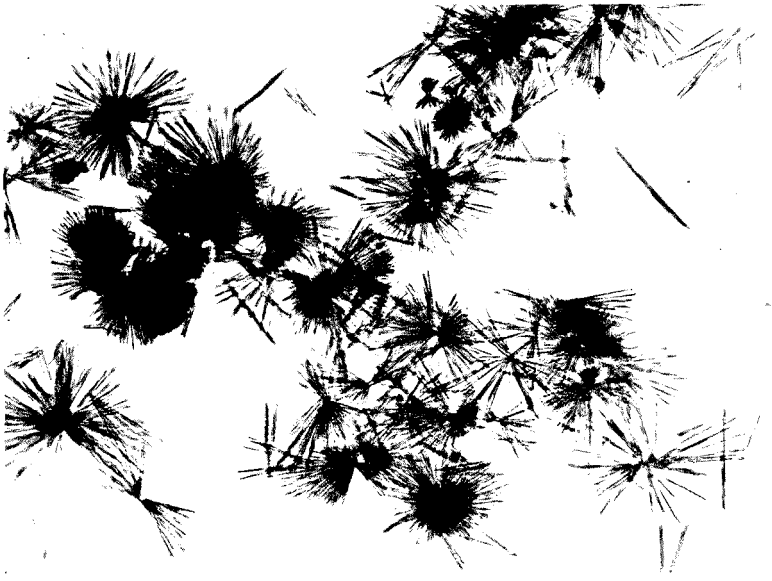

FIG. 2.—Pyranisamine maleate with platinic chloride (×200).

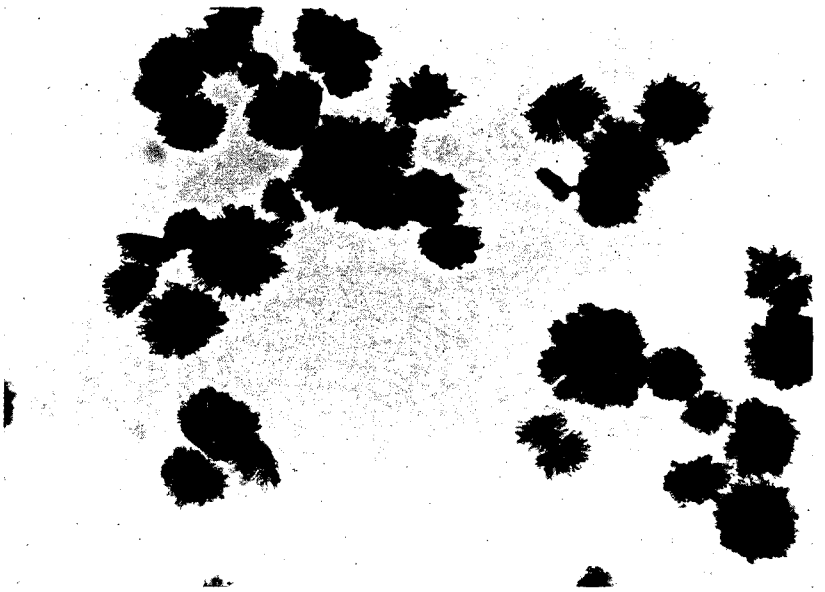

FIG. 3.—Pyribenzamine hydrochloride ® with platinic chloride (×200).

to 1 drop of glycerin-alcohol or distilled water on a microscope slide, and stir into the drop.

Identification.—To a drop of the test soln, draw in 1 drop of platinic chloride reagent, and stir. (Or the dry substance may be added directly to a drop of the reagent.) Needles are formed in rosette aggregates, sheaves, and singly. The needle crystals show second order blue and green and first order red and yellow polarization colors and exhibit parallel extinction and negative elongation.

Sensitivity.—1:20,000.

TRIPELNNAMINE HYDROCHLORIDE (PYRIBENZAMINE HYDROCHLORIDE ®)

Reagents.—5% aqueous soln of platinic chloride ($H_2PtCl_6 \cdot 6H_2O$); glycerin-alcohol soln (1+1).

Preparation of sample.—Add a very minute amount of the pure substance or tablet material to 1 drop of glycerin-alcohol or distilled water on a microscope slide, and stir into the drop.

Identification.—To a drop of the test soln, draw in 1 drop of platinic chloride reagent, and stir. Small needles and bladed crystals are formed in dense rosette aggregates and singly. The needles show white and first order yellow polarization colors and exhibit parallel extinction and positive elongation.

Sensitivity.—1:20,000.

Excipients in the tablet material (lactose and corn starch) do not interfere when present in the test drop.

RESULTS AND COMMENTS

Albert H. Tillson, U. S. Food and Drug Administration, Washington, D. C.—No difficulty was encountered in the identification of the three

substances. More characteristic crystalline precipitates are obtained with test solutions of the substances in glycerin-alcohol or in water than when the solid substances were added directly to the reagent.

A. E. Schulze, U. S. Food and Drug Administration, Washington, D. C.—Characteristic crystalline precipitates were readily obtained by following the directions.

RECOMMENDATIONS*

It is recommended that the described microchemical tests for Diphenhydramine Hydrochloride (Benadryl Hydrochloride ®), Pyranisamine Maleate, and Tripelennamine Hydrochloride (Pyribenzamine Hydrochloride ®) be adopted, first action.

REPORT ON GLYCOLS AND RELATED PRODUCTS

By HARRY ISACOFF (Food and Drug Administration, Federal Security Agency, New York, 14, N. Y.), *Associate Referee*

Propylene glycol is used in many pharmaceutical products and during the last war its use was permitted to a limited extent in official U.S.P. and N.F. preparations as a replacement for glycerol. Methods for determining the presence and amount of propylene glycol in drug products are limited and no collaborative work has been done by the A.O.A.C. on methods.

Malaprade (1) has shown that periodates have a selective oxidizing action on 1, 2 glycols and certain other alpha-hydroxy compounds. Shupe (2) has reported the application of the periodate reaction to cosmetic ingredients including propylene glycol. Metayer and co-workers (3) studied co-distillation and entrainment of glycols by a number of hydrocarbons and reported that propylene glycol could be distilled quantitatively. Bruening (4) has reported on a method for determining propylene glycol in vanilla extracts. A similar method (5), utilizing the co-distillation procedure, was the subject of preliminary work by the Associate Referee, and this year the method has been expanded to include the submission of samples to other laboratories for collaborative study.

Propylene glycol is entrained by the hydrocarbons and collected in a receiver tube fitted with a stopcock to facilitate the separation and removal of the glycol from the entraining fluid. Cyclohexane is found to have a sufficiently low boiling point (81°C.) to be easily handled on a hot plate, and it will quantitatively entrain propylene glycol. Glycerol, if present, does not interfere, although some glycerol will be entrained with the propylene glycol. Decomposition of sugar and other ingredients of pharmaceutical preparations is held to a minimum by the low boiling point of cyclohexane, and it will dissolve volatile oils and other volatile

* For report of Subcommittee B and action of the Association, see *This Journal*, 35, 48 (1952).

substances. This reduces the contamination of the glycol layer. Alcohol does not interfere.

The periodate oxidation method was used to determine the amount of propylene glycol present in the distillate. Preliminary work on an elixir containing propylene glycol established that satisfactory recoveries could be obtained with cyclohexane.

METHOD

APPARATUS

Condenser—West type with drip tip and ⅌ inner joint.

Distilling Flask—250 ml Erlenmeyer, narrow mouth with ⅌ joint.

Distilling Receiver—Barrett type, 20 ml capacity, with ⅌ stopcock and ⅌ joints.

REAGENTS

Cyclohexane (Practical).—Boiling point 81°C.

Potassium Periodate 0.02M.—Dissolve 4.6 grams of KIO_4 in ca 500 ml hot water. Dilute to about 900 ml with water, cool to room temperature and make to 1000 ml. Filter through sintered glass and store in amber glass bottles in a cool place.

Potassium Arsenite 0.02N.—Prepare a 0.1*N* $KAsO_2$ solution as directed in the U.S.P. and dilute 200 ml to 1000 ml with water.

Starch Indicator.—Mix 0.5 g soluble starch with 10 ml cold water and add 90 ml boiling water. Boil for ca 5 minutes and cool before use.

Bromcresol Purple Indicator Solution U.S.P.—Dissolve 50 mg of bromcresol purple in 100 ml of alcohol and filter if necessary.

DISTILLATION

Take an aliquot of the sample solution containing the equivalent of ca 1 g of propylene glycol, and transfer to a 250 ml. Erlenmeyer flask. Add sufficient water to make the volume a total of 10 ml. Add 75 ml of cyclohexane and some glass beads. Connect the flask to the distilling tube of the receiver and connect both to the condenser. Fill the receiver with cyclohexane and heat the contents of the flask with a variable heat hot plate and distill at such a rate that a rapid stream of distillate flows from the condenser tip. Distill ca 8 hours and cool.

Open the stopcock and transfer the aqueous-glycol layer to a 200 ml volumetric flask. Wash down the condenser with several successive portions of water (ca 15 ml each time) collecting the wash water in the receiver tube and draining it off into the volumetric flask. Dilute to volume with water and mix well.

OXIDATION

Transfer an aliquot of the aqueous solution containing not more than 45 mg of propylene glycol (or its equivalent if glycerin is present) to a 100 ml volumetric flask. (The sample solution should be neutral to bromcresol purple.)* Add 50 ml of 0.02 *M* KIO_4 solution. Make up to 100 ml with water and allow to stand for one hour. (Excess periodate must be present in the oxidation mixture. A preliminary test on an unknown sample can be made to determine proper sample size. Excess periodate will release iodine from a sodium bicarbonate-potassium iodide mixture). Take a 50 ml aliquot of the oxidized mixture and transfer to an Erlenmeyer flask. Add 1 g of sodium bicarbonate and 0.5 g of potassium iodide, and titrate at once with a 0.02 *N* $KAsO_2$ solution until the color of the solution fades to a light yellow. Add 3 ml of starch solution and titrate to the disappearance of the blue color.

* The sample solution is titrated with 0.02 *N* NaOH and bromcresol purple indicator solution to correct for any acidic substances that may carry over in the distillation. Usually the distillate is neutral in reaction.

Standardize 25 ml of the 0.02 *M* KIO_4 by the same titration procedure. The difference between the two titrations is a measure of the amount of periodate reduced.

1 ml of 0.02 *N* $KAsO_2$ = 0.46 mg glycerin.

1 ml of 0.02 *N* $KAsO_2$ = 0.76 mg propylene glycol.

Take a separate 25 ml aliquot of the oxidized mixture remaining in the volumetric flask and add 2 drops of the bromcresol purple indicator. Titrate with 0.02 *N* NaOH until a light purple colored solution is obtained. Apply a correction for any acidity in the 0.02 *M* KIO_4 solution (titrate 25 ml of 0.02 *M* KIO_4 with 0.02 *N* NaOH using bromcresol purple indicator).

1 ml 0.02 *N* NaOH = 1.84 mg glycerin.

A = 2(ml $KAsO_2$ for blank—ml $KAsO_2$ for 50 ml oxidation aliquot).

B = 4(ml NaOH for 25 ml oxidation aliquot)—2(ml NaOH for blank correction).

Propylene glycol in sample aliquot = (A − 4B) × .00076 g.

EXPERIMENTAL

The above method has been applied to known amounts of glycerol and propylene glycol incorporated in a medicinal elixir base. The reported recoveries are based on calculations from the individual determinations. Table 1 shows the relationships that form the basis for calculations on mixtures.

TABLE 1.—*Comparative volumes of 0.02 N reagents required by individual alcohols*

	0.02 *N* NaOH (FORMIC ACID)	0.02 *N* ARSENITE (REDUCED KIO_4)
Glycerol	1	4
Propylene Glycol	0	1

With pure glycerol, the formic acid titer in ml of 0.02 *N* NaOH will be 1/4 that of the reduced potassium periodate in terms of 0.02 *N* arsenite. In a mixture containing glycerol and propylene glycol, four times the formic acid titer is subtracted from the volume of 0.02 *N* arsenite to correct for the effect of glycerol. The balance of the arsenite can then be calculated to propylene glycol.

TABLE 2.—*Composition of mixtures containing glycerol and propylene glycol*

	(1)	(2)	(3)
Propylene Glycol	39.2 g	18.8 g	28.8 g
Glycerol	—	16.8 g	8.2 g
Phenobarbital	0.4 g	0.4 g	0.4 g
Amaranth Soln (1%)	1.0 ml	1.0 ml	1.0 ml
Orange Oil	0.1 ml	0.1 ml	0.1 ml
Alcohol	14.0 ml	14.0 ml	14.0 ml
Syrup	15.0 ml	15.0 ml	15.0 ml
Water to make	100.0 ml	100.0 ml	100.0 ml

TABLE 3.—*Recovery of propylene glycol from propylene glycol and glycerol mixtures*

HRS. DISTILLED	MIXTURE (1)			MIXTURE (2)			MIXTURE (3)		
	MG IN ALIQUOT		PER CENT RECOVERY	MG IN ALIQUOT		PER CENT RECOVERY	MG IN ALIQUOT		PER CENT RECOVERY
	TAKEN	RECOVERED		TAKEN	RECOVERED		TAKEN	RECOVERED	
3	29.4	26.3	89.5	23.5	22.7	96.6	36.0	33.5	93.1
6	24.5	23.5	95.9	23.5	23.5	100.0	36.0	35.5	98.6
8	29.4	28.5	96.9	23.5	23.6	100.4	36.0	35.8	99.4
8	29.4	28.5	96.9	23.5	23.5	100.0	30.6	30.6	100.0
8	23.5	22.9	97.4	23.5	23.5	100.0	28.8	28.0	97.2
8	23.5	23.0	97.9						

The following mixtures (Table 2) were prepared and analyzed to determine the accuracy of the method.

Table 3 shows the recovery of propylene glycol using the method described and Table 4 illustrates the relative rate of distillation of glycerol.

Collaborative samples, whose composition is given in Table 5, were pre-

TABLE 4.—*Recovery of glycerol from known mixtures of propylene glycol and glycerol*

HRS. DISTILLED	MIXTURE (2)			MIXTURE (3)		
	MG IN ALIQUOT		PER CENT RECOVERY	MG IN ALIQUOT		PER CENT RECOVERY
	TAKEN	RECOVERED		TAKEN	RECOVERED	
3	21.0	0.33	1.6	10.3	0.3	2.9
6	21.0	1.3	6.2	10.3	0.38	3.7
8	21.0	2.9	13.8	10.3	1.2	11.7
8	21.0	3.0	14.3	8.7	1.0	11.5
8				8.2	1.1	13.4

pared and submitted to collaborators. Recoveries by the Associate Referee are given in Table 6 and by four collaborators in Table 7. Fairly good results were obtained, except for one high value reported on Sample 2.

The following comments were offered by one of the collaborators: (1) Use the word "reflux" instead of "distil" in the method at the point "distil at such a rate that a rapid stream of distillate."

(2) The calculation for glycerin should be amplified.

SUMMARY

The method for the determination of propylene glycol in medicinal mixtures has given fairly satisfactory results in the hands of the collaborators. It is recommended that more collaborative work be done.*

* For report of Subcommittee B and action of the Association, see *This Journal*, 35, 48 (1952).

TABLE 5.—*Composition of mixtures, containing glycerol and propylene glycol, prepared for collaborative analysis*

	(1)	(2)
Propylene Glycol	49.6 g	70.0 g
Glycerol	48.4 g	26.5 g
Phenobarbital	1.0 g	1.0 g
Alcohol	35.0 ml	35.0 ml
Amaranth Soln (1%)	2.5 ml	2.5 ml
Orange Oil	0.25 ml	0.25 ml
Syrup	37.5 ml	37.5 ml
Water to Make	250.0 ml	250.0 ml

TABLE 6.—*Recovery of propylene glycol from mixtures* prepared for collaborative study*

HRS. DISTILLED	MIXTURE 1			MIXTURE 2		
	MG IN ALIQUOT		PER CENT RECOVERY	MG IN ALIQUOT		PER CENT RECOVERY
	TAKEN	RECOVERED		TAKEN	RECOVERED	
8	19.8	19.3	97.5	28.0	27.9	99.6
8	24.8	24.2	97.6	35.0	34.2	97.7

* The amount of glycerin co-distilled ranged from 7.1% to 11.3%.

TABLE 7.—*Recovery of propylene glycol. Collaborative results*

COLLABORATOR	MIXTURE 1		MIXTURE 2	
	RECOVERED	RECOVERED	RECOVERED	RECOVERED
	mg/ml	*per cent*	*mg/ml*	*per cent*
D. W. Johnson, Food & Drug Adm., Denver	194.6 197.2	98.1 99.4	272.1 274.1	97.2 97.9
E. Sarnoff, Food & Drug Adm., New York	196.0 202.0	98.8 101.8	268.2 275.0	95.7 98.2
The Upjohn Co.	A 197.6 198.8 197.6	99.6 100.2 99.6	A 294.3 293.7 294.9	105.1 104.9 105.3
	B 200.0 196.4 198.8	100.8 99.0 100.2	B 297.3 296.1 295.5	106.2 105.8 105.5
R. Crisaffio, Food & Drug Div., Dept. of National Health, Ottawa, Canada	200.6	101.1	272.0	97.1

REFERENCES

(1) L. MALAPRADE, *Compt. rend.*, **186**, 382 (1928); *C.A.*, **22**, 1332 (1928).
(2) I. S. SHUPE, *This Journal*, **26**, 249 (1943).
(3) G. L. METAYER, *Ann. Chim.* **(12)2**, 790–843 (1947).
(4) C. F. BRUENING, *This Journal*, **33**, 103 (1950).
(5) H. ISACOFF, *This Journal*, **33**, 218 (1950).

REPORT ON ASSAY OF MERCURY PREPARATIONS

By ROBERT L. HERD (Food and Drug Administration, Federal Security Agency, St. Louis 1, Mo.), *Associate Referee*

This report on the assay of mercury preparations is a continuation of the work reported by Green (1) in 1949. No report was made to the Asso-

TABLE 1-A.—*Pure chemicals*

	SAMPLE	FOUND	RECOVERY
	gram	*gram*	*per cent*
Mercuric Oxide	.0131	.0131	100.0
	.0131	.0130	99.5
	.0262	.0261	99.6
Mercurous Chloride	.0173	.0169	97.8
	.0164	.0163	99.4
	.0433	.0428	98.8
	.0192	.0192	100.0
	.1597	.1593	99.7
	.0506	.0506	100.0
	.0506	.0503	99.4
	.0506	.0505	99.8
Mercuric Chloride	.0269	.0264	98.2
	.0269	.0265	98.4
	.0536	.0536	100.0
	.0500	.0498	99.6
	.0400	.0399	99.8
	.0500	.0498	99.6
Mercuric Iodide	.1025	.1016	99.1
	.1389	.1386	99.8
Mercury Ammoniated	.1000	.0996	99.6
	.1000	.0997	99.7
Phenylmercuric Chloride	.1000	.0994	99.4
	.1245	.1240	99.6
Merbromin	.2000	25.05[1]	
	.2105	24.28[1]	

[1] Calculated as Hg.

TABLE 1-B.—*Mercury preparations*

	THEORETICAL	FOUND	RECOVERY
	per cent	*per cent*	*per cent*
Calomel Tablet Material	15.0	14.98	99.9
		14.93	99.5
Calomel Tablet Material	5.35	5.17	96.6
		5.14	96.1
		5.28	98.8
		5.30	99.1
Ammoniated Mercury Ointment	5.10	5.04	98.8
		5.02	98.4
		4.98	97.6
		5.04	98.8
Merbromin Solution Calculated as Hg	.50	.494	98.8
		.497	99.4
Nitromersol Solution Calculated as Hg	.113	.118	104.4
		.117	103.5

ciation last year, but a recommendation was made and adopted for the continuation of the problem.

This year's work involved modification of the Rotondaro (2) method in order to make it generally applicable to a wide variety of organic and inorganic mercurials. This modification included a direct transfer of the sample to the digestion flask and oxidation of the mercury with bromine when necessary. This method was used by the associate referee on several mercurials and the results are tabulated below (Tables 1-A and 1-B).

The samples prepared for collaborative study were calomel tablet material, ammoniated mercury ointment, merbromin solution, and nitromersol solution. These samples were sent to eleven collaborators for study and nine reports have been received (Table 2).

A review of the results show that a continuation of the work is indicated. There is too wide a variation in the results to justify tabulation, and instead the highest, lowest, and average figures are included in this report.

TABLE 2.—*Collaborative results*

PREPARATION	THEORETICAL	LOWEST RESULT	HIGHEST RESULT	AVERAGE
	per cent	*per cent*	*per cent*	*per cent*
Calomel	15.0	4.16	15.37	98.0
Calomel	5.35	4.90	5.60	97.4
Ammoniated Mercury Ointment	5.10	4.43	5.40	94.9
Merbromin Calculated as Hg	.50	.456	.510	96.4
Nitromersol Calculated as Hg	.113	.070	.370	

Many helpful suggestions and criticisms were received from the various collaborators which indicate that the method can be improved both in text and technique.

CONCLUSIONS AND RECOMMENDATIONS

1. Results of collaborators are too variable on all samples, with a trend on the low side of the theoretical.
2. It is indicated that the method was not written in sufficient detail and can be improved in text and technique.
3. It is recommended* that the work on the method for mercury preparations be continued.

ACKNOWLEDGEMENT

The associate referee wishes to express his appreciation to the following collaborators for their willingness to assist on this problem and for their suggestions and criticisms:

John F. Weeks, Jr., New Orleans, La.
Garland L. Reed, Baltimore, Md.
James D. Curphey, Philadelphia, Pa.
H. J. Meuron, San Francisco, Calif.
Curt Rupke, Buffalo, N. Y.
Harold F. O'Keefe, Chicago, Ill.
H. Rogavitz, New York, N. Y.
J. H. Cannon, St. Louis, Mo.
M. L. Dow, St. Louis, Mo.

REFERENCES

(1) MELVIN W. GREEN, *This Journal*, **32**, 552 (1949).
(2) F. A. ROTONDARO, *J. Am. Pharm. Assoc.*, **33**, 353 (1944).

No report was given on organic iodides and separation of halogens alkali metals, preservatives and bacteriostatic agents in ampul solutions or on methyl alcohol.

REPORT ON SOILS AND LIMING MATERIALS

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

The work done by each of the three Associate Referees is described in their respective reports.

The intensive three-year studies on slags by W. M. Shaw, Associate Referee on liming materials, culminated in a recommendation that the neutral calcium acetate procedure be made official, first reading. A second recommendation is for further study of quantitative procedures to determine the limestone requirement necessary to raise a soil's reaction to a desired practical *p*H value.

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* For report of Subcommittee B and action of the Association, see *This Journal*, **35**, 48 (1952).

The Associate Referee on methods for determination of exchangeable potassium in soils, A. Mehlich, obtained collaborative findings that he and R. J. Monroe of the North Carolina Agricultural Experiment Station are presenting, with the suggestion that there be prepared "a detailed outline" of a procedure that would "be considered as a standard reference." That suggestion is being furthered through current recommendation No. 8 of the Referee.

L. J. Hardin, Associate Referee on the determination of fluorine in soils, is presenting the findings obtained through collaborative studies and is repeating recommendation No. 6 of the 1950 report* of the Referee, that the stipulated procedure be made official.

The recommendations of 1950 that were not furthered through reports are being repeated with expression of hope that the designated associate referees will be able to function.

RECOMMENDATIONS†

It is recommended—

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

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

(3) That the utilization of carmin as an indicator in the determination of 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 in that determination.

(4) That further studies on the *p*H of soils at a moisture content approximating the field capacity be carried out.

(5) That the neutral calcium acetate procedure for the replacement of the exchangeable hydrogen of soils and determination thereof be adopted as official, first action.

(6) That further studies be made of methods for the determination of the limestone requirement to raise a soil's reaction to a desired practical *p*H value.

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

(8) That the survey and comparisons of methods for the determination of exchangeable K in soils be continued (*This Journal*, **30**, 44, (1949)) and that the Associate Referee and his collaborators implement their findings into a detailed procedure to be recommended for verification and ultimate adoption as official.

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* *This Journal*, **34**, 587 (1951).

† For report of Subcommittee A and action of the Association, see *This Journal*, **35**, 45 (1952).

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

(10) That the double distillation procedure for fluorine in soils, as set forth in the paper by MacIntire, *et al.*, **34**, 602 (1951) be made official, and that work on this subject be continued.

REPORT ON POTASSIUM ANALYSES BY MEANS OF FLAME PHOTOMETER METHODS

By A. MEHLICH, *Associate Referee*, and R. J. MONROE (North Carolina Agricultural Experiment Station, Raleigh, N. C.)

The collaborative studies in 1950 on exchangeable potassium revealed large variations in results by different analysts and by different analytical procedures on extracts which were known to be quite uniform. In view of this it was recommended that the reasons for the lack of agreement be investigated. These investigations were divided into studies with (a) solutions and (b) soils.

STUDIES WITH SOLUTIONS

The first part of the study involved the use of *N* ammonium acetate solutions containing potassium together with 100 p.p.m. lithium as LiCl. Seven 50 ml portions containing 0 to 40 p.p.m. K, labeled as standards, and 10 portions varying from 0.5 to 35 p.p.m. K, labeled as unknowns, were made available to collaborators at 9 Experiment Stations.

The K content of the referee solutions and the measured values obtained by the flame photometer at the various stations are shown in Table 1. To evaluate these data, a regression line was fitted using the true value of the unknown solution as an independent variable, and the average deviation from the regression line was regarded as an estimate of error. Under these conditions it is clear that a slope of 1 and an intercept of 0 are the desirable characteristics of the regression line. An appreciable deviation from a slope of 1 must be viewed with suspicion, because this indicates that the measurement is not being done properly at all levels. An intercept deviation from 0 can be caused by an error in slope, an error in calibration, or both. Since the regression line must pass through the mean value of the dependent variable, we can also look at the deviation of the observed mean from the true value of 14 to detect the presence of constant errors.

Table 2 shows the results of this analysis. The residual error for Station *g*, using the Perkin Elmer, Model 18, is far out of line, and was omitted in calculating the average error used in obtaining the limits on slope, intercept, and means. Stations *f* and *h* (P.E. 52A) have the lowest residual errors, but *h* is off on the slope and the mean is too low. Stations *e* (P.E. 52A) and *i* (P.E. 18) are examples of a calibration error. Since the slope is

TABLE 1.—*K content of referee solutions measured with different instruments at different stations—p.p.m. K*

NO.	K PRESENT P.P.M.	FLAME PHOTOMETER AND STATIONS										
		PERKIN ELMER 52A							PERKIN ELMER 18		BECKMAN	UNIVERSAL
		a	*b*	*c*	*d*	*e*	*f*	*h*	*g*	*i*	*k*	*l*
U- 1	1.0	0.8	1.0	0.8	0.5	1.8	0.8	1.3	0.0	1.6	1.3	1.0
U- 2	35.0	34.8	35.0	35.0	34.8	35.0	35.0	33.3	29.0	34.2	32.0	—*
U- 3	25.0	24.6	26.0	24.8	24.5	26.5	25.0	24.9	23.0	24.8	23.6	26.0
U- 4	0.5	0.5	0.0	0.2	0.0	1.8	0.5	0.7	0.0	1.2	0.6	0.5
U- 5	3.5	3.0	4.5	3.5	3.8	5.0	3.6	4.1	3.0	4.2	3.8	4.0
U- 6	10.0	9.8	10.0	10.0	9.7	11.3	10.0	10.2	11.0	11.0	9.6	10.5
U- 7	20.0	18.9	20.0	18.6	18.8	20.5	19.5	19.0	19.0	21.2	19.3	20.0
U- 8	15.0	15.1	15.0	15.0	14.2	17.0	15.3	15.2	16.0	16.8	15.4	15.5
U- 9	15.0+200 p.p.m. Ca	14.7	14.5	14.7	14.0	16.5	15.1	14.7	13.0	17.0	15.0	16.0
U-10	15.0+200 p.p.m. Mg	15.1	16.5	15.0	14.0	17.0	15.2	14.9	14.0	17.1	15.3	15.0

* Sample lost.

TABLE 2.—*Results of statistical analysis of data from table 1*

STATION	INSTRUMENT	RESIDUAL ERROR PER DETERMINATION	RESIDUAL ERROR PER CENT OF MEAN	SLOPE	INTERCEPT	MEAN
a	P.E. 52A	0.400	2.9	1.001	−0.243	13.73
b	P.E. 52A	0.714	5.1	1.008	+0.144	14.25
c	P.E. 52A	0.451	3.2	0.996	−0.185	13.76
d	P.E. 52A	0.468	3.3	0.992	−0.461	13.43*
e	P.E. 52A	0.624	4.5	0.978	+1.551*	15.24*
f	P.E. 52A	0.234	1.7	0.999	+0.011	14.00
h	P.E. 52A	0.314	2.2	0.947*	+0.566	13.83
g	P.E. 18	1.509	10.8	0.875*	+0.553	12.80*
i	P.E. 18	0.919	6.6	0.968	+1.358*	14.91*
h	Beckman	0.476	3.4	0.918*	+0.741	13.59
i	Universal	0.435	3.1	1.005	+0.281	14.35

Limits on Slope 0.956–1.044 (95% limits).
Limits on Intercept −0.77–+0.77 (95% limits).
Limits on Mean 13.54–14.46 (95% limits).
* Observed value outside limits (95% limits).

satisfactory an average correction to all readings would bring the intercept into line. The results for Stations *a, b, c, f* (P.E. 52A), and *i* (Universal) appear satisfactory on all counts, although *b* has a higher residual error than any of the others. Stations *g* and *h* (P.E. 52A and Beckman) are off in slope and all have a value less than 1, indicating a falling off at the higher levels of K.

In only one case (Station *g* with P.E. 18) did the addition of Ca or Mg to the solutions appear to have any effect (compare Nos. 9 and 10 with 8).

ANALYSTS AND DAY TO DAY VARIATION

The results listed in Table 1 are averages of all the measurements made at any given station. Station *c*, for example, reported 3 successive measurements. The variations of those were negligible. Station *a* reported results obtained by three analysts, and in the case of one analyst, also data obtained on 2 different days. Station *h* likewise reported analyses made on different days with two instruments. These results are shown in Table 3.

Several kinds of comparisons were possible from the data in Table 3, and two types of statistical analyses were made. In cases where a valid error could be obtained the analysis of variance technique was used, but in cases where several factors such as instrument, station, and analyst were confounded, a regression technique was employed to get an internal estimate of the analytical error.

Different analysts.—At Station *a* where three analysts used the same instrument the mean p.p.m. K were: 13.46; 13.87; and 13.98 for analysts 1, 2, and 3 respectively. The average error per determination was 0.38 p.p.m., and for the average of 10 samples any mean which departed from

TABLE 3.—*K content of referee solutions measured by different analysts or on different days by the same analyst—p.p.m. K*

NO.	K P.P.M.	MEASURED BY DIFFERENT ANALYSTS (P.E. 52A)*			MEASURED BY THE SAME ANALYST ON DIFFERENT DAYS					
					PERKIN ELMER 52A†				BECKMAN	
		ANALYST			ANALYST 1		ANALYST 4		ANALYST 4	
		1	2	3	1ST DAY	2ND DAY	1ST DAY	2ND DAY	1ST DAY	2ND DAY
U- 1	1.0	0.7	1.0	0.5	0.7	1.0	1.2	1.4	1.4	1.2
U- 2	35.0	34.5	35.0	35.0	34.5	34.5	33.3	33.3	32.7	31.2
U- 3	25.0	24.0	25.0	25.5	24.0	24.0	23.7	26.0	24.4	22.8
U- 4	0.5	0.0	0.7	0.4	0.0	0.8	0.7	0.8	0.5	0.6
U- 5	3.5	3.0	2.0	3.5	3.0	3.3	3.9	4.3	3.7	3.8
U- 6	10.0	9.5	10.0	10.3	9.5	9.5	9.7	10.7	10.0	9.2
U- 7	20.0	18.8	19.0	19.0	18.8	18.8	18.5	19.5	20.3	18.3
U- 8	15.0	15.0	15.5	15.0	15.0	15.0	14.4	16.0	16.3	14.6
U- 9	15+200 p.p.m. Ca	14.3	15.0	15.3	14.3	14.3	14.2	15.1	15.8	14.3
U-10	15+200 p.p.m. Mg	14.8	15.5	15.3	14.8	14.8	14.0	15.8	16.3	14.3
Mean	14.0	13.46	13.87	13.98	13.46	13.60	13.36	14.29	14.14	13.03

* Station *a*.
† Analyst 1, Station *a*; analyst 4, Station *h*.

TABLE 4.—*Reproducibility of exchangeable K analyses due to repeated extractions, repeated measurements and different instruments (stations)*

SOIL NO.	EXCHANGEABLE K, M.E. PER 100 G SOIL									
	EXTRACTION 1						EXTRACTION 2			
	STATION *h*				STATION *f*		STATION *f*		STATION *h*	
	1ST RUN		2ND RUN							
	1	2	1	2	1	2	1	2	1	2
1	.028	.027	.021	.017	.043	.035	.040	.040	.031	.031
2	.208	.210	.207	.213	.236	.236	.240	.235	.244	.236
3	.290	.279	.284	.284	.350	.330	.325	.325	.333	.333
4	.159	.159	.171	.171	.226	.210	.185	.185	.199	.196
5	.244	.239	.239	.239	.270	.258	.254	.238	.270	.247
6	.202	.202	.213	.213	.252	.250	.230	.225	.233	.227
7	.114	.108	.135	.126	.130	.130	.113	.113	.128	.134
8	1.420	1.390	1.480	1.450	1.540	1.540	1.460	1.480	1.510	1.540
9	1.170	1.180	1.170	1.170	1.170	1.170	1.180	1.140	1.180	1.150
10	1.320	1.270	1.310	1.280	1.330	1.330	1.340	1.330	1.350	1.320
11	.153	.153	.156	.153	.185	.192	.168	.168	.165	.162
12	.318	.318	.318	.318	.345	.334	.330	.330	.333	.333
13	.065	.063	.048	.044	.067	.065	.066	.066	.060	.058
14	.139	.148	.128	.134	.185	.185	.172	.170	.171	.162
15	.036	.038	.027	.030	.045	.045	.047	.047	.046	.050
Mean	.392	.386	.394	.389	.425	.420	.410	.406	.417	.412

the true value of 14.00 p.p.m. by more than 0.24 p.p.m. was regarded as suspect. Thus Analyst 1 above appears to be too low on the average.

Day to day variation.—The average residual error for Analyst 1 was 0.236 p.p.m., for Analyst 4, it was 0.594 p.p.m. and 0.551 p.p.m. for the P.E. 52A and Beckman instruments, respectively. In this case the standard error of the mean of 10 determinations is 0.154 p.p.m., and, if two standard errors are taken as reasonable limits, any mean of 10 lying outside the range 13.69 to 14.31 is suspect.

It appears that Analyst 1 is consistent from day to day, more so than Analyst 4, but he is consistently low in estimating K as well. Analyst 4 had a higher residual error and more day to day difference, but 2 of the 4 runs gave means within the accepted limits.

SOIL STUDIES

Similar studies to the above were made on extracts obtained from the 15 soils reported on in 1950 (*This Journal*, **34,** 589 (1951)). Two sets of extracts were made approximately one month apart at Station *f*, and 50 ml portions were sent to Station *h*. The extracts were obtained according to procedure A as outlined in the 1950 report. A Perkin Elmer, Model 52A, was used at both stations. The results of this study are shown in Table 4.

Two runs with the same instrument on different days.—The "instrumental" error was 0.0098 m.e., while the real analytical error measured by the failure of the two runs to give the same answer from sample to sample was 0.0184 m.e. On the average, the two runs agreed remarkably well—the only differences between runs could be ascribable to chance errors of about 0.0184 m.e. per determination.

Instrument tests at two stations.—The "instrumental" error was 0.0097 m.e., while the real analytical error measured by the failure of the labs to agree from sample to sample was 0.0155 m.e. The agreement of the two laboratories on the average was fairly good, as the average difference between the laboratories of 0.003 m.e. may be reasonably ascribed to chance variations.

Extraction tests.—Two different extractions were run in duplicate on the same instrument. The "instrumental" error was 0.0077 m.e. but the analytical error measured by the failure of the extracts to agree from sample to sample was 0.0186 m.e. There was a real difference between extracts, with "Extract 1" being 0.015 m.e. higher on the average than "Extract 2."

FLAME PHOTOMETER VERSUS CHEMICAL METHODS

In a further experiment, Station *c* obtained a duplicate set of extracts based on a slightly modified procedure B (20 g soil were shaken for 30 minutes with 100 ml *N* ammonium acetate, filtered, and leached with 100 ml ammonium acetate in small portions). The extract, after having been

made up to 200 ml was then divided into 3 portions. Approximately 50 ml portions each were used at Stations *c* and *f* to be analyzed flame photometrically. One hundred ml portions were evaporated to dryness, the ammonium acetate and organic matter removed, K precipitated with sodium cobaltinitrite and finally K was determined by a titration method using ceric sulfate and ferrous ammonium sulfate. These results together with those obtained with the flame photometer are shown in Table 5.

TABLE 5.—*Exchangeable K analyses obtained by cobaltinitrite titration, and flame photometer methods in the same extract from each soil and at 2 stations (photometer only)*

SOIL NO.	EXCHANGEABLE POTASSIUM IN M.E. PER 100 GRAMS OF SOIL					
	CHEMICAL METHOD		FLAME PHOTOMETER METHOD			
	STATION *c*		STATION *c*		STATION *f*	
	1	2	1	2	1	2
1	0.028	0.031	0.009	0.009	0.025	0.023
2	0.292	0.294	0.282	0.280	0.265	0.260
3	0.325	0.348	0.336	0.343	0.327	0.332
4	0.216	0.226	0.220	0.220	0.225	0.225
5	0.301	0.300	0.192	0.186	0.268	0.254
6	0.279	0.270	0.256	0.261	0.271	0.271
7	0.210	0.205	0.211	0.188	0.195	0.186
8	1.458	1.440	1.492	1.510	1.510	1.530
9	1.010	1.078	1.283	1.280	1.260	1.260
10	1.188	1.182	1.413	1.402	1.430	1.430
11	0.248	0.219	0.230	0.230	0.245	0.243
12	0.420	0.422	0.403	0.417	0.385	0.391
13	0.113	0.109	0.068	0.062	0.071	0.068
14	0.184	0.175	0.160	0.180	0.155	0.155
15	0.047	0.038	0.031	0.031	0.029	0.029
Mean	0.420	0.422	0.439	0.440	0.444	0.444

The "instrumental" errors or failure of duplicates to agree for each method are: chemical method 0.0147 m.e.; photometer method, Station *c*, 0.0076 m.e.; Station *f*, 0.0051 m.e. The average error for the three methods measured by the failure of the methods to agree for all samples is 0.0388 m.e. The two photometer methods gave, with some exceptions, essentially the same results, and the chemical method gave slightly higher values on the low K soils and lower values on the very high K soils.

INFLUENCE OF EXTRACTING SOLUTION ON K ANALYSES

The extracting solution used in most of the previous work was *N* ammonium acetate. Investigators, particularly those engaged in "short test" work, employ other extracting solutions as well. Generally, a narrower

soil solution ratio and a shorter period of extraction is employed than in the more quantitative methods. Several collaborators have made comparisons between several of these methods, and the results are recorded in Table 6.

Method A is one of the conventional leaching methods used in the 1950 study, and the values obtained may be considered as a standard with which the results of the other methods may be compared. Consideration

TABLE 6.—*K values obtained with different methods*
m.e. K per 100 g soil

SOIL NO.	METHOD*							
	A	G	H	I	J	K	L	M
	COLLABORATOR							
	10	10	10	10	1	3	7	7
1	.04	.03	.04	.02	.06	.07	.05	.05
2	.24	.21	.22	.24	.22	.38	.24	.21
3	.33	.27	.31	.31	.31	.51	.28	.30
4	.16	.16	.20	.16	.19	.32	.16	.22
5	.24	.20	.25	.27	.26	.33	.25	.27
6	.24	.17	.21	.18	.23	.31	.16	.25
7	.13	.09	.12	.04	.18	.18	.07	.16
8	1.46	.74	1.17	.68	1.41	1.53	1.35	1.52
9	1.23	.60	.94	.49	1.06	1.11	1.02	1.23
10	1.40	.55	.95	.60	1.23	1.43	1.14	1.45
11	.23	.14	.17	.13	.17	.22	.12	.22
12	.39	.27	.31	.29	.32	.49	.23	.34
13	.09	.05	.09	.09	.10	.13	.04	.06
14	.19	.13	.16	.12	.17	.23	.15	.17
15	.04	.03	.04	.04	.08	.05	.06	.04
Mean	.43	.24	.35	.24	.40	.49	.35	.43
Mean (Less Nos. 8, 9, 10)	.19	.15	.18	.16	.19	.27	.15	.19

* A—1 *N* NH_4OAc (1:10 soil solution ratio) Flame photometric.
G—1 *N* NH_4OAc (1:2 soil solution ratio) 30 minute extraction. Flame photometric.
H—1 *N* NH_4OAc (1:2 soil solution ratio) digested at 90°C. for 1 hour. Flame photometric.
I—1 *N* $NaNO_3$ (1:2 soil solution ratio) turbidimetric.
J—NH_4OAc-HOAc pH 4.8 buffer (1:4 soil solution ratio). Flame photometric.
K—25% $NaNO_3$ (1:2 soil solution ratio) turbidimetric.
L—1 *N* $NaNO_3$ in 0.1 *N* HCl (1:2 soil solution ratio) Flame photometric.
M—$BaCl_2$-Triethanolamine (1:10 soil solution ratio). Flame photometric.

of the mean values show methods J and M to replace essentially the same amounts of potassium, whereas method K replaces substantially more, and the other methods substantially less, than the standard. A large part of this major difference is caused by the very high K-soils (Nos. 8, 9, and 10). By omitting these results from the mean, most of the data fall more in line, except with method K where the results are still too high, and methods G, I, and L where they are somewhat too low.

SUMMARY AND RECOMMENDATIONS

1. When the flame photometer was used on standard solutions containing from 0.5 p.p.m. to 35.0 p.p.m. K, the average residual error was calculated to be 0.49 p.p.m. K (excepting the Perkin Elmer, Model 18, which gave an average residual error of 1.24 p.p.m. K).

2. When the flame photometer was used on *N* ammonium acetate extracts of soils containing from 0.02 m.e. K to 1.50 m.e. K, the average residual error including day to day variations was 0.0184 m.e. K, and the average residual error including replicated soil extractions was 0.0186 m.e. K.

3. When the flame photometer was compared with the chemical method (cobaltinitrite titration) on the same soil extracts, the "instrumental" errors for the photometer averaged 0.0064 m.e. K and for the chemical method averaged 0.0147 m.e. K. The failure of the methods to agree is represented by an average error of 0.0388 m.e.

4. The presence of Ca and Mg does not influence the K values excepting when determined by the Perkin Elmer, Model 18, flame photometer.

5. With soils of low to medium K content (0.02 to .3 m.e./100 g) ammonium acetate buffered at *p*H 4.8 with acetic acid, and $BaCl_2$ buffered at *p*H 8.1 with triethanolamine extracted substantially the same amount of K as *N* ammonium acetate at *p*H 7. *N* $NaNO_3$ at *p*H 7 and *N* $NaNO_3$ in 0.1 *N* HCl at *p*H 2 extracted somewhat less, and 25 per cent $NaNO_3$ extracted substantially more *K* than *N* ammonium acetate.

6. The Committee on Chemical Analyses of SSSA together with the referee and collaborators should prepare a detailed outline of a standard procedure to be followed. Once the procedure is agreed upon, future programs should be initiated to determine the following:

(a) The cause of systematic errors in the flame photometer (as evidenced by errors in slope) due either to instrument, analysts, or procedure.

(b) The cause of calibration errors (as evidenced by errors in intercept) again due to instruments, analysts, or procedure.

(c) The magnitude of day-to-day variations, eliminating differences between instruments, between analysts, and between procedures.

Grateful acknowledgement is made to the following for their active participation in this study: O. S. Attoe, University of Wisconsin; N. Gammon, Jr., University of Florida; W. L. Garman, formerly of Oklahoma, now at Cornell University; E. R. Graham, Missouri; R. S. Holmes and R. F. Reitemeier, U.S.D.A., Beltsville, Maryland; J. D. Lancaster, Mississippi Agricultural Experiment Station; S. W. Melsted, University of Illinois; R. V. Olson, T. Tucker, and D. Bouldin, Agricultural Experiment Station of Kansas; and R. Reed, Oklahoma Agricultural Experiment Station.

REPORT ON EXCHANGEABLE HYDROGEN IN SOILS

INTERRELATIONSHIP BETWEEN CALCIUM SORPTION, EXCHANGEABLE HYDROGEN AND *p*H VALUES OF CERTAIN SOILS AND SUBSOILS

By W. M. SHAW (Agricultural Experiment Station, Knoxville 16, Tenn.), *Associate Referee*

In his 1950 report,* the Associate Referee presented a procedure for the quantitative determination of the exchangeable hydrogen of soils, which consisted of the extraction and leaching of an appropriate soil charge with neutral 0.5 *M* Calcium-acetate to a total volume of 250 ml and the titration of the extract with 0.1 *N* barium hydroxide to *p*H 8.8. Concordance with the ammonium acetate procedure, simplicity of technique, and a high degree of precision commended this procedure for the routine determination of exchangeable hydrogen in soil. As a practical consideration, the neutral Ca-acetate solution was chosen in preference to electrolytes at higher *p*H values because it is nearer to *p*H 6.5. This has been recognized by leading soil scientists as the ideal reaction of soils for general crop production (4, 13, 29).

NEUTRAL CALCIUM ACETATE METHOD FOR DETERMINATION OF EXCHANGEABLE HYDROGEN

REAGENTS

(a) 0.5 *M Ca-acetate.*—Prepare 0.5 *M* calcium acetate by dissolving 176.1 g $Ca(C_2H_3O_2)_2 \cdot H_2O$ in 2 liters of H_2O; adjust to *p*H 7 by titration with 1 *N* acetic acid, using the glass electrode as indicator. (Ca 4 ml of the acid will be required per 2 liters.) Make supply needed for a week at a time, store in bottle provided with siphon and soda-lime tube.

(b) 0.1 *N* $Ba(OH)_2$—Dissolve 1/20 molar wt of $Ba(OH)_2 \cdot 8H_2O$, C.P. per liter, allowing a 2% excess for impurities and CO_2 contamination. Let stand 24 hours. Prepare a storage bottle connected with siphon and soda-lime guard tube and freed of CO_2. Draw $Ba(OH)_2$ soln into the prepared bottle by applying suction at the soda-lime tube and drawing the soln thru a sintered glass filter. Connect the $Ba(OH)_2$ soln with a 25 ml pinch cock burette, provided with side tube for gravity feed, and a soda-lime tube at the top.

DETERMINATION

Weigh soil charges calculated to contain 1–2 m.e. of exchangeable hydrogen; place in 250 ml Erlenmeyer flasks; introduce ca 100 ml of the Ca-acetate soln; stopper and shake several times during first hour. Let stand overnight. Filter soil suspension thru 12.5 cm filter papers, placed on 3-inch funnels resting upon 250 ml volumetric flasks. Transfer soil onto filter by the aid of stream of the soln; wash the soil with small quantities of the soln until the volume is just below the 250 ml mark. Remove funnel, discard soil, and make filtrate to volume with same soln. Transfer to 400 ml beaker; rinse flask with H_2O and titrate potentiometrically with $Ba(OH)_2$ to *p*H 8.8. Obtain also the titration value of 250 ml of the replacing

* *This Journal*, 34, 595.

soln. Calculate the exchangeable hydrogen in m.e. per 100 g of soil from the formula: ((Tu-Tb)/Wt.)×10, in which—

Tb equals titration value of replacing soln;
Tu equals titration value of soil extract, both expressed in ml of 0.1 *N* $Ba(OH)_2$;
Wt. equals weight of soil charge.

The acceptance of the Ca-acetate method for the determination of the exchangeable hydrogen content of soils to *p*H level of 7 still leaves a number of practical questions unanswered. First, will liming at a rate equivalent to the determined hydrogen content, and of such fineness as to effect complete reaction with the soil, produce a reaction of exactly 7. Second, if the soil reaction induced or anticipated from such liming rate is not the one desired, how can the rate of liming that would produce the desired *p*H be computed, say one of 6.0, upon basis of the determined content of exchangeable hydrogen? Third, if the liming rate for any desired *p*H can be calculated accurately from the determined exchangeable hydrogen, how can this information be translated into tons of ground limestone per acre to produce the desired *p*H, with due consideration for variation in chemical composition and fineness of liming materials? The evaluation of a limestone on the basis of its chemical and mechanical composition would require knowledge of the specific reaction rates of various limestone particles. Although this problem is important to practical agriculture and has been dealt with in a number of contributions (3, 12, 13, 25), the present report will concern itself with questions 1 and 2 only.

To supply the answer to the first question it would be necessary only to add $CaCO_3$ to a number of soils in quantities equivalent to their determined exchangeable hydrogen contents and to observe the resultant *p*H values after an appropriate period of incubation. The answer to the second question calls for a knowledge of the buffer properties of the soils, which are usually obtained through titration with either NaOH, $Ba(OH)_2$, or $Ca(OH)_2$ on soil suspensions (1, 5, 9, 17, 18, 19). In the present study, the titration was obtained through the interaction of solid $CaCO_3$ with soils for a period of 4 weeks, under moist conditions at 30°C. The plan of the present investigation and its broad objectives may be presented in the following outline:

1. The calcium build-up of a number of typical soils and subsoils will be accomplished through additions of finely divided calcite in increasing quantities from 10 to nearly 200 per cent of the estimated cation exchange capacities.

2. The Ca-built-up soils will be analysed for residual carbonate so that the Ca-sorptions can be ascertained, and these, rather than the calcite additions, correlated with resultant *p*H-values, and with exchangeable hydrogen contents. Also observations will be made on the effect of increases in added calcite upon the speed of attainment of maximal Ca-sorption, or Ca-saturation.

3. The *p*H values of the Ca-built-up soils will be plotted against Ca-sorptions to detect characteristic curves and transition points for the respective materials. The Ca-acetate method will be evaluated in terms of the Ca-sorptions at *p*H 7.0.

4. The exchangeable hydrogen will be determined on soils built up to different degrees of Ca-saturation through the use of the proposed Ca-acetate procedure, and the determined values will be compared with the computed values, as these are obtained from the exchangeable hydrogen in the starting soils, minus the determined Ca-sorptions. Upon the assumption that all the sorbed calcium was exchanged for hydrogen, each increase in the Ca-sorption should effect a corresponding decrease in hydrogen content of the soil.

5. The maximal Ca-sorptions from $CaCO_3$ additions will be compared with the exchange capacities obtained on the acidic soils, both by summation of exchangeable hydrogen and exchangeable Ca, and by NH_4-sorption from ammonium acetate.

6. Investigations will be made as to what extent the exchangeable hydrogen determinations may be affected by normal variations in technique of the Ca-acetate procedure, such as variation of soil charge, leaching volume, and duration of contact. Similarly an attempt will be made to determine the causes of decreasing *p*H values of soil systems during their incubation.

SOILS AND THEIR CALCIUM BUILD-UP IN RELATION TO CALCITE ADDITIONS

As in a previous study on exchangeable hydrogen methods (28), the following soils and subsoils were used as representative of several types of adsorption complexes:

1. A Hartsells fine sandy loam, obtained in Crossville, Tennessee, in 1947 which contained a moderate amount of undecomposed vegetative matter and kaolinitic clay material.

2. A Cumberland clay subsoil, obtained on the University farm at Knoxville in 1950, composed largely of kaolinitic clay high in hydrated ferric oxide and of meager content of organic matter.

3. A Susquehanna clay subsoil of 36–42 inch horizon, obtained from the Alabama Agricultural Experiment Station in 1950 through the courtesy of Dr. L. E. Ensminger, and containing about 32 per cent clay of Montmorillonitic type (22).

4. A Portsmouth sandy muck, obtained in 1950 from the Florida Agricultural Experiment Station through the courtesy of Dr. J. R. Neller, the adsorption complex of which was due almost entirely to organic matter content.

Technique of Ca-build-up.—The adsorbed calcium contents of these materials were determined through leaching with NH_4-acetate, and the exchangeable hydrogen contents were determined by means of extractions

TABLE 1.—*Residual carbonate, calcium sorption, and degree of saturation resultant of increasing additions of 325-mesh calcite to certain soils and subsoils, following a 4-week period of moist contact at 30°C.*

SOILS AND SUBSOILS, AND THEIR EXCHANGEABLE CATION VALUES	II CALCITE ADDITIONS PER 100 G	III CALCIUM SUPPLY, AS DEGREE OF SATURATION*	IV RESIDUAL $CaCO_3$ PER 100 G	V CALCIUM SORPTION PER 100 G	VI ADSORBED CALCIUM STATUS PER 100 G	VII ADSORBED CALCIUM STATUS AS DEGREE OF SATURATION*
	m.e.	*per cent*	*m.e.*	*m.e.*	*m.e.*	*per cent*
Hartsells fine sandy loam, 1947, with high content of undecomposed vegetative matter	0.0	10	0.0	0.0	1.0	10
	1.0	20	0.0	1.0	2.0	20
	3.0	40	0.0	3.0	4.0	40
Exchangeable:	5.0	60	0.0	5.0	6.0	60
Ca 1.0 m.e.	7.0	80	0.0	7.0	8.0	80
H 9.0 m.e.	9.0	100	0.3	8.7	9.7	97
Capacity 10.0 m.e.	11.0	120	0.6	10.4	11.4	114
	13.0	140	1.4	11.6	12.6	126
	15.0	160	2.6	12.4	13.4	134
	17.0	180	4.8	12.2	13.2	132
	20.0	220	7.5	12.5	13.5	135
Cumberland clay subsoil; Kaolinitic type virtually devoid of organic matter	0.0	12	0.0	0.0	1.0	12
	0.6	20	0.0	0.6	1.6	20
	2.2	40	0.0	2.2	3.2	40
Exchangeable:	3.8	60	0.0	3.8	4.8	60
Ca 1.0 m.e.	5.4	80	0.0	5.4	6.4	80
H 7.0 m.e.	7.0	100	0.0	7.0	8.0	100
Capacity 8.0 m.e.	8.6	120	0.2	8.4	9.4	118
	10.2	140	0.6	9.6	10.6	132
	11.8	160	1.7	10.1	11.1	139
	13.4	180	3.3	10.1	11.1	139
	15.0	200	5.0	10.0	11.0	138
	17.5	231	7.2	10.3	11.3	141
	20.0	262	9.5	10.5	11.5	144
Susquehanna clay subsoil, Montmorillonitic type of clay, virtually devoid of organic matter	0.0	9	0.0	0.0	2.6	9
	3.2	19	0.0	3.2	5.8	19
	9.0	39	0.0	9.0	11.6	39
	14.8	58	0.0	14.8	17.4	58
Exchangeable:	20.6	77	0.0	20.6	23.2	77
Ca 2.6 m.e.	26.4	97	2.0	24.4	27.0	90
H 27.4 m.e.	32.2	116	4.3	27.9	30.5	102
Capacity 30.0 m.e.	38.0	135	9.8	28.2	30.8	103
	43.8	155	14.4	29.4	32.0	107
	49.6	174	20.7	28.9	31.5	105
	55.4	193	27.1	28.3	30.9	103
Portsmouth sandy muck; adsorption complex nearly entirely of organic nature	0.0	4	0.0	0.0	2.3	4
	7.5	19	0.0	7.5	9.8	19
	17.3	37	0.0	17.3	19.6	37
Exchangeable:	27.1	56	0.0	27.1	29.4	56
Ca 2.3 m.e.	36.9	75	0.2	36.7	39.0	75
H 50.0 m.e.	47.7	96	0.8	46.9	49.2	94
Capacity 52.3 m.e.	56.5	112	2.6	53.9	56.2	107
	66.3	131	7.5	58.8	61.1	117
	76.1	150	16.3	59.8	62.1	119
	86.0	169	26.9	59.1	62.4	119
	100.0	196	38.6	61.4	63.7	122

* The computed degrees of base saturation are based upon the cation exchange capacities of the respective materials obtained by summation of the adsorbed Ca-content and exchangeable hydrogen by the Ca-acetate procedure of the original samples.

and leachings with neutral .5 *M* Ca-acetate. The sums of Ca and H were taken as the exchange capacities of the materials, and their values are given in column 1 of Table 1. The Mg and K contents of these samples were disregarded because the quantities involved were deemed unlikely to affect the over-all results of this experiment.

Calcite of 325-mesh was added to 100 g air-dry soil or subsoil in increments of approximately 20 per cent of the cation exchange capacities of the respective soils, inclusive of their initial calcium content and in range from 20 to 200 per cent of exchange capacity, or at least 20 m.e. per 100 g of soil. The actual quantities of calcite added and the approximate degrees of saturation which such additions were capable of effecting are given in columns II and III, respectively, of Table 1. The systems of the first two soils were contained in 150 ml beakers, whereas the other two systems were in 250 ml beakers. Water was added to complete saturation, overall weights were recorded, and water losses were replenished on Mondays and Thursdays of each week in the four-week period during which the beakers were kept immersed in water that was held constant at 30°C. The mixtures then were removed from the beakers, spread out on sheets of paper, and dried in a well ventilated room, free of laboratory fumes. The air-dry soils were ground to pass a 0.5 mm screen, mixed thoroughly, and stored in air-tight containers. The findings for residual carbonate contents of the various mixtures are given in column IV. The Ca-sorption data given in column V were obtained by subtracting each residual carbonate value from its corresponding calcite addition. The calcium-status data, column VI, differ from the calcium sorption data in that they are inclusive of the calcium native to the soil.

Results of Ca-build-up.—The data of Table 1 serve to supply the Ca-sorption status of the Ca-built-up soils in terms of m.e. per 100 g and as percentage of base saturation capacities. These data will be used further in conjunction with determinations of exchangeable hydrogen and *p*H values. First, however, several observations will be made with respect to the extent of calcite reactions as affected by rate of addition under the given experimental conditions.

Reliance for complete reaction with soils of 325-mesh calcite as affected by rate of addition can be seen from the residual carbonate data, Table 1. These data show complete carbonate decomposition of all additions up to 80 per cent of the estimated exchange capacities of each of the soils. Appreciably higher degrees of calcite decompositions were attained by the Cumberland subsoil (120 per cent) and the Hartsells soil (nearly 100 per cent). With increasing additions of calcite, the decompositions and Ca-sorptions progressed beyond the 100 per cent exchange capacity and showed a definite tendency for maximal Ca-sorptions, as can be seen best in the curves in Figure 1. The most consistent results on Ca-sorption were

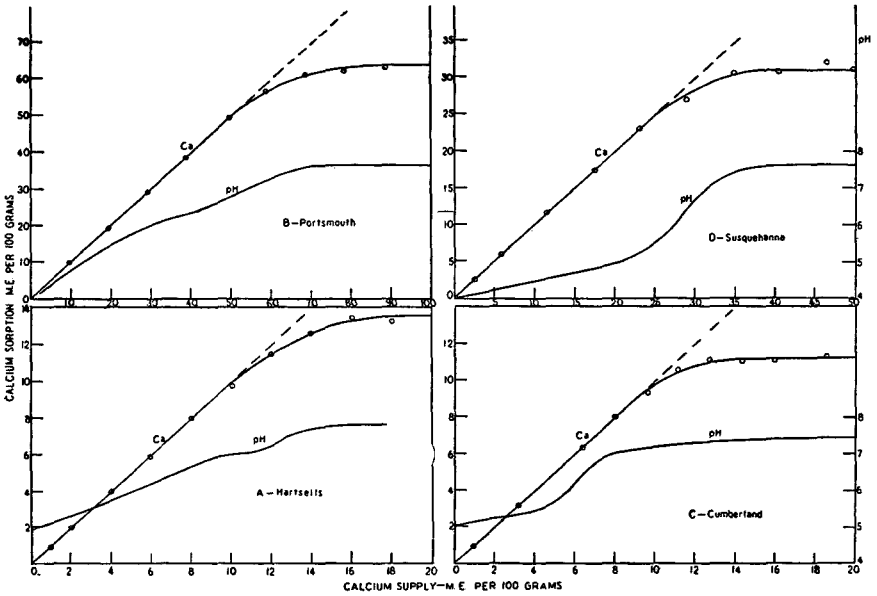

FIG. 1.—Calcium sorptions and *p*H values resultant from increasing addition of 325-mesh calcite after a 4-week moist contact period at 30°C.

A.—Hartsells sandy loam;
B.—Portsmouth muck;
C.—Cumberland clay subsoil;
D.—Susquehanna clay subsoil.

obtained on the Cumberland clay subsoil. The last appreciable gain in Ca-sorption by this subsoil was from the 11.8 m.e. calcite addition, which showed a calcite residue of 1.8 m.e. The four additional calcite increments resulted in $CaCO_3$ residues of 3.3, 5.0, 7.2, and 9.5 m.e., whereas the average increase in Ca-sorption was only .1 m.e. per each 2 m.e. Calcium carbonate excess. It appears that an excess of 5.0 m.e. of 325-mesh calcite per 100 g is adequate to attain maximal Ca-sorption of the Cumberland clay loam.

The last appreciable gain in Ca-sorption by the Susquehanna clay subsoil was at the point of 32.2 m.e. calcite addition, which showed a residue of 4.3 m.e. of calcite. Small, indecisive gains in Ca-sorption were effected by four additional 5.8 m.e. calcite increments which showed respective residues of 9.8, 14.4, 20.7, and 27.1 m.e. It appears that the Susquehanna subsoil effected a maximal Ca-sorption from an excess of 5 m.e. of calcite per 100 g and that this value was not appreciably changed by calcite excesses up to 27.0 m.e.

The last appreciable gain in Ca-sorption of the Hartsells soil was in the

range between the 12 and 15 m.e. additions of calcite. Gains of .2 to .4 m.e. were induced by succeeding additions of 17 and 20 m.e. but are deemed of little significance. The maximal Ca-sorption by the Hartsells soil was about 12.4 m.e. when calcite excess was 5 to 10 m.e.

The Ca-sorption by the Portsmouth muck with reference to increased calcite addition appears to reach a maximum of 59 m.e. at the point of 66.3 m.e. addition, giving a calcite residue of 7.5 m.e. The maximal Ca-sorption attributable to calcite additions of 76.1, 86.0, and 100 m.e. are within 1 m.e., plus or minus, of a total of 62 m.e.

The conclusion from the results on Ca-sorptions from 325-mesh calcite by soils and subsoils in moist contact at 30°C. may be stated as follows:

1. Calcite additions up to 80 per cent of the base saturation capacities of soils, inclusive of native Ca supplies, will be decomposed completely within a period of four weeks.

2. Calcite additions above the soil's saturation capacity will effect Ca-sorptions beyond the cation exchange capacity as determined by neutral salt methods, but will leave variable residues of calcite.

3. Maximal Ca-saturation of soils can be attained in four weeks' contact, provided the calcite excess after contact is between 5 and 10 m.e. per 100 g of soil.

4. The increased Ca-sorptions from contact with calcite over that of cation exchange capacities differ according to the type of exchange complex.

a. The kaolinitic Cumberland clay subsoil registered an increase of 40 per cent.

b. The Montmorillonitic Susquehanna clay subsoil showed about 4 per cent increase.

c. The Organic Portsmouth muck registered a 20 per cent increase.

d. The Kaolinitic-organic Hartsells soil registered an increase of about 32 per cent.

RELATIONSHIP BETWEEN *p*H VALUES AND PERCENTAGES OF BASE SATURATION OF EXPERIMENTAL SOILS

The *p*H values were determined by means of the glass electrode on soil-water suspensions of pasty consistency after one hour's contact. The results are given in Table 2 alongside the computed values of percentage base saturation of the Ca-built-up soils. Although maximal values for calcium sorptions were induced by the excessive additions of calcite, the *p*H values registered by such soils were only between 7.5 and 7.8. The *p*H determinations were carried out in a room with free outdoor air circulation, but no attempt was made to equilibrate the soil-water systems with air of .03 per cent carbon dioxide tension that would have resulted in much higher values (7, 8).

The primary objective in the determination of the *p*H values of the Ca-built-up soils was to determine whether or not those soils with Ca-

TABLE 2.—*Effect of gradual build-up of adsorbed calcium upon the exchangeable hydrogen contents, exchange capacities and pH values of certain soils and subsoils*

SOILS AND SUBSOILS		I	II	III	IV	V	VI	
		ADSORBED CALCIUM BUILD-UP, DEGREE OF SATURATION	REACTION IN H_2O PASTE	EXCHANGEABLE HYDROGEN CONTENT		APPARENT EXCHANGE CAPACITY (Ca+H)	DEVIATION IN EXCHANGE CAPACITY FROM INITIAL STATUS	
TYPE	PREDOMINANT COLLOID			COMPUTED*	DETERMINED			
		per cent	*pH*	*m.e.*	*m.e.*	*m.e.*	*m.e.*	*per cent*
Hartsells fine sandy loam, 1947	Organic-Kaolinitic	10	5.1	9.0	9.1	10.1	0.1	1
		20	5.3	8.0	8.1	10.1	0.1	1
		40	5.8	6.0	6.5	10.5	0.5	5
		60	6.2	4.0	5.1	11.1	1.1	11
		80	6.7	2.0	3.8	11.8	1.8	18
		97	7.0	0.3	2.7	12.4	2.4	24
		114	7.4	− 1.4	1.6	13.0	3.0	30
		126	7.7	− 2.6	1.0	13.6	3.6	36
		134	7.8	− 3.4	0.8	14.2	4.2	42
		132	7.8	− 3.2	0.8	14.0	4.0	40
		135	7.8	− 3.5	0.7	14.2	4.2	42
Cumberland clay subsoil, 1950	Kaolinitic	12	5.2	7.0	7.5	8.5	0.5	6
		20	5.2	5.4	6.8	8.4	0.4	5
		40	5.3	3.8	5.2	8.4	0.4	5
		60	5.6	2.2	3.6	8.4	0.4	5
		80	5.8	0.6	2.6	9.0	1.0	12
		100	7.0	− 1.0	1.5	9.5	1.5	20
		118	7.4	− 2.4	0.8	10.2	2.2	28
		132	7.6	− 3.6	0.2	10.8	2.8	35
		139	7.7	− 4.1	0.0	11.1	3.1	39
		139	7.7	− 4.1	0.0	11.1	3.1	39
		138	7.7	− 4.0	0.0	11.0	3.0	38
		141		4.3	0.0	11.3	3.3	41
		144	7.8	− 4.5	0.0	11.5	3.5	44
Susquehanna clay subsoil, Alabama, 1950	Montmorillonitic	9	4.1	27.4	27.2	29.8	−0.2	−1
		19	4.3	24.2	23.6	29.4	−0.6	−2
		39	4.6	18.4	16.9	28.5	−1.5	−5
		58	4.8	12.6	11.0	28.4	−1.6	−5
		77	5.2	6.8	5.8	29.0	−1.0	−3
		90	6.4	3.0	2.9	29.9	−0.1	0
		102	7.5	− 0.5	0.7	31.2	1.2	4
		103	7.5	− 0.8	0.5	31.3	1.3	4
		107	7.5	− 2.0	0.1	32.1	2.1	7
		105	7.5	− 1.5	0.0	31.5	1.5	5
		103	7.5	− 0.9	0.0	30.9	0.9	3
Portsmouth sandy muck, Florida, 1950	Organic	4	4.1	50.0	48.6	50.9	−1.4	−3
		19	4.7	42.5	39.8	49.6	−2.7	−5
		37	5.3	32.7	31.7	51.3	−1.0	−2
		56	6.0	22.9	22.8	52.2	−0.1	0
		75	6.3	13.3	15.2	54.2	1.9	4
		94	6.7	3.1	8.6	57.8	5.5	10
		107	7.2	− 3.9	5.0	61.9	9.6	9
		117	7.6	− 8.8	3.1	64.2	11.9	23
		119	7.6	− 9.8	2.1	64.2	11.9	23
		119	7.6	−10.1	1.8	64.2	11.9	23
		122	7.6	−11.4	1.5	65.2	12.9	25

* The computed values for exchangeable hydrogen content were obtained by difference between the Ca-sorption, column V, and the hydrogen content of the respective initial soil or subsoil, column I, both of Table 1.

sorptions equivalent to the original exchangeable hydrogen content would show a reaction of *p*H 7. Of more general usefulness, however, would be the correlation of *p*H with the varying percentages of Ca-saturation afforded by these data on the four representative types of adsorption complexes. The data of Table 2 were plotted as curves in Figure 2 to

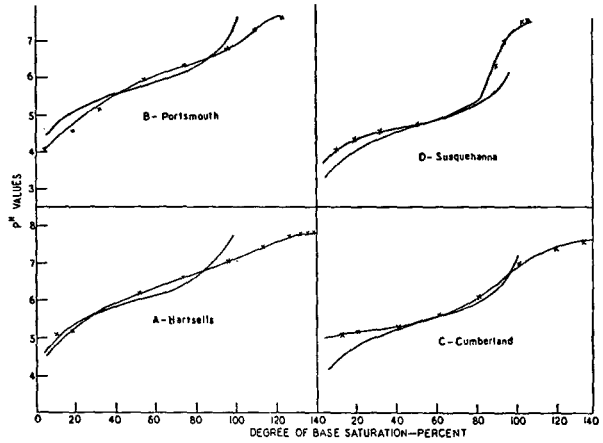

FIG. 2.—*p*H vs. percentage base saturation curves: experimental, after reaction with calcite during a period of 4 weeks at 30°C., and theoretical, based on estimated *p*K values of the materials (fine line).

A.—Hartsells sandy loam;
B.—Portsmouth muck;
C.—Cumberland clay subsoil;
D.—Susquehanna clay subsoil.

show the *p*H values as continuous functions of the percentage base saturation.

Inspection of *p*H values at points of 100 per cent base saturation (Figure 2) reveals that three of the four soils that had reacted with calcite to the full extent of the exchangeable hydrogen indications have shown *p*H of 7.0 or 7.1, whereas the fourth, the Susquehanna subsoil, registered a *p*H of 7.4. The discrepancy in terms of base saturation for this particular clay amounts to 6 per cent, or 1800 pounds in 30,000 pounds of calcium carbonate per 2,000,000 pounds of clay. These experiments indicate that liming with calcium carbonate in equivalence of the exchangeable hydrogen content as determined by the Ca-acetate procedure will result in soil reactions of or close to *p*H 7, provided the calcium carbonate is of sufficient fineness to react completely in the experimental interval.

Upon assumption that the exchangeable hydrogen determination gives the true value of the calcium carbonate required to raise the soil's *p*H to

7, the problem arises as to how this determination can be utilized in calculating the quantities of limestone required to attain *p*H values other than 7. Theoretically, the exchangeable hydrogen value of a particular soil may be utilized to give specific values in the generalized formula that expresses relationship between *p*H values of a soil and its percentage base saturation. Such a formula was suggested by Bradfield (6), further developed by Peech (23), and later presented by Peech and Bradfield (24) in the form:

$$p\text{H} = \text{constant} + \log \frac{[\text{Exchange capacity} - \text{Exchangeable H}]}{[\text{Exchangeable Hydrogen}]}$$

After the constant of a particular soil type has been once established, a curve may be constructed to show the general relationship between the *p*H and the percentage of base saturation. It may be noted that, due to the method of computation, the contour of this theoretical curve is identical for all soil types. Differences in soil type will result only in the horizontal displacement of the entire curve with reference to *p*H scale. The position on the *p*H scale is governed entirely by the constant, *p*K, which is defined as the *p*H value at the point of 50 per cent base saturation, and is characteristic of the predominant adsorption complex of the soil. Such theoretical curves with *p*K values corresponding to the *p*H values at the 50 per cent base saturation are given in Figure 2. By means of these theoretical curves, we shall attempt to calculate the calcium needs of the same four soils and subsoils to effect reactions of *p*H 5.5 and 6.5, as indicated below.

The initial *p*H for the Hartsells soil was 5.1 and the exchangeable hydrogen was 9.0 m.e.; also, at *p*H 5.1 (figure 2A), the corresponding percentage saturation is 12. It follows that the 9.0 m.e. exchangeable hydrogen is equivalent to 100 minus 12, or 88 per cent base saturation, from which the exchange capacity is found equal to $(9/88)\times100$, or 10.2 m.e. At *p*H 5.5, the base saturation on the theoretical curve (Figure 2A) is 22 per cent; the computed calcium need to induce *p*H 5.5 is expressed by the values of $(.22-.12)\times10.2$, or 1.02 m.e. At *p*H 6.5, the percentage base saturation is 77; the computed calcium need to induce *p*H 6.5 is $(.77-.12)\times10.2$, or 6.63 m.e.

Similar computations for the Portsmouth muck (Figure 2B), with *p*H 4.1 and exchangeable hydrogen of 50 m.e., give an exchange capacity of $(50/100\text{–}2)\times100$, 51.0 m.e., and the computed requirement for *p*H 5.5 is $(.35-.02)\times51$, or 16.8 m.e.; and for *p*H 6.5, $(.85-.02)\times51$, or 42.3 m.e.

For the Cumberland subsoil (Figure 2C) with *p*H 5.2 and exchangeable hydrogen of 7.0 m.e., the exchange capacity becomes $(7.0/100\text{–}38)\times100$, or 11.3 m.e.; the calcium need for *p*H 5.5, $(.55-.38)\times1.92$, and for *p*H 6.5, $(.93-.38)\times11.3$, or 6.22 m.e.

For the Susquehanna subsoil (Figure 2D) with *p*H 4.1 and exchangeable

hydrogen 27.4 m.e., the exchange capacity becomes (27.4/100–20) ×100, or 34.2 m.e.; the calcium requirement for *p*H 5.5 is (.87 − .20) ×34.2, or 27.0 m.e.

The calculated calcium carbonate requirements for *p*H 5.5 and 6.5 according to theoretical titration curves are summarized in Table 3 in parallel with corresponding values found on the experimental curves. The

TABLE 3.—*Lime requirements for p*H *5.5 and 6.5 according to theoretical and experimentally established titration curves*

SOIL AND SUBSOIL	INITIAL STATUS				LIME REQUIREMENTS, M.E./100G			
	PERCENTAGE SATURATION			EXCH.	THEORETICAL FOR		EXPERIMENTAL FOR	
	*p*H	THEOR.	EXP.	H	*p*H 5.5	*p*H 6.5	*p*H 5.5	*p*H 6.5
				m.e.				
Hartsells sandy loam	5.1	12	10	9.0	1.02	6.63	1.20	5.6
Portsmouth muck	4.1	2	4	50.0	16.80	42.30	17.80	41.8
Cumberland subsoil	5.2	38	12	7.0	1.92	6.22	2.64	6.32
Susquehanna subsoil	4.1	20	9	27.4	22.90	27.00	21.90	24.30

greatest differences between the experimental and the theoretical *p*H curves occur in the initial and last stages of base saturation. In the experimental curve, the *p*H values fail to make the sharp downward trend from 20 to 0 and the sharp upward trend from 80 to 100 per cent base saturation. It follows that, where the initial base saturation of the soil is less than 25 per cent, considerable error may be incurred through the evaluation of exchange capacity upon basis of the determined *p*H value and the quantity of exchangeable hydrogen.

It may be of interest to compare saturation curves obtained in this experiment with curves on similar materials obtained by Mehlich (17, 18). The Kaolinitic subsoil, Fig. 2C, shows a distinct inflexion point at about the 90 per cent base saturation, whereas the curve for kaolinitic obtained by Mehlich (17, Figure 1) shows no such inflexion point. The Montmorillonitic Susquehanna clay shows a distinct inflexion point at the 90 per cent base saturation, whereas in the Bentonite curve obtained by Mehlich no such inflexion point is indicated. The curves obtained by the use of 325-mesh calcite in the two subsoils resemble closely the titration curves that were obtained by Marshall and Krinbill (16). Our Portsmouth muck gave a titration curve quite similar to that given by Mehlich (17, Figure 2). The differences in the presently reported types of curves and those obtained by Mehlich on the Kaolinitic and Montmorillonitic clays may be due to difference in the method of neutralization. Mehlich neutralized his acid clays with $Ba(OH)_2$ in 1 to 2 suspensions of 48 hours contact, whereas the present curves were based upon reaction of the acid clay with calcite during a period of 4 weeks.

From the foregoing discussion of the experimental data, it is evident that the calcium carbonate needs for any desired *p*H of a soil can be determined from the *p*H value and exchangeable hydrogen determination by the calcium acetate procedure, provided the *p*K value of the soil type is known.

RELATION BETWEEN Ca-BUILD-UP THROUGH CALCITE ADDITIONS AND EXCHANGEABLE HYDROGEN CONTENT BY THE Ca-ACETATE PROCEDURE

The principal objective of this phase of the study was to subject the results on exchangeable hydrogen to an accuracy test, as this may be affected by variation of degree of Ca-saturation of soils. More specifically, the purpose was to check upon the capabilities of the procedure to evaluate correctly larger as well as smaller quantities of exchangeable hydrogen, within the prescribed limits of 2 m.e. per determination and a 250 ml leaching volume. Moreover, the determined exchangeable hydrogen values plus the Ca-sorption on a series of calcite-treated soils afford an opportunity to observe the effect of increasing Ca-sorption upon the apparent exchange capacities of the several materials. This particular test of correctness is based upon the premise of equivalent exchange of calcium ions for the hydrogen ions. If this premise is valid, the sums of the Ca-sorptions and of the exchangeable hydrogen should yield a constant for any particular soil which had been neutralized to varying degrees of base saturation—at least within systems whose *p*H is below 7. It is to be expected that within a series of increasing calcite additions some Ca-sorptions would exceed the cation exchange capacities of the materials by reason of the higher *p*H values of the systems containing excesses of calcium carbonate (7, 12).

The results of the exchangeable hydrogen determinations by the Ca-acetate procedure are given in column IV, Table 2, in parallel with the computed values of same in column III. The computed values were arrived at by the determination of the exchangeable hydrogen on the starting materials and corrected by subtraction for the determined Ca-sorption after contact with calcite, as given in column V of Table 1. The negative values in column III, Table 2, indicate the extent of Ca-sorptions in excess of the determined exchangeable hydrogen of the original soil or subsoil. To facilitate the observations on effects of increasing Ca-sorptions and rise in *p*H values upon the decreasing hydrogen contents and upon the combined values of both Ca+H, portions of the data of Table 2 are presented graphically in Figure 3 (a, b, c, and d).

Each section of Figure 3 contains four types of curves: (a) the Ca-sorption, (b) the hydrogen content, (c) the sum of Ca and H—all expressed in m.e. per 100 g of soil—and (d) the *p*H curve. The general trends of these values are: first, the rapid rise of Ca-sorptions in direct proportion to the additions in the regions below 80 or 90 per cent of cation exchange capacity; next, a slackening in the rate of adsorption until maximal values are at-

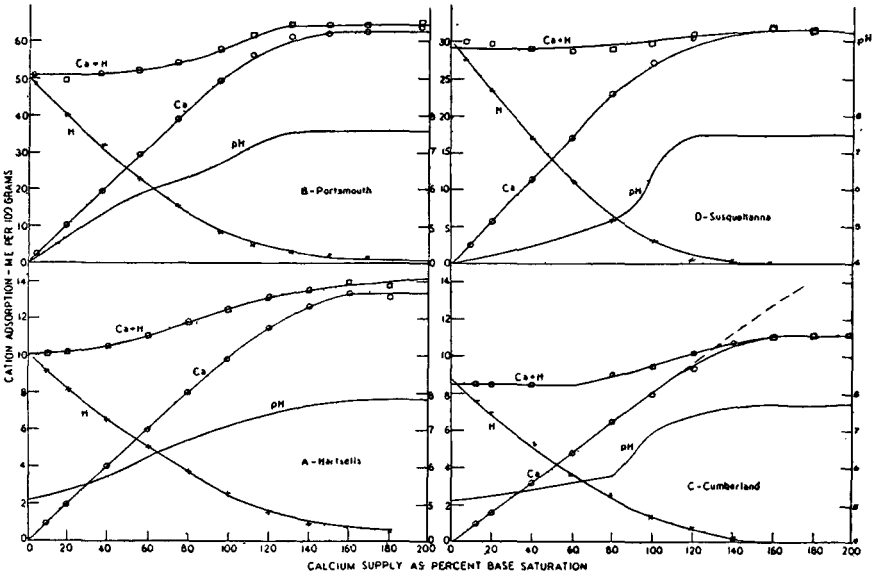

FIG. 3.—Calcium sorptions, exchangeable hydrogen, sum of Ca+H, and *p*H values, as affected by calcite additions, expressed in percentage base saturation of the respective materials.

A.—Hartsells sandy loam;
B.—Portsmouth muck;
C.—Cumberland clay subsoil;
D.—Susquehanna clay subsoil.

tained in the regions of 105 to 140 per cent Ca supplies; a corresponding descent of exchangeable hydrogen values approaching or attaining zero values at points corresponding to maximal Ca-sorption; and the Ca+H curve which starts on the acid side in a horizontal line that extends to points of 40 per cent base saturation and beyond, followed by an upward trend to a new horizontal line of maximal sorption.

Closer inspection of the several curves reveals certain features relative to the Ca-H interchange that deserve a more detailed examination. In the case of the Cumberland (Kaolinitic) clay subsoil, Figure 3C, the horizontal line formed by the four points from the acid side on the Ca+H curve supports the validity of the postulation of equivalent calcium-hydrogen interchange, and proves that the Ca-acetate procedure yields results in accord with the computed values for exchangeable hydrogen. On the other hand, the constant value for the three points on the alkaline side of the same curve indicates a Ca-sorption capacity of 11.2 m.e., or an increase of 2.8 m.e. over the apparent cation exchange capacity as indicated on the acid side of the same curve. Furthermore, the transition towards

the higher apparent exchange capacity (Ca+H), begins long before the exchangeable hydrogen has been completely replaced and when the *p*H of the system is not quite 6. At the point of 100 per cent Ca-supply and *p*H 7, the Ca-sorption is .4 m.e. short of 100 per cent cation exchange capacity; while, at the same time, the system still shows an exchangeable hydrogen content of 1.5 m.e. At the point of 120 per cent Ca-supply, the *p*H is 7.4, the Ca-sorption is 9.4 m.e., or 1.0 m.e. in excess over the cation exchange capacity, while the exchangeable hydrogen residue is still .8 m.e.

The several features regarding the Ca-H interchange discussed in connection with the kaolinitic clay subsoil prevail also with even greater emphasis in the Ca-H relationships of the Portsmouth muck (Fig. 3B) and the Hartsells sandy loam (Fig. 3A). In the Portsmouth muck, the increased sorption capacity (Ca+H) begins with points 80 and 100 per cent Ca-supply and at *p*H values between 6.0 and 6.7. In the Hartsells soil, this increase extends over the points of 60, 80, and 100 per cent Ca-supply and in the *p*H range of 6.2 to 7.0. At the last point, the increased sorption capacity is 2.0 m.e. above that normally shown at *p*H values below 5.8, and in the presence of an exchangeable hydrogen residue of 2.7 m.e. In both of these soils the calcium sorptions increased progressively with increase in calcite addition and paralleled the rise in *p*H above 7 until attainment of constants for the four-week period of both *p*H and Ca-sorption values. The susquehanna subsoil, representing the Montmorillonitic type of clay, alone showed no increase in sorption capacity at *p*H below 7, and only a meager (4 per cent) increase in sorption capacity in the *p*H regions above 7.

The above observations with respect to Ca-H interchange, as applying to organic and kaolinitic complexes, give emphasis to two phenomena that require further interpretations: (a) the increased sorption capacity (Ca+H) of soils in the *p*H regions between 6 and 7; and (b) the continued presence of exchangeable hydrogen in the Ca-sorption systems above *p*H 7. The combined effect of these phenomena is an anomalous situation in which the soil has attained a calcium sorption in equivalence of the hydrogen indication by neutral salt replacement and has a *p*H value of 7 or above, yet upon renewed contact with neutral Ca-acetate solution manifests further release of exchangeable hydrogen. One explanation that may be offered to account for these phenomena is that the heterogeneity of the calcite-soil system is of such nature that Ca-sorption at higher *p*H than 7 and Ca-H interchange at lower *p*H than 7 can take place simultaneously at different micro-zones of the reaction system while the averaged *p*H of the mixture is still below 7.

The findings that bear on the relationship of Ca-sorption build-up and exchangeable hydrogen may be summarized as follows:

1. On organic and kaolinitic complexes, the Calcium built-up soils up to

*p*H 6 yield exchangeable hydrogen values which, when added to the Ca-sorption values, give constants that express cation exchange capacities.

2. Beyond the *p*H 6, the added values of the sorbed Ca+H increasingly exceed the determined cation exchange capacity of the original materials.

3. The increased Ca-sorption above *p*H 7 does not preclude the presence of residual exchangeable hydrogen as determined by means of neutral Ca-acetate extraction.

4. The simultaneous Ca-sorption beyond the cation exchange capacity and the presence of exchangeable hydrogen at *p*H values about 7 is explained by the probable micro-heterogeneity of the soil-calcite system which permits reactions above and below *p*H 7 at different points.

5. The Montmorillonitic Susquehanna subsoil shows only a slight increase in sorption capacity in the alkaline range and no increase whatsoever in the *p*H range of 6 to 7.

6. Further inquiry will be made as to the nature of the excessive Ca-sorption outside the limits of ordinary cation exchange capacity of organic and kaolinitic soils.

ON THE NATURE OF CALCIUM SORPTION AT *p*H BELOW 7

The demonstrated phenomenon of increasing Ca-sorption from calcite by organic and kaolinitic materials at or below *p*H 6 has the effect of increasing the exchange capacity of the soil when such is obtained by the summation of the sorbed Ca and H. According to Bradfield (5), acid soils undergo two types of reactions upon titration with bases: one, that of neutralization of the exchangeable hydrogen, and the other, the disintegration of the colloidal material. In the titration of electrodialyzed clay, characterizing the first type of reaction, $Ba(OH)_2$ gave an inflexion point at *p*H 7, whereas a similar titration with NaOH gave a transition between *p*H 8.2 and 8.5. On the other hand, Kelley and Brown (11) reported experiments in which a number of soils were treated with $Ca(OH)_2$, and the exchange capacities were measured through NH_4 adsorption from neutral NH_4Cl before and after such treatment. Their results showed that although the adsorbed Ca, resulting from $Ca(OH)_2$ treatment, was from 2 to 3 times as great as the adsorbed NH_4, the exchange capacities of the soils before and after such treatment showed little change. Commenting upon the above findings sometime later, Kelley (12, p. 77) postulated, "Had the replacement been effected with an ammonium salt solution, the *p*H of which was as high as that of the soil after having been treated with $Ca(OH)_2$, it is highly probable that much greater amounts of NH_4 would have been adsorbed." Kelley did not envision any destructive effect upon the soil complex as result of the highly alkaline $Ca(OH)_2$ solution.

Replaceability of sorbed Ca by NH_4.—The NH_4 adsorptions from neutral ammonium acetate were determined on the several experimental materials which were built up to different degrees of base saturation falling below

TABLE 4.—*Comparison of adsorbed (Ca+H) with that of NH_4 as affected by degree of base saturation and pH value*

SOIL OR SUBSOIL	CATION EXCHANGE CAPACITY	Ca SUPPLIED	ADSORBED Ca			ADSORBED Ca+H	ADSORBED NH_4
			QUANTITY	DEGREE OF SATURATION	pH		
	m.e.	*me.*	*m.e.*	*per cent*		*m.e.*	*m.e.*
Hartsells sandy loam, 1947	10.0	1.0	1.0	10	5.1	10.1	10.7
		6.0	6.0	60	6.2	11.1	10.6
		10.0	9.7	97	7.0	12.5	10.8
		16.0	13.4	134	7.8	13.8	11.3
Cumberland clay subsoil, 1950	8.0	1.0	1.0	12	5.2	8.4	9.7
		4.8	4.8	60	5.6	8.4	9.9
		9.6	9.4	118	7.4	10.2	9.7
		12.8	11.1	139	7.7	11.1	9.8
Susquehanna clay subsoil, 1950	30.0	2.6	2.6	9	4.1	29.5	28.1
		17.4	17.4	58	4.8	28.5	28.6
		29.0	27.0	90	6.4	29.5	29.5
		34.8	30.5	102	7.5	31.0	29.8
Portsmouth muck, 1950	52.0	9.8	9.8	19	4.3	50.5	48.0
		29.4	29.4	56	6.0	51.0	51.2
		50.0	49.2	94	6.7	53.5	52.0
		68.6	61.1	117	7.6	62.5	48.2

and above *p*H 7. The results are given in Table 4 in comparison with the sums of adsorbed Ca+H, *p*H values and degrees of base saturation. Although the Ca+H of the Hartsells soil increased from 10.1 to 13.8 with increase in *p*H from 5.1 to 7.8, no appreciable change is indicated in the NH_4 sorption of these soils. Similarly, as result of reaction with calcite, the Portsmouth muck showed increases of Ca+H from 50.5 m.e. to 62.5 m.e., whereas the adsorbed NH_4 remained practically constant at about 50 m.e. The Cumberland clay subsoil showed increases in the Ca+H values from 8.4 to 11.1 and *p*H changes from 5.2 to 7.7, whereas the NH_4 adsorption remained constant at 9.8 m.e. With the exception of the Cumberland clay subsoil, the NH_4 adsorptions checked perfectly with the sums of Ca+H on all of the original acidic materials. The untreated Cumberland subsoil showed a sorption of NH_4 distinctly greater than the sum indicated by summation of the Ca and H values. These data on the Ca-sorbed soils from calcite confirm in a general way the findings reported by Kelley and Brown for $Ca(OH)_2$-treated soils to the effect that increased Ca-sorptions from calcite at *p*H 7 or above are not reflected in any increased NH_4 adsorption from *neutral* ammonium acetate solution.

The most alkaline soils of each group given in Table 4 then were extracted with an especially prepared ammonium acetate solution which

was adjusted to *p*H 8, following the aforementioned postulation by Kelley (12), as to probable higher NH_4 adsorption from ammonium acetate of higher *p*H value. The NH_4 adsorptions from the acetate at *p*H 7 on the Hartsells, Cumberland, Susquehanna, and Portsmouth soils were respectively 11.3, 9.8, 29.8, 48.2 m.e. against corresponding values of 10.5, 9.7, 29.5, 43.4 m.e. from the acetate at *p*H 8. In no instance has the acetate solution at *p*H 8 caused any increase in the NH_4 adsorption; on the contrary, it has caused a decrease in NH_4-sorption on the soils with high content of organic matter. Since most surface soils do contain organic matter, we may conclude that the use of ammonum acetate solutions at *p*H 8 will result in lower NH_4 adsorptions than would be obtained from solutions at *p*H 7.

THE INDEFINITE EXTENT OF THE HYDROGEN REPLACEMENT THRU LEACHING WITH Ba- AND Ca-ACETATE SOLUTIONS AT *p*H 7

The practical advantages from the use of Ca-acetate solution for the replacement of exchangeable hydrogen and its determination have been described in an earlier publication (28). The accuracy of the procedure then was established through the concordance of the results by it and those obtained by the use of ammonium acetate on a number of soils. The ammonium acetate method is rated as standard for exchange capacity determinations at *p*H 7 and, therefore, also for the replacement of exchangeable hydrogen at same *p*H. Peech and Bradfield (24, p. 39) pointed out that "because of the high concentration of ammonium ions, the amount of exchangeable hydrogen replaced by leaching the soil with neutral *N* ammonium acetate solution corresponds more closely to that given by the potentiometric titration of the hydrogen saturated soil with NH_4OH to about *p*H 8.2, which represents approximately the equivalence point." Parker (21) has shown that the hydrogen replaced through the leaching of acid soils with neutral Ba-acetate solution is equivalent to the amount of $Ba(OH)_2$ required in the titration to raise the *p*H of these soils to 7. Because of the increased Ca+H sorption from reaction with calcite at or below *p*H 7, it appeared pertinent to inquire whether the neutral Ca-acetate also had the capacity to effect increased hydrogen replacements under conditions other than those followed by the routine procedure. Accordingly, laboratory experiments were set up for the extraction and the titration of exchangeable hydrogen in typical organic-kaolinitic soils under conditions of larger volume of leachate, longer time of contact, and decrease in weight of analytical sample.

Effect of Leaching Volume and Time of Contact.—The effects of increase in volume of leaching with neutral Ba-acetate solution and variation in preliminary contact period are given in Table 5. The effect of time of contact is indicated at two stages: first, in the difference of results between the 1-hour and the 16-hour contacts prior to the first 100-ml. extraction; secondly, in a similar difference in the results upon the fifth 100 ml

TABLE 5.—*Effect of contact period and volume of extraction with neutral barium acetate solution upon exchangeable hydrogen results*

SOIL AND EXTRACTION TECHNIQUE		EXCHANGEABLE HYDROGEN FROM SUCCESSIVE 100 ML EXTRACTIONS—M.E./100 G					
		1	2	3	4	5	TOTAL
Hartsells sandy loam, 1947	1	6.2	1.2	0.6	0.3	0.8*	9.1
10 g air dry, 1 hr. in	2	6.0	1.4	0.6	0.3	0.8	9.1
contact with 50 ml solu-	3	6.2	1.2	0.6	0.3	0.8	9.1
tion, leachings on Shimer filter.	Av.	6.1					9.1
Same	1	8.0	0.4	0.3	0.2	0.2	9.1
10 g preliminary contact	2	7.6	0.4	0.3	0.2	0.2	8.7
16 hrs., otherwise same as	3	7.6	0.4	0.3	0.2	0.2	8.7
above	Av.	7.7					8.8
Portsmouth muck, 1940	1	36.6	10.8	6.0	3.4	5.4*	62.2
5 g air dry; 1 hr. contact	2	37.0	10.6	6.4	3.2	5.4	62.6
with 50 ml solution; leach-	3	37.0	10.4	6.4	3.2	5.4	62.4
ings on Shimer filter.	Av.	36.9					62.4
Same	1	48.4	6.6	3.2	2.5	1.6	62.3
5 g air dry; 16 hr. prelimi-	2	46.4	5.8	2.8	2.5	1.6	59.1
nary contact, otherwise	3	47.6	6.0	3.0	2.5	1.6	60.7
same as above.	Av.	47.5					60.7

* These results were effected by a 16-hour contact, instead of 1-hour which obtained for others in this column.

extraction. The proportion of the total extracted from the Hartsells soil by the first 100 ml of the 1-hour contact was 67, whereas that from the 16-hour contact was 85 per cent. The proportions of exchangeable hydrogen extracted from the Portsmouth muck in the two corresponding contact periods were 58 and 78 per cent, respectively. The larger initial hydrogen replacement in the first 100 ml from the longer contact period prior to the first 100 ml extraction resulted in smaller hydrogen recoveries in the subsequent 100-ml extractions. Although it is true that each following extraction yielded less and less hydrogen, it is also equally true that there is no indication of hydrogen exhaustion in the fifth 100 ml extraction. This phenomenon is more obvious on the high hydrogen-containing Portsmouth muck than on the Hartsells soils of much less hydrogen content. Moreover, in the fifth extraction, where the indications were .2 and 1.6 m.e. for respective hydrogen replacement from the Hartsells and the Portsmouth soils, the additional intervening 15-hour contact period resulted in a boost of these values to .8 and 5.4 m.e., respectively. From the results

in Table 5, it is obvious that the process of hydrogen replacement by neutral solutions of barium acetate is of continuous nature, and that the final results are influenced by the volume of leachings and by the duration of contact prior to and during the leaching process.

It is also of interest to note that, in the five 100-ml extractions with the neutral Ba-acetate solution, the Portsmouth muck yielded between 61 and 62 m.e. of exchangeable hydrogen, a quantity which equals the maximal Ca-sorption attained by contact with excessive amounts of 325-mesh calcite in a period of 4 weeks. The important fact in this comparison is that in the hydrogen replacement with Ba-acetate solution the reactions of the system never exceeded the *p*H of 7, whereas in the soil-calcite system the *p*H was somewhere between 7.6 and 8.4. The five 100 ml extractions of the Hartsells soil failed to duplicate the record of hydrogen replacement on the Portsmouth soil in that the maximal value by the Ba-acetate was only 9.1 m.e. against the 12.4 m.e. from the 4-week reaction with calcite. Repeated extractions with Ba-acetate of the Cumberland clay subsoils was attempted but could not be carried out because the tightening of the clay precluded leaching beyond the 200 ml volume.

Effect of Variation of Sample Weight.—In Table 6 are given results of hydrogen replacement with neutral Ca-acetate solution of the Hartsells and the Portsmouth soils, as affected by weight of analytical sample and also as affected by filtration technique. The smaller sample-weights are considered normal, being within the stipulated 1 to 2 m.e. of adsorbed hydrogen per determination, whereas the larger weights might be con-

TABLE 6.—*Effect variation of sample weight upon the exchangeable hydrogen results by the Ca-acetate procedure*

SOIL	FILTRATION METHOD	SAMPLE WEIGHT AIR-DRY	EXCHANGEABLE HYDROGEN—M.E. PER 100 G					
			REPLICATE DETERMINATION					
			1	2	3	4	5	AVERAGE
		g						
Hartsell sandy loam, 1947	Gravity	25	8.37	8.31	8.40	8.26	8.18	8.30 ± .07
	Gravity	10	9.27	9.31	9.31	9.37	9.33	9.33 ±
Portsmouth muck, 1950	Gravity	10	46.2	44.8	46.8	47.8	45.8	46.28 ± .82
	Gravity	4	50.9	50.3	50.3	50.3	48.2	50.00 ± .72
Same	55 m.m. Büchner	10(a)	45.2	44.7	46.1	46.4	46.4	45.8 ± .66
	55 m.m. Büchner	(b)	3.7	3.5	3.8	3.7	3.7	3.7 ± .08
			48.9	48.2	49.9	50.1	50.1	49.4 ± .74

(a) 1st 250 ml volume, leachings.
(b) 2nd 250 ml volume of Ca-acetate.

sidered excessive. Quintuplet determinations were found necessary for the Portsmouth muck because of the segregating tendencies of the mixture of sand and organic matter which led to somewhat erratic results when comparisons were made upon the basis of single determinations. The 250 ml extraction and gravity leachings of the 10 g sample of the Portsmouth muck (Table 6) gave a hydrogen replacement on 100 g basis only 3.7 m.e. less than the replacement from the 4 g sample. Hence, when the charge of the Portsmouth muck was 2.5 times that prescribed for maximum hydrogen load, there was only 7 per cent decrease in the effected replacement of exchangeable hydrogen by the neutral Ca-acetate solution. A similar increase in analytical charge of the Hartsells soil caused a 1 m.e. decrease in the hydrogen release per 100 g, or a percentage decrease of 11. The 250 ml volume of leachings on the 55 mm Büchner filter with slight suction failed to effect any greater hydrogen replacement on the 10 g sample of the Portsmouth muck than that obtained by gravity leaching. The additional 250 ml suction leaching of the 10 g sample of the muck boosted the total hydrogen replacement per 100 g to a value close to the one obtained through gravity leachings of the 4 g charge.

The foregoing findings as to the action of neutral Ba- and Ca-acetate solutions in the replacement of exchangeable hydrogen from acidic soils show that:

1. Five 100-ml successive extractions of 5 g charges of a Portsmouth muck and of 10 g charges of a Hartsells sandy loam failed to give any indication of completeness of the hydrogen replacement process.

2. Considerable increases in hydrogen replacement were induced by 16-hour contact periods in the first and fifth 100 ml extractions, in comparison with contacts of 1 hour.

3. Based upon results from five replicate determinations, the hydrogen replacement by the proposed routine Ca-acetate procedure from a 10 g charge of the Portsmouth muck was 7 per cent less than that from a 4 g charge of that soil. A similar extraction from a 25 g charge of the Hartsells soil yielded 11 per cent less hydrogen than that obtained from a 4 g charge.

4. The aggregate of the hydrogen replacement affected by five 100-ml successive neutral Ca-acetate extractions of the Portsmouth muck were equal to the Ca-sorption obtained from the 4-week reaction of that soil and an excess of 325-mesh calcite.

RELIABILITY OF *p*H VALUE AS A MEASURE OF DEGREE OF BASE SATURATION

The *p*H value has become established as the most popular single criterion in the evaluation of the lime status of soils, though opinions differ as to the most desirable *p*H for best crop production. Lyon and Buckman state, "With crops that respond noticeably to lime, the *p*H should be raised to at least 6 or perhaps above."(13, p. 366). Truog suggests that,

"Under most general farming conditions in the northern states, it is advantageous to regulate the application of lime so that the reaction of the soil will be maintained nearly at the neutral point or close to *p*H 6.5" (29, p. 570). On the other hand, Miles (20) reported that excellent crops of alfalfa, corn, cotton, and peanuts were being produced on a number of soils representative of the Southeastern Coastal Plain region whose *p*H was only about 5.5.

A more technical application of *p*H determination is that in connection with the estimation of the degree of base saturation and the computation of lime requirement of soils in the manner already indicated. Such use of the *p*H value of the soil postulates that this property is a fairly stable characteristic of the soil, except when modified by the addition of a liming material or the removal of calcium by plant growth or through leaching. In reality, however, the determination of soil *p*H is subject to many disturbing influences, some of which are of technical nature, whereas others are attributable to forces inside and outside of the soil. Volk and Bell (30) investigated *p*H readings as they are affected by the manner of soil suspension, type of electrode, and time of standing. Baver (2), Puri and Puri (26), and Hester and Shelton (10) called attention to the effect of electrolyte solutes and to seasonal variations. Bear and Toth (4) found that, after incubation periods of 1, 2, 3 and 4 weeks, limed soils showed progressive lowerings in *p*H values, which were attributed to the nitric acid engendered during the incubation of the soil. The data presented by Bear and Toth (their table 4) reveal disparities between the expected and induced *p*H values from the 6000-pound calcium carbonate application (amounting to 2000 pounds calcium carbonate equivalence) but the reported nitrate formation accounted for only about 700 pounds of this deficiency. There is a possibility that the 100-mesh limestone incorporation had not fully reacted with the soil at the time of the analysis.

Experimental data concerning the effect of incubation period upon *p*H value of calcite-treated soils and subsoils are given in Table 7. These soil systems were incubated 1, 2, 4, and 8 weeks according to the described procedure, and each periodic sample comprised an independent experiment. The *p*H values were determined by means of the glass electrode, on unleached and H_2O-leached soils. The results on the unleached sample reveal progressive decreases in *p*H values on the surface soils in the periods from 1 week to 8 weeks, but no such decreases in *p*H occurred in either the Cumberland clay subsoil or the Susquehanna subsoil, both of which were almost entirely of inorganic nature. From these results it is obvious that the decreases in *p*H values during incubation are due to the activities of micro-organisms in the presence of organic matter and the resultant formation of nitrates and sulfates.

It is quite likely that these observed differences in *p*H after different periods of incubation and after leaching could be duplicated in field soils by

TABLE 7.—*Effect of period of contact upon pH value of certain types of soils and subsoils, unleached and leached*

SOIL	$CaCO_3$ ADDED PER 100 G	pH AFTER CONTACT PERIODS IN WEEKS							
		UNLEACHED				LEACHED			
		1	2	4	8	1	2	4	8
	m.e.								
Hartsells sandy loam, 1950	15.0	7.4	7.3	7.0	6.8	7.7	7.6	7.5	7.6
Same	10.0	6.6	6.4	5.9	5.6*	7.3	7.1	6.8	6.6*
Claiborne silt loam	6.0	7.5	7.5	7.1	7.0	7.8	7.7	7.6	7.6
Cumberland clay subsoil, 1950	10.0	7.6	7.6	7.7	7.7	7.7	7.8	8.0	7.9
Susquehanna clay subsoil, 1950	25.0	5.8	5.9	5.9	6.0	6.1	6.0	6.0	6.1
Portsmouth muck, 1950	50.0	7.1	7.0	6.8	6.6	7.5	7.3	7.2	7.4

* These had 13-week contact period.

samplings at various seasons of the year, or shortly after a hot dry spell and again after a drenching rain (30). There is always the possibility of securing .5 to 1.0 *p*H differences in soils that are well supplied with organic matter. Just how such fluctuations in *p*H values may have vitiated the estimated base saturation percentage will be governed by the buffer property of the soil. As read from the graphs in Figure 3, an error of .5 *p*H between 5.0 and 5.5 *p*H will result in the following errors in the estimated percentage base saturation: on the Hartsells soil, 13; on the Cumberland clay subsoil, 26; on the Susquehanna clay subsoil, 19; and on the Portsmouth muck, 16. A similar disparity in the *p*H region of 6.0 to 6.5 will produce errors of estimated percentage saturation of 27, 13, 4, and 20 for the respective systems in the order given above.

These data demonstrate the futility of attempting to estimate the percentage base saturation of a soil upon basis of casual *p*H determinations with accuracy of better than within 10 to 20 per cent of the true value. Many determinations of nitrate in limed soils incubated at 30°C. have shown that the NO_3 formation is insignificantly low in the 1st and 2nd weeks of incubation. Interferences of biochemical end products in a series of a *p*H-titration curve by means of solid $CaCO_3$ can be made negligible when the incubation period is limited to two weeks.

SUMMARY

A Montmorillonitic clay subsoil, a kaolinitic subsoil, a kaolinitic-organic surface soil, and a muck were used as exchange materials in study of the interrelationships between the Ca-sorption build-up, the exchangeable hydrogen and *p*H value. The calcium contents of the several acidic materials were built up thru additions of increasing quantities of 325-

mesh calcite, and the incubation of the mixtures 4 weeks in moist condition at 30°C. The Ca-sorptions were determined by difference between the calcite inputs and residual $CaCO_3$. The exchangeable hydrogen content was determined by extraction and leaching of a 10-g charge of the materials with neutral .5*M* Ca-acetate solution to a combined volume of 250 ml. The *p*H values were determined by the glass electrode on H_2O suspensions of pasty consistency. Special studies were made on: (a) the replaceability of the sorbed Ca by NH_4 through leaching with NH_4OAc solutions of *p*H 7 and of *p*H 8; (b) the effects that variations of the extraction technique, such as duration of contact, volume of leaching, and sample size, exert upon results registered for exchangeable hydrogen by neutral Ba- and Ca-acetate solutions; and (c) the effect of incubation period upon reciprocal relationship of soil *p*H and nitrate formation.

Within a period of 4 weeks of moist contact at 30°C., maximal Ca-sorptions were attained with an excess of 325-mesh calcite of 5 m.e. per 100 g. of soil. In comparison with exchangeable (Ca+H) of the original samples, maximal Ca-sorption resulted in increased cation sorption capacities of the organic and the kaolinitic materials, but not of the Montmorillonitic clay. These increases in sorption capacities began to develop when the *p*H of the systems was considerably below 7.

Calcite additions resulting in Ca-sorptions equivalent to the quantity of exchangeable hydrogen determination produced soil reactions close to *p*H 7. With allowance for natural fluctuations in soil reaction, the simple determintions of *p*H value and exchangeable hydrogen may be utilized for computing the lime requirement to raise the soil to any desired *p*H value, provided one of the following values is also known: the metal cation content, the exchange capacity, or the *p*K value.

On organic and kaolinitic materials, the calcium build-up is in equivalent exchange for the adsorbed hydrogen in the systems with *p*H below 6. In systems with *p*H above 6, the Ca-sorptions exceed the determined diminutions of exchangeable hydrogen. Increased Ca-sorptions above the cation exchange capacities of the original materials do not preclude the presence of exchangeable hydrogen that can be determined thru extraction with neutral Ca-acetate solutions in the Ca-sorbed soil.

The use of the neutral ammonium acetate solution in the extraction of Ca-saturated soils failed to yield any increase in NH_4 sorption above that found for the acidic soils. The raising of the *p*H of the ammonium acetate to 8 failed to give any increase in the NH_4 sorption of the Ca-sorbed soils; on the contrary, the higher *p*H of the ammonium acetate caused appreciable decrease in the sorption of NH_4 by the organic soils.

On organic soils, increases in volume of leachings, prolongation of the contact period, and diminution of analytical charge have contributed to increased replacement of hydrogen by neutral solutions of either Ba- or Ca-acetate. Five successive 100 ml extractions of a 5 g charge of a Ports-

mouth muck with neutral Ba-acetate solution resulted in a hydrogen replacement equivalent to the maximal Ca-sorption from calcite in four weeks of contact.

Through special experiments that were set up to determine the effect of time of incubation on *p*H values under conditions where the reaction with calcite was complete in the shortest period, it was found that *p*H lowering with extension of time occurred in surface soils only; and that lowering begins with the third week of incubation. Both the Ca-replacement by the generated hydrogen and nitrate could contribute to the observed lowering of *p*H values in soils.

CONCLUSIONS

The Ca-acetate procedure for the determination of exchangeable hydrogen in soils affords satisfactory indications of the $CaCO_3$ required to raise the soil to *p*H 7, and can be utilized jointly with the *p*H determination and one other soil constant for the determination of calcium needs of the soil for attainment of *p*H values other than 7. To obtain concordant results by means of the Ca-acetate procedure, the stipulations as to size of sample, duration of contact, and volume of leaching should be adhered to strictly.

The soil-calcite reaction at 30°C. should be limited, for purposes of *p*H-Ca-sorption curves, to a period of two weeks, to preclude vitiations from biologically engendered nitrates and sulfates.

RECOMMENDATIONS

It is recommended—

(1) That the neutral calcium acetate procedure for the replacement and the determination of the exchangeable hydrogen of soils be adopted as official, first action.

(2) That further studies be made of methods for the determination of the limestone requirement to change a soil's reaction to practical *p*H values other than 7.

REFERENCES

(1) ANDERSON, M. S., and BYERS, H. G., "Neutralization Curves of the Colloids of Soils, Representative of the Great Soil Groups." United States Department of Agriculture Tech. Bulletin 542 (1936).
(2) BAYER, L. D., *Journal American Society of Agronomy*, **23**: 587–605 (1931).
(3) BEAR, F. E., and ALLEN, L., *Ind. Eng. Chem.*, **24**: 998–1001 (1932).
(4) BEAR, F. E., and TOTH, S. J., "*p*H Values and Lime Requirements of Twenty New Jersey Soils," New Jersey Agricultural Experiment Station Circular 446 (1944).
(5) BRADFIELD, R., First Congress International Soc. Soil Science, Proc., IV: 858–868 (1927).
(6) BRADFIELD, R., "Some Chemical Reactions of Colloidal Clay." *Jour. Phys. Chem.*, **35**: 360–373 (1931).

(7) BRADFIELD, R., and ALLISON, W. B., International Soc. Soil Science, Trans. 2nd Commission, Vol. A: 63–79 (1933).
(8) BRADFIELD, R., Soil Science Society America, Proceedings, 6: 8–15 (1941).
(9) DUNN, L. E., *Soil Science*, 56: 341–351.
(10) HESTER, J. B., and SHELTON, F. A., *Journal American Society of Agronomy*, 25: 299–350 (1933).
(11) KELLEY, W. P., and BROWN, S. M., "Replaceable Bases in Soils." California Agricultural Experiment Station Tech. Paper 15 (1924).
(12) KELLEY, W. P., "Cation Exchange in Soils." Reinhold Pub. Corp., New York (1948).
(13) LYON, T. L., and BUCKMAN, H. O., "The Nature and Properties of Soils." 4th Edition. The McMillan Company, New York (1943).
(14) MACINTIRE, W. H., and SHAW, W. M., *Soil Science*, 20: 403–417 (1925).
(15) MACINTIRE, W. H., and SHAW, W. M., *Journal American Society of Agronomy*, 22: 272–276 (1930).
(16) MARSHALL, C. E., and KRINBILL, C. A., *Jour. Phys. Chem.*, 46: 1077–1090 (1942).
(17) MEHLICH, A., Soil Science Society of America, Proceedings, 6: 150–156 (1941).
(18) ——, *Ibid*, 7: 167–174 (1942).
(19) ——, *Ibid*, 7: 353–361 (1942).
(20) MILES, J., *Soil Science*, 65: 97–102 (1948).
(21) PARKER, F. W., *Journal American Society of Agronomy*, 21: 1030–1039 (1929).
(22) PEARSON, R. W. and ENSMINGER, L. E., Soil Science Society of America, Proceedings 13: 153–156 (1948).
(23) PEECH, M., "Chemical Studies on Soils from Florida Citrus Groves." Florida Agricultural Experiment Station, Tech. Bulletin 340 (1939).
(24) PEECH, MICHAEL, and BRADFIELD, R., *Soil Science*, 65: 35–56 (1948).
(25) PIERRE, W. H., and SCARSETH, G. D., *Soil Science*, 31: 99–114 (1931).
(26) PURI, A. N., and PURI, A. G., *Soil Science*, 46: 247–257 (1938).
(27) SALTER, R. M., and SCHOLLENBERGER, J., "Criteria for the Evaluation of Agricultural Ground Limestone." American Soc. Testing Materials Limes Symposium, Columbus, Ohio (1939).
(28) SHAW, W. M., and MACINTIRE, W. H., *Jour. A.O.A.C.*, 34: 471–492 (1951).
(29) TRUOG, E., United States Dept. Agr. Yearbook, 563–580 (1938).
(30) VOLK, G. M., and BELL, C. E., "Soil Reaction." Florida Agricultural Experiment Station Tech. Bulletin 400 (1944).

REPORT ON THE DETERMINATION OF FLUORINE CONTENT OF SOILS

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There is an increased recognition of the importance of the fluorine content of livestock forage and of human food, which has raised the question of the soil as a source of such fluorine (3, 4). This consideration has emphasized the necessity for improved analytical methods for determination of fluorine in soils. Through the $HClO_4$-steam distillation modification of the Willard and Winter method (8) full recovery of fluorine was not obtained from all types of soils. The fluorine contained in certain minerals,

such as micas and topaz (7), may not be released completely by that procedure. Consequently, the values obtained for the fluorine content of soils containing appreciable amounts of those and similar minerals may be considerably lower than actual. Soils of high organic matter content also have yielded inconsistent values.

The addition of CaO, to prevent loss of fluorine during incineration, has been adopted (1, 2) as a step preparatory to separation through distillation. It was found (5), however, that fluorine losses occurred when CaF_2 was incinerated at 900°C., the temperature stipulated in the *Methods of Analysis*, 6th Ed., 1945 (1), or even at 500°C. This procedure was revised in the 7th edition, 1950 (2), to stipulate 500°C. as the incineration temperature. Discrepancies between results by the official and by the direct double distillation procedures led to the proposal of a direct double distillation method (6). This proposal was supported by comparison of results that were obtained by application of the two methods to the analysis of some 26 selected soils at two laboratories.

Because of these and other considerations, it was deemed necessary to modify the present method. After exploratory studies, the two modifications described herein were selected for collaborative study.

OBJECTIVES

The following objectives were presented to the collaborators:

(1) To compare the over-all recovery of fluorine through use of the two described procedures.

(2) To compare results obtained by the several analysts.

(3) To determine recovery of the fluorine native to the soil and that added as CaF_2.

(4) To observe the precision and reproducibility obtained by the two methods.

(5) To ascertain loss of fluorine from that added as CaF_2 due to volatilization or other causes.

SAMPLES

The following soil samples were ground to pass a 100-mesh sieve, mixed thoroughly, and quantities sufficient for 50 determinations were sent to each collaborator:

No. 1. Muck from North Carolina. High organic matter content.
No. 2. Same as No. 1, with addition of approximately *200* p.p.m. F as CaF_2.
No. 3. Hartsells Loam I. Medium organic matter content.
No. 4. Same as No. 3, with addition of approximately *200* p.p.m. F as CaF_2.
No. 5. Maury Silt Loam. Medium organic matter content.
No. 6. Same as No. 5, with addition of approximately *200* p.p.m. F as CaF_2.

A quantity of special "Fluorine free" CaO, sufficient for several soil incinerations also was included with each set of soil samples.

DIRECTIONS TO COLLABORATORS

The collaborators received detailed directions on the two methods to be compared and were instructed to make at least one preliminary determination on the soil samples by both methods as guidance to the characteristics and approximate fluorine content of the sample, and then to make the triplicate determinations which were to be reported.

DETERMINATION OF FLUORINE IN SOILS

METHOD I, DIRECT DOUBLE DISTILLATION

Transfer a 0.50-g charge of the 100-mesh soil into a clean 125 ml Claisen flask, previously fitted with thermometer and steam inlet tube. Connect the flask to a water-cooled condenser, preferably of the worm type, and provided with a delivery tube to dip below the surface of a few ml of dilute NaOH in a 600 ml beaker. Moisten the soil charge with 5 ml of water and add 50 ml of 1+1 H_2SO_4. Close the flask, apply heat and raise temperature to 150°C. Introduce the steam current, then raise the temperature to 165°C., and at that temperature collect 500 ml of distillate at the rate of about 4 ml per minute. Keep the distillate slightly alkaline to phenolphthalein throughout the collection by means of dilute NaOH. Evaporate the distillate to 10–15 ml and transfer it to a Claisen flask for the second distillation.

Add 25 ml of 70% $HClO_4$ containing 0.2% Ag_2SO_4 (6 g Ag_2SO_4 in 2 liters $HClO_4$ or the equivalent of silver perchlorate). Use a few ml of the $HClO_4$ to aid in the transfer of the contents of the beaker to the flask. Connect the Claisen flask to condenser and steam-distill at 130°–135°C. until 200 ml is collected (4 ml per minute) in a wide-mouth Erlenmeyer flask. Make distillate to volume in a volumetric flask.

METHOD II, INCINERATION OF SAMPLE

Transfer a 0.50-g charge of the 100-mesh soil to a small (100 ml) nickel or platinum dish. Add 1 g of fluorine-free, powdered CaO (Fisher special No. C116-1), mix, slurry with 5 to 10 ml of water and dry in a low temperature oven (60° to 75°C.) at least 8 hours. Place dish in a controlled electric furnace, raise temperature to 500°C. and maintain at that temperature 30 minutes.

Remove dish from furnace and allow to cool. Proceed with the single perchloric acid distillation as follows:

SINGLE PERCHLORIC ACID DISTILLATION OF INCINERATED CHARGE

Transfer the incinerated charge into a 125 ml Claisen flask. Moisten with water, add 30 ml of 70% $HClO_4$ containing 0.2% Ag_2SO_4 (or the equivalent of silver perchlorate), and proceed with the distillation as prescribed in the second phase of the double distillation procedure, with the exception that *500 ml is collected.*

Transfer an appropriate aliquot of the final distillate, obtained by means of either distillation procedure, to a 150 ml beaker and proceed with the titration in accordance with the directions.

DETERMINATION OF FLUORINE BY MEANS OF THORIUM NITRATE TITRATION

Reagents:

Sodium Hydroxide.—0.05 *N.*

Alizarin Red S Indicator.—Make stock solution of 1 g per liter concentration and dilute 10 times, for use as required.

Buffer Solution.—Dissolve 2 g sodium hydroxide in 50 ml H_2O. Add this solution to a solution of 9.44 g monochloracetic acid and dilute the total to 100 ml.

Thorium Nitrate.—2.761 g dry $Th(NO_3)_4 \cdot 4H_2O$ per liter (0.02 N).

Sodium Fluoride.—2.21 g per liter for standard.

Standardization:

Standardize the thorium nitrate solution against aliquots of the standard Sodium Fluoride solution, in range from 0 to 200 micrograms of fluorine, in 20-microgram increments. From the titers, plot a standard curve for the solution using micrograms of fluorine as the abcissa and ml of .02 *N* $Th(NO_3)_4$ as the ordinate. In plotting the curve, apply the titration blank, i.e. the titer required for the end-point in absence of any fluoride.

Determination of Fluorine Content of Sample:

(A satisfactory burette for making the titration is a 5ml reservoir micro-burette with 0.01 ml calibrations. A Sargent fluorescent illuminator or other source of white light is placed back of a white base burette support. Helpful, though not essential, is a titration stand, made from a 5″×12″ shelf of clear plastic, or of glass, and supported 1¾ inches above the white porcelain base of the burette support. The transparent stand permits the light to pass both through and under the beaker; thus giving the effect of a modified Nessler tube.)

Transfer a 100 ml aliquot of the sample distillate into a 150 ml beaker. Add two ml of Alizarin Red S; neutralize the solution to a faint pink with 0.05 *N* NaOH and then add 1 ml of the buffer solution. Titrate by addition of the 0.02 *N* thorium nitrate solution drop by drop, and with constant stirring, until a light pink or salmon-pink end-point is just reached.

By reference to the standardization curve, and after deduction on any blank due to reagents, etc., convert the burette reading into micrograms of fluorine and calculate the parts per million of fluorine in the sample. Repeat the titration on another aliquot, using the first titer as the basis to determine the volume of the second aliquot, and take a smaller aliquot in case the titer required for 100 ml was too large. Report the fluorine content of the sample as calculated from the average of the two titrations.

NOTES

The strength of the $Th(NO_3)_4$ solution used should be commensurate with the amount of fluorine in the aliquot. For a wide range of samples, two concentrations of $Th(NO_3)_4$ solutions are required, preferably .002 *N* for samples of low fluorine content, and .02 *N* for those in the intermediate range. However, the .02 *N* titrating solution is adequate for all fluoride levels in the soil samples included in this study. (This was later changed to permit use of weaker solution for samples of lower fluorine content.) A titration in the range of 0.5 to 0.6 ml of .02 *N* $Th(NO_3)_4$ is preferable. If a 50 ml aliquot of a 200-ml distillate from a 0.5-g charge of soil is used (0.5/200/50), and the fluorine content of the sample is assumed to be 1600 p.p.m., a 1.00 ml titration would be required. Because this titer is somewhat excessive, a 25 ml aliquot of the sample should be taken.

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COMMENTS BY COLLABORATORS

The collaborators have made several helpful suggestions, some of which should be the objective of further study. Problems relating to the titration phase were mentioned frequently. Variations in results due to factors which affect the end-point were widespread and may be difficult to eliminate, due, in some cases, to the personal equation.

The blanks reported as due to reagents or other causes have varied widely, as did the manner of statement of the blank. Some of the collaborators used milliliters of solution, whereas others stated the blank as micrograms of fluorine.

The comments are quoted as follows:

1. I am unable to send you any results on the referee analysis of the soil samples. I did, however, have the soil residues left after distillation checked spectrographically. No measurable trace of fluorine was found. The limit of spectrographic detection is about 10 p.p.m. F. This would mean that there is less than 5 micrograms F left in the residues after distillation.

5. Sample No. 1 appeared to have two very distinct ranges of recoveries. A single distillation following the incineration at 500°C. does not liberate the fluorine completely. A similar series at 500°C. followed by a double distillation seems to give much higher results, which were comparable to the direct double distillation.

Sample No. 1 did not give as consistent results as the other five.

6. We encountered considerable trouble with the direct double distillation procedure on sample No. 2. In general, we obtained much better checks and encountered less trouble with the incineration-single distillation procedure. In the direct double distillation procedure (samples 1 and 2) there was an excessive amount of foaming during the initial distillation, especially at the rate of 4 ml/min. In our opinion 250 ml Claissen distillation flasks would be better for the work than the 125 ml. The chance of mechanical carry-over due to foaming and agitation by the steam

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[a] Did not send complete report.

[b] With supervision of John Jewell.

[c] With supervision of W. H. Knox, Jr.

[d] With supervision of Dr. M. L. Moss.

would be lessened. Both distillates from the direct double distillation procedure contained an oily substance that tended to float on the surface and adhere to the glassware. This was most noticeable in Samples 1 and 2, "Muck Soil from North Carolina," and less noticeable in Samples 5 and 6, "Maury Silt Loam."

Some of the titers were quite small even with 0.01 *N* $Th(NO_3)_4$. Although we did not have an opportunity to try a weaker solution, we feel that a 0.005 *N* solution would be desirable. While we worked some with both 0.02 and 0.01 *N* solutions, our final work was done with the 0.01 *N*. We prefer the colorimetric or back titration procedure as given under "The Determination of Fluorine in Foods," page 389, *Methods of Analysis*, 7th Edition. We used a light pink or the first permanent color change as the end point. We used a standard for comparison in all the titrations. The standard was prepared by titrating a known amount of fluoride to a matched point on the standardization curve.

7. The direct titration using .02 *N* thorium nitrate lacks accuracy. Using a .5 g charge and collecting 500 ml distillate a .01 ml drop of thorium makes a difference of 20 p.p.m. in the results.

8. We were interested in reading in your "Directions to Collaborators" that the steam distillation method is not satisfactory for the recovery of fluorine from all types of soil, for example, micas and topaz, and that the values obtained on soil containing these minerals may be quite low. We are in agreement with this opinion and feel that alkaline fusion is necessary in order to convert the fluoride in the soil to a form from which it can be liberated under conditions of the steam distillation.

10. Suggest that, if the double distillation procedure is accepted as a routine method, a calcination be made with lime after evaporating the first distillate. The possibility of interference by organic material, which might distill from both acids, would seem difficult to ignore. We noted the formation of a film, which later dissolved, on the top of our sulfuric acid distillates.

11. We believe that more uniform results may be obtained by the use of larger samples in distillation. Advantages resulting therefrom would be:

(1) A smaller factor in the conversion to p.p.m. (2) A small blank in ratio to total volume of $Th(NO_3)_4$.

Our experience with the soils submitted indicates that the greatest error is in titration of the sample. If the sample is large, so that .01 ml of $Th(NO_3)_4$ may represent only a few p.p.m. of F (i.e. small factor), the strongest $Th(NO_3)_4$ consistent with the above requirement should be used. In that way, the end-point is more easily seen. A reduction of volumes to the micro-scale might be advantageous. The differences reported in the two distillation procedures, amounting to .01 ml of 0.2 *N* $Th(NO_3)_4$, are within the range of experimental error of the operation. It appears to me that the determination of the end point leaves much to be desired.

COMMENTS ON RESULTS

The results reported by the collaborators are shown in Table 1.

The deviations between the mean value reported by each collaborator and the over-all average are shown in Table 2. Of the 54 individual means reported by each method, 44 per cent of those by Method I and 50 per cent by Method II were within 10 p.p.m. of the over-all average. The deviation between the averages of the results obtained by the individual collaborators and the over-all averages was less than 10 per cent of the total fluorine by both methods on two-thirds of the samples. The largest percentage deviation by all collaborators was on sample 1.

Analysis of variance was made on several samples and showed that:

TABLE 1.—*Fluorine content of soils as determined by two methods, A.O.A.C. collaborative studies, 1951*

COLLABORATOR	METHOD I DIRECT DOUBLE DISTILLATION		METHOD II INCINERATION WITH 1 GRAM CaO PRIOR TO DISTILLATION	
	p.p.m.	*average p.p.m.*	*p.p.m.*	*average p.p.m.*
		Sample No. 1		
2	40, 40, 60	47	30, 40, 30	33
3	25, 19, 18	21	23, 23, 18	21
4	36, 38, 40	38	42, 31, 40	38
5	60, 61, 62	61	35, 30, 33	33
6*	48, 53, 48	50	41, 41, 41	41
7	34, 40, 44	39	32, 32, 38	34
8	55, 66, 38	53	33, 30, 20	27
9*		60		50
10	47, 51, 47	48	54, 29, 52	48
11	39, 46, 46	44	32, 41, 32	38
13	20, 28, 12	20	20, 10, 21	17
Average		41		32
		Sample No. 2		
2	230, 210, 220	220	220, 200, 200	207
3	190, 195, 195	193	215, 200, 205	207
4	200, 200, 196	199	189, 202, 196	196
5	214, 220, 228	221	213, 214, 217	215
6*	205, 208, 208	207	209, 214, 209	210
7	211, 212, 220	214	190, 200, 200	197
8	194, 261, 219	225	204, 204, 192	200
9*		210		220
10	212, 211, 203	209	224, 227, 231	227
11	203, 223, 197	208	197, 246, 246	230
13	196, 224, 224	215	168, 200, 170	179
Average		212		206
		Sample No. 3		
2	190, 210, 190	197	80, 90, 85	85
3	151, 149, 145	148	140, 138, 138	139
4	144, 136, 140	140	106, 119, 110	112
5	143, 146, 150	146	99, 101, 102	101
6*	140, 140, 140	140	127, 127, 127	127
7	160, 188, 192	180	130, 130, 140	133
8	145, 155, 148	149	118, 100, 112	110
9*		220		140
10	151, 151, 151	151	129, 132, 126	129
11	151, 138, 144	144	82, 115, 82	93
13	161, 204, 174	180	120, 130, 121	123
Average		159		114

* Not included in average.

TABLE 1.—*Continued*

COLLABORATOR	METHOD I DIRECT DOUBLE DISTILLATION		METHOD II INCINERATION WITH 1 GRAM CaO PRIOR TO DISTILLATION	
	p.p.m.	*average p.p.m.*	*p.p.m.*	*average p.p.m.*
		Sample No. 4		
2	360, 380, 380	367	275, 285, 275	278
3	380, 370, 355	368	340, 320, 300	320
4	338, 324, 336	333	296, 284, 322	301
5	307, 308, 316	310	299, 305, 306	303
6*	350, 345, 345	347	303, 306, 306	305
7	332, 332, 336	333	310, 320, 330	320
8	358, 306, 323	329	310, 324, 308	314
9*		330		300
10	302, 278, 298	293	301, 304, 295	300
11	367, 347, 347	354	344, 311, 377	344
13	332, 352, 329	337	290, 300, 320	303
Average		336		309
		Sample No. 5		
2	280, 280, 260	273	220, 230, 210	220
3	275, 275, 275	275	220, 220, 220	220
4	216, 200, 236	217	242, 229, 217	229
5	228, 238, 248	238	195, 197, 199	197
6*	228, 233, 233	231	235, 240, 235	237
7	256, 256, 264	259	230, 240, 240	237
8	238, 232, 250	240	218, 198, 188	201
9*		270		230
10	239, 234, 236	236	239, 250, 239	243
11	249, 269, 249	256	278, 246, 246	257
13	212, 200, 196	203	220, 208, 237	222
Average		244		225
		Sample No. 6		
2	410, 410, 410	410	360, 360, 360	360
3	451, 451, 451	451	420, 400, 420	413
4	410, 404, 430	415	396, 386, 404	395
5	404, 409, 420	411	347, 354, 364	354
6*	425, 430, 430	428	405, 405, 405	405
7	380, 404, 408	397	390, 390, 420	400
8	406, 426, 426	416	382, 398, 378	386
9*		410		390
10	395, 400, 379	391	423, 414, 418	418
11	433, 433, 452	439	475, 541, 459	492
13	404, 404, 400	403	425, 392, 410	409
Average		415		403

* Not included in average.

TABLE 2.—*Comparison between collaborators' average fluorine determinations and over-all average values*

COLLABORATOR NO.	METHOD I						METHOD II					
	SAMPLE						SAMPLE					
	1	2	3	4	5	6	1	2	3	4	5	6
	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.*	*p.p.m.*	*p.p.m.*	*p.p.m.*
2	+ 6	+ 8	+38	+31	+29	− 5	+ 1	+ 1	+29	−31	− 5	−43
3	−20	−19	−11	+32	+31	+36	−11	+ 1	+25	+11	− 5	+10
4	− 3	−13	−19	− 3	−27	0	+ 6	−10	− 2	− 8	+ 4	− 8
5	+20	+ 9	−13	−26	− 6	− 4	+ 1	+ 9	−13	− 6	−28	−49
7	− 2	+ 2	+21	− 3	+15	−18	+ 2	− 9	+19	+11	+12	− 3
8	+12	+13	−10	− 7	− 4	+ 1	− 5	− 6	− 4	+ 5	−24	−17
10	+ 7	− 3	− 8	−42	− 8	−23	+16	+21	+15	− 9	+18	+15
11	+ 3	− 4	−15	+18	+12	+24	+ 6	+24	−21	+25	+32	+89
13	−21	+ 3	+21	+ 1	−41	−12	−15	−27	+27	− 6	− 3	+ 6
Av.	41	212	159	336	244	415	32	206	114	309	225	403
Av. Dev.	10	8	17	18	19	15	7	12	18	9	13	27

(1) for a given sample, there was a significant difference among the individual collaborators; (2) there was significant agreement in the replications by the same collaborator; and (3) the average of the results obtained by Method I (direct double distillation) were significantly higher on samples 1, 3, 4, and 5, and higher, although not significantly so, on samples 2 and 6.

Other comparisons are summarized in Table 3.

The percentage recovery of that fluorine added as CaF_2 to the soil samples (objective 3) was calculated (Table 4) from the difference between the average fluorine content of the original soil reported by each analyst and his average value obtained on the sample augmented by 200 p.p.m. fluorine added as CaF_2. This was done also upon the basis of the over-all average value. As indicated by these computations, the recovery of the added fluorine ranged from 2 per cent to 14 per cent less than the theoretical content, although some of the individual averages showed greater variations from the theoretical. This loss may be attributed partially to retention of fluoride in the distillation, a condition which is well known and difficult to prevent even when dealing with a pure fluoride.

The generally lower values reported by use of Method II may indicate some volatilization due to the incineration, but the somewhat higher recovery of added fluorine by Method II, as shown by the calculations, would not substantiate that interpretation in the case of the samples to which CaF_2 had been added.

TABLE 3.—*Summary of collaborative results*

COMPARISON APPLIED	SOIL SAMPLE					
	1	2	3	4	5	6
Av. value, Method I,[a] p.p.m.	41	212	159	336	244	415
Av. value, Method II,[b] p.p.m.	32	206	114	309	225	403
Av. value increase by Method I, p.p.m.	9	6	45	27	19	12
No. of collaborators reporting mean value higher by Method I	6	6	9	8	5	5
No. of collaborators reporting mean value higher by Method II	0	3	0	1	4	4
No. of collaborators reporting mean value identical by both methods	3	0	0	0	0	0
Maximum individual difference in triplicates, Method I, p.p.m.	28	67	43	52	36	24
Maximum individual difference in triplicates, Method II, p.p.m.	25	49	33	66	32	82
Standard Deviation, Method I, p.p.m.	14.4	15.9	31.4	27.1	24.7	40.3
Standard Deviation, Method II, p.p.m.	9.8	18.6	19.1	22.0	20.4	41.2
Average Deviation, Method I, p.p.m.	10	8	17	18	19	15
Average Deviation, Method II, p.p.m.	7	12	18	9	13	27

[a] Direct, double distillation of 0.5 gram charge.
[b] Single $HClO_4$ distillation of 0.5 gram charge incinerated with 1 gram CaO.

TABLE 4.—*Recovery of fluorine added to three soil samples*

COLLABORATOR	METHOD I			METHOD II		
	SAMPLE NUMBERS[a]			SAMPLE NUMBERS[a]		
	2	4	6	2	4	6
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
2	87	85	69	88	97	70
3	86	110	88	93	91	97
4	81	97	99	79	95	83
5	80	82	87	91	101	79
6	79	104	98	85	89	84
7	88	77	69	82	94	82
8	86	90	88	87	102	93
9	75	55	70	85	80	80
10	81	71	58	90	86	83
11	82	105	87	96	126	118
13	98	79	100	81	90	94
Average	86	89	86	87	98	89

[a] Samples 2, 4, and 6 were the same soils as their counterparts, 1, 3, and 5 respectively. Samples 2, 4, and 6, however, had 200 p.p.m. of F as CaF_2 added and thoroughly incorporated.

The calculated recovery for each collaborator was based on the difference between his own average value for the original soil and his average value for its counterpart containing the incorporated 200 p.p.m. of F.

FUSION OF SOIL SAMPLE PRELIMINARY TO ANALYSIS

In connection with the determination of fluorine in soils, the possibility of alkali fusion to effect the release of soil-contained fluorine, regardless of its combination, has been advocated by several chemists. Several collaborators volunteered to make exploratory analyses by application of a fusion technique to the 1951 samples. No directions were sent out by the associate referee; the fusion procedure followed was the choice of the collaborator. The general practice was to fuse a 0.5 g charge of soil with 5 g of NaOH pellets, using a nickel crucible and a temperature of 550°–600°C. The melt was dissolved in water, transferred to a distillation flash, and the fluorine determinations were made through titration of an aliquot of the 500 ml collection from a single $HClO_4$ distillation.

The results reported by the volunteer collaborators are shown in Table 5.

TABLE 5.—*Fluorine content of soil samples determined by $HClO_4$ distillation of NaOH-fusion*[a]

COLLABORATOR	SAMPLE NUMBERS											
	1		2		3		4		5		6	
	p.p.m.	av. p.p.m.	p.p.m.	av. p.p.m.	p.p.m.	av. p.p.m.	p.p.m.	av. p.p.m.	p.p.m.	av. p.p.m.	p.p.m.	av. p.p.m.
7[b]	24	31	192	197	130	137	302	313	220	229	432	429
	42		198		138		314		232		434	
	28		202		142		322		236		450	
8[c]	45	27	206	198	138	124	324	303	244	226	400	404
	17		178		120		280		218		418	
	18		212		115		306		216		384	
9	40	40	230	220	130	135	270	280	240	245	380	380
	40		210		140		290		250		380	
10[d]					161	160	316	310	253	231	416	396
					157		320		217		393	
					162		293		222		379	
13	91	67			176	160	341	353	260	245		
	44				144		359		254			
	65				159		358		220			
Average		41		192		144		312		235		402

[a] These results were submitted voluntarily by several collaborators as representative of methods in use in various laboratories. The usual procedure was direct fusion of 0.5 gram charge with 5.0 grams NaOH pellets followed by single $HClO_4$ distillation to collect 500 ml.

[b] Charge slurried with 1 gram CaO prior to fusion.

[c] This collaborator also reported values obtained by double distillation following Na_2CO_3 fusion. Average values reported for samples 1 to 6 respectively were 23, 190, 125, 276, 222, 362.

[d] 0.5 gram charge slurried, dried and ashed with 0.42 gram CaO, fused with 5 grams NaOH distilled from 20 ml excess $HClO_4$, 750 ml collected.

The average for fluorine content obtained by means of the fusion of the charge was lower than the average obtained by Method I on five of the six samples. Identical average values were obtained by both methods on sample number 1. The results obtained by the fusion procedure and those by Method II were generally in close agreement, although the values for samples 1 and 3 were somewhat higher by fusion.

Through use of the fusion method, the individual collaborators obtained good agreement among their replications on a given sample, but the values obtained by the various collaborators differed widely. In these respects, the findings by the fusion method correspond to those by Methods I and II.

No particular comments have been made by collaborators as to the fusion procedure. The opinions usually have been that certain fluorine-bearing minerals require alkali fusion to effect the disintegration necessary for total distillation recovery of the fluorine content. As has been pointed out, the collaborative results indicate, although not conclusively, that fluorine recovery is actually lower when fusion is applied. Two explanations are advanced for this apparent contradiction. Volatilization of fluorides may occur during sodium hydroxide fusion, or the mass of gelatinous silica released by the fusion effects occlusion of fluorides and thereby increases the tendency of retention of fluorides against distillation. Obviously, additional work should be done on this point.

SUMMARY AND CONCLUSIONS

Samples of soils of three types, with and without addition of 200 p.p.m. of fluorine as CaF_2, were analyzed by two methods. Nine collaborators reported their results.

Method I consisted of direct double distillation of a 0.5-gram soil charge to collect 500 ml of distillate from the first (H_2SO_4) distillation and 200 ml from the second ($HClO_4$). Method II consisted of a 500°C. incineration of a 0.5-gram charge of soil mixed with 1 gram of CaO, followed by a single $HClO_4$ distillation to collect 500 ml. Fluorine content was determined in an aliquot of the distillate from each method by means of titration with thorium nitrate solution, using alizarin red indicator.

Deviation between collaborators was relatively high on a given sample, although there was good agreement among the replications by the same analyst. The results and the comments by collaborators indicate that the poor agreement between their findings may be due to conditions inherent in the titration end-point phase, rather than to disparities in the recovery of the fluorine in the distillation step.

Based on the results submitted, (a) higher recovery of fluorine from the soil was obtained by Method I; (b) there was an indicated recovery of about 90% of the fluorine added as CaF_2; and (c) no particular difficulty

was reported in the direct double distillation of the non-incinerated samples, except in the case of the muck soil (example 1).

Certain collaborators submitted data that were obtained after applying alkali fusion to the samples. These exploratory values were generally lower than those by Method I and were in close agreement with those obtained by Method II.

It seems obvious that the titration phase of the procedure should be improved to assure greater accuracy and precision.

ACKNOWLEDGMENTS

The collaborators and their assistants are commended for the large amount of laboratory work done by them. Also, the cooperation of the staff of the Tennessee Experiment Station, especially of Doctor W. H. MacIntire* for editorial suggestions and of Doctor S. H. Winterberg for the statistical, and other interpretations, made by him.

RECOMMENDATIONS

It is recommended† that the work be continued on samples of soil without addition of fluoride and that multiple determinations be made by the collaborators to obtain a statistical evaluation of the accuracy of the analytical procedure or procedures.

REFERENCES

(1) *Methods of Analysis*, 6th Ed. (1945), p. 13, 1.32.
(2) *Methods of Analysis*, 7th Ed. (1950), p. 40, 3.32.
(3) HART, E. B., PHILLIPS, P. H., and BOHRSTEDT, G. *Amer. Jour. Pub. Health*, **24**, 936–940 (1934).
(4) MACINTIRE, W. H., *et al.*, "The Fluorine Content of Plants Fertilized With Phosphates and Slags Containing Fluorides." *Ind. and Eng. Chem.*, **34**, 1469–1479 (1942).
(5) MACINTIRE, W. H., JONES, L. S., and HARDIN, L. J., *This Journal*, **33**, 653 (1950).
(6) MACINTIRE, W. H., HARDIN, L. J., and JONES, L. S., *This Journal*, **34**, 597 (1951).
(7) ROBINSON, W. O., and EDGINGTON, GLEN. *Soil Science*, **61**, No. 5, May, 1946.
(8) WILLARD, H. H., and WINTER, O. B., *Ind. and Eng. Chem.*, Anal. Ed., **5**, 7–10 (1933).

No reports were given on hydrogen-ion concentration of soils, boron, zinc and copper, exchangeable calcium and magnesium, phosphorus, or molybdenum.

* For the method as edited by the Referee, see *This Journal*, **35**, 62 (1952).
† For report of Subcommittee A and action of the Association, see *This Journal*, **35**, 45 (1952).

TUESDAY AFTERNOON SESSION

REPORT ON SUGARS AND SUGAR PRODUCTS

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

Uniform and accurate methods for the analysis of sugars and sugar products are essential in regulatory work as well as in trade transactions involving these materials. In the study and adoption of such methods the Association is indebted to the members of the staff of the New York Sugar Trade Laboratory, and especially to Dr. F. W. Zerban, the Director, for their many contributions and their close cooperation.

The United States National Committee of the International Commission for Uniform Methods of Sugar Analysis, at its meeting in Boston, April 5, 1951, adopted certain analytical sugar methods. In some cases these methods differ in certain respects from the official methods of this Association. It is proposed that efforts be made to unify such methods by collaborative means. It is hoped that this may be accomplished within the next year or two, to permit necessary changes to be incorporated in the next edition of the Book of Methods.

Increased interest in methods of analysis of maple products shows the desirability of obtaining an associate referee on this subject.

RECOMMENDATIONS*

It is recommended—

(1) That the study of methods for the determination of moisture be continued.

(2) That the study be continued on tables of density of solutions of sugar at various temperatures.

(3) That the study of methods for the detection of adulteration of honey be continued.

(4) That the study of methods for the determination of reducing sugars, including those employing chromatographic separation, be continued.

(5) That the Zerban and Martin values for refractive indices of dextrose and invert sugar solution, **41.10**, be made official.

(6) That the study of methods, **29.132–29.154**, incl., be continued.

(7) That until a satisfactory rapid method for the determination of color and turbidity in unfiltered solutions is developed, the color of sugar products be determined at wavelength 560 mμ by the method given in this year's report of the Associate Referee, with Celite Analytical Filter Aid, and expressed as absorbancy index.

* For Report of Subcommittee D and action of the Association, see *This Journal*, **35**, 59 (1952).

(8) That the study of the transmittancy of sugar solutions be continued, by determining the precision of the method, having each collaborator make at least three complete replicate experiments with the same raw sugar, all collaborators agreeing to carry out the work at a predetermined period of time.

(9) That the Folin and Wu micro methods (*Biol. Chem.*, **41**, 367, 1920) for the determination of dextrose be adopted first action.

(10) That the refractive index values for raffinose hydrate solutions reported by Zerban and Martin* be adopted, first action.

REPORT ON MICRO METHODS OF SUGAR ANALYSIS

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

Folin and Wu's method for the determination of dextrose was devised to analyze for blood sugar, and, consequently, has been of particular interest to biochemists and medical technicians. However, because of its advantages of speed, ease and reproducibility it has been under collaborative study as a micro method for the analysis of dextrose solutions (other than blood) where the total sample is small.

This report describes an adaption which employs an instrument for reading, rather than the visual matching, of colors. (Photoelectric colori-

Table 1

	5 mg/100 cc (0.1 mg)	10 mg/100 cc (0.2 mg)	15 mg/100 cc (0.3 mg)	20 mg/100 cc (0.4 mg)	25 mg/100 cc (0.5 mg)	30 mg/100 cc (0.6 mg)
Analyst A	0.10	0.19	0.30	standard	0.50	0.60
	.09	.23	.33	standard	.50	.60
	.09	.21	.31	standard	.51	.60
Analyst B	.08	.19	.31	standard	.49	.55
	.10	.18	.30	standard	.47	.60
	.10	.18	.29	standard	.52	.59

meters are widely used in analysis at present, and so are generally available).

Details of the method[1] are given in *This Journal*, **35**, 88 (1952).

The method is applicable to concentrations ranging from 5 to 30 mg dextrose per 100 ml, and is most accurate at about 10 to 25 mg per 100 ml. Standard dextrose samples should cover the range of the solutions to be analyzed. Solutions were made up to contain 5, 10, 15, 20, 25, and 30 mg

* *This Journal*, **34**, 808 (1951); **35**, 89 (1952).

[1] Folin, O., and Wu, H., *J. Biol. Chem.*, **41**, 367–74 (1920).

dextrose per 100 ml with the 20 mg/100 being used as the standard. By making up the solutions with ¼ per cent benzoic acid, they were stable over an indefinite period of time. Duplicate runs were made on 2 ml samples of each strength, representing respectively, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 mg dextrose. Table 1 shows agreement between two analysts as well as agreement between runs of the same analyst. The average overall deviation was only 0.01, and in no case did the dextrose found vary from the destrose present by more than 0.05 mg. It varied by that in only one case, which was in the highest concentration studied. The average deviation within runs of each analyst was 0.01 mg.

RECOMMENDATIONS*

In view of the apparent accuracy and reproducibility of the method, as well as its simplicity, it is recommended that it be made first action.

Additional methods for micro analysis of sugars are being considered for study. Among these are paper chromatographic methods and additional reducing sugar methods such as that of Hagedorn-Jensen. The Associate Referee would appreciate any comments, suggestions, or results of previous studies.

REPORT ON TRANSMITTANCY OF SUGAR SOLUTIONS

By F. W. ZERBAN, (New York Sugar Trade Laboratory, New York, N.Y.), *Associate Referee*

Color determination in sugar products was first placed on the program of the Association in 1941 (1), with J. F. Brewster as Associate Referee. In 1944 he presented a brief report (2) in which he recommended that asbestos and diatomaceous earth be used in collaborative work as filtering materials, and that complete absorption curves between 420 mμ and 700 mμ be reported. This general plan has been adopted for the collaborative work reported here.

Three raw sugars were used in this investigation, a Cuban, furnished by George P. Meade, of the Cuban-American Sugar Company, a Puerto Rican, supplied by Rafael Pol Méndez, of Central Los Caños, P. R., and a Hawaiian, provided by T. R. Gillett, of the California and Hawaiian Sugar Refining Corporation. The writer wishes to express his thanks to them and to the collaborators for their cooperation. Portions of the well mixed samples were sent to each collaborator, with the following directions:

Directions.—The principal object of this year's work is to ascertain whether asbestos and kieselguhr, or either of them, when used to filter solutions of raw cane sugars prior to transmittancy determinations, give reproducible results in different laboratories. According to the enclosed article by Zerban and Sattler (*Ind. Eng.*

* For report of Subcommittee D and action of the Association, see *This Journal*, 35, 59 (1952).

Chem., Anal. Ed., **8**, 168 (1936)), both filtering materials remove coloring matter as well as turbidity, depending on the particle size of either, and the proportion removed varies not only with the relative amount of filter aid, but also with the individual sugar, and other procedural conditions used. This makes it necessary to use a standard procedure which must be strictly adhered to. The Associate Referee has chosen the method of asbestos filtration, as published by the National Bureau of Standards, and the method of Celite filtration, as described by Zerban and Sattler, for this year's work. Three raw cane sugars, distributed by the associate referee, are to be tested by both methods.

Asbestos filtration method.—The method of Peters and Phelps (*Bur. Standards Technologic Paper*, No. 338 (1927)), as modified by Brewster and Phelps (*Bur. Standards J. Research*, **10**, 365 (1933)) is to be used, except that the filtrations are to be carried out at ordinary temperature, as recommended by Peters and Phelps, because of the well known effect of prolonged heating on the color of sugar solutions. Asbestos, Grade XXX, marketed by Powhatan Mining Corporation, Woodlawn, Baltimore, Md., shall be used, and purified according to the directions of Brewster and Phelps (*Ind. Eng. Chem.*, Anal. Ed., **2**, 373 (1930)). For the Jena filters specified by Brewster and Phelps, there will be substituted the equivalent Pyrex Büchner funnels with fritted glass disk, of Ace Glass, Inc., Vineland, N. J., Cat. No. 7305, 40 mm diameter of disk, 50 mm high above disk, capacity 60 ml, porosity B. Two filters with pads of purified asbestos are prepared in these funnels for each color determination, before the sugar solution is made up. The fritted glass filter disk is covered with a well fitting disk of 200-mesh bolting silk, obtainable from Kressilk Products, Inc., 71 Murray Street, New York, N. Y. To form the pad for both filters, the prepared asbestos is shaken up with distilled water, sucked down on the silk disk by means of a vacuum pump and packed tightly by tamping with a blunt stirring rod to produce a layer 5 mm thick. The pad is then washed several times with water, and the excess water is sucked off.

To prepare the sugar solution, 60 grams of the sugar sample is placed in a flask, the flask is tared on a rough balance, and boiling distilled water is added from a wash bottle in small quantities. The flask is shaken to promote quick solution and to insure high concentration at all times. The flask is occasionally placed on the balance pan, and the additions of water are stopped when slightly less than 40 grams water has been added, to produce a solution of not less than 60° Brix. When solution is complete, 0.5 gram of purified asbestos is added to the solution, the flask is closed with a clean rubber stopper and vigorously shaken by hand for 15 minutes. A few milliliters of the mixture of sugar solution and asbestos is poured on one of the two asbestos pads previously prepared, to displace the residual water. The main portion of the solution, or as much as the filter will hold, is added, and after a few milliliters have passed through, the suction is stopped, and a clean, dry receiver is substituted. The pad is to be kept covered with liquid during the filtration, at the end of which the suction is stopped before the pad becomes uncovered. The receiver is now detached. The *preliminary* filtrate thus obtained is now filtered a second time through the other previously prepared asbestos pad, but no further asbestos is added to it. This second filtration is performed exactly like the first. The clear, main portion of the filtrate is collected in a clean receiver, but the filtration rate is regulated by adjusting the vacuum in such a way that the solution filters drop by drop, never in a steady stream. The bottle containing the perfectly clear final filtrate is closed with a clean, dry stopper, and shaken to mix the contents thoroughly. The refractometric Brix of the solution is determined, and the concentration c (grams dry substance per 100 ml solution) is calculated by multiplying the Brix by the true density. The solution is now ready for spectrophotometric analysis, as described later. With properly prepared solutions and filters, further

filtration is of doubtful value, unless some scattered particles of asbestos have passed through. If the final filtrate appears turbid by showing a pronounced Tyndall cone upon oblique examination with a bright light source, it is best to repeat the entire procedure with a fresh sample of sugar, paying close attention to details.

The fritted glass filters are easily cleaned, for use in subsequent tests, by removing the pads and drawing water and air through the pores by means of vacuum suction alternately through the mouth and the stem of the funnel. Treatment of the glass filters with chromic-sulfuric acid mixture removes waxy or greasy film deposited by sugar solutions.

Celite filtration method.—The Celite to be used is the Celite Analytical Filter Aid of the Johns-Manville Corporation, marketed by Fisher Scientific Company, 711–723 Forbes Street, Pittsburgh, Pa., and 633–635 Greenwich Street, New York, N. Y. It is used as received. The solution of the raw sugar is prepared by placing 60 grams of the sugar in a flask, adding 40 ml of distilled water, and rotating the flask until the sugar is all dissolved. 6 grams of Celite Analytical Filter Aid is added to the sugar solution in a flask or bottle, and the mixture is vigorously shaken. To insure complete separation of the first turbid runnings from the clear portion of the filtrate without breaking the vacuum and thus disturbing the filter bed already formed, the fractional filtration apparatus of Sattler, illustrated on page 173 of the enclosed article, is used. The Coors porcelain Büchner funnel, size 2, diameter of filtering plate 75 mm, is placed by means of a 1-hole rubber stopper on the top of a filtering tube (dimensions shown in Figure 1), which has a side arm near the top, and a glass stopcock at the lower end. The side arm of the filtering tube is connected by means of the straight part of a T-tube with the side arm of the 250-ml filtering flask, and the side outlet of the T-tube with the suction pump. After the filtering apparatus has been assembled, with the stopcock open, a circle of Schleicher & Schüll filter paper No. 589, blue ribbon, 7 cm diameter, is placed on the filtering surface of the Büchner funnel, wetted with water, and the excess water is sucked down by vacuum through the filtering tube into the filtering flask underneath. The stopcock is now closed, and the well shaken mixture of sugar solution and Celite poured evenly over the filter paper. About 5 to 10 ml of the filtrate, which is somewhat turbid, is collected in the filtering tube, and then run into the filtering flask by opening the stopcock. The stopcock is closed again, and two or three more portions of 5 to 10 ml filtrate are collected as previously in the filtering tube and then run down into the filtering flask, to wash the inner wall of the filtering tube free of any small particles of turbidity. It is essential that during the entire filtration process the bed of Celite must be kept covered with sugar solution, and not allowed to run dry. The final clear filtrate is collected in the filtering tube, and transferred to a small bottle in which it is thoroughly mixed. The refractometer Brix is determined and converted to concentration, as described previously for the asbestos filtrate. The remainder of the solution is used for the transmittancy determinations.

It is presupposed that the collaborators are well versed in spectrophotometry and are familiar with the exacting optical cleanliness required. Our studies on raw sugars have shown that if only one cell is to be used to construct the entire transmittancy curve from 400 to 700 mμ, a thickness of about 2.5 mm is most appropriate. With this thickness the transmittancies for the darkest raw sugar tested range from about 3% at 400 mμ to 83% at 700 mμ; for the lighest raw sugar from 60% to 97%, and for a medium colored raw sugar from 55% to 89% over the same range. From these figures the ranges for thicker or thinner cells can be estimated. The transmittancies are to be determined at 13 points throughout the visible spectrum, viz. 400, 415, 430, 445, 460, 480, 500, 530, 560, 590, 620, 660, and 700 mμ for each of the three samples, for both asbestos and Celite filtrates, and also the concentration of the final filtrate measured in the spectrophotometer, in grams

dry substance per 100 ml of solution. These figures are to be reported to me, and also the thickness of the cell or cells used.

Each collaborator was requested to make the measurements as soon as possible because raw sugars are liable to darken if kept for any length of time.

Reports were received from seven collaborators who used the instruments and experimental conditions specified, during the month indicated:

(1) Carl Erb, New York Sugar Trade Laboratory; Coleman Universal Spectrophotometer with diffraction grating; cells of 5.10 mm thickness; Brix of final solutions varying from 60.5 to 61.5; tests made at end of January.

(2) James Martin, New York Sugar Trade Laboratory; same instrument and cells used as by Carl Erb; Brix of final solutions varying from 61.4 to 62.6; tests made beginning of February.

(3) V. R. Deitz, National Bureau of Standards; Beckman DU spectrophotometer; cells of 2 mm thickness; Brix of final solutions varying from 59.4 to 61.3; tests made in April.

(4) R. Winston Liggett, American Sugar Refining Company Research Laboratory, Philadelphia, Pa.; Beckman DU Spectrophotometer; cells of 0.98 mm thickness for lower wavelength range, 10.00 mm thickness for higher wavelength range; Brix of final solutions varying from 61.5 to 65.2; tests made in June.

(5) I. W. Lohman and Miss M. D. Murray, results reported by L. F. Martin, Head of Agricultural Chemical Research Division, Southern Regional Research Laboratory, New Orleans, La.; Cary Recording Spectrophotometer; cells of 10 mm thickness; Brix of final solutions 56.7 to 60.0; tests made in June.

(6) R. T. Balch, U. S. Department of Agriculture, Houma, La.; Coleman Junior Spectrophotometer. (a) Test tube cells of 12 mm diameter; Brix of final solutions varying from 59.4 to 61.4; tests made in April. (b) Cells of 1 mm thickness for lower wavelength range, and of 8 mm thickness for higher wavelength range; Brix of final solutions 59.0 to 65.2; tests made in August.

(7) T. R. Gillett, California and Hawaiian Sugar Refining Corp., Crockett Calif.; photoelectric filter photometer of own design; thickness of cells and Brix of final solutions not given, but results reported as absorbancy indexes; tests made in May.

Results with Spectrophotometers.—Table 1 gives the absorbancy indexes reported by collaborators 1 to 6.

The absorbancy indexes found for each wavelength show large discrepancies, especially in the low wavelength range. The reasons for this may be:

1. Instrumental errors in some of the instruments used.

2. Errors in transmittancy readings near the zero and 100 per cent points. Collaborators 5 and 6, using cells of 10 and 12 mm respectively, obtained such low readings, some of them below 1 per cent transmittancy. Collaborators 1 and 2, with 5.1 mm cells, also recorded some readings below 10 per cent transmittancy. The readings of collaborator 3, with 2 mm cells, were all above 12 per cent. The readings of collaborator 4, with two different cell thicknesses, are the most reliable. Near the zero point small errors in the transmittancy reading cause large discrepancies

TABLE 1.—*Absorbancy indexes found with spectrophotometers*

WAVELENGTH, mμ	COLLABORATOR						
	1	2	3	4	5	6a	6b
Cuban Sugar, Asbestos							
400			5.651	6.111	3.595	1.740	4.868
405	3.497	3.713					
415	3.390	3.495	4.380	4.589	3.361	1.874	4.152
430	2.974	3.160	3.505	3.618	2.960	1.959	3.455
445	2.552	2.699	2.890	2.914	2.559	1.740	2.844
460	2.118	2.284	2.415	2.374	2.134	1.781	2.337
480	1.679	1.833	1.893	1.829	1.689	1.502	1.819
500	1.342	1.440	1.501	1.432	1.332	1.228	1.402
530	0.959	1.048	1.076	1.029	0.961	0.901	0.976
560	0.703	0.773	0.780	0.741	0.706	0.660	0.724
590	0.529	0.586	0.574	0.562	0.521	0.495	0.549
620	0.406	0.444	0.423	0.425	0.392	0.375	0.416
660	0.285	0.322	0.264	0.274	0.253	0.270	0.292
700	0.197	0.225	0.164	0.177	0.166	0.199	0.208
Cuban Sugar, Celite							
400			5.769	6.376	3.497	1.618	5.445
405	3.515	3.496					
415	3.409	3.324	3.986	4.853	3.497	1.762	4.623
430	3.049	2.975	3.199	3.809	3.107	1.806	3.853
445	2.634	2.588	2.629	3.058	2.792	1.762	3.181
460	2.280	2.200	2.156	2.467	2.402	1.618	2.609
480	1.811	1.751	1.608	1.920	1.937	1.383	2.005
500	1.451	1.389	1.359	1.487	1.555	1.132	1.547
530	1.043	0.989	0.990	1.060	1.127	0.829	1.080
560	0.769	0.733	0.740	0.787	0.822	0.615	0.809
590	0.536	0.556	0.561	0.592	0.610	0.465	0.601
620	0.412	0.422	0.425	0.443	0.454	0.352	0.451
660	0.309	0.300	0.286	0.285	0.295	0.252	0.320
700	0.215	0.217	0.175	0.185	0.187	0.185	0.225
Puerto Rican Sugar, Asbestos							
400			4.955	5.446	3.756	1.712	4.401
405	3.469	3.534					
415	3.272	3.364	3.867	4.225	3.511	1.839	3.799
430	2.897	2.976	3.171	3.428	3.092	1.891	3.203
445	2.571	2.589	2.649	2.827	2.673	1.816	2.678
460	2.199	2.216	2.209	2.329	2.254	1.643	2.225
480	1.745	1.786	1.765	1.810	1.811	1.390	1.752
500	1.403	1.424	1.396	1.427	1.455	1.133	1.360
530	1.015	1.017	1.001	1.014	1.057	0.812	0.959
560	0.747	0.765	0.722	0.772	0.772	0.608	0.699
590	0.565	0.584	0.530	0.563	0.585	0.461	0.541
620	0.435	0.442	0.387	0.429	0.436	0.346	0.405
660	0.307	0.322	0.246	0.282	0.285	0.246	0.288
700	0.217	0.220	0.150	0.176	0.194	0.184	0.206

TABLE 1.—(*Continued*)

WAVELENGTH, mμ	COLLABORATOR						
	1	2	3	4	5	6a	6b
Puerto Rican Sugar, Celite							
400			4.094	6.061	3.512	1.774	4.512
405	3.439	3.373					
415	3.250	3.284	3.264	4.099	3.512	1.894	3.831
430	2.900	2.938	2.667	3.310	3.120	1.952	3.254
445	2.520	2.535	2.256	2.748	2.728	1.734	2.732
460	2.148	2.145	1.898	2.265	2.373	1.696	2.271
480	1.746	1.733	1.502	1.738	1.911	1.421	1.777
500	1.389	1.390	1.203	1.393	1.519	1.163	1.387
530	1.005	1.004	0.870	0.970	1.111	0.843	0.970
560	0.748	0.746	0.646	0.760	0.826	0.619	0.721
590	0.569	0.556	0.482	0.563	0.615	0.462	0.550
620	0.418	0.428	0.352	0.423	0.461	0.351	0.407
660	0.279	0.296	0.243	0.271	0.302	0.256	0.289
700	0.194	0.219	0.153	0.176	0.195	0.187	0.208
Hawaiian Sugar, Asbestos							
400			2.698	3.087	2.670	1.420	2.533
405	2.331	2.336					
415	2.084	2.068	2.080	2.377	2.116	1.402	2.108
430	1.756	1.751	1.642	1.883	1.725	1.268	1.760
445	1.470	1.447	1.348	1.516	1.416	1.106	1.432
460	1.223	1.221	1.107	1.237	1.187	0.942	1.146
480	0.967	0.955	0.870	0.954	0.933	0.763	0.919
500	0.783	0.769	0.701	0.792	0.757	0.616	0.746
530	0.591	0.580	0.514	0.595	0.562	0.455	0.551
560	0.453	0.445	0.387	0.455	0.428	0.347	0.424
590	0.355	0.352	0.291	0.340	0.327	0.272	0.330
620	0.288	0.280	0.213	0.270	0.255	0.213	0.258
660	0.203	0.213	0.140	0.175	0.171	0.156	0.188
700	0.152	0.149	0.081	0.118	0.114	0.124	0.137
Hawaiian Sugar, Celite							
400			2.187	3.216	2.763	1.388	2.540
405	2.246	2.299					
415	2.012	2.058	1.670	2.453	2.239	1.388	2.134
430	1.677	1.725	1.332	1.898	1.814	1.286	1.747
445	1.390	1.446	1.083	1.539	1.498	1.116	1.429
460	1.152	1.200	0.901	1.221	1.258	0.961	1.157
480	0.921	0.950	0.715	0.962	1.001	0.784	0.923
500	0.740	0.759	0.575	0.810	0.814	0.632	0.745
530	0.546	0.574	0.429	0.611	0.619	0.468	0.555
560	0.415	0.439	0.330	0.469	0.474	0.361	0.421
590	0.324	0.343	0.259	0.364	0.370	0.285	0.332
620	0.257	0.278	0.196	0.272	0.290	0.226	0.257
660	0.186	0.212	0.134	0.176	0.198	0.168	0.190
700	0.134	0.164	0.079	0.119	0.159	0.130	0.138

in the absorbancy index, and the same is true of readings near the 100 point as discussed below.

3. In spite of the fact that the ratio of weight of filtering material to weight of sugar, and the conditions of filtration were standardized, unavoidable differences in the speed of filtration and other experimental conditions are liable to have affected the amount of colloidal material remaining in the solutions.

4. During the time from the end of January to the month of June the sugars may have changed in color and in the amount and character of the colloidal material.

It has been shown by Peters and Phelps (3) and confirmed by the writer and associates (4) that the absorbancy index at 560 mμ of a sugar in filtered solution is equivalent to the color as determined by monochromatic analysis, and the results obtained at that wavelength will be discussed in greater detail.

To gain an idea of the effect which errors in the transmittancy readings exert on the precision of the corresponding absorbancy indexes, it must be considered that with a sugar having an absorbancy index (a) of, e.g., 0.730 at wavelength 560 mμ (the approximate figure for both the Cuban and the Puerto Rican sugars) a plus error of 1 per cent transmittancy (T) causes a decrease in a of 0.192 at 5 per cent T, of 0.030 at 10 per cent T, of 0.022 at 20 per cent T, of 0.020 at 30 and 40 per cent T, of 0.021 at 50 per cent T, of 0.024 at 60 per cent T, of 0.029 at 70 per cent T, of 0.041 at 80 per cent T, and of 0.077 at 90 per cent T.

TABLE 2.—*Comparison of absorbancy indexes at 560 mμ, found in different months*

	JAN./FEB. AVERAGE OF COLLABORATORS 1 AND 2	APRIL AVERAGE OF COLLABORATORS 3 AND 6A	JUNE AVERAGE OF COLLABORATORS 4 AND 5	AUGUST COLLABORATOR 6B
Cuban Sugar				
Asbestos filtration	0.738	0.720	0.724	0.724
Celite filtration	0.751	0.678	0.805	0.809
Puerto Rican Sugar				
Asbestos filtration	0.756	0.665	0.772	0.699
Celite filtration	0.747	0.633	0.793	0.721
Hawaiian Sugar				
Asbestos filtration	0.449	0.367	0.442	0.424
Celite filtration	0.427	0.346	0.472	0.421
Av., Asbestos	0.648	0.584	0.646	0.616
Av., Celite	0.642	0.552	0.690	0.650
Av. for both	0.645	0.568	0.668	0.633

TABLE 3.—*Results of asbestos filtration versus Celite filtration*

COLLABORATOR	a, ASBESTOS FILTRATION, MINUS a, CELITE FILTRATION	
	Cuban Sugar	
1		−0.066
2	+0.040	
3	+0.040	
4		−0.046
5		−0.116
6a	+0.045	
6b		−0.085
	Puerto Rican Sugar	
1		−0.001
2	+0.019	
3	+0.076	
4	+0.012	
5		−0.054
6a		−0.011
6b		−0.022
	Hawaiian Sugar	
1	+0.038	
2	+0.006	
3	+0.057	
4		−0.014
5		−0.046
6a		−0.014
6b	+0.003	—
Sums	+0.336	−0.475
Averages	+0.034	−0.043

If we compare the average results at 560 mμ, obtained in different months, we find the data given in Table 2.

These figures do not indicate progressive darkening of the sugars, although the absorbancy indexes average higher in June than in January /February, and higher in August than in April. Because of these discrepancies the results cannot be used to draw conclusions about the comparative precision of either method. However, the results of asbestos infiltration vs. Celite filtration, obtained by each single collaborator at the same period of time, permit conclusions to be drawn on this basis alone. This comparison is made in Table 3.

The table shows that in ten cases asbestos filtration gave a higher absorbancy index than Celite filtration, and that in the other eleven cases the reverse is true. The average amount of change is nearly the same and the Celite filtration gives an only slightly higher value. It would appear that if a filtration method is used, either asbestos or Celite may be employed as filtering material. Filtration with Celite is much easier to execute and is so much more rapid that this filtering material can be recommended as preferable to asbestos. Collaborator 6

has stated that filtration with asbestos was found to be very troublesome and that an extremely long time was required to obtain sufficient filtrate for the color measurements.

Results with photoelectric filter photometer (Collaborator 7). The results of this collaborator are shown in Table 4.

TABLE 4.—*Results of collaborator 7 with photoelectric filter photometer*

	ABSORBANCY INDEXES				
COLOR FILTER NO.	1	2	3	4	5
Approximate dominant wavelength (mμ)	*420*	*535*	*585*	*640*	*720*
Cuban Sugar					
Method A	2.39	1.09	0.60	0.31	0.01–0.06
B	2.32	1.06	0.56	0.29	0.01–0.05
C	2.53	1.10	0.61	0.32	0
D	2.94	1.23	0.71	0.37	0.01–0.05
Puerto Rican Sugar					
Method A	2.16	1.00	0.57	0.27	0.00–0.03
B	2.16	1.00	0.54	0.27	0.01–0.02
C	2.33	0.98	0.54	0.30	0
D	2.86	1.20	0.65	0.35	0.01–0.04
Hawaiian Sugar					
Method A	1.26	0.57	0.30	0.18	0
B	1.28	0.58	0.33	0.18	0
C	1.31	0.59	0.36	0.22	0
D	1.65	0.70	0.43	0.24	0.00–0.02

Filters:
1. Blue (Corning #554, 4 mm)
2. Green (Corning #401, 4 mm)
3. Yellow (Corning #351 and #398, 2 mm each)
4. Red (Corning #243, 4 mm)
4. Red (Corning #243, 4 mm)
5. Infrared (Wratten /88-A, 2 mm)

Methods:
A. Asbestos filtration (no *p*H adjustments)
B. Celite filtration (no *p*H adjustments)
C. C and H method (no *p*H adjustment)
D. C and H method (includes *p*H adjustment)

The filter No. 1, with an indicated effective wavelength of 420 mμ gave results corresponding to those of an actual wavelength of about 450 to 460 mμ, while the effective wavelengths of 535, 585, and 640 mμ for filters Nos. 2, 3, and 4 are approximately correct.

The results of this collaborator lead to the same conclusions as those of the others, namely that it makes little difference whether asbestos filtration or Celite filtration is used for raw sugars. He states that:

"There is exceptionally good agreement between the colors (absorbancy indexes) of the solutions which were prepared by various filtration

methods. This indicates that from a practical standpoint it does not matter too much what particular method is employed as long as there is no appreciable color removal and a clear sample is obtained. Apparently the two methods you specify, namely asbestos filtration and Celite filtration, as well as the method which we regularly employ in this laboratory using Filter-Cel, all accomplish about the same results. This is shown by the aforementioned good agreement in color (absorbancy index) values, and especially by the low absorbancies in the infrared which indicate extremely good clarity of the solutions."

Collaborator 7 also calls attention to the fact that for comparative results the solutions of all factory products should be adjusted to the same *p*H. But in the work reported here it was decided not to adjust the *p*H in order not to complicate matters unduly.

Monochromatic analyses.—The writer is greatly indebted to Louis Sattler, of this laboratory, who has drawn thirty-six transmittancy curves for 1 mm thickness and a solids concentration of 1 gram per milliliter of solution. He has calculated the brightness, excitation purity, and dominant wavelength according to the system of Hardy (5), with ten selected wavelengths and Illuminant C, for the solutions of the three sugars filtered with asbestos, and with Celite, by collaborators 1, 2, 3, 4, 5, and 6b, that is, in the order of time when the tests were made. (The values reported by collaborator 6a were not used, because the transmittancies reported for a 12 mm thickness were too low and too erratic in the lower range to give a reliable curve.) The results are given in Table 5.

The results show small, irregular changes in brightness and purity due to errors discussed previously. Collaborators 4 and 5 (June) obtained slightly lower average brightness and somewhat higher average purity for all samples than collaborators 1 and 2 (January-February). This indicates some darkening of the samples and a higher proportion of coloring matter of dominant wavelength about 576 mμ. The average brightness of the Celite filtrate of the Cuban sugar is somewhat lower, and the purity somewhat higher than those of the asbestos filtrate. The opposite is true for the Puerto Rican and Hawaiian sugars. This again indicates that both filtering materials cause selective adsorption of colloidal coloring matter, but the differences are small.

Louis Sattler also calculated the transmittancies for a concentration of 60 Brix, and a cell thickness of 2.46 mm at wavelength 560 mμ by equation 2 computed previously by Zerban and associates (4), and compared them with those actually found and computed on the same basis (Table 5). There was again very good agreement between the found and calculated values, except in two cases where the differences were 0.74 and 0.87.

Reliability of filtration methods.—The writer, with Louis Sattler, showed years ago (6) that filtration methods are not satisfactory because no

TABLE 5.—*Monochromatic analyses*

COLLABORATORS	BRIGHTNESS	PURITY	DOMINANT WAVE LENGTH	T. 560 2.46 MM. 60 BRIX EQUATION	T. 560 2.46 MM. 60 BRIX FOUND
		Cuban Sugar—Asbestos			
1	83.43	19.86	575.4	73.61	73.62
2	81.94	21.07	575.7	71.33	71.29
3	81.71	23.00	576.0	71.24	71.09
4	82.41	23.72	575.2	72.51	72.33
5	83.47	20.37	575.4	73.77	73.43
6b	83.01	23.19	574.8	73.42	72.86
		Cuban Sugar—celite			
1	82.07	21.07	576.3	71.65	71.55
2	82.69	20.48	576.0	72.47	72.50
3	82.72	21.10	576.5	72.69	72.35
4	81.59	24.34	574.6	71.17	70.89
5	81.02	21.82	576.3	69.88	69.82
6b	81.26	25.61	574.9	70.83	70.20
		Puerto Rican Sugar—Asbestos			
1	82.47	19.65	576.0	72.14	71.98
2	82.07	20.02	575.6	71.17	71.33
3	83.13	21.42	576.6	73.45	72.92
4	82.35	22.63	575.3	72.23	71.36
5	82.02	20.85	575.6	71.38	71.36
6b	83.26	21.80	575.0	73.63	73.66
		Puerto Rican Sugar—Celite			
1	82.65	19.38	575.6	72.14	71.98
2	82.83	19.70	575.5	72.79	72.57
3	84.77	18.04	576.9	75.71	75.38
4	82.52	21.71	575.2	72.36	71.73
5	81.07	21.20	576.1	69.85	69.70
6b	83.05	21.95	575.0	73.29	72.95
		Hawaiian Sugar—Asbestos			
1	89.28	11.11	575.3	82.04	82.16
2	89.35	11.33	575.1	82.56	82.30
3	90.63	11.14	576.2	84.54	84.42
4	89.50	12.32	574.8	82.71	81.97
5	89.94	11.49	575.0	83.33	83.00
6b	89.80	11.46	574.4	83.05	83.08
		Hawaiian Sugar—Celite			
1	89.80	10.90	574.5	83.43	83.18
2	89.36	11.14	574.5	82.56	82.73
3	91.92	8.97	575.9	86.36	86.57
4	89.02	12.08	575.1	81.86	81.44
5	88.73	11.87	575.1	81.33	81.28
6b	89.84	11.28	574.7	83.10	83.18

filtering material has been found that will completely remove colloidal matter without removing coloring matter also, and that it is necessary to work with unfiltered solutions, determining the transmittancy and Tyndall beam intensity of the solution, and correcting the transmittancy to obtain that of the solution perfectly free from colloidal matter. V. R. Deitz (collaborator 3) has also concluded from his investigations (7) that the problem of determining color and turbidity in sugar products must be solved by working with unfiltered solutions. R. Winston Liggett (collaborator 4) has concluded from the results obtained by him on the three sugars used in the present study that "both types of filtration were unsatisfactory in that selective removal of color stuff occurred." On the other hand, the filtrates still contained turbidity, as shown by the results of collaborator 7, obtained at wavelength 720 mμ.

Until the problem of color and turbidity determinations in unfiltered solutions is completely solved, filtration methods will have to be used. The present study has shown that filtration with asbestos and with Celite under the experimental conditions selected give approximately the same results. Filtration with Celite is generally preferred because it is much easier to handle and is more rapid.

RECOMMENDATIONS*

It is therefore recommended—

(1) That until a satisfactory rapid method for the determination of color and turbidity in unfiltered solutions is developed, the color of sugar products be determined at wave length 560 mμ by the method given, with Celite Analytical Filter Aid, and expressed as absorbancy index.

(2) That the study of this subject be continued, by determining the precision of the method, having each collaborator make at least three complete replicate experiments with the same raw sugar, all collaborators agreeing to carry out the work during a predetermined period of time.

REFERENCES

(1) *This Journal*, **25**, 211 (1942).
(2) *Ibid.*, **28**, 540 (1945).
(3) Bur. Standards Technologic Paper No. 338 (1927).
(4) *Anal. Chem.*, **23**, 308 (1951).
(5) ARTHUR C. HARDY, "Handbook of Colorimetry." The Technology Press, 1936.
(6) *Ind. Eng. Chem.*, Anal. Ed., **8**, 168 (1936).
(7) Paper presented at the Boston Meeting of the American Chemical Society, April 1–5, 1951.

* For Report of Subcommittee D and action of the Association, see *This Journal* **35**, 62 (1952).

REPORT ON REDUCING SUGAR METHODS OF ANALYSIS

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

The report of the Associate Referee on reducing sugar methods is presented as a progress report. No recommendations are being made, but it is hoped that procedures discussed will stimulate work on these or related methods.

The DeWhalley method[1] for the determination of invert sugar in refined white sugars finds wide application in industry on the Continent but has not been adopted to any extent in this country. The method has been under study because it is believed that its simplicity and speed will make it of interest to some laboratories.

It is a colorimetric method which depends on the visual matching of colors. Once the standards are set up, they are good indefinitely. The sample is matched to the standard immediately upon removal from the boiling water bath; the total operation takes about 3 minutes.

METHOD

APPARATUS

Test tubes.—White glass 6 in. × ¾ in., of uniform weight (about 9.4 to 9.6 g). Large rubber rings are fitted around the top so that they can be supported in the water bath.

Water bath.—Sheet copper, 7 in. cube, with three holes 1 in. diameter; the central hole is used for the test and the other two act as vents. Water level is held constant 2 in. below the top of the bath. Heat is applied by gas burner, protected from draft, with a manometer on the gas supply to insure a pressure of 3.5 to 3.75 in. of water maintained by use of a gas pressure regulator.

REAGENTS

(1) 20% methylene blue.

(2) 3 *N* NaOH (between 2.90 and 3.10 *N*).

(3) $CuSO_4 \cdot 5H_2O$ soln for standards—19.5 g made up to 500 ml with boiled, distilled water.

(4) NH_4OH for standards—32.9% NH_4OH by titration.

DETERMINATION

Seven g of the ground sample are weighed out to within 0.05 g, poured into a clean, drained test-tube, and 6 ml of distilled water, 1 ml of the methylene blue solution, and 1 ml of the caustic soda solution (micro-burette) are added. The tube is stoppered with a rubber stopper and shaken vigorously for 15 seconds. The stopper is removed, and the tube is immersed in the boiling water bath for 120 seconds. It is then removed and compared with the row of standard tubes. About 5 seconds or less are required to match it. If the invert per cent is above 0.015, invert-free sucrose plus sufficient of sample to make 7 g can be used for the test with the same set of standards and the per cent of invert present calculated.

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[1] *International Sugar Journal*, 39, 300 (1937).

PREPARATION OF STANDARDS

Measure the following volumes of the $CuSO_4$ soln into a series of the test tubes, add 10 ml of the NH_4OH and make to 50 ml with boiled, distilled water. After preparation, the tubes are sealed off. (Mixtures are designed to match samples of the tabulated invert sugar content.)

INVERT SUGAR	$CuSO_4$ SOLN	NH_4OH
per cent	*ml*	*ml*
0.001	40.00 (40.00)	10
0.002	24.60 (40.00)	10
0.003	16.40 (24.60)	10
0.004	10.66 (20.50)	10
0.005	7.18 (16.40)	10
0.006	4.92 (13.53)	10
0.007	2.97 (9.50)	10
0.008	2.26 (7.18)	10
0.009	1.74 (6.62)	10
0.010	1.33 (5.49)	10
0.015	0.50 (1.33)	10

Standards prepared from invert-free sucrose and invert sugar change rapidly as do methylene blue and water standards; hence the use of ammoniacal copper solutions.

In a study of the method, it was found that the steps between two adjacent color standards were in many cases too great. Standard color solutions were, therefore, prepared to fill these gaps and to better match the colors produced by the invert solutions of known sugar content. It is believed that differences in test-tube color, water-bath arrangement and unavoidable individual variations in manipulation account for the necessity for the modified color standards. Volumes of copper solution found, in our hands, to give better color matches are given in the table (in parentheses).

Matching the sample to the standard is most easily accomplished around the middle of the scale (0.005%), and is most difficult at the upper limit. It takes practice to match them within the specified 5 second time interval, and it is to be expected that they cannot be matched closer than 0.002%. For example, the difference between 0.005% and 0.007% is easily distinguished, but it is extremely difficult to differentiate between 0.005% and 0.004%.

Three brands of locally available commercial sucrose were analyzed by this method. The results are given on the following page.

The method was quick and easy to perform, and this should make it of interest to laboratories where analyses are run on high purity sucrose samples.

In the work on an adsorption method for the analysis of sugar mixtures

SAMPLE	I	II	III
	per cent invert	*per cent invert*	*per cent invert*
Run 1 8/ 6/51	0.018	0.023	0.032
Run 2 8/28/51	0.021	0.028	0.038
Run 3 8/28/51	0.018	0.028	0.038

a procedure has been described for the determination of dextrose, maltose, and dextrins in corn sirups (Nat. Bur. Standards J. Research, **47**, 363 1951). The analysis of sugar mixtures, especially when incorporated in food products, is one of the most difficult problems the sugar analyst meets. Continued studies are being made to extend the adsorption technique to other sugar mixtures.

No reports were given on drying methods, densimetric and refractometric methods, honey, corn sirup and corn sugar, or starch conversion products.

The contributed paper "Refractive Indices of Raffinose Hydrate Solutions," by F. W. Zerban and James Martin, was published in *This Journal*, **34**, 808 (1951).

REPORT ON CACAO PRODUCTS

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

A large amount of work was done this year on methods for lecithin and pectic acid and a limited amount on sugars and cacao constituents. The writer has noted some new procedures for theobromine which appear promising, but has not tried them as yet. Associate Referee Meyers has obtained special apparatus and is ready to proceed with work on a chromatographic procedure for sugars (an adaptation of that reported last year by Dr. E. J. McDonald of the National Bureau of Standards), but does not have a report this year.

Lecithin

The Associate Referee on lecithin, Mr. Bornmann, has conducted a collaborative study of three commercial samples of milk chocolate, two labeled with a declaration of added lecithin which is required under the standards for cacao products, and one with no such declaration. The results given in the Associate Referee's report show a marked difference

TABLE 1.—*Pectic acid in samples of ground nib (shell free) expressed on fat-free dry basis*

SAMPLE NO.	SOURCE OF CACAO BEAN	PECTIC ACID (PER CENT)		CACAO RESIDUE (GRAMS)	FACTOR FAT-FREE CHOCOLATE CACAO RESIDUE*
1	Brazil	0.22	0.21	2.4878	2.01
2	Brazil	0.27	0.23		
3	Cameroon (Fr.)	.07		2.7190	1.84
4	Cameroon (Fr.)	.07		2.6500	1.89
5	Dom. Republic	.04		2.3528	2.13
6	Dom. Republic	.09		2.7344	1.83
7	Ecuador	.06	.06	2.6306	1.93
8	Ecuador	0.11	0.13	2.6112	1.89
9	Gold Coast	0.10		2.5633	1.95
10	Gold Coast	0.11		2.4174	2.07
11	Guiana (Spanish)	0.14		2.2322	2.24
12	Guiana (Spanish)	0.13		2.2005	2.27
13	Haiti	0.06	.05	2.5538	1.96
14	Haiti	0.07			1.88
15	Ivory Coast	0.10		2.6100	1.92
16	Ivory Coast	0.10		2.700	1.85
17	Mexico	0.05		2.664	1.88
18	Nigeria (Lagos)	.05		2.2252	2.24
19	Nigeria	.04		2.527	1.98
20	Nigeria	.05		2.595	1.93
21	Trinidad	0.15	0.18	2.6514	1.89
22	Trinidad	0.21	0.22	2.6776	1.87
23	Venezuela	.02	.05	2.6509	1.89
24	Venezuela	0.05		2.5288	1.98
25	Blend	0.17		2.680	1.86

* See text.

between the samples labeled to show added lecithin and the other with no such declaration. Results obtained by the collaborators are in fair agreement but difficulty was experienced by some collaborators in obtaining a clear aliquot. The Associate Referee recommends an amendment of the method to take care of this difficulty, and the Referee concurs in this recommendation.

Shell by Pectic Acid

Extensive work has been done this year on the method for pectic acid. In collaboration with a team of chemists from Great Britain the first action method was edited carefully to eliminate all ambiguities. No material changes in procedure were made. It is recommended that the method which was subjected to this editing be published in the *Journal*,* The method was used in the examination of numerous authentic samples

* The edited method was adopted and appears in *This Journal*, 35, 71 (1952).

of hand picked, shell-free nib and shell of various varieties of beans, to obtain authentic data. This work was done by the N.Y. District and the Division of Food of the Food and Drug Administration.

The authentic samples were prepared by roasting cacao beans in a laboratory-size drum type roaster at the usual roasting temperatures (100–135°C.) for a period of ½ hour. The roasted beans were cracked and after screening into several sizes they were air separated several times by slowly pouring the cracked beans from a position a little above an electric fan past the front of the fan. This procedure effected a fairly good separation of shell and nib. The separation of shell was completed by pouring the material down a blotting paper on an inclined plane and finally by hand picking until nib-free shell, and shell-free nibs were obtained. The samples of nibs were finely ground, first in a Quaker mill, then by passing through a roll refiner several times. The shell samples were ground in a high speed hammer-mill micro-pulverizer.

Results of determination of pectic acid on these samples by the edited method are given in Tables 1 and 2.

TABLE 2.—*Pectic acid found in samples of pure ground cacao shell expressed on fat-free dry basis*

SAMPLE NO.	ANALYST	SOURCE	PECTIC ACID (PER CENT)
1	B	Trinidad	4.62
2	B	Trinidad	4.73
3	D	Brazil	4.90
4	D	Haiti	5.08
5	B	Gold Coast	5.17
6	B	Gold Coast	5.12
7		Ecuador	5.31
8		Brazil	5.59
9	B	Ivory Coast	5.43
10	C	Brazil	5.63
11	C	Ecuador	5.64
12	A	Nigeria	5.72
13	A	Nigeria	5.75
14	C	Venezuela	5.77
15	B	French Cameroon	5.88
16		Lagos	5.90
17		Haiti	6.31
18	A	Spanish Guiana	6.56
19	A	Spanish Guiana	6.58
20	B	Ivory Coast	5.44
21	A	Dom. Republic	6.31
22	A	Haiti	6.34
23	A	Haiti	6.46
24	B	French Cameroon	6.52
25	A	Dom. Republic	6.61
26		Venezuela	7.00

The pectic acid content found in the nib varies from a few hundredths per cent to a maximum of 0.27 per cent. Results on shell vary from 4.62 per cent to 7.00 per cent. The large spread of pectic acid between nib and shell makes pectic acid a good criterion of the quantity of excess shell. The spread in the pectic acid content of shell-free nib is reduced considerably when a "blank" is obtained by determining pectic acid on samples containing known added quantities of shell and correcting for the pectic acid in the shell added. (This "true blank" on nib will be discussed more fully later in this report.)

Table 1 also lists the factors obtained on the various liquor samples to convert the dry residue remaining after the various extractions to the fat-free dry cacao in the original sample.

Results on a few samples of raw beans and nibs are given in Table 3. Results on the same samples after roasting show no significant difference on the dry, fat-free basis.

TABLE 3.—*Pectic acid in samples of raw (unroasted) ground shell-free nibs and raw (unroasted) ground shell (nib free) on fat-free dry basis*

SAMPLE NO.	ORIGIN	PECTIC ACID (GRAMS)	PECTIC ACID (PER CENT)
	Unroasted Ground Nib (Shell Free)		
1	Gold Coast	.0020	.04
2	Gold Coast	.0026	.05
3	Trinidad	.0020	.04
4	Trinidad	.0026	.05
	Unroasted Pure Ground Shell		
1	Gold Coast	.1036	5.18
2	Gold Coast	.1036	5.18
3	Trinidad	.0925	4.63
4	Trinidad	.0916	4.58

COLLABORATIVE STUDY

Two chocolate samples, one of bitter chocolate, and one of sweet chocolate containing known amounts of added shell, were prepared. The nibs used to obtain the liquor for the samples were a blend of Accra, Lagos, Seasons Arriba, and La Gayra Caracas beans. Eighty pounds of nibs were collected from the factory nib stream during a period of a half hour. This quantity was reduced by dividing on a riffle until about 10 pounds were obtained. This sample was then rendered shell free by means of a small fanning mill and finally by hand picking. The two samples were prepared from the shell-free liquor obtained by grinding these nibs. The sample of liquor containing 97 per cent of the shell-free liquor and 3 per cent of added cacao shell was identified as FD 1266-B.

The other sample was the authentic sweet chocolate (FD 1267-B), which contained 40 per cent liquor with 2 per cent added shell, 45 per cent sugar and 15 per cent cacao butter. The shell used was a blend. These samples were submitted to collaborators who were instructed to determine pectic acid by the first action method which had been edited as described above.

Two of the collaborators in addition to the referee were asked also to run the sweet chocolate sample by a double boiling modification of the first action method in which the pectic acid precipitate, after the first boiling, is separated by centrifuging and filtering. It is then rehydrolyzed, precipitated, and boiled again, after which it is filtered as in the regular method. Results obtained by all of the collaborators are given in Table 4.

TABLE 4.—*Pectic acid found in chocolate samples of known composition. (Results expressed as percentage of dry fat free cacao)*

COLLABORATOR NO.	PECTIC ACID (PER CENT)					
	FD 1266-B		FD 1267-D		DOUBLE BOILING FD 1267-D	
1	0.71	0.66	0.37	0.39	—	—
2	0.63	0.64	0.47	0.48	0.46	0.47
3	0.64	0.63	0.48,0.49,0.44			0.39
4	0.71	0.74	0.50	0.51		
5	0.64	0.62	0.46	0.46	0.44	0.43

(FD 1268B, Shell, 5.27% as is, 5.90% f.f.d. basis.)

COMMENTS

Agreement among collaborators is considered good, particularly so on sample FD 1266-B. Three of the collaborators show excellent agreement on both samples. These were analysts who had had more experience with the method than the others. The shell used in these samples contained 5.27 per cent pectic acid on the "As is" basis (5.90 on fat free dry basis). The liquor sample, FD 1266-B, contained 54.38 per cent fat and 1.87 per cent moisture. From these values it is apparent that the shell present contributed 0.362 per cent pectic acid to the dry, fat-free liquor (FD 1266-B) leaving 0.27 per cent (author's figure) as contributed by the liquor itself. This blank is higher than is usually found in a shell-free liquor to which no shell is added. It is somewhat reduced by the double boiling method but not as much as expected. It appears therefore that there is either a small solubility factor for the nib pectin or a small quantity of the nib material is carried down by the pectic acid from the shell. The usable blank on shell free liquor would therefore have to be obtained by determination of pectic acid on samples containing some shell and correcting for the shell pectic acid present.

In pursuit of this thesis a number of determinations of pectic acid were made on liquors containing known amounts of added shell of known pectic acid content. These are given in Table 5.

TABLE 5.—*Pectic acid (blank) contributed by the liquor after correction for pectic acid from shell*

SAMPLE	PER CENT SHELL IN LIQUOR	PECTIC ACID FOUND	PECTIC ACID CONTRIBUTED BY SHELL	PECTIC ACID IN LIQUOR CORRECTED
1	3.64	0.67	0.42	0.25
2	3.64	0.69	0.42	0.27
3	6.17	0.94	0.77	0.17
4	6.02	1.04	0.76	0.28
5	3.96	0.51	0.39	0.12
6	4.0	0.52	0.44	0.08
	8.0	0.97	0.86	0.11
7	3.96	0.59	0.39	0.20
FD 1266-B	3.0	0.63	0.36	0.27
FD 1267-B	2.0	0.46	0.24	0.22
			Average	0.21

The results in Table 5 show a small increase over the results obtained on the shell free liquor when run alone.

The higher "blank" obtained on the nibs when shell is added and allowed for in the manner stated appears to be due to the precipitation of additional material from the nibs under those conditions and not to the solubility of the pectic acid in the shell. The following experiment points to this conclusion. Four portions of varying amounts of the same (nib free) shell sample were used for pectic acid determination by the method herein. The quantities used and the results obtained are given in the following Table 6.

The results in the table show that even on samples of shell as small as 0.25 gram (in which the pectic acid precipitated is only about 13 mg) the percentage pectic acid obtained is only a little less than with larger samples of one gram or more, and does not increase with the size of sample.

TABLE 6.—*Pectic acid found on the same sample of shell using various quantities for determination*

SHELL	SIZE OF SAMPLE	PER CENT PECTIC ACID FOUND (AV. OF 2 DETERMINATIONS)
NY 29893	1 gram	4.91
NY 29893	0.5 gram	5.06
NY 29893	0.25 gram	4.68
NY 29893	2.0 gram	5.11

In order to form an exact basis for shell calculation, the blank should be obtained by determinations on liquors containing some shell and correcting for the portion of pectic acid contributed by the shell. Samples of liquor containing about 2 or 3 per cent shell (1.75 per cent is specified in the standards for cacao products) should give results which will furnish a sound basis for the calculation of shell in all liquor samples that might be examined.

RECOMMENDATIONS*

It is recommended—

(1) That the study of methods for the determination of lactose and maltose in the presence of other reducing sugars be continued.

(2) That the method for lecithin as revised in this year's report of the Associate Referee be studied collaboratively.

(3) That the method for pectic acid discussed in the Referee's report for chocolate liquor, cocoa, and sweet chocolate (without milk solids), which is an editorial revision of the first action pectic acid method (sec. 12.15, *Methods of Analysis*, A.O.A.C. 1950), be made official and be substituted for the first action method in sec. 12.15.

(4) That work on the determination of pectic acid in products containing milk solids be continued and that work on the method of conversion of pectic acid to galacturonic acid be continued.

(5) That work on characteristic cacao constituents such as theobromine, cacao red, tannins, etc., be continued.

REPORT ON LECITHIN IN CACAO PRODUCTS

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

Subcommittee D recommended† that the method for lecithin be studied further collaboratively.

Three samples of well-known brands of milk chocolate were bought at retail. Samples 1 and 3 declared added lecithin, while No. 2 did not. The samples were grated and thoroughly mixed before sending them to collaborators. This was done to insure uniformity of samples. Collaborators were instructed to determine lecithin in duplicate by 12.36 (*Methods of Analysis*, 7th edition p. 187). The results are given in Table 1.

COLLABORATORS' COMMENTS

Yarnall, Kansas City: After samples had stood overnight with the solvent some difficulty was experienced in obtaining a clean filtrate when using a setup similar to device No. 2 (*Methods of Analysis*, 7th edition,

* For report of Subcommittee D and action of the Association, see *This Journal*, **35**, 56 (1952).
† *This Journal*, **34**, 53 (1951).

page 187). The first two results on Sample 1 were obtained in this way and the filtrate was not entirely clear. All other subs were handled in the following manner: After standing overnight in the volumetric flasks, mixtures were transferred to 250 ml centrifuge bottles. (Evaporation was guarded against by covering the opening of the bottle with a thin rubber diaphragm.) The bottles were centrifuged at 1600 r.p.m. until a clear liquid was obtained (ca. 20 min.). The pipet was inserted

TABLE 1.—*Lecithin in sweetened milk chocolate*

ANALYST	SAMPLE 1	SAMPLE 2	SAMPLE 3
	per cent	*per cent*	*per cent*
1	0.30	0.11	0.30
	0.31	0.14	0.28
	0.30		
	0.29		
2	0.334	0.127	0.296
	0.216	0.114	0.273
	0.296		
	0.325		
3	0.25	0.050	0.21
	0.22	0.072	0.22
	0.22	0.48	0.21
4	0.409	0.148	0.262
5	0.38	0.15	0.34
	0.41	0.15	0.35
6	0.290	0.086	0.282
	0.282	0.092	0.282
7	0.284	0.114	0.284
	0.284	0.114	0.291
8	0.300	0.095	0.287
	0.299	0.095	0.288

thru a two-hole stopper and filled (without filter paper) by pressure supplied by a bulb attached to the other hole of the stopper. A perfectly clear solution was easily obtained in this way.

Perlmutter, Minneapolis: First values on Sample 1 did not check, and since we use a phosphate detergent it was thought contamination had taken place. On re-running the color development, check values were obtained. We cannot account for one low value.

Borker, Hoboken, N.J.: Replicate determinations were made simultaneously; however, the digestion of each sample was made on a different

day. Color development of all the samples was made at the same time. There is no known cause of error for the value 0.48 on Sample 2.

The heteropoly blue color did not develop the first time the determination was made. The results reported were obtained by using the normal sulfuric acid-sodium sulfite procedure described in the "Notes" (*Methods of Analysis*, 7th ed., p. 337). We would like to inquire if other microphosphorus methods have been studied. The molybdate method described in *Methods of Analysis*, 7th ed., 6.38, 39, 40 (p. 104–5), seems to us to be much easier to handle.

Offutt, New York: The P_2O_5 determination was made also on Samples 1 and 3 by the volumetric method under "Fertilizers," **2.13**(a), *Methods of Analysis*, which is shorter. Lecithin found was:

Sample 1—0.44%, Sample 3—0.28%.

Theper, St. Louis: The only difficulties encountered with the method involved filtering the samples. Probably the use of some type of pressure filtration device would simplify this step.

McCarthy, Cincinnati: Filtering device No. 3 was used and found impractical with filter paper wrapped around tip of pipet. However, when one operator guided the pipet (without filter paper) well away from the residue in the flask and a second handled the separators, the portions for analysis were neatly withdrawn.

LIST OF COLLABORATORS

1. F. E. Yarnall, Kansas City District, Food and Drug Administration.
2. Sam H. Perlmutter, Minneapolis District, Food and Drug Administration.
3. E. Borker, General Foods Corporation, Hoboken, N. J.
4. M. L. Offutt, New York District, Food and Drug Administration.
5. Frank P. Colten, Walter Baker Chocolate & Cocoa, Dorchester, Mass.
6. H. E. Theper, St. Louis District, Food and Drug Administration.
7. W. J. McCarthy, Cincinnati District, Food and Drug Administration.
8. J. H. Bornmann, Chicago District, Food and Drug Administration.

The associate referee wishes to thank the collaborators for their assistance.

DISCUSSION

The results reported by five of the eight collaborators are in fairly good agreement. It appears that some had difficulty because of inexperience with the molybdenum blue phosphate method. Since another micro method is available the Associate Referee believes that the directions should be amended to permit its use. High results may be encountered in case glassware is contaminated with phosphate detergent.

In formulating this method the Associate Referee had in mind the extraction of the lecithin with a minimum amount of labor and the determination of the P_2O_5 by an existing micro method. Since some collaborators had difficulty with the withdrawal of an aliquot, it might be well to amend the directions to permit pouring the mixture into a centrifuge bottle, stoppering, centrifuging until clear, and pipetting a portion without

filtering. Elaborate precautions to prevent evaporation are not necessary. Evaporation from a 1-inch bottle mouth is only ca 10 mg per minute. It is believed that for regulatory purposes the method is adequate for detecting added lecithin. (The ideal method would be one which enabled the analyst to differentiate cacao lecithin from that of another source.)

RECOMMENDATIONS*

It is recommended—

1. That the method be amended as follows: Delete **12.36,** Apparatus, including Fig. 21 and change **12.37,** Determination, to read:

Weigh 5 g of prepared sample, 12.1, into 200 ml volumetric flask, add ca 150 ml of $CHCl_3$-absolute alcohol (1+1), and shake occasionally during day. At end of day dilute to vol with same solvent, pour into 250 ml centrifuge bottle, stopper, and allow to stand overnight. Next day centrifuge with stopper on until clear (ca 15 min at 1800 r.p.m.) and pipet 100 ml of clear liquid into 500 ml Kjeldahl flask. Place Kjeldahl flask on steambath, remove solvent with current of air, and det. P_2O_5 as directed in 20.49 and 20.50. $P_2O_5 \times 11.37 =$ lecithin.

P_2O_5 may be det. by 6.40, in which case concentrate the 100 ml clear liquid in a 250 ml beaker, then wash into small crucible with solvent, evaporate, and proceed with 6.39. After digesting on the steambath, the crucible must be heated cautiously on a gauze until dry, and the heating continued until frothing ceases and most of the fat has smoked off before ashing in the furnace.

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

REPORT ON THE COLORIMETRIC DETERMINATION OF PECTIC ACID IN CACAO PRODUCTS

By HARRY SHUMAN (Food and Drug Administration, Federal Security Agency, Philadelphia 6, Pennsylvania), *Associate Referee*

The purpose of this work is to render more specific the method for pectic acid (1) in cacao products as a measure of cacao shell content.

Much of the time this year was devoted to the examination of the literature covering cacao products, pectic substances, pectic enzymes, and related subjects. Experimental work was largely devoted to the study of the colorimetric carbazole method for uronic acids (2).

Several approaches to the problem have been considered. The employment of commercially available pectic enzymes for the conversion of the cacao pectic substances to galacturonic acid, with subsequent separation by ion exchange has been suggested. However the carbazole method is applicable to pectic acid as well as to galacturonic acid, and since it appears that isolation of the pectic acid in highest purity may not be essential, study of enzymic methods are not contemplated at the present time.

* For report of Subcommittee D and action of the Association, see *This Journal*, **35**, 56 (1952).

Study of the carbazole method as applied by Stark (3) on galacturonic acid standards has shown that while plotted points for any one set of standards yields an essentially straight line, the line is not readily reproducible even with carefully duplicated conditions. Differences in room temperature appeared to influence rate of color development; but, use of a carefully controlled water bath at 25°C. failed to show expected improvement.

A sample of purified citrus pectic acid kindly supplied by the California Fruit Growers Exchange Research Department with an assay of 96.0 per cent (by titration) on moisture and ash free basis yielded by the carbazole method 95.0 per cent on same basis. The sample was prepared for colorimetric analysis by hydrolyzing in 60 ml 0.1 *N* NaOH in the steam bath for 15 minutes. The cooled solution was then nearly neutralized with acetic acid and diluted to suitable volume. Standards contained the same concentration of NaOH and acetic acid.

A sample of chocolate liquor was analyzed for pectic acid by the Winkler method (1). A second subdivision was carried through the final filtration and the pectic acid-asbestos mat was washed into a flask and analysis completed colorimetrically by the carbazole method. Results by the two methods did not check well and further study will be required. It is recommended* that—

(1) Study of the carbazole method for pectic acid be continued.

(2) Suitable modifications for isolation of pectic acid for colorimetric determination be studied.

REFERENCES

(1) *This Journal*, **35**, 71 (1952).
(2) DISCHE, Z., *J. Biol. Chem.*, **167**, 189 (1947).
(3) STARK, S. M., JR., *Anal. Chem.*, **22**, 1158 (1950).

No report was given on malt solids or on lactose.

REPORT ON FRUITS AND FRUIT PRODUCTS

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

The collaborative results on recoveries of small amounts of tartaric acid, as reported by Associate Referee Ferris, indicate that the procedure should be studied further. Additional work may help to explain the variations in the amounts of tartaric acid found by the different analysts.

The collaborative results on the determination of citric acid, given in a previous report of Associate Referee Ferris (*This Journal*, **34**, 74–75 and 445 (1951)) served as the basis for a recommendation for adoption of the procedure, first action. It is now recommended that the method be

* For report of Subcommittee D and action of the Association, see *This Journal*, **35**, 56 (1952).

adopted as official after correcting an omission of the strength of the nitric acid used to liberate the acids and a misplaced decimal point.

There is no report from the Associate Referee on the determination of malic acid. It is recommended that work on this subject be continued.

During the past year, associate referees were appointed for work on methods for the examination of frozen fruit. Mr. Fallscheer is studying procedures for the quantitative determination of fruit, sugar, and water in frozen fruit mixtures. Mr. Wallace is concerned with the development of a suitable procedure for the determination of the fill of the frozen fruit container. During the current packing season the two Associate Referees and the Referee planned work on these projects and participated in the preparation of a number of packs of frozen fruit in Washington and Oregon. Additional packs were put up in California by personnel of the San Francisco District of the Food and Drug Administration. Fruits which have been packed include strawberries, red and black raspberries, cherries, boysenberries, blackberries, loganberries, peaches, and apricots. All packs will be stored under usual commercial conditions for several months before examination. Plans have been made for a collaborative study of procedures and a report may be expected from the two Associate Referees at the next annual meeting of this Association.

RECOMMENDATIONS*

It is recommended—

(1) That the first action method for citric acid described in *This Journal*, **34**, 74–75 (1951) be adopted as official with the following corrections: In paragraph headed "Removal of Pectin," line 3, insert "N" between "ml" and "HNO_3." Last paragraph, last line, change "64" to "0.64."

(2) That the work on the separation and determination of fruit acids be continued.

(3) That work on methods for the examination of frozen fruits and fruit products for fill of container be continued.

(4) That work on methods for the examination of frozen fruits and fruit products for fruit, sugar, and water be continued.

REPORT ON TARTARIC ACID IN FRUIT PRODUCTS

By L. W. FERRIS (Food and Drug Administration, Federal Security Agency, Buffalo 10, N.Y.), *Associate Referee*

The A.O.A.C. method for tartaric acid in fruit products in the 7th Edition of *Methods of Analysis* is the same as that in the two previous editions except that directions for speed and time of centrifuging are omitted and a flask is substituted for a beaker in the final precipitation.

* For report of Subcommittee D and action of the Association, see *This Journal*, **35**, 58 (1952).

The complete recovery of small amounts of tartaric by this procedure has been questioned. In an article entitled "Polybasic Acids of Fruits and Fruit Products," *This Journal*, **26**, 446, Hartmann suggested the addition of a fixed amount of Rochelle salt to a product in which tartaric acid is to be determined—"because of the reluctance with which the cream of tartar reaction goes to completion in the lower concentrations." Dr. Osborn, Referee on Fruit Products, suggested that this matter be given further study, since the addition of an acid which is to be determined is undesirable from a regulatory official's point of view.

One sample of grape juice and two samples of grape and apple juices were prepared for collaborative study by the method for tartaric acid given in *Methods of Analysis*, 7th Edition. The tartaric acid in the grape juice used in making samples 1 and 2 was determined before mixing it with the apple juice. Sample No. 3 was pure grape juice but not identical with that used in making the mixtures. The amount of sample taken for analysis was sufficient to give, in the aliquot for precipitation of the acid tartrate, the following amounts of tartaric acid: No. 1, 10.8 mg; No. 2, 51.0 mg; No. 3, 92.8 mg. These amounts were calculated from the tartaric acid found in the grape juice as analyzed by the Associate Referee. The calculated amount in mixture No. 1 was 0.03 g/100 ml and in mixture No. 2, 0.23 g/100 ml. Results by collaborators are tabulated:

Tartaric acid g/100 ml

ANALYST	SAMPLES		
	1	2	3
No. 1	0.14	0.21	0.64
	0.16	0.19	0.62
No. 2	0.05	0.24	0.58[1]
	0.05	0.24	0.58[1]
			0.18
			0.18
			0.57[1]
No. 3	0.15	0.16	0.26
	0.16	0.17	0.27
			0.48
			0.47
No. 4	0.04	0.23	0.58
	0.03	0.23	0.58
	0.03		

[1] Sample placed in deep freeze overnight.

COMMENTS

Results on Sample No. 1, which contained only about 10 mg of tartaric acid in the aliquot for precipitation, not only indicate complete precipitation, but two of the analysts obtained much higher results than expected. All results on Sample No. 2 were in fairly good agreement. Results on Sample No. 3, a pure grape juice, were very unsatisfactory. There is at present no explanation for the great variation in tartaric acid found.

RECOMMENDATION

It is recommended* that further study be given to details of precipitation and washing, and, if a satisfactory explanation of the varying results is found, that a revised procedure be again studied collaboratively.

Appreciation is expressed to J. Marder and C. Hatmaker of the Food and Drug Administration, Washington, D.C., and to M. L. Dow of the St. Louis District, for their collaborative work.

No report was given on frozen fruit or malic acid.

REPORT ON FERTILIZERS

By F. W. QUACKENBUSH (Purdue University Agricultural Experiment Station, Lafayette, Indiana), *Referee*

Reports were received from the Referees on nitrogen, phosphoric acid, potash, sulfur, magnesium, and manganese. The recommendations are approved. No reports were received from Referees on moisture, sampling, acid-base balance, inert materials, boron, copper, and zinc.

REPORT ON MAGNESIUM IN FERTILIZERS†

By JOHN B. SMITH, *Associate Referee*, and Charles E. Olney, (Agricultural Experiment Station, Kingston, R. I.)

A single-value method for available magnesium in fertilizers seems impracticable because of the many variables involved in the rate of decomposition of dolomitic limestone in soils and consequent entrance of the element into the plant. A solvent such as acid ammonium citrate *p*H 4; *This Journal*, **27**, 500 (1944)) might be arbitrarily prescribed but its efficacy over a wide range of soil and climatic conditions would probably prove unsatisfactory. Such a single solvent might be accepted as a compromise in the interest of general agreement, but that does not appear to be likely at present. The method for water-soluble magnesium in use in many states is not interpreted as a measure of availability,

* For report of Subcommittee D and action of the Association, see *This Journal*, 35, 58 (1952).
† Contribution No. 784 of Agri. Experiment Sta., Kingston, R. I.

but indicates the very active fraction needed for acute deficiencies. The most frequent and most justified criticism of present magnesium methods is directed against the inclusion of the magnesium from coarse particles of dolomitic limestone in the determination of acid-soluble magnesium. This report discusses a preliminary study of a mechanical separation of coarse material with a view to the estimation of magnesium in this less-active fraction for the information of the professional agronomist and the consumer.

A similar distinction was made by Horat (*This Journal*, **21**, 296 (1938)) in a study of the acid- and base-forming quality of fertilizers. Choosing 20 mesh sieve size as the critical limit, he found that samples of unground fertilizer as large as 100 g were necessary for satisfactory duplication of results. Allen and Gault continued the study and apparently accepted the necessity for the large samples, but they finally discarded the method because of limited usage. (Quantities as large as 100 grams are difficult to manipulate and require larger reserve samples than are customary. Despite the heterogeneity of unground fertilizers, smaller samples must serve if the method is to be practicable.)

Although it is not intended that the coarse fraction be labeled as inert filler, the choice of sieve size is important. From the data available in 1940 the Associate Referee reported: "It seems conservative to estimate that ordinary dolomites in the average soil at *p*H 5–5.5 for two or three months will decompose at about the following rate: 20–40 mesh, 15 per cent; 40–60 mesh, 25 per cent; 60–100 mesh, 50 per cent; 100–200 mesh, 60 per cent; finer than 200 mesh, 75 per cent." (*This Journal*, **23**, 247.) With increasing use of liming materials and the westward movement of fertilizers, average soils may soon have *p*H values greater than 5–5.5. Higher values will decrease the rate of decomposition of dolomite.

To study the feasibility of relatively small samples for the separation, magnesic limestone in 10–20, 20–35, 35–40, and −40 mesh separates was mixed with a 6-8-8 fertilizer containing coarse tankage and granulated concentrated superphosphate along with other ingredients of smaller particle size. Most of the material passed a 10 mesh sieve and was somewhat finer than a representative commercial fertilizer, but was much coarser than a ground analytical sample. The separates of magnesic limestone were washed thoroughly to remove dust particles.

After mixing, the fertilizer was spread on oil cloth, remixed with a spatula, and samples of 5, 10, and 15 grams were weighed, using a plastic spoon to transfer small random portions to the balance. Margins of the layer were avoided. The samples were boiled with 100 ml of water for 45 minutes to disintegrate lumps and remove soluble salts. The suspension was poured on a 35 mesh sieve, 3 inches in diameter, and washed with a $\frac{1}{4}$-inch stream of tap water. (The 35 mesh sieve was selected in lieu of the 40 mesh because the latter was at hand only in larger sizes, and

TABLE 1.—*Recovery of magnesium in magnesic limestone coarser than 35 mesh after wet-sieving of fertilizer**

SAMPLE WT. (GRAMS)	LIMESTONE IN FERTILIZER						
	10–20 MESH		20–35 MESH FOUND ON 35 MESH SIEVE		4/5 20–35 MESH 1/5 35–40 MESH		FINER THAN 40 MESH
	MG PER CENT	RECOVERED PER CENT	MG PER CENT	RECOVERED PER CENT	MG PER CENT	RECOVERED PER CENT	MG PER CENT
5	1.98	109.4	1.70	93.9	1.37	92.6	Trace†
	1.52	84.0	1.66	91.7	1.51	102.0	Trace
	1.45	80.1	1.59	87.8	1.41	95.3	Trace
10	1.79	98.9	1.57	86.7	1.32	89.1	Trace
	1.83	101.1	1.54	85.1	1.48	100.0	Trace
	1.33	73.5	1.62	89.5	1.48	100.0	Trace
15	1.85	102.2	1.65	91.2	1.35	91.2	Trace
	1.93	106.6	1.63	90.0	1.37	92.6	Trace
	1.93	106.6	1.71	94.5	1.36	91.9	Trace

* 6-8-8 grade containing 1.81 per cent magnesium. Formula: coarse tankage, 150 lbs.; sulfate of ammonia, 540 lbs.; coarse granulated concentrated superphosphate, 155 lbs.; normal superphosphate, 435 lbs.; muriate of potash, 270 lbs.; magnesic limestone, 450 lbs. (The ingredients other than limestone supplied only insignificant quantities of magnesium.)

† Approximately 0.01 per cent.

the 3 inch size was believed to be more convenient and exact for the manipulation of small samples. Small 40 mesh sieves will be used if the study is continued.) The residue on the screen was washed into a porcelain evaporating dish; the lumps were then further disintegrated with a small rubber pestle, and again washed on the screen. This process was repeated until only a few particles appeared in the washings when they were collected in a beaker. Finally, acid-soluble magnesium was determined in the residue and calculated as per cent of the entire fertilizer. A similar procedure with the fertilizer without limestone showed only insignificant traces of magnesium in the portion remaining on the sieve.

The results are listed in Table 1. As expected, the greatest discrepancies among successive runs occurred with the coarsest limestone and the smallest samples. Where 10–20 mesh limestone was used results from the 5 and 10-gram samples are too variable. Results on the 15-gram samples, however, are as consistent as those on the ground samples. The fact that slightly more than 100 per cent of the coarser separate was recovered is probably due to manipulation and to minor errors of analysis, but much of the apparent loss from the 20–35 mesh size is ascribed to interaction with phosphates during the boiling period. In these samples none of the 40 mesh material remained in the residues, and a mixture of 20–35 mesh with 35–40 mesh particles was satisfactorily separated.

As a preliminary trial, this method of separating a coarse fraction of dolomite is promising, but factory-made fertilizers with coarser granules

may not bear out these findings. The method could provide information that could be interpreted in terms of specific uses and soil conditions. However, this might not imply the label, "available."

RECOMMENDATIONS*

It is recommended—

(1) Method 2.57 (b), first action (Methods, 1950), has been used for many years in a number of laboratories for the determination of soluble magnesium in mixed fertilizers. It was last studied collaboratively in 1940 (*This Journal*, **24**, 268), and was advanced to first action status in 1949 (*This Journal*, **33**, 37). The method is now recommended to be made official.

(2) That the study be continued.

REPORT ON PHOSPHORIC ACID IN FERTILIZERS

EFFECT OF PARTICLE SIZE ON CITRATE SOLUBILITY OF CALCIUM METAPHOSPHATE

By K. D. JACOB, *Associate Referee*, J. H. CARO, and R. M. MAGNESS
(Division of Fertilizer and Agricultural Lime, Bureau of Plant Industry, Soils, and Agricultural Engineering, Agricultural Research Administration, U. S. Department of Agriculture, Beltsville, Maryland)

The report on phosphoric acid presented at the last meeting of this Association indicated that the citrate solubility and the fertilizer efficiency of vitreous calcium metaphosphate are markedly affected by the particle size of the material (2). Further investigation of these relationships has been made. The data of a collaborative study of the citrate solubility of a typical sample of metaphosphate, as affected by its degree of fineness, are given herein.

SAMPLES AND METHODS

The sample of calcium metaphosphate (No. 2741, TVA No. 64665) was supplied by the Division of Chemical Engineering, Tennessee Valley Authority, Wilson Dam, Ala. It was from the same batch, but from a different shipment, as the identically numbered sample used in the previous study (2). Analysis by the TVA laboratory showed 26.6 and 63.6 per cent of CaO and total P_2O_5, respectively. The chemical composition and the particle-size range of the material as received were stated to be typical of the product planned for distribution by the Authority in 1950. It contained about 3 per cent of ground limestone as a conditioning agent.

In preparing the samples for the collaborative study, separate portions of the original metaphosphate were ground to pass U. S. standard sieves

* For report of Subcommittee A and action of the Association, see *This Journal*, **35**, 61 (1952).

of 20, 35, 60, and 80 square meshes per linear inch. In each case, the material coarser than the specified mesh was removed by screening, carefully ground in a horizontal-plate mill to pass the sieve, and then thoroughly mixed with the previously separated portion. The particle-size distribution in the original material and in the prepared samples is given in Table 1. As one would expect, the distributions in the several grinds differed from those in the comparable grinds used in the previous investigation. The grinds used in the present study were generally characterized by higher percentages of material in the coarser fractions.

TABLE 1.—*Mechanical composition of calcium metaphosphate*

SAMPLE WEIGHT (GRAMS)	FINENESS	COMPOSITION, MESH[a]						
		−20, +35	−35, +60	−60, +80	−80, +100	−100, +150	−150, +200	−200
	mesh	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
[b]	−10	33.3	17.2	7.4	5.2	5.8	4.2	10.4
1	−20	39.3	21.9	10.8	5.3	6.0	4.7	12.0
2	−35	—	37.5	18.2	8.2	10.1	7.1	18.9
3	−60	—	—	23.8	14.2	18.1	13.0	30.9
4	−80	—	—	—	18.3	20.7	17.0	44.0

[a] Screen openings in sieve series are 840, 420, 250, 177, 149, 105, and 74 microns, respectively.
[b] Original sample as received; contained 0.1% of +10 mesh (1,680 microns) and 16.4% of −10, +20 mesh material.

Citrate-insoluble P_2O_5 was determined in the four prepared samples by each of the two official procedures for the citrate digestion, (a) with manual shaking at 5-minute intervals and (b) with the aid of continuous agitation, constant-temperature devices. The several types of devices used by the collaborators are listed in Table 2.

TABLE 2.—*Type of continuous agitation, constant-temperature apparatus used in determining citrate-insoluble P_2O_5*

COLLABORATOR	APPARATUS	
	TYPE	REVOLUTIONS OR OSCILLATIONS PER MINUTE
2, 4, 5, 6, 7, 8, 9	End-over-end[a]	20–22
10	End-over-end[a]	40
11	End-over-end[b]	18
3	Stirring	1,150
12	Shaker[c]	240
1	Shaker[d]	120

[a] MacIntire-Marshall-Meyer type (3). Catalog No. 65960, Precision Scientific Co., Chicago, Ill.
[b] Apparatus made in the laboratory shop.
[c] "Wrist action" type. Catalog No. 75-775, Burrell Technical Supply Co., Pittsburgh, Pa.
[d] Apparatus made in the laboratory shop; motion imparted to the flasks is similar to that given by the Ross-Kershaw shaker (4).

Collaborators were given the following directions:

(1) Determine citrate-insoluble P_2O_5 as directed in *Methods of Analysis*, 6th Ed., 1945, p. 24, sec. **2.16(b).** Dissolve the citrate-insoluble residue by boiling for 15 minutes in 15–30 ml. of HCl plus 3–10 ml. of HNO_3 (p. 22, sec. **2.8(b)**) and determine P_2O_5 as directed on p. 23, sec. **2.12(a)** or **(b).**

(2) Repeat the determination of citrate-insoluble P_2O_5 as follows: Proceed as directed in sec. **2.16(b)** through the point where the flask is first shaken vigorously to reduce the filter paper to a pulp. Next place the flask in a continuous agitation apparatus provided with means for maintaining the contents of the flask at 65°C., and agitate for exactly 1 hour from the time the sample was introduced into the flask. Then proceed with the determination as before.

The sample for the citrate digestion should be placed on a *dry* paper and the initial agitation of the flask's contents should be such as to effect thorough dispersion of the sample in the citrate solution.

As some difficulty may be experienced in obtaining good agreement among the replicate determinations, especially with the −20 and −35 mesh materials, special care should be taken to insure uniformity in the treatment of the sample during the citrate digestion and the filtering and washing of the residue.

COLLABORATORS

(1) Allen, H. R., Dept. Feed and Fertilizer, Ky. Agr. Expt. Sta., Lexington, Ky.

(2) Bidez, P. R., and Richburg, Rex, State Chem. Lab., Ala. Dept. Agr. and Ind., Auburn, Ala.

(3) Blackwell, A. T., and Hoffmaster, F. G., The Davison Chem. Corp., Baltimore, Md.

(4) Caro, J. H., Div. Fertilizer and Agr. Lime, Bur. Plant Ind., Soils andAgr. Eng., Beltsville, Md.

(5) Hardin, L. J., and Williams, J. Earl, Tenn. Agr. Expt. Sta., Knoxville, Tenn.

(6) Hollington, W. H., Plant Products Div., Canada Dept. Agr., Ottawa, Ontario, Canada.

(7) Lawton, J. K., and Pasley, H. N., Chem. Div., Fla. Agr. Dept., Tallahassee, Fla.

(8) Luff, B. B., Div. Chem. Eng., Tenn. Valley Authority, Wilson Dam, Ala.

(9) Magness, R. M., Div. Fertilizer and Agr. Lime, Bur. Plant Ind., Soils and Agr. Eng., Beltsville, Md.

(10) Tannehill, B. K., Miss. State Chem. Lab., State College, Miss.

(11) Wetherbee, Robert T., Feed and Fertilizer Regulatory Service, Mass. Agr. Expt. Sta., Amherst, Mass.

(12) Willis, R. L., Chem. Dept., N. J. Agr. Expt. Sta., New Brunswick, N. J.

COMMENTS OF COLLABORATORS

Collaborator 1.— We have always had difficulty in getting closely agreeing checks on citrate-insoluble P_2O_5 in metaphosphate when the sample was coarser than 60 or 80 mesh. I think it should be specified that samples be ground to pass a 35-mesh screen.

Collaborator 4.—Digestion of the citrate-insoluble residue was done in 25 ml of hydrochloric acid and 5 ml of nitric acid. Care must be exercised to avoid loss by bumping.

Collaborator 5.—The citrate-insoluble residue was digested with 20 ml of hydrochloric acid and 5 ml of nitric acid. Difficulty in obtaining check values was experienced in the case of the −20 mesh sample with the intermittent shaking procedure. The grade of paper to be used in transferring the sample to the citrate digestion flask is not stipulated in *Methods of Analysis*, but Whatman's No. 3 is recommended as one that disintegrates readily.

Collaborator 6.—Following agitation at 5-minute intervals, it was noted that large particles of citrate-insoluble residue adhered to the inside of the flasks.

Collaborator 7.—Numerous analyses still gave wide variation in results on No. 1 and No. 2. The reported data are the most precise that were obtained in a single series of replications.

Collaborator 8.—The citrate-insoluble P_2O_5 in the −35 mesh sample is somewhat higher than we had expected, in view of the results obtained on the −35 mesh material used in the investigation reported at the 1950 Meeting of the A.O.A.C. However, a screen analysis of the present sample showed that it contains substantially more of the −35, +60 mesh fraction than the previously used, similar grind. The variability of replicate determinations on the coarsely ground samples was confirmed by other triplicated determinations.

Collaborator 11.—With all samples, less variation in the results was obtained by shaking at 5-minute intervals during the citrate digestion.

DISCUSSION

The collaborators' results for citrate-insoluble P_2O_5 in the four grinds of calcium metaphosphate are summarized in Table 3. They show, in general, that:

(1) Lower values were obtained with continuous agitation than with intermittent agitation during the citrate digestion, especially on the coarser grinds (−20 and −35 mesh).

(2) With continuous agitation the particle-size effect was practically eliminated by grinding the sample to −60 mesh, or, with intermittent agitation, by grinding to −80 mesh.

(3) With all types of agitation the precision of results, as to both the replicate determinations and the average values between collaborators, was very poor for the −20 mesh sample. The precision improved with increase in the fineness of the grind, and it was better with continuous agitation than with intermittent agitation.

For continuous agitation during the citrate digestion, nine of the collaborators used end-over-end rotation devices, while the others accomplished this operation by stirring or shaking. Owing to the much greater use of end-over-end rotation and the considerable variation among the results of the collaborators who employed this type of apparatus, the data do not warrant conclusions as to the relative efficiencies of the several continuous agitation procedures.

The results generally confirm those previously reported by Jacob *et al.* (2). It should be noted, however, that in the present study grinding the sample to −60 mesh, together with continuous agitation during the citrate digestion, or to −80 mesh with intermittent agitation, was usually

TABLE 3.—*Effect of particle size and type of agitation during citrate digestion on results for citrate-insoluble P_2O_5 in calcium metaphosphate*

COLLABORATOR	CITRATE-INSOLUBLE P_2O_5 BY—				DIFFERENCE IN AVERAGE RESULTS[c]
	INTERMITTENT AGITATION[a]		CONTINUOUS AGITATION[b]		
	RANGE	AVERAGE	RANGE	AVERAGE	
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
			Sample 1, −20 mesh		
1	16.60–18.05	17.23	5.00– 5.72	5.30	11.93
2	7.85– 8.00	7.95	4.45– 5.55	5.07	2.88
3	7.72–11.80	9.43	7.36–10.56	9.17	0.26
4	9.79–10.62	10.19	4.21– 4.76	4.49	5.70
5	9.25– 9.90	9.64	2.50– 2.62	2.56	7.08
6	11.80–18.55	14.85	3.95– 4.80	4.30	10.55
7	9.15– 9.45	9.28	4.90– 5.50	5.20	4.08
8	7.82– 9.52	8.72	4.43– 5.31	4.91	3.81
9	8.45–11.42	9.72	3.37– 4.52	3.94	5.78
10	11.23–12.12	11.57	4.52– 5.80	5.32	6.25
11	9.38–10.08	9.73	7.69–10.16	9.14	0.59
12	9.40–11.43	10.70	4.35– 4.75	4.61	6.09
Group	7.72–18.55	10.75	2.50–10.56	5.33	5.42
			Sample 2, −35 mesh		
1	8.78–10.05	9.39	1.20– 2.30	1.68	7.71
2	3.30– 3.60	3.45	1.05– 1.10	1.08	2.37
3	2.36– 3.64	2.84	1.24– 4.56	2.89	−0.05
4	3.26– 3.45	3.34	1.46– 1.64	1.53	1.81
5	2.90– 3.02	2.97	0.78– 0.83	0.80	2.17
6	6.10– 8.00	7.10	1.00– 1.20	1.10	6.00
7	3.10– 3.40	3.27	1.65– 1.75	1.70	1.57
8	2.73– 3.12	2.88	1.35– 1.51	1.44	1.44
9	2.38– 2.52	2.44	0.80– 1.06	0.92	1.52
10	3.95– 5.17	4.39	1.32– 1.58	1.49	2.90
11	3.04– 3.71	3.44	2.43– 4.22	3.04	0.40
12	4.70– 4.92	4.81	1.15– 1.38	1.23	3.58
Group	2.36–10.05	4.19	0.78– 4.56	1.57	2.62
			Sample 3, −60 mesh		
1	2.85– 3.42	3.21	0.90– 0.95	0.92	2.29
2	0.90– 1.15	1.05	0.75– 0.90	0.82	0.23
3	0.92– 1.00	0.96	0.76– 0.96	0.84	0.12
4	0.95– 1.00	0.98	0.71– 0.75	0.73	0.25
5	0.98– 0.99	0.98	0.73– 0.75	0.74	0.24
6	1.00– 2.20	1.47	0.70– 0.80	0.77	0.70
7	0.80– 0.90	0.85	0.70– 0.80	0.75	0.10

TABLE 3—(*Continued*)

COLLABORATOR	CITRATE-INSOLUBLE P_2O_5 BY—				DIFFERENCE IN AVERAGE RESULTS[c]
	INTERMITTENT AGITATION[a]		CONTINUOUS AGITATION[b]		
	RANGE	AVERAGE	RANGE	AVERAGE	
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
8	0.81– 0.84	0.83	0.69– 0.73	0.71	0.12
9	0.76– 0.79	0.77	0.69– 0.71	0.70	0.07
10	1.03– 1.07	1.04	0.72– 0.75	0.74	0.30
11	0.80– 0.88	0.83	0.88– 1.26	1.01	−0.18
12	1.05– 1.15	1.09	0.70– 0.76	0.73	0.36
Group	0.76– 3.42	1.17	0.69– 1.26	0.79	0.38
	Sample 4, −80 mesh				
1	1.00– 1.15	1.08	0.72– 0.82	0.76	0.32
2	0.80– 0.85	0.82	0.75– 0.80	0.78	0.04
3	0.68– 0.72	0.69	0.72– 0.88	0.79	−0.10
4	0.74– 0.77	0.75	0.74– 0.76	0.75	0.00
5	0.71– 0.75	0.74	0.71– 0.71	0.71	0.03
6	0.70– 1.30	1.07	0.60– 0.90	0.77	0.30
7	0.70– 0.75	0.72	0.65– 0.70	0.68	0.04
8	0.65– 0.66	0.65	0.67– 0.70	0.69	−0.04
9	0.67– 0.71	0.69	0.68– 0.69	0.68	0.01
10	0.77– 0.78	0.77	0.72– 0.75	0.73	0.04
11	0.74– 0.78	0.76	0.76– 0.86	0.81	−0.05
12	0.64– 0.70	0.67	0.62– 0.73	0.67	0.00
Group	0.64– 1.30	0.78	0.60– 0.90	0.73	0.05

[a] Manual shaking at 5-minute intervals during the citrate digestion.
[b] Collaborators 2, 4, 5, 6, 7, 8, 9, 10 and 11 used end-over-end rotation at 18–40 r.p.m.; Collaborator 3 used continuous stirring at 1,150 r.p.m.; and Collaborators 1 and 12 used shakers having 120 and 240 oscillations per minute, respectively.
[c] The minus sign denotes value by continuous agitation is higher than that by intermittent agitation.

necessary to eliminate the effect of particle size on the values for citrate-insoluble P_2O_5. In the previous work, practical elimination of the particle-size effect was accomplished by grinding the sample to −35 and −60 mesh, respectively. These differences in the results of the two investigations may be attributed, at least in part, to the higher percentages of material and its rate of solution under the conditions of the determination in the coarser particle-size fractions of the current grinds.

The data reported at the 1950 Meeting of this Association (2), together with the results of subsequent agronomic experiments, indicate that the fertilizer efficiency of calcium metaphosphate increases as the particle size of the material is decreased from −10 mesh (the fineness of the commercial product) to −35 mesh. At the latter fineness the effect of the metaphosphate in promoting plant growth is approximately equal to

that of triple superphosphate. Likewise, the solubility of the metaphosphate in neutral ammonium citrate solution and the precision of the analytical results are markedly dependent on the fineness of the material and its rate of solution under the condition of the determination. With continuous agitation during the citrate digestion, relatively little increase in the solubility value is usually obtained, however, by grinding the sample finer than −35 mesh. Also the precision of the analysis is much improved by decreasing the particle size to this level. With intermittent agitation, still finer grinding of the sample is necessary to obtain similar solubility values and precision of results.

For the determination of available P_2O_5 in calcium metaphosphate, practical considerations point to a fineness of −35 mesh for the analytical sample and continuous agitation during the citrate digestion. In this connection, it should be noted that commercial calcium metaphosphate contains ground limestone as a conditioning agent, and that the metaphosphate itself tends to undergo segregation because of the wide range in its particle size. Hence, this material would seem to fall in the class of products for which grinding to pass a 35-mesh sieve is specified in the Official Procedure for preparing fertilizer samples for analysis (1).

PRESERVATION OF MOLYBDATE SOLUTIONS

Tartaric acid was effective in preserving molybdate solutions against discoloration and deposition of precipitate over a considerable period of time. In itself, however, the prevention of such deterioration had little or no influence on the results for P_2O_5 in fertilizers, as determined by the volumetric method. On the other hand, interference of the sulfate ion in the hot-precipitation volumetric method for P_2O_5 appeared to be eliminated by the presence of tartaric acid. (Results of this investigation will be presented in a separate paper.)

ACKNOWLEDGMENT

The Associate Referee wishes to express his appreciation of the cooperation given by the collaborators and their respective organizations. Assistance in certain phases of the work was also rendered by D. V. Bennett, Division of Fertilizer and Agricultural Lime.

RECOMMENDATIONS*

It is recommended—

(1) That determination of citrate-insoluble P_2O_5 in calcium metaphosphate be made on samples ground to pass the 35-mesh sieve, with continuous agitation during the citrate digestion.

(2) That work on methods for phosphoric acid in fertilizers be continued, with study of:

* For report of Subcommittee A and action of the Association, see *This Journal*, 35, 44 (1952).

(1) Use of perchloric acid in preparation of solutions for analysis.

(2) Preparation of solutions of calcium metaphosphate for determination of total P_2O_5.

(3) Use of tartaric acid for preventing interference of the sulfate ion in the hot-precipitation volumetric method.

LITERATURE CITED

(1) *Methods of Analysis, A.O.A.C.*, 1945, par. **2.2**, p. 6.

(2) JACOB, K. D., ARMIGER, W. H., CARO J. H., and HOFFMAN, W. M., *This Journal*, **34**, 624–640 (1951).

(3) MACINTIRE, W. H., MARSHALL, H. L., and MEYER, T. A., *ibid.*, **27**, 272–283 (1944).

(4) ROSS, W. H., and MERZ, A. R., *Wiley's Principles and Practice of Agricultural Analysis*, 3d ed., vol. II, 1931, p. 178.

REPORT ON NITROGEN IN FERTILIZERS

By H. A. DAVIS (New Hampshire Agricultural Experiment Station, Durham, New Hampshire), *Associate Referee*

The report of the Associate Referee is brief due to the fact that unexpected circumstances prevented the preparation of samples for collaborative work. It was recommended in reports made at the 1950 meeting of the Association that "the study of the Shuey method for the determination of nitrogen in high nitrate-high chloride mixtures be continued."

When it was found that samples would not be available in time to allow collaborative work, a letter was sent to twenty-five persons who had expressed an interest in the work, stating that no samples would be available for collaborative work this year. Each collaborator was asked to express his opinion regarding further work and to offer any comments concerning the determination of nitrogen in fertilizers. Thirteen replies were received, and the Associate Referee has reached the following conclusions:

Further collaborative work with the Shuey method is desirable. The samples should be made up of c.p. or equivalent chemicals so that the nitrogen content may be closely known. The practicability of the Shuey method in routine use is questioned. Its use may be limited to the analysis of samples high in nitrate nitrogen or chlorides that have been screened by the usual method.

It is recommended* that the study of the Shuey method be continued. The Associate Referee wishes to thank those who have assisted in any way with this work. Any suggestions pertinent to the problem will be appreciated.

* For report of Subcommittee A and action by the Association, see *This Journal*, **35**, 44 (1952).

REPORT ON POTASH IN FERTILIZERS

By O. W. Ford (Purdue University Agricultural Experiment Station, Lafayette, Indiana), *Associate Referee*

In accordance with the recommendations approved by the Association in 1950 (*This Journal*, **34**, 41, 1951), additional collaborative studies were made on methods for the determination of potash in fertilizers. A copy of the proposed work was sent to each chemist who had expressed a willingness to collaborate. Twenty-six chemists representing the fertilizer industry, commercial and control laboratories, found time to do the work and report to the Associate Referee.

DIRECTIONS TO COLLABORATORS

1. Prepare a double amount of solution as follows: weigh and transfer 5 grams of each sample to a 500 ml pyrex volumetric flask. Add 100 ml of ammonium oxalate solution, 250 ml water and digest as in A.O.A.C. Method.* When cool, add ammonium hydroxide, make to volume and filter. This should be a sufficient volume of solution for all potash determinations.
2. Using the Official Method for Potash, make 3 individual determinations on each sample on solution prepared in 1 above.
3. Also, using the slightly modified Perrin wet-digestion method, copy of details enclosed, make 3 individual potash determinations on each sample on solution prepared in 1 above.
4. Also, if a flame photometer is available, make 3 different readings on each sample on solution prepared in 1 above.
   a. Report potash values obtained without correction for sodium and magnesium.
   b. Report potash values obtained with correction for sodium and magnesium.

Report all results as soon as possible and not later than August 1, 1951, so the report of the General Referee can be completed for the Fall meeting.

Please list any comments or criticisms.

The samples sent to you have been ground and prepared for analysis, and *should be analyzed as received.*

RAPID WET-DIGESTION METHOD

REAGENTS

(a) *Platinum soln.*—Use a Pt soln containing the equivalent of 0.5 g of Pt (1.05 g H_2PtCl_6) in every 10 ml.

(b) *Diglycol stearate soln.*—Dissolve 20 g of diglycol stearate (tech.) in 1 liter of equal parts of benzene and ethyl alcohol.

PREPARATION OF SOLN

Place 2.5 g or the factor weight 2.425 g of a sample in a 250 ml volumetric flask. Add 125 ml of H_2O, 50 ml of saturated NH_4 oxalate soln, and 1 ml of diglycol stearate soln when necessary to prevent foaming. Boil 30 min, add slight excess of NH_4OH and, after cooling, dilute to 250 ml. Mix and pass thru dry filter.

DETERMINATION†

Place a 50 ml aliquot of soln (or a 25 ml aliquot and 25 ml H_2O, if sample con-

* *Methods of Analysis, 7th* Ed. (1950), par. 2.40(a), p. 17.
† Perrin, *Anal. Chem.*, 21, 984 (1949), slightly modified.

tains over 20% K_2O) in a 500 ml Kjeldahl flask. Add 10 ml HNO_3 and a silica granule (about 1 cm long) previously weighed along with a prepared Gooch or medium fritted crucible (Pyrex M porosity). Boil 2 min and add 10 ml HCl. Boil down to ca 25 ml and add 5 ml HCl and excess Pt soln. Boil down to 10–15 ml, rotating flask occasionally, and then add 5 ml HCl. Reduce heat and boil down to 3–5 ml (depending on amount of precipitate), rotating flask frequently near the end of the evaporation. Remove flask from heat and swirl to dissolve any soluble residue on walls. After cooling, immediately add 25 ml of 95% alcohol so that it washes down neck of flask. Chill under tap, swirl and allow to stand for at least 5 min. Decant into the tared crucible and transfer precipitate and granule with the aid of a stream of 95% alcohol. Wash 5 or 6 times with 10 ml portions of NH_4Cl soln (2.39(a)) to remove magnesium and sodium salts from ppt. Wash again thoroly with alcohol and dry ppt. for 30 min at 100°C. Weigh and subtract weight of crucible plus the silica granule. $K_2PtCl_6 \times 0.19376 = K_2O$.

THE COMPOSITION OF SAMPLES

Five samples of fertilizer were analyzed by three methods: (a) the official A.O.A.C. method, (b) the Perrin wet digestion method modified to include an ammonium chloride wash, and (c) the flame photometer. All samples were prepared at the Purdue laboratory in a Micro-Samplmill with the $\frac{1}{8}$ inch screen and were thoroughly mixed before shipment to the collaborators. The five samples had the following composition:

1. Mixture of 360 grams of c.p. potassium chloride, 350 grams c.p. dicalcium phosphate, 300 grams of c.p.-ammonium sulphate and 350 grams of c.p. monocalcium phosphate. The theoretical value for this sample is 16.72% K_2O.
2. Mixture of 100 grams c.p. monocalcium phosphate, 350 grams c.p. potassium chloride, 765 grams c.p. sodium nitrate and 145 grams c.p. dicalcium phosphate. The theoretical value for this sample is 16.24% K_2O.
3. Mixture of 200 grams c.p. monocalcium phosphate, 175 grams c.p. potassium chloride, 765 grams c.p. sodium nitrate, and 220 grams c.p. dicalcium phosphate. The theoretical value for this sample is 8.12% K_2O.
4. Mixture of 175 grams c.p. dicalcium phosphate 175 grams c.p. monocalcium phosphate, 350 grams c.p. potassium chloride, 660 grams c.p. magnesium nitrate. Theoretical value for this sample is 16.24% K_2O.
5. Mixture of 1012 grams (Sulfo-Mag-22% K_2O content), 116 grams concentrated superphosphate (0-46-0), 116 grams c.p. sodium nitrate and 116 grams c.p. sodium chloride. The theoretical value for this sample is 16.37% K_2O.

Notes

Samples 2 and 3 contain sodium
Sample 4 contains magnesium
Sample 5 contains sodium and magnesium

COMMENTS

(1) The results on the samples by the A.O.A.C. and modified Perrin methods showed very good agreement (Table 1). Likewise, the results by these two methods compared favorably with the values obtained with a flame photometer, where proper correction was made. The grand average of the results obtained by the three methods as within about 0.1% K_2O of theory. The modified Perrin and A.O.A.C. methods averaged

TABLE 1.—*Summary of results (per cent K_2O)*

METHOD	NUMBER OF COLLABORATORS	SAMPLE NO. 1			SAMPLE NO. 2			SAMPLE NO. 3			SAMPLE NO. 4			SAMPLE NO. 5		
		HIGH	LOW	AV.	HIGH	LOW	AV.	HIGH	LOW	AV.	HIGH	LOW	AV.	HIGH	LOW	AV.
A.O.A.C.	25[1]	17.67	16.41	16.84	17.10	15.82	16.45	8.66	8.09	8.44	16.93	15.38	16.07	17.04	16.17	16.61
MODIFIED PERRIN	25	17.03	16.16	16.76	16.81	16.13	16.48	8.57	8.06	8.32	16.99	15.87	16.15	16.79	15.69	16.49
FLAME PHOTOMETER																
Corrected	8	16.93	16.35	16.62	17.15	16.50	16.67	8.76	8.00	8.40	16.60	14.20	15.92	17.25	16.10	16.54
Not Corrected	4[2]	17.80	16.84	17.16	18.20	16.88	17.34	9.35	8.42	8.90	15.95	14.20	15.22	17.40	14.84	16.26
Grand Average[3]		16.74			16.53			8.39			16.05			16.55		
Theory		16.72			16.24			8.12			16.24			16.37		

[1] 22 for Sample 4.

[2] 3 for Samples 1 and 4.

[3] Excluding uncorrected flame results.

respectively 0.10% and 0.14% K_2O higher than theory while the flame value (corrected) was 0.09% K_2O higher than theory.

(2) Higher results than theory would have been reported on samples 2, 3, and 5 by the Perrin method unless modified to include the Lindo-Gladding wash. Collaborator #10 reported values of 21.48, 10.43, and 18.46% K_2O before the Lindo-Gladding wash and 16.54, 8.41 and 16.38% K_2O after the wash on these three samples. All three of these samples were high in sodium, and #5 contained, in addition, a high percentage of magnesium, and the phosphate content was so low that not all of the magnesium was removed in the preparation of the original solution. A Lindo-Gladding wash is definitely needed on samples of this kind.

(3) Results on sample #4 were lower than theory by both the A.O.A.C. and modified Perrin methods in all cases except one. This is what could be expected from a mixture of this kind. This sample was high in magnesium nitrate and unless analyzed soon after it was received it deliquesced and caked so that results tended to be low.

(4) Analysis of the results, and the reactions of the collaborators would indicate that the modified Perrin method might safely be used as an alternate method to the official A.O.A.C. method for potash in fertilizers.

(5) Examination of the results obtained by the flame photometer indicates that this new analytical tool may be used to advantage in the determination of potash in fertilizers. Of particular interest was sample #3 which contained a large amount of sodium. Here the flame values were closer to theory than those obtained by the A.O.A.C. or the modified Perrin methods. However, it should be noted that high concentrations of sodium or magnesium salts may result in false potash values if their presence is not recognized and corrections made (Table 1). The flame photometric procedure appears to be very promising, but the limited number of collaborators reporting flame values makes a critical evaluation of this method impossible at this time. However, work should certainly be continued.

LIST OF COLLABORATORS

(1) Allen, H. R., Kentucky Agricultural Experiment Station, Lexington, Kentucky.

(2) Austin, W. R., and Buford, Madelane, Armour Fertilizer Works, Nashville, Tennessee.

(3) Avens, A. W., Bigelow, C. R., and Rickey, G. F., New York State Agricultural Experiment Station, Geneva, New York.

(4) Batton, H. C., Swift & Company, Plant Food Division, Norfolk, Virginia.

(5) Blackwell, A. T., Davison Chemical Corporation, Baltimore, Maryland.

(6) Bopst, L. E., Inspection and Regulatory Service, College Park, Maryland.

(7) Caldwell, Paul, and Paulson, C. E., Darling & Company, Chicago, Illinois.

(8) Charlton, R. C., Kilpatrick, K. F., Layton, J. A., and Simpson, W. N., The American Agricultural Chemical Company, New York, New York.

(9) Etheredge, M. C., State Chemist, Mississipppi State Chemical Laboratory, State College, Mississippi.

(10) Geagley, W. C., O'Mera, P., and Sprague, R. B., Bureau Chemical Laboratories, State of Michigan Department of Agriculture, State Office Bldg., Lansing, Michigan.

(11) Gehrke, C. W., University of Missouri, Columbia, Missouri.

(12) Gilbertson, Harold, State of Minnesota Department of Agriculture, Dairy and Food, St. Paul, Minnesota.

(12A) Howes, C. Cliffton, Davison Chemical Corp., Nashville, Tennessee.

(13) Kocaba, Helen, and Fisher, H. J., Connecticut Agricultural Experiment Station, New Haven, Connecticut.

(14) Koch, R. C., Swift & Company, 150 Marble Street, Hammond, Indiana.

(15) Koehler, L. A., and Baird, R. O., State Laboratories Department, Bismarck, North Dakota.

(16) Kuzmeski, J. W., Weatherby, R. T., and Smith, C. T., University of Massachusetts, Agricultural Experiment Station, Amhurst, Massachusetts.

(17) Leary, J. W., Rabourn, W. J., and Kazeniac, S. J., Department of Agricultural Chemistry, Agricultural Experiment Station, Purdue University, West Lafayette, Indiana.

(18) Marshall, H. L., Mathieson Chemical Corporation, Baltimore, Maryland.

(19) Miller, R. D., Spencer Chemical Company, Pittsburg, Kansas.

(20) Morgan, W. A., E. I. duPont de Nemours and Company, Polychemicals Department, Experiment Station, Wilmington, Delaware.

(21) Perrin, C. H., Canada Packers Limited, Toronto, Canada.

(22) Pikosky, D. J., and Connellan, M. M., General Scientific Equipment Company, Hamden, Connecticut.

(23) Randle, S. B., State Chemist, Rutgers University, New Jersey Agricultural Experiment Station, New Brunswick, New Jersey.

(24) Smith, R. M., and Taylor, J. J., State Chemist, State of Florida, Department of Agriculture, Chemical Division, Tallahassee, Florida.

(25) Smith, W. A., Smith-Douglass Company, Inc., Streator, Illinois.

(26) Smith, G. R., Director, Chemistry, Soils and Fertilizer Service, Truro, Nova Scotia, Department of Agriculture and Marketing.

COMMENTS OF COLLABORATORS

(1) Sample #4 received in poor mechanical condition. We use 250 ml Erlenmeyer flasks for the Perrin method. We think the Perrin method should be an alternate official method.

(2) Many of the Armour laboratories depend on the Perrin method modified by use of the Lindo-Gladding wash, for all potash work except check work.

(3) All of the potash work with the Perkin-Elmer flame photometer has employed lithium as the internal standard. Our experience has been, with plant materials, that there has been little interference from sodium and magnesium in concentrations found in the solutions from plant materials.

(7) We like the accuracy and reproducibility of the modified Perrin method. We do not like to use the Kjeldahl flask; its shape does not lend to policing out the precipitate. The Perrin method requires close attention in various operations—in a small laboratory this interferes with other work which normally could be carried on during the evaporations in the regular A.O.A.C. potash method.

(8) We obtained good agreement between the A.O.A.C. and the modified Perrin methods.

(10) Samples 2, 3, and 4 show need of the Lindo-Gladding wash for removal of large amounts of sodium in both the A.O.A.C. and Perrin methods for potash.

(11) The Perrin method was too time consuming and awkward for our laboratory. Flame results reported were not corrected for sodium and magnesium.

(14) Perrin method is excellent for a quick check on a sample, but in regular routine was not as time saving as the regular A.O.A.C. method.

(15) Samples 1, 2, and 3 were analyzed in April shortly after they were received. Samples 4 and 5 were not analyzed until July. Sample number 4 had caked badly by the time it was analyzed.

(16) Perkin-Elmer flame photometer used. No correction was made for sodium content.

(18) It is difficult to remove the precipitate from the Kjeldahl flask. The Perrin method gave slightly higher results.

(19) The Perrin method is more rapid and easier to do in our laboratory than the A.O.A.C. method. The only drawback was that it was hard to remove the precipitate from the Kjeldahl flask.

(20) Better results were obtained by the modified Perrin than the A.O.A.C. method. The flame results are by the Beckman Model DU spectrophotometer.

(21) We made flame tests by the Perkin-Elmer photometer and results were reported without Na and Mg corrections. Since variations from the chemical averages were just within the normal tolerance for flame photometers, it did not seem necessary to make such corrections. Comparison of direct intensity and internal standard techniques is very badly needed.

(22) The Barclay flame photometer was used.

(23) Good agreement between the two methods is our experience. We feel that the flame photometer method can be adopted as an alternate method.

(24) With the Perrin method our production would drop 50-60% of that which we now obtain with the A.O.A.C. method. Our new Beckman was received only recently and the results reported on it for the flame are probably not as good as they would have been had we had more experience with it. No correction was made for Na on these flame values.

RECOMMENDATION*

It is recommended—

(1) That the modified Perrin method be adopted as an alternate to the A.O.A.C. method for potash in fertilizers.

(2) That additional studies be made towards adopting the flame photometer for use in the determination of potash in fertilizers.

ACKNOWLEDGMENT

The Associate Referee wishes to express his thanks to the many collaborators for their fine cooperation and to Dr. F. W. Quackenbush and Dr. E. D. Schall for their suggestions and criticisms in the development of this report.

* For report of Subcommittee A and action of the Association, see *This Journal*, **35**, 44 (1952).

REPORT ON SULFUR IN MIXED FERTILIZERS

By GORDON HART (Department of Agriculture, Tallahassee, Fla.),
Associate Referee

The referee did some work on burning free sulfur in a bomb instead of extracting with carbon disulfide. Results were lower than obtained by the extraction process and did not recover all sulfur present in known samples, as the extraction method did. The other method reported on last year, briefly, "boiling with di-sodium phosphate, washing, transferring to flask, boiling with sodium sulfite, and titrating with potassium iodate," was tried further, but for lack of time was neither perfected nor rejected; it is not thought to be as promising as it appeared last year.

The referee makes no recommendations for the next year.

The results on free sulfur were run by the following method using a Parr bomb, by Dan Long, Analyst.

METHOD FOR FREE SULFUR IN FERTILIZERS

1. Digest 1 gram of sample in 100 ml dilute hydrochloric acid (1+9) for 15 minutes; filter, and wash with hot water on #609 ED paper.
2. Dry paper and residue in oven, brush off residue into mortar, add 0.5 g of benzoic acid, and grind.
3. Add 1 g of accelerator and one scoop of sodium peroxide, and mix with spatula. (Do not grind.)
4. Transfer to Parr calorimeter chamber, and explode as in coal analysis.
5. Take up residue in HCl and hot water, filter, and ppt sulphate with $BaCl_2$.
6. Filter off $BaSO_4$ after standing over night, and calculate to S.
7. Correction if necessary: Extract 1 g of sample with carbon disulfide until free sulfur is removed, then proceed as above. Deduct sulfur found from free S found above.

COMPARISON OF RESULTS

SAMPLE	GUARANTEE (*per cent*)	PARR BOMB (*per cent*)	EXTRACTION WITH CS_2 (*per cent*)
Official Sample 1	49.50	46.29	50.51
		47.87	
		42.37	
2	49.00	46.42	48.88
		43.70	
3	5.00	4.35	4.84
		3.80	
		4.00	
4	9.00	6.82	7.95
		5.47	
Sublimed Sulfur	—	88.20	—
		91.59	

No report was given on free water, acid-forming quality in fertilizers, sampling and preparation of sample, copper and zinc, boron, or inert material.

The three contributed papers on fertilizers are given on pages 757, 791, and 764, respectively. They are:

"Application of Flame Photometry to the Determination of Potash in Fertilizers," by E. D. Schall and R. R. Hagelberg;

"The Determination of Copper and Zinc in Fertilizers with the Polarograph," by Noah J. Halbrook;

"Methods for Direct Determination of Phosphoric Acid in Fertilizers," by H. R. Allen, Elizabeth Swift, R. Hays, and Z. F. Kaufman.

REPORT ON CEREALS

By V. E. MUNSEY (Food & Drug Administration, Federal Security Agency Washington 25, D. C.), *Referee*

CEREAL FOODS

It is recommended*—

(1) That the studies on the determination of starch in raw and cooked cereals be continued.

(2) That the method for sugar in baked products be further studied as recommended by the Associate Referee (essentially **13.30–13.32**, inclusive, on flour).

(3) That the method for the determination of lactose in bread as reported by the Associate Referee, be adopted, first action, and study continued.

(4) That the work on the determination of proteolytic activity of flour be discontinued.

(5) That the study of methods on soy bean flour for moisture, ash, nitrogen, crude fiber, and oil be continued.

(6) That the method referred to in *This Journal*, **25**, 83–84, for the determination of unsaponifiable matter and sterols in noodles, be studied for its application to bakery products containing eggs.

(7) That the method for water soluble protein-nitrogen precipitable by 40% alcohol (albumen) in noodle and macaroni products **13.34** be deleted and the method as reported by the Associate Referee, be adopted, first action, and study continued.

(8) That the methods for solids, **13.86**, ash **13.87**, protein **13.88**, fat **13.89**, and crude fiber **13.90** be adopted as official for all baked products not containing fruit, and the study discontinued.

(9) That the study on the determination of bromates in flour be continued.

(10) That the method for the determination of acetic and propionic acid in bread, *This Journal*, **34**, 64–68 (1951), be adopted as official, and the study continued on other types of bread such as whole wheat, milk, etc.

* For report of Subcommittee D and action of the Association, see *This Journal*, **35**, 56 (1952).

(11) That Method I on nitrites, *This Journal*, **34**, 68 (1951), be adopted as official.

(12) That the method for carotene in noodles, *This Journal*, **34**, 68–69 (1951), be adopted as official.

(13) That the work on the determination of lipoid and lipoid P_2O_5 in noodles, as reported this year, be continued.

REPORT ON SUGARS IN BAKED PRODUCTS

By R. P. SMITH (National Biscuit Company, New York, N. Y.), *Associate Referee*

At the recommendation of the Association, collaborative study was continued on the determination of sugars in baked products. This included the preparation of a standard invert and sucrose conversion table and the analysis of three cookie samples containing different levels of invert sugar and sucrose. The method employed in the determination of sugars in the prepared samples was as follows:

DETERMINATION OF REDUCING AND NON-REDUCING SUGARS IN BAKED PRODUCTS

REAGENTS

(a) *Acid buffer soln.* Make 3 ml of acetic acid, 4.1 g anhydrous sodium acetate and 4.5 ml sulfuric acid to 1 liter with water.

(b) *Sodium tungstate soln.* 12%. Make 12 g $Na_2WO_4 \cdot 2H_2O$ to 100 ml with water.

(c) *Ferricyanide soln (alkaline), 0.1 N.* 33.0 g of pure, dry $K_3Fe(CN)_6$ plus 44.0 g Na_2CO_3 per liter.

(d) *Acetic acid-salt mixture.* Make up 200 ml of acetic acid, 70 g KCl and 40 g $ZnSO_4 \cdot 7H_2O$ to one liter with water.

(e) *Soluble starch-potassium iodide soln.* Add 2 g soluble starch to a small quantity of cold water and pour slowly into boiling water with constant stirring. Cool thoroly (or resulting mixture will be dark colored), add 50 g KI and make up to 100 ml with water. Add 1 drop of NaOH soln (1 + 1).

(f) *Thiosulfate soln 0.1 N.* 24.82 g $Na_2S_2O_3 \cdot 5H_2O$ and 3.8 g $Na_2B_4O_7 \cdot 10H_2O$ per liter.

DETERMINATION

(a) *Preparation.*—Introduce 5 to 15 g. of ground sample (depending upon its sugar content) into a 500 ml volumetric flask. Tip flask so that all the sample is on one side. Wet sample with just enough alcohol (5.0 to 12.5 ml, depending upon size of sample) to dampen so that sample will adhere to upper side of flask when turned. Add approximately 250 ml of the acid buffer soln, preventing the soln from coming in contact with the sample until all has been added to the flask. Add 10 ml Na_2WO_4 soln, make to mark with acid buffer soln and mix vigorously for 1 min. Filter immediately through a Whatman #4 filter paper, discarding the first 10 ml of filtrate.

(b) *Reducing Sugars.*—Pipet 12.5 ml of the filtrate into a 250 ml Erlenmeyer flask. Add 25 ml alkaline ferricyanide soln, immerse in a vigorously boiling water bath to a depth of about 2 inches for exactly 20 min. Remove and cool at once under running cold water. Add 62.5 ml of acetic acid salt soln, 1 ml of starch KI

soln and titrate with standardized 0.1 *N* $Na_2S_2O_3$ to complete disappearance of the blue color. (The addition of the acetic acid salt soln and of the indicator must immediately precede the titration.) When several samples are being analyzed, the acetic acid salt soln is added to each sample immediately prior to the titration. Subtract the titer from a blank, which is obtained by placing 2.5 ml of C_2H_5OH and 2 ml of Na_2WO_4 soln in a 100 ml vol. flask, making to mark with acid buffer soln and subjecting 12.5 ml of this soln to the treatment outlined above. This difference represents the quantity of ferricyanide reduced, and may be converted to weight of invert sugar by consulting the standard invert sugar table.

(c) *Non-Reducing Sugars (Sucrose).*—Pipet 12.5 ml of the filtrate into a 250 ml Erlenmeyer flask. Immerse the flask in a boiling water bath to a depth of about 2 inches for exactly 15 min. After removing the flask from the bath, cool at once under running cold water and proceed as outlined under (b) for reducing sugars, beginning with, "Add 25 ml alkaline ferricyanide soln." Ml of 0.1 *N* thiosulfate reduced after hydrolysis less ml of 0.1 *N* thiosulfate reduced by the reducing sugars is equal to the ml of 0.1 *N* thiosulfate reduced by the inverted sucrose. The weight of sucrose present is ascertained by consulting the standard sucrose table.

The standard invert and sucrose curves were prepared according to the following directions:

PREPARATION OF STANDARD INVERT SUGAR CURVE

Prepare a standard invert sugar soln containing 10 mg of invert sugar per ml as directed in *Methods of Analysis*, 7th Edition, under 29.32 (6th Edition 34.33). Prepare solns in 100 ml volumetric flasks containing respectively 65, 60, 55, 50, 45, 40, 35, 30, 25, 20, 15, 10, and 5 mg of invert sugar per 12.5 ml as follows:

ML OF STANDARD INVERT SOLN MADE UP TO 100 ML	MG OF INVERT SUGAR PER 100 ML	MG OF INVERT SUGAR PER 12.5 ML
52	520	65
48	480	60
44	440	55
40	400	50
36	360	45
32	320	40
28	280	35
24	240	30
20	200	25
16	160	20
12	120	15
8	80	10
4	40	5

To each of the above flasks, add 2 ml Na_2WO_4 soln and 2.5 ml of C_2H_5OH and make to mark with acid buffer soln. Take 12.5 ml of each soln and subject it to the analysis for reducing sugars with alkaline ferricyanide, using as a blank 12.5 ml of a mixture of 2 ml Na_2WO_4 soln, 2.5 ml of C_2H_5OH and 95.5 ml of acid buffer soln. Subtract the titers with 0.1 *N* thiosulfate from the blank and plot the differences against the respective weight of invert sugar present in each 12.5 ml aliquot.

PREPARATION OF STANDARD SUCROSE CURVE

Prepare a standard sucrose soln containing 10 mg of sucrose per ml by placing 10 g of sucrose in a 1 liter volumetric flask and making up to mark with H_2O. Using

TABLE 1.—*Ferricyanide invert-sucrose table*

ML. 0.1 *N* FERRICYANIDE REDUCED	MG. INVERT PER 12.5 ML.	MG. SUCROSE PER 12.5 ML.	ML. 0.1 *N* FERRICYANIDE REDUCED	MG. INVERT PER 12.5 ML.	MG. SUCROSE PER 12.5 ML.
0.2	.50	.45	12.8	31.49	28.80
0.4	.98	.90	13.0	31.98	29.25
0.6	1.48	1.35	13.2	32.47	29.70
0.8	1.97	1.80	13.4	32.96	30.15
1.0	2.46	2.25	13.6	33.46	30.60
1.2	2.95	2.70	13.8	33.95	31.05
1.4	3.44	3.15	14.0	34.44	31.50
1.6	3.94	3.60	14.2	34.93	31.95
1.8	4.43	4.05	14.4	35.42	32.40
2.0	4.92	4.50	14.6	35.92	32.85
2.2	5.41	4.95	14.8	36.41	33.30
2.4	5.90	5.40	15.0	36.90	33.75
2.6	6.40	5.85	15.2	37.39	34.20
2.8	6.89	6.30	15.4	37.88	34.65
3.0	7.38	6.75	15.6	38.38	35.10
3.2	7.87	7.20	15.8	38.87	35.55
3.4	8.37	7.65	16.0	39.36	36.00
3.6	8.86	8.10	16.2	39.85	36.45
3.8	9.35	8.55	16.4	40.34	36.90
4.0	9.84	9.00	16.6	40.84	37.35
4.2	10.33	9.45	16.8	41.33	37.80
4.4	10.82	9.90	17.0	41.82	38.25
4.6	11.32	10.35	17.2	42.31	38.70
4.8	11.81	10.80	17.4	42.80	39.15
5.0	12.30	11.25	17.6	43.30	39.60
5.2	12.79	11.70	17.8	43.79	40.05
5.4	13.28	12.15	18.0	44.28	40.50
5.6	13.78	12.60	18.2	44.77	40.95
5.8	14.27	13.05	18.4	45.26	41.40
6.0	14.76	13.50	18.6	45.76	41.85
6.2	15.25	13.95	18.8	46.25	42.30
6.4	15.74	14.40	19.0	46.74	42.75
6.6	16.24	14.85	19.2	47.23	43.20
6.8	16.73	15.30	19.4	47.72	43.65
7.0	17.22	15.75	19.6	48.22	44.10
7.2	17.71	16.20	19.8	48.71	44.55
7.4	18.20	16.65	20.0	49.20	45.00
7.6	18.70	17.10	20.2	49.69	45.45
7.8	19.19	17.55	20.4	50.18	45.90
8.0	19.68	18.00	20.6	50.68	46.35
8.2	20.17	18.45	20.8	51.17	46.80
8.4	20.66	18.90	21.0	51.66	47.25
8.6	21.16	19.35	21.2	52.15	47.70
8.8	21.65	19.80	21.4	52.64	48.15
9.0	22.14	20.25	21.6	53.14	48.60
9.2	22.63	20.70	21.8	53.63	49.05
9.4	23.12	21.15	22.0	54.12	49.50
9.6	23.62	21.60	22.2	54.61	49.95
9.8	24.11	22.05	22.4	55.10	50.40
10.0	24.60	22.50	22.6	55.60	50.85
10.2	25.09	22.95	22.8	56.09	51.30
10.4	25.58	23.40	23.0	56.58	51.75
10.6	26.08	23.85	23.2	57.07	52.20
10.8	26.58	24.30	23.4	57.56	52.65
11.0	27.06	24.75	23.6	58.06	53.10
11.2	27.55	25.20	23.8	58.55	53.55
11.4	28.04	25.65	24.0	59.04	54.00
11.6	28.54	26.10	24.2	59.53	54.45
11.8	29.03	26.55	24.4	60.02	54.90
12.0	29.52	27.00	24.6	60.52	55.35
12.2	30.01	27.45	24.8	61.01	55.80
12.4	30.50	27.90	25.0	61.50	56.25
12.6	31.00	28.35			

this standard, prepare solns containing respectively 65, 60, 55, 50, 45, 40, 35, 30, 25, 20, 15, 10 and 5 ml of sucrose per 12.5 ml as outlined for invert sugar above.

Pipet 12.5 ml of each soln into a 250 ml Erlenmeyer flask and subject this aliquot to the analysis for non-reducing sugars with alkaline ferricyanide. Prepare a blank like that used in the invert sugar curve procedure. Subtract the titers with 0.1 *N* thiosulfate from the blank, and plot the difference against the respective weights of sucrose present in each 12.5 ml aliquot.

Table 1 is derived from the arithmetical average of four collaborators (different laboratories) of the standard invert sugar and sucrose tables. Individual collaborative results did not deviate appreciably from the arithmetical average shown in the table.

The prepared cookie samples submitted to the collaborators for analysis contained the following calculated percentages of invert sugar and sucrose:

	Invert Sugar *per cent*	*Sucrose* *per cent*
A	1.5	1.6
B	5.5	5.9
C	9.0	9.6

Collaborative results of invert sugar and sucrose determinations on the samples by the above method are shown in Table 2 and are compared with the results obtained by the Munson-Walker method.[1]

TABLE 2.—*Collaborative results of sugar determinations by alkaline ferricyanide method*

COLLABORATOR	SAMPLE A		SAMPLE B		SAMPLE C	
	INVERT	SUCROSE	INVERT	SUCROSE	INVERT	SUCROSE
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	2.40	4.86	7.04	12.92	10.72	19.92
2	2.17	4.52	5.93	11.89	8.99	17.52
3	2.12	2.79	5.94	7.37	9.13	10.79
4	2.53	3.09	7.27	7.84	10.92	11.78
Method 22.32[1]	1.87	2.75	5.70	7.15	8.77	10.87

[1] *Methods of Analysis, A.O.A.C.* 7th Ed. (1950) par. 22.35.

DISCUSSION

The results would indicate that the method should prove feasible in spite of the observed discrepancies. It will be noted that two of the collaborators were in fairly close agreement with each other although their results are slightly higher than those obtained by the Munson-Walker method.

RECOMMENDATION

It is recommended* that collaborative study be continued on this method and its application with a view toward further refinement.

[1] *Methods of Analysis*, A.O.A.C. 7th Ed. (1950) par. 29.35.

* For report of Subcommittee D and action of the Association, see *This Journal*, 35, 56 (1952).

ACKNOWLEDGMENT

The Associate Referee is very grateful to the following persons for their contributions in this study:

Arthur L. Brandon, Anheuser Busch, Inc., St. Louis, Mo.

Edward F. Steagall, Food & Drug Administration, Federal Security Agency, Washington D. C.

E. F. Budde, The Quaker Oats Company, Chicago, Illinois.

W. V. Lyons & W. Woods, National Biscuit Company, New York City, New York.

REPORT ON BAKED PRODUCTS OTHER THAN BREAD (NOT CONTAINING FRUIT)

SOLIDS, ASH, PROTEIN, FAT, CRUDE FIBER

By FRANK H. COLLINS (Food and Drug Administration, Federal Security Agency, Cincinnati 2, Ohio), *Associate Referee*

This Association has for many years had under consideration the study of methods for the above named constituents of cereal products. In the 1915 *Journal* (**1,** 195) the following appears, "At a meeting of Committee C of this Association, held in 1912, the following recommendations relative to methods of analysis of wheat flour, were adopted—(2) That methods for the estimation of moisture by the use of the vacuum oven . . . be referred to the next referee for immediate consideration." In 1920 (*This Journal,* **4,** 253) it was recommended and approved—"That the referee for the coming year study methods for the determination of fat in baked cereal products."

In the years following, reports on baked products were given (see Journal references below*) and tentative methods were adopted and later made official for bread. In the case of other baked products work has been continued, resulting in the adoption of official methods for bread, and tentative for other baked products. Some of these tentative methods have been adopted as official and later changed back to "tentative" or "official, first action," methods.

The following methods have at one time been official for this class of products: moisture—vacuum oven and air oven at 130°C. (**17,** 65), crude fiber (**18,** 76), fat by acid hydrolysis (**19,** 83). In the past, collaborative work has been done on the following products: soda crackers, graham crackers, cake, pretzels, vanilla wafers, short bread, and ginger cookies. The present study is concerned with chocolate flavored cookies, cocoanut bars, and graham crackers. Ground subsamples (one each of these products) were sent to collaborators with the request that they be

* *This Journal,* **4,** 253, 577; **5,** 341; **6,** 236; **13,** 76; **16,** 63, 72, 513, 518; **17,** 58, 65, 403, 404; **18,** 59, 76; **19,** 64, 83; **20,** 351; **23,** 83, 537; **24,** 624; **32,** 62 and **33,** 79.

TABLE 1.—*Chocolate flavored cookies*

DETERMINATION	SOLIDS (PER CENT)		SOLIDS (PER CENT)		ASH (PER CENT)		PROTEIN (PER CENT)		FAT (PER CENT)		CRUDE FIBER (PER CENT)	
METHOD	VACUUM OVEN		AIR OVEN, 130°						ACID HYDROLYSIS			
Analyst 1	94.92	94.92	94.55	94.59	2.13	2.14	6.54	6.48	18.12	18.04	.58	.60
2	94.95	94.96	94.93	94.68	1.95	1.94	6.50	6.44	18.1	18.2	.59	.56
3	95.11	95.09	94.45	94.43	1.85	1.93	6.50	6.50	18.03	18.42	.65	.65
4	94.97	94.95	—	—	2.17	2.16	6.58	6.63	17.77	18.04	.61	.57
5	95.03	95.11	94.84	94.92	2.39	2.38	6.50	6.55	18.01	18.11	.64	.55
6	95.65	95.76	94.77	94.62	2.06	2.10	6.27	6.27	18.36	18.18	.52	.54
7	—	—	94.70	—	2.13	—	6.44	—	18.15	—	—	—
8	—	—	94.95	94.95	—	—	6.44	—	—	—	—	—
Average	95.12		94.72		2.10		6.47		18.12		.59	
Maximum Deviation from Average	.64		.29		.29		.20		.35		.07	

TABLE 2.—*Cocoanut bars*

DETERMINATION	SOLIDS (PER CENT)		SOLIDS (PER CENT)		ASH (PER CENT)		PROTEIN (PER CENT)		FAT (PER CENT)		CRUDE FIBER (PER CENT)	
METHOD	VACUUM OVEN		AIR OVEN, 130°						ACID HYDROLYSIS			
Analyst 1	95.34	95.35	95.22	95.11	1.54	1.57	4.34	4.37	18.58	18.43	.37	.38
2	95.31	95.34	95.29	95.39	1.46	1.48	4.33	4.33	18.6	18.8	.40	.38
3	95.61	95.60	95.12	95.13	1.42	1.38	4.33	4.39	18.59	18.74	.39	.30
4	95.32	95.33	—	—	1.62	1.63	4.40	4.42	18.51	18.34	.40	.39
5	95.71	95.82	95.40	95.45	1.72	1.64	4.62	4.62	18.21	18.30	.38	.37
6	95.64	95.57	95.96	95.74	1.61	1.65	4.33	4.28	18.45	18.51	.40	.41
7	—	—	95.27	—	1.58	—	4.36	—	18.45	—	—	—
8	—	—	95.63	95.64	—	—	4.39	—	—	—	—	—
Average	95.49		95.41		1.56		4.49		18.50		.38	
Maximum Deviation from Average	.33		.55		.18		.16		.30		.03	

TABLE 3.—*Graham crackers*

DETERMINATION	SOLIDS (PER CENT)		SOLIDS (PER CENT)		ASH (PER CENT)		PROTEIN (PER CENT)		FAT (PER CENT)		CRUDE FIBER (PER CENT)	
METHOD	VACUUM OVEN		AIR OVEN, 130°						ACID HYDROLYSIS			
Analyst 1	94.79	94.76	94.43	94.44	2.52	2.46	6.59	6.57	10.57	10.63	.69	.66
2	94.78	94.77	94.57	94.58	2.45	2.46	6.50	6.56	10.8	10.5	.70	.61
3	94.97	94.99	94.37	94.34	2.40	2.37	6.61	6.50	10.78	10.68	.66	.68
4	94.92	94.90	—	—	2.52	2.50	6.60	6.64	10.57	10.74	.68	.72
5	94.99	95.00	94.99	94.69	2.53	2.60	6.50	6.50	10.47	10.43	.70	.75
6	95.04	95.09	95.21	95.19	2.50	2.52	6.38	6.33	10.80	10.83	.60	.65
7	—	—	94.72	—	2.51	—	6.60	—	10.50	—	—	—
8	—	—	95.01	95.06	—	—	6.50	—	—	—	—	—
Average	94.92		94.74		2.49		6.53		10.64		.67	
Maximum Deviation from Average	.17		.47		.12		.20		.21		.08	

run and reported in duplicate by each of the following First Action methods as listed on page 212, *Methods of Analysis*, 7th Edition.

13.86 Solids (Official Cereal Methods: **13.3** Vacuum oven and **13.4** air oven at 130°C. Methods)
13.87 Ash (Official **13.6**)
13.88 Protein (Official **13.80–2.24**)
13.89 Fat (Acid hydrolysis, official **13.19**)
13.90 Crude Fiber (Official **22.31**)

As was stated to the collaborators, "It is desired that collaborative work, sufficient and satisfactory, can be done so that these first action methods can be made official, final action."

Some collaborators did not report results by all methods. The reported results are tabulated in Tables 1, 2 and 3.

DISCUSSION

The collaborators, for the most part, obtained close agreement in their duplicate determinations and with each other. The greatest maximum deviation from the average occurred in the moisture methods. It was noted that these maximum deviations were no greater than those experienced in the early collaborative work used as a basis for making these methods official for wheat flour. By the vacuum oven method in the earlier studies (*This Journal*, **8**, 640) the maximum deviation from the average was 0.63 per cent and in the air oven method (*This Journal* **10**, 456) the maximum deviation was 0.64 per cent.

RECOMMENDATIONS*

It is recommended that the above listed official first action methods be made official, final action, for all baked products not containing fruit.

ACKNOWLEDGMENT

The Associate Referee expresses his appreciation to the following collaborators: Thomas E. Hollingshead, The Technical Institute, Independent Biscuit Manufacturer's Company, Chicago 26, Illinois; L. S. Thompson, Strietman Biscuit Company, Cincinnati, Ohio; and the following of the Food and Drug Administration:

D. W. Johnson, Denver
George E. Keppel, Minneapolis
John A. Thomas, New Orleans
Theodore E. Byers, Cincinnati
Thomas F. Osberger, Cincinnati

* For report of Subcommittee D and action of the Association, see *This Journal*, **35**, 56 (1952).

REPORT ON STARCH IN CEREAL PRODUCTS

By E. F. STEAGALL (Food and Drug Administration, Federal Security Agency, Los Angeles 15, California), *Associate Referee*

Early during the past year, further work was done in an attempt to improve and modify the Rask-McVey method as given by V. E. Munsey, Referee on Cereals (1). There was no improvement in the results reported previously. An attempt was then made to adopt the Hoffpauir procedure (2) to cereal products. The results were consistently low.

Attention was then turned to the use of enzymes. Various types and combinations of enzymes were tried, and again with one exception the results were low. The exception was the diastase method (3). Accordingly, the following procedure was adapted to cereals and submitted to collaboration.

THE DETERMINATION OF STARCH IN RAW AND COOKED CEREALS

REAGENT

Malt Extract.—Use clean, new barley malt of known efficacy and grind, only as needed, to pass thru 20 mesh sieve. Prepare infusion of the freshly ground malt just before it is to be used. For every 80 ml of the malt extract required, digest 5 g of the ground malt with 100 ml of H_2O 3 hrs. at room temperature, 20 min. in electric malted milk stirrer or 2 min. in Waring blender. Centrifuge 15 min. at ca 1500 rpm and filter. Mix infusion well before use.

DETERMINATION

Weigh 2 g of sample, ground to an impalpable powder, into 50 ml round bottomed centrifuge tube with lip. Wash twice with 10 ml ether to remove fat, centrifuging and decanting each time. Wash with 10 ml of ca 65% by weight of alcohol ($d_{20}=0.88$) and stir thoroly with glass rod. Centrifuge and pour off soln. Repeat washing until 60 ml of wash liquid has been used, stirring each time with the same rod.

Transfer residue to 150 ml beaker with 50 ml H_2O, immerse beaker in boiling H_2O and stir constantly 15 min.; cool to 55°C., add 20 ml of the malt extract, and maintain at this temperature 1 hr. Heat again to boiling and stir constantly for 15 min., cool to 55°C., add 20 ml of the malt extract and maintain at this temperature 1 hr. or until residue treated with I soln shows no blue color upon microscopic examination. Cool, make up directly to 250 ml, and filter. (This makes a convenient stopping place if the determination is to be carried on the next day.)

Place 200 ml of filtrate in a 500 ml flask, add 20 ml HCl (sp. gr. 1.125) connect with reflux condenser, and heat in a boiling water bath 2.5 hrs. Cool, nearly neutralize with 10% NaOH soln, finish neutralization with Na_2CO_3 soln, and make up to 500 ml. Mix soln thoroly, pour thru dry filter and determine dextrose in 50 ml aliquot as directed under **29.36,** *Methods of Analysis,* A.O.A.C., 7th Ed. (**34.39,** Sixth Edition). Conduct blank determination on same volume of the malt extract as used with the sample and correct weight of dextrose accordingly. Weight of dextrose obtained × 0.90 equals weight of starch.

Three samples consisting of graham crackers, air dried bread, and flour were sent each collaborator. Their results are reported in the following table.

TABLE 1.—*Per cent starch by the modified diastase method*

ANALYST	GRAHAM CRACKERS	AV.	BREAD	AV.	FLOUR	AV.
1	50.90		62.20		71.60	
	51.40	51.15	62.40	62.30	71.90	71.75
2	45.70		53.30		63.80	
	45.10	45.40	52.30	52.80	63.00	63.40
3	58.08		68.31		70.86	
	57.60		68.71		70.54	
	57.90	57.86	67.93	68.32	70.52	70.64
4	47.59		58.40		66.43	
	47.53	47.56	59.51	58.96	66.43	66.43
5	40.30		53.40		59.30	
	40.50	40.40	53.60	53.50	58.80	59.05
6	48.40		59.90		68.00	
	48.80	48.60	60.40	60.25	68.20	68.10
7	49.11		61.65		68.40	
	49.11	49.11	61.48	61.58	68.10	68.25
Max.	58.08		68.71		71.90	
Min.	40.30		53.40		58.80	
Av.	49.20		60.23		67.06	

As can be seen by these results there is poor agreement between collaborators. Only analysts 4, 6, and 7 came within a maximum deviation of 5 per cent. The cause is either due to failure of the analysts to adhere strictly to the directions or due to limitations of the method itself.

REFERENCES

(1) *This Journal*, **34**, 270 (1951).
(2) *Ibid.*, **33**, 810 (1950).
(3) *Methods of Analysis*, 7th Ed., **22.35**, p. 348.

COLLABORATORS

The assistance of the following collaborators is greatly appreciated:

Rae H. Harris, Agricultural Experiment Station, Fargo, N. D.
Westley W. Bath, Best Foods, Inc, New York, N. Y.
Niles H. Walker, Arnold Bakers, Inc., Port Chester, N. Y.
Arthur H. Brandon, Anheuser-Busch, Inc., St. Louis, Mo.
F. N. McMillan, Florida Department of Agriculture, Tallahassee, Fla.
V. E. Munsey, Food & Drug Administration, Washington, D. C.

It is recommended* that the study of method for starch in cereals be continued.

* For report of Subcommittee D and action of the Association, see *This Journal*, **35**, 56 (1952).

REPORT ON LIPOID AND LIPOID P_2O_5 IN NOODLES

By V. E. Munsey (Food & Drug Administration, Federal Security Agency, Washington 25, D. C.), *Associate Referee*

The methods for lipoid and lipoid P_2O_5 in noodles (*Methods of Analysis*, 7th Ed., **13.110, 13.35** and **13.36**), have been in the official status since 1926 (*This Journal*, **9**, 89 (1926). From time to time reports of poor agreement are obtained for lipoid P_2O_5. There have also been statements that considerable loss of platinum occurs in this determination. The method directs to "char well," and various lengths of ashing periods have been used under this direction. Recommendations have been made for a second ashing period for complete recovery of all the lipoid P_2O_5. Generally, alcoholic sodium hydroxide, as a fixative, ashes with less carbon remaining than with the use of alcoholic potassium hydroxide. The P_2O_5 is determined by the use of molybdate solution under sec. **2.8** (*Methods of Analysis*, 7th Edition) which has a relatively short period of stability. The directions for determination under sec. **2.13** are not as specific as under sec. **20.47** and the molybdate solution under sec. **20.46** has the advantage of stability. In order to compare the two molybdate solutions (sec. **2.8** and sec. **20.46**) samples have been analyzed for P_2O_5 in flour, noodles, and eggs. The results follow in Table 1.

The method for lipoids and lipoid P_2O_5 in eggs (sec. **16.12**) directs the use of Pyrex beakers. It seems logical that beakers could be used for lipoids in noodles in place of platinum as directed under sec. **13.35.** Five noodles were analyzed, using both beakers and platinum dishes, by E. F. Steagall.

Product	*Firm*	*Per cent Lipoids*	
		Beaker	*Platinum*
Yolk Noodle	A	5.80	5.84
Yolk Noodle	B	5.95	5.92
Yolk Noodle	C	5.04	5.05
Whole Egg Noodle	D	4.72	4.72
Whole Egg Noodle	E	5.57	5.56

These results indicate that either molybdate solution may be used in this determination for flour, noodles and eggs and that lipoids are satisfactorily determined by substitution of a beaker for a platinum dish.

Results on yolk and whole egg noodles analyzed for lipoid P_2O_5 after ashing for 1 hour at 500°C. showed no increase in lipoid P_2O_5 upon a second ashing, either in beakers or platinum. However, in view of the comments on the present method (sec. **13.36**) the following changes were made: use of alcoholic NaOH instead of alcoholic KOH, change of "char well" to "ash 1 hr. at 500°C.," inclusion of a second ashing, use of Pyrex beaker instead of platinum dish and use of molybdate solution under sec. **20.46** with directions under **20.47,** instead of molybdate solution

TABLE 1.*—*Comparison of two molybdate solutions under Sections 2.8 and 20.46 for P_2O_5 determination*

PRODUCT		PER CENT TOTAL P_2O_5		PER CENT LIPOID P_2O_5	
		MOLYBDATE SOLUTION		MOLYBDATE SOLUTION	
		SEC. 2.8	SEC. 20.46	SEC. 2.8	SEC. 20.46
			(*as is basis*)		(*as is basis*)
Flour		0.219	0.212		
Egg Noodle	A	0.475	0.475		
Whole Dried Egg		1.89	1.89		
Salad Dressing		0.076	0.078		
Yolk Noodle	B			0.124	0.124
Yolk Noodle	C			0.126	0.126
Whole Egg Noodle	D			0.096	0.096
Yolk Noodle	E			0.124	0.123
Yolk Noodle	F			0.136	0.136
Whole Egg Noodle	G			0.111	0.110
Whole Egg Noodle	H			0.104	0.103
Yolk Noodle	I			0.122	0.121
Egg Yolk	97-765K	1.15	1.15	.87–.87	.84–.84
	21-066L	1.16	1.16		
	28-005L	1.28	1.28		
	91-556F	1.15	1.14	.83	.82
	89-762K	1.18	1.16		
	18-781L			.83	.82
	46-515K			.86	.86
	89-894K			.87	.87
	9-671L			.83	.83
	82-337K			.83	.83
	95-528K			.93	.93

* Most of these results were obtained by E. F. Steagall, Food & Drug Administration.

under sec. **2.8** with directions under sec. **2.13.** These changes require also a change from platinum dish to beaker (sec. **13.35**).

Two samples of noodles, one yolk noodle #1 and one whole egg noodle #2, each containing 5.5 per cent egg yolk and whole egg solids respectively according to the supplier, were submitted to collaborators for analysis for lipoid and lipoid P_2O_5. The collaborators were asked to use the following two methods, designated Method I and Method II. Method I is the same as the method for lipoids, sec. **13.35** and lipoid P_2O_5, sec. **13.36.** Method II contains the four modifications referred to in the previous part of this report.

METHOD I

LIPOID AND LIPOID P_2O_5 IN NOODLES

Analyze the samples by following the method under sec. **13.35** and **13.36** specifically following directions with the following exception. After line 4, sec. **13.36,** reading "with hot water," return filter paper to Pt dish, reash, add 5 ml

HNO_3 (1+9), warm on steam bath and filter, collecting in the flask with previous filtrate. Make soln slightly alkaline to litmus paper with NH_4OH and then slightly acid with HNO_3 (1+3), add 10g NH_4NO_3, dilute to 75 ml, add 20 ml molybdate soln (or sufficient to ensure complete precipitation) under sec. 2.11, and proceed as under 2.13 (b). Dissolve and titrate with 0.1 *N* alkali and acid resp. Report ashing time used under this procedure.

METHOD II

In sec. **13.35** substitute 100 ml Pyrex beaker for the platinum dish.

LIPOID P_2O_5

Dissolve lipoids in 5–10 ml of $CHCl_3$, add 5–10 ml alc. NaOH soln (sec **16.10** (b), p. 277) evap. to dryness on steam bath, ash 1 hr. at 500°. Cover beaker with watch glass, add sufficient HNO_3 (1+9) to make soln acid, warm on steam bath and filter into 300 ml Erlenmeyer flask. Wash residue and filter well with hot H_2O. Return filter to beaker, ash 1 hr., cool, add 5 ml HNO_3 (1+9), warm on steam bath, filter and wash, collecting in flask with previous filtrate. Make soln slightly alkaline

TABLE 2.—*Lipoid, lipoid P_2O_5 and calculated egg content in yolk noodle*

COLLABORATOR	SAMPLE I					
	METHOD I			METHOD II		
	LIPOID	LIPOID P_2O_5	EGG SOLIDS	LIPOID	LIPOID P_2O_5	EGG SOLIDS
	(as is basis) *per cent*	*(as is basis)* *per cent*	*(mfb)* *per cent*	*(as is basis)* *per cent*	*(as is basis)* *per cent*	*(mfb)* *per cent*
1	5.5–5.5 av. 5.5	.097–.095 av. .096	3.6–3.4 av. 3.5	5.4–5.5 av. 5.5	.099–.099 av. .099	3.7–3.7 av. 3.7
2	5.6–5.7 av. 5.7	.115–.116 av. .116	4.9–4.9 av. 4.9	5.9–5.9 av. 5.9	.120–.120 av. .120	5.2–5.2 av. 5.2
3	5.7–5.7 av. 5.7	.114–.111 av. .113	4.7–4.5 av. 4.6	5.8–6.0 av. 5.9	.109–.110 av. .110	4.4–4.5 av. 4.5
4	5.1–5.1 av. 5.1	.124–.126 av. 0.125	5.4–5.4 av. 5.4		.125–.124 av. .125	5.4–5.4 av. 5.4
5	5.7–5.8 av. 5.8	.112–.116 av. .114	4.6–4.9 av. 4.8	5.6–5.7–5.7 av. 5.7	.102–.104 av. .103	3.9–4.1–4.8 av. 4.3
6	5.9–5.9 av. 5.9	.125–.126 av. .126	5.4–5.5 av. 5.5		.125–.129 av. .127	5.4–5.7 av. 5.6
7	5.8–6.0 av. 5.9	.122–.124 av. 0.123	5.3–5.4 av. 5.4	6.1–5.9 av. 6.0	.120–.120 av. .120	5.1–5.1 av. 5.1
8	5.6	.122	5.2	5.6	.121	5.2
9	6.1–6.1 av. 6.1	.124–.126 av. .125	5.5–5.6 av. 5.6	6.2–6.0 av. 6.1	.118–.119 av. .119	5.1–5.1 av. 5.1
10	5.8	.114	4.7	5.4	.109	3.1
Max.	6.1		5.6	6.1		5.6
Min.	5.1		3.5	5.5		3.1
Av.	5.7		5.0	5.8		4.7

TABLE 3.—*Lipoid, lipoid P_2O_5 and calculated egg content in whole egg noodle*

COLLABORATOR	SAMPLE II					
	METHOD I			METHOD II		
	LIPOID	LIPOID P_2O_5	EGG SOLIDS	LIPOID	LIPOID P_2O_5	EGG SOLIDS
	(as is basis) *per cent*	*(as is basis)* *per cent*	*(mfb)* *per cent*	*(as is basis)* *per cent*	*(as is basis)* *per cent*	*(mfb)* *per cent*
1	5.1–5.1 av. 5.1	.095–.091 av. .093	4.8–4.8 av. 4.8	5.1–5.0 av. 5.1	.084–.092 av. .088	3.1–3.7 av. 3.4
2	4.5–4.7 av. 4.6	.094–.095 av. .095	4.5–4.5 av. 4.5	5.4–5.1 av. 5.3	.098–.098 av. .098	4.8–4.8 av. 4.8
3	5.1–5.2 av. 5.2	.095–.098 av. .097	4.5–4.8 av. 4.7	5.2–5.0 av. 5.1	.095–.091 av. .093	4.5–4.1 av. 4.3
4	4.2–4.3 av. 4.3	.101–.103 av. .102	4.9–5.0 av. 5.0		.102–.102 av. .102	5.0–5.0 av. 5.0
5	4.7–4.9 av. 4.8	.099–.100 av. .100	4.8–5.0 av. 4.9	5.0–4.9–5.0 av. 5.0	.084–.093–.098 av. .092	4.2–4.3–4.8 av. 4.4
6	5.1–5.1 av. 5.1	.104–.105 av. .105	5.2–5.3 av. 5.3	5.1–5.1 av. 5.1	.102–.102 av. .102	5.0–5.0 av. 5.0
7	5.2–5.1 av. 5.2	.105–.103 av. .104	5.4–5.2 av. 5.3	5.0–5.3 av. 5.2	.101–.101 av. .101	5.0–5.0 av. 5.0
8	4.7	.104	5.2	4.7	.103	5.2
9	5.9–5.6 av. 5.8	.117–.115 av. .116	6.5–6.5 av. 6.5	5.5–5.1 av. 5.3	.103–.103 av. .103	5.2–5.3 av. 5.3
10	4.5–4.8 av. 4.7	.095–.099 av. .097	4.1–4.4 av. 4.3	4.6–4.5 av. 4.6	.094–.090	4.0–3.7 av. 3.9
Max.	5.8		6.5	5.3		5.3
Min.	4.3		4.3	4.6		3.4
Av.	5.0		5.1	5.0		4.6

to litmus paper with NH_4OH and then slightly acid with HNO_3 (1+3), add 10 ml HNO_3 (1+9), dilute to 50–60 ml, and proceed as on p. 335, sec. **20.47**, line 4 "Add 20 ml of the NH_4NO_3." Use molybdate soln under **20.46**. Run blank and report % P_2O_5 and lipoids by each method.

The collaborators were requested to analyze one sample by one ashing and another by second ashing to determine the increase in P_2O_5. They were also asked to make blank determinations and report per cent lipoid and lipoid P_2O_5 on "as is" basis and egg solids on moisture free basis. The egg solids were calculated by the formula given in *This Journal*, **7**, 407 (1924). The results obtained by 10 collaborators are given in Tables 2 and 3.

Three collaborators reported an increase in lipoid P_2O_5 on second ashing of the range of 0.7, 1.3, 3, 3, and 4 per cent while the other 7 reported no

increase. Assuming that a variation in blank does not account for the increase reported on second ashing, for maximum recovery it appears desirable in some instances to include a second ashing. One collaborator reported losses in platinum dishes of 0.4, 9.5, 10.3 and 10.5 mg, respectively, while another reported losses from 0.3–0.9 mg per determination.

The results for lipoids in Tables 2 and 3 indicate that either platinum or Pyrex beakers may be used, which confirms earlier work in this laboratory. There is more variation in lipoid P_2O_5 than desired, by both methods. The greatest variation occurred for collaborators 1 and 10 in the same laboratory. Collaborator 9 reported unusually high values for lipoid P_2O_5 on sample 1 by Method I, and collaborator 1 was especially low on sample 1 by both methods and on sample 2 by Method II. Collaborator 10 was likewise very low on both samples by Method II.

While this data may not definitely indicate the choice of Method II over Method I, a careful consideration of all the changes warrants the use of Method II. However, further work is intended.

There does not appear to be clear cut evidence that a second ashing is necessary if the first ashing period of 1 hour is followed. In order to cover all possibility of a low recovery, a second ashing period is indicated. One collaborator suggested it may be helpful to add more alcoholic NaOH prior to the second ashing.

COLLABORATORS

The assistance of the following collaborators is very much appreciated.

J. J. Winston, Jacobs-Winston Lab., Inc., New York City
Charles H. Coleman, Q.M. Subsistence Testing Lab., Chicago, Ill.
Mary Zenk, U.S.D.A., Grain Branch, Washington, D. C.
Frank J. Kokoski, State of New York, Department of Agriculture & Markets, Albany, N. Y.
Louis C. Weiss, L. W. Ferris, E. F. Steagall, A. L. Weber, and L. Auerbach, all of Food & Drug Administration.

It is recommended* that this study be continued.

REPORT ON LACTOSE IN BREAD

By V. E. MUNSEY (Food and Drug Administration, Federal Security Agency, Washington 25, D. C.), *Associate Referee*

The determination of lactose in bread has been studied collaboratively by this Association with unsatisfactory results, as last reported in *This Journal*, **25**, 628 (1942). R. P. Choi et al., published a method for the determination of lactose in bread in *Cereal Chemistry* **27**, 398 (1950) which appeared to be an improvement over previous methods. Results by this method on laboratory breads of known lactose content were in-

* For report of Subcommittee D and action of the Association, see *This Journal*, 35, 57 (1952).

consistent and low as indicated by recoveries of 85, 74, 72 and 80 per cent. An investigation of the various steps of the procedure revealed that complete recovery was obtained with definite known quantities of lactose added to the fermentation flask. This led to the suspicion of incomplete extraction. Following up this lead, extraction was carried out on a hot plate in a Soxhlet extractor, using alcohol-water mixture. Recoveries of 99, 105, 92, 97 and 96 per cent were obtained. On the basis of these results, the extraction procedure of Choi was changed to call for extraction with alcohol-water mixture. Also more specific directions were given for the

TABLE 1.—*Per cent lactose and estimated content of nonfat milk solids in bread crumb*

COLLABORATOR		SAMPLE 1	SAMPLE 2	SAMPLE 3
		per cent	*per cent*	*per cent*
1	lactose	0.45–0.45–0.40	1.82–1.87	3.05–3.00–3.13–3.11
	nonfat milk solids	av. 0.86	2.84	5.28
	recovery	—	101	93.7
2	lactose	0.45	1.94–1.94	3.32–3.32
	nonfat milk solids	0.90	2.98	5.74
	recovery	—	106	102
3	lactose	0.30–0.35	1.55–1.63	2.85–2.72
	nonfat milk solids	0.66	2.52	4.92
	recovery	—	89.4	87.2
4	lactose	0	1.45–1.45 1.45–1.47 (av. 1.45)	2.40–2.40–2.45–2.50 (av. 2.44)
	recovery	—	103	86.6
5	lactose	0	0.90	1.70
	recovery		63.8	60.2
6	lactose	0	1.43–1.38 (av. 1.41)	2.60–2.72 2.66
	recovery	—	100	94.4
7	lactose	0.24 0.24	1.48–1.47 1.48–1.48	2.86–2.62 2.60–2.80
	nonfat milk solids	0.48 0.48	2.96	5.4
		—	88.0	86.7
8	lactose	.56–.53–.50 .53–.51–.45 .47–.50–.48 (av. 0.50)	1.78–1.73 1.73–1.80 1.76–1.72 (av. 1.75)	3.19–3.09–3.15 3.26–3.22–3.13 3.07–3.20 (av. 3.15)
	nonfat milk solids	1.0	3.50	6.30
	recovery	—	88.6	94.0
Max. lactose		0.50	106 recovery	102 recovery
Min. lactose		none	63.8 recovery	60.2 recovery
Av. lactose		0.39	92.5 recovery	88.1 recovery

Collaborator #3 also reported the following results by the method as published in *Cereal Chemistry* 27, 398 (1950):

Sample	1	2	3
% Lactose	0.41–0.50	2.04	2.85–2.90

On sample No. 1, containing an added 300 mg lactose, a recovery of 283 and 286 mg was reported.

reduction and titration. The same problem of correction for a significant blank on a bread containing no added lactose still exists. In an attempt to decrease or eliminate this reducing non-fermentable substance, the technic of using ion-exchange resins as published by Williams et al., *This Journal*, **33**, 986 (1950), was employed. A treatment of the aqueous extract with a combination of Duolite 4A and Amberlite 1R120, and also Amberlite 1R120 and Amberlite 1R4B, results in no decrease in blank on bread containing no lactose.

TABLE 2.—*Recovery results on 300 mg lactose added directly to 10 g of sample 1*

COLLABORATOR	LACTOSE	RECOVERY
	per cent	*per cent*
1	3.60–3.60–3.60 3.17–3.17–3.17*	105
2	3.58–3.58 3.13–3.13*	104
3	3.40–3.53 3.07–3.20*	104
4	3.0 –2.95*	99.4
5	2.60*	87
6	3.22–3.88–2.78 2.70*	97
7	2.63–2.63 2.39–2.39*	80
8	3.56–3.53–3.56 3.73–3.72–3.53 3.52–3.56–3.66 3.10*	103

Recovery maximum 105 per cent, minimum 80 per cent, average 97 per cent.
* Corrected for titration value on bread.

Three samples of air dry bread crumb were sent the collaborators; #1 contained no lactose, #2 contained 1.41 per cent lactose and #3 2.82 per cent lactose. They were requested to add 300 mg of lactose to a 10 g weighed out portion of sample 1 for determination of their recovery. These breads were made in one pound loaves, using a common commercial formula including malt syrup.

Details of the method as submitted to the collaborators are given in *This Journal*, **35**, 73 (1952).

The results reported by 8 collaborators are given in Table 1.

The results for the recovery of added lactose by each collaborator appears in Table 2.

Mr. Choi has recently suggested that the reference curve be made by adding the pure lactose to the fermentation flask, followed by addition of yeast nutrient and yeast as in the procedure for the bread. Recovery experiments do not indicate that this is necessary. E. F. Steagall of this laboratory has prepared reference curves as directed in the submitted procedure and the above modification. He obtained identical reference curves.

Collaborator 5 has reported very low recoveries. While this collaborator cited a few variations from the procedure, one would not expect them to be the cause of the low results. On the whole, the results of the other collaborators are in fair agreement and closely represent the amount of lactose in bread.

One collaborator packed the 25 per cent yeast suspension in ice and held in ice box overnight in an attempt to meet the specification of 0–4°C. The temperature of 4°C. apparently is low enough and the method should be changed to read "approximately 4°C." instead of "0–4°C."

Several laboratories commented on regulations against overnight extractions, which necessitated 2-day extraction periods. Our results show that an 8-hour extraction gives a complete recovery, and further work may indicate specification of this shorter period.

ACKNOWLEDGEMENT

Acknowledgement is herein expressed for the generous assistance of the following chemists.

W. B. Bradley, American Institute of Baking, Chicago, Ill.
W. Holmes, Purity Bakeries Corp., Chicago, Ill.
D. L. Kinnally, Ward Baking Company, New York City
R. P. Choi, American Dry Milk Institute, Chicago, Ill.
R. T. Bohn, General Baking Company, New York City
William Luckow, W. E. Long Company, Chicago, Ill.
E. F. Steagall, Food & Drug Administration, Washington, D. C.

RECOMMENDATION

It is recommended* that this procedure be adopted as first action and that study be continued.

* For report of Subcommittee D and action of the Association, see *This Journal*, 35, 56 (1952).

REPORT ON WATER-SOLUBLE PROTEIN-NITROGEN PRECIPITABLE BY 40 PER CENT ALCOHOL

By V. E. MUNSEY (Food and Drug Administration, Federal Security Agency, Washington 25, D. C.), *Associate Referee*

The method for water-soluble protein-nitrogen precipitable by 40 per cent alcohol, *Methods of Analysis*, 7th Ed., **13.34,** called albumin N in this report for brevity, was adopted first action (*This Journal*, **33,** 78 (1950)) on the basis of limited collaborative study. This method has been proposed for the differentiation of whole egg noodles from egg yolk noodles. Since the last collaborative results were obtained, the temperature of drying has been increased to higher temperatures varying up to about 150°F. in some instances. Samples of both yolk and whole egg noodle, reported as dried at from 120–150°F., were analyzed with the following results. The yolk noodle, with determined yolk solids content of 4.8 per cent (m.f.b.), had 0.067 per cent albumin N and 2.11 per cent total N. The whole egg noodle with 5.0 per cent whole egg solids (m.f.b.) contained 0.19 per cent albumin N and 2.22 per cent total N. Nitrogen figures are on as is basis. These values do not indicate that the drying temperature of 120–150°F. denatured the albumin to the extent of rendering it insoluble in water.

In an attempt to find a substitute for the albumin method that might more clearly differentiate whole egg noodle from a yolk noodle, the procedure published in *Mitt. Lebensm. Hyg.*, **21,** 205 (1930) and **25,** 313

TABLE 1.—*Per cent albumin N in semolina, flour, and noodles. (As is basis)*

SEMOLINA	GERMAN PROCEDURE	A.O.A.C. PROCEDURE
1	.02	.05
2	.02	.05
3	.03	.06
4	.03	.05
5	.02	.06
6	.03	.06
7	.02	.09
8	.03	.06
9	.03	.05
10	.03	.04
11	.03	.08
12	.03	.06
Average	.03	.06
Flour 1	.01	.04
2	.01	.04
Whole egg noodle	0.13, .13, .11, .13 (av. 0.13)	0.20, .21, .18, .19 (av. 0.19)
Yolk noodle	.04, .04, .03, .03 (av. 0.04)	.07, .07, .07, .07 (av. 0.07)

(1934) was applied to 12 semolinas, 2 flours, a laboratory whole egg noodle, and yolk noodle. The method consists, briefly, of treating an aqueous extract with a relatively large amount of $MgSO_4$, filtering, treating the filtrate with $CuSO_4$ at boiling temperature of water, and filtering off the precipitate for nitrogen determination. Results by the so-called German procedure and by the A.O.A.C. procedure as determined by E. F. Steagall are given in Table 1.

For the differentiation of whole egg noodle from yolk noodle one method appears as good as the other. Laboratory application, background of use, and experience warrants the use of the A.O.A.C. procedure.

The A.O.A.C. method directs shaking gently by hand or on a slowly revolving wheel for 1 hr. A revolving wheel is not generally available and shaking by hand is laborious and time consuming. The results below on a few samples of noodles, obtained by shaking by hand, in horizontal shaker, and in wrist action type shaker, indicate no difference in type of shaker. It should be mentioned that Alfend (*This Journal*, **14**, 500 (1931)) points out that too vigorous agitation may cause coagulation of the albumin.

	Per cent Albumin N		
	Hand shaking	*Horizontal shaking*	*Wrist type shaker*
Macaroni	0.06	0.06	
Yolk noodle A	0.12	0.12	
Yolk noodle B		0.13	0.13
Whole egg noodle		0.25	0.25

The filtrate after precipitation of albumin may be frequently of varying degrees of turbidity. A clear filtrate has been obtained by filtering by suction on an asbestos pad, covered with a layer of filtercel, in a Büchner funnel, instead of thru a fluted filter paper as specified in **13**, 34. However, this change was not made in the method because it might affect comparison with previous results obtained by use of the filter paper.

The A.O.A.C. method is an indirect one and requires two digestions. A convenient, direct method would be preferable. Accordingly, a yolk and whole egg noodle were analyzed by determination of nitrogen, directly on the precipitated albumin, for comparison with the A.O.A.C. method.

	Per cent Albumin N (as is basis)	
	Direct Method	*A.O.A.C. Indirect Method*
Yolk noodle dried at max. 150°F.	.08	.07
Whole Egg Noodle dried at max. 150°F.	0.20	0.21

While similar results may be obtained by either procedure, the amount of time involved in filtration and digestion of the direct method does not warrant its choice over the A.O.A.C. method. Alfend (*This Journal*, **14**,

500 (1931)) also obtained similar results by the direct and indirect A.O.A.C. method.

A salt solution (1.2%) instead of water has been tried on a yolk and whole egg noodle with these results:

	Per Cent Albumin N	
	H_2O extraction	*1.2% salt solution*
Yolk noodles	0.08	0.13
Whole egg noodles	0.20	0.26

These results do not indicate that salt extraction should replace water extraction.

The few minor changes made in the method submitted for collaborative study were intended to improve the mechanics and at the same time obtain results to supplement those obtained previously. These changes include the use of centrifuge bottles instead of nursing bottles, Büchner funnel instead of Hirsch funnel, specification of time for digestion and more option in shaking procedure.

Two samples of noodles, one yolk noodle with 5.4 per cent egg solids (m.f.b.) #2 and one whole egg noodle with 5.8 per cent egg solids (m.f.b.) #1 were submitted to collaborative study using the following procedure.

WATER-SOLUBLE PROTEIN—NITROGEN PRECIPITABLE BY 40 PER CENT ALCOHOL

Weigh 20 g sample (20 mesh or finer) into a 250 ml centrifuge bottle. Add 100 ml H_2O from pipet, shaking bottle to prevent lumping of sample. Add 100 ml more H_2O from pipet. Shake contents of stoppered bottle one hour in shaking machine or by hand. (Preferably a horizontal shaker with bottle lengthwise. If a vertical wrist type motion machine is used, shake by hand 5 min. after the 1 hour shaking.) Temp. of H_2O should not exceed 30°C. Centrifuge at 1200 r.p.m. for ca 15 min. and filter into 500 ml suction flask thru pad of asbestos (fine) on Büchner funnel (ca 2 in. diameter) using suction. Determine *N* in 50 ml of filtrate as directed under **2.22, 2.23, 2.24,** with a glass bead in each flask, and distilling the NH_3 into 20 ml of 0.1 *N* acid. Digest 1 hr. after clear. Correct for blank on reagents used in digestion.

Pipet 100 ml of above filtrate into 200 ml volumetric flask, add 15 ml NaCl soln (28 g diluted to 300 ml), fill nearly to mark with alcohol, mix well, cool to room temp., make to mark, mix and allow to stand overnight. Pipet off supernatant liquid and filter thru 18½ cm fluted filter paper (S & S 588 or equivalent). Determine *N* in 100 ml of filtrate as above, using a glass bead to avoid bumping. Add the H_2SO_4 mix and carefully boil off the alcohol before adding the Na_2SO_4-HgO mixture. Rinse Na_2SO_4—HgO mixture down neck of flask. Digest 1 hr. after clear. (Watch for foaming before clearing and keep contents out of neck of flask.) Distill into 20 ml of 0.1 *N* acid as before. Correct for blank or reagent used in digestion. Subtract this number of ml of acid used from the number of ml of acid used for water-soluble *N* and convert to per cent of water-soluble *N* precipitable by 40% alcohol.

RESULTS OF COLLABORATORS

The results received from nine collaborators are reported in Table 2. These results show some undesirable variation; however, in this de-

TABLE 2.—*Water soluble protein-nitrogen precipitable by 40 per cent alcohol, and total nitrogen in noodles (as is basis)*

COLLABORATOR	SAMPLE I		SAMPLE II	
	WATER SOL. *N* PPT'D BY 40 PER CENT ALCOHOL	TOTAL *N*	WATER SOL. *N* PPT'D BY 40 PER CENT ALCOHOL	TOTAL *N*
	(per cent)	*(per cent)*	*(per cent)*	*(per cent)*
1	0.21 –0.22 (av. 0.22)	2.19 –2.20 (av. 2.20)	0.11 –0.12 (av. 0.12)	2.14 –2.16 (av. 2.15)
2	0.169–0.211 (av. 0.19)	2.27 –2.21–2.21–2.21 (av. 2.23)	0.108–0.119 (av. 0.110)	2.19 –2.15–2.12–2.17 (av. 2.16)
3	0.19 –0.19 (av. 0.19)	2.23 –2.25 (av. 2.24)	0.09 –0.09 (av. 0.09)	2.17 –2.16 (av. 2.17)
4	0.21 –0.22 (av. 0.22)	2.20	0.076–0.071 (av. 0.07*)	2.12
5	0.20 –0.19 (av. 0.20)	2.25	0.082–0.088 (av. 0.09)	2.16
6	0.193–0.226 (av. 0.21)	2.25 –2.22 (av. 2.24)	0.092–0.098 (av. 0.10)	2.15 –2.18 (av. 2.17)
7	0.244–0.248 (av. 0.25)	2.17 –2.16 (av. 2.17)	0.082–0.096 (av. 0.09)	2.08 –2.09 (av. 2.09)
8	0.179–0.177 (av. 0.18)	2.25	0.088–0.092 (av. 0.09)	2.11
9	0.22 –0.22 (av. 0.22)		0.10 –0.10 (av. 0.10)	
Max.	0.25	2.25	0.12	2.17
Min.	0.18	2.17	0.07*	2.09
Av.	0.21	2.22	0.10	2.14

* Reported murky filtrate.

termination, involving this amount of nitrogen, they may be as good as can be expected. The modification referred to above to obtain a clear filtrate offers a possibility of improving the agreement among collaborators. One collaborator suggested centrifuging the 40 per cent alcohol solution after standing overnight, to make the precipitate more compact.

ACKNOWLEDGMENT

The assistance of the following collaborators is greatly appreciated:

J. J. Winston, Jacobs-Winston Laboratory, Inc., New York City, E. F. Budde, The Quaker Oats Company, Chicago, Ill., Mary Zenk, U. S. Dept. of Agriculture, Grain Branch, Washington, D. C., and the following, of the Food & Drug Administration: Mary Offutt, New York, N. Y., N. W. Conroy, Kansas City, Mo., P. M. Sanders, Cincinnati, Ohio, J. H. Cannon, St. Louis, Mo., and E. F. Steagall, Washington, D. C.

RECOMMENDATION

It is recommended* that the method be adopted, first action, and study be continued.

No report was given on proteolytic activity of flour, soybean flour, bromates in flour, or mold inhibitors.

REPORT ON BAKING POWDER

By V. E. MUNSEY (Food & Drug Adm., Fed. Sec. Agency, Washington 25, D. C.), *Referee*

It is recommended:†

(1) that the work as reported in *This Journal*, **34**, 60 (1951) on the neutralizing value of phosphates be continued.

(2) that the qualitative test, Method II for aluminum, *This Journal*, **34**, 61 (1951), be adopted as "official."

REPORT ON VITAMINS

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

There has been unusual activity on the part of the Associate Referees on vitamin methods during the past year. Work on two new methods has been reported at this meeting. Other reports have dealt largely with changes in methods that were adopted, first action, last year. It is gratifying to see the progress that has been made by the Associate Referees.

The determination of vitamin A and carotene in feeds continues to be

* For report of Subcommittee D and action of the Association see *This Journal*, **35**, 56 (1952).
† For report of Subcommittee D and action of the Association, see *This Journal*, **35**, 57 (1952).

a problem, as indicated by the Associate Referees on these subjects, but, as judged from their reports, progress is being made. The Referee concurs in their recommendations.*

The report on vitamin D in poultry feeds was different this year in that an attempt was made to determine the precision of the A.O.A.C. method for the determination of vitamin D_3. This has been the subject of much discussion in the past, and the data presented by the Associate Referee serve to re-evaluate the validity of the method.

The report on nicotinic acid dealt with a minor change in the method adopted, first action, last year and the Referee concurs in the recommendation of the Associate Referee on this subject.

A new chemical method for pyridoxine has been described, and it is hoped that during the coming year its practical application can be demonstrated. The Referee concurs in the recommendation of the Associate Referee on pyridoxine.

The Associate Referee on pantothenic acid has reported on the results of his collaborative study conducted the past year, and it appears that progress is being made with this complex problem. The Referee concurs in the recommendations of the Referee on pantothenic acid.

Interest in a method for the determination of vitamin B_{12} has continued to grow as B_{12} is being added to more and more products, including feed supplements and feeds. The U.S.P. adopted a method for the determination of this vitamin in U.S.P. products this year, and our Associate Referee has made considerable progress with the method for the determination of vitamin B_{12} in feed materials. You have heard his report, and the Referee concurs with his recommendation* that study be continued.

REPORT ON VITAMIN A IN MIXED FEEDS

By MAXWELL L. COOLEY (General Mills, Inc., Minneapolis, Minnesota), *Associate Referee*

The importance and general use of true vitamin A in animal nutrition have increased efforts to perfect a suitable chemical procedure for the measurement of this vitamin in mixed feed. A number of papers (1, 3, 4, 5, 9) have been written on this subject and in 1949 the A.O.A.C. instigated a collaborative study on vitamin A in mixed feeds (7). The work was continued in 1950, and following the report and recommendations of the Associate Referee (2), the method was adopted first action (8). The report of the 1951 collaborative study provides information which will aid those interested in this type of assay. Details of the procedure used for the collaboration are given in previous publications (2, 8). Briefly, it is as follows:

* For report of Subcommittee A and action of the Association, see *This Journal*, **35**, 46 (1952).

PROCEDURE

Weigh 10 g of feed directly into a fat extraction or refluxing flask. Weigh approximately 1 g of dehydrated alfalfa meal into the same flask. This serves as a source of beta carotene which facilitates locating by visual examination the carotene band in the chromatogram. Add exactly 100 ml of hexane and reflux for 30 minutes. Remove refluxing flask, cover, cool, and allow to settle. Draw a 50 ml aliquot of the supernatant liquid through a magnesia-Super Cel adsorption column under suction. Elute with enough 10% acetone in hexane to bring the beta carotene band to the bottom of the column and just begin to elute this pigment. The colorless vitamin A band precedes the beta carotene band, and elution of some of the latter band indicates complete elution of vitamin A. Evaporate the solvent from a suitable aliquot of the eluate, under reduced pressure and mild heat, and take residue up in chloroform. The Carr-Price reaction is applied in a spectrophotometer or colorimeter tube; the antimony trichloride reagent is added rapidly to the solution of vitamin A in chloroform, and the maximum blue color is read at 620 mμ. Readings are referred to a standard curve established with the U.S.P. Vitamin A Reference Standard.

DISCUSSION OF PROCEDURE AND COMMENTS BY COLLABORATORS

Extraction Equipment.—The procedure indicates that either a Goldfisch, Bailey-Walter, or Soxhlet fat extractor may be used. The soxhlet extractor has been reported by several collaborators to be unsuitable; therefore "soxhlet" should be omitted from the description of extraction apparatus, and the words "any suitable ground glass-joint refluxing apparatus" should be inserted.

Reagents: Antimony Trichloride Reagent.—Several collaborators indicated that emphasis should be placed on Note 1 of the 1950 report (2) concerning the precaution in making the 20 per cent antimony trichloride solution in chloroform. (See Note 2, under Recommendations, at end of this report.)

Adsorbent.—The procedure specifies equal parts by weight of diatomaceous earth (Johns-Manville Hyflo Super Cel) and magnesia (Micron brand no. 2641, Westvaco Chlorine Products Corporation, Newark, Calif.). According to comments from several of the collaborators, some difficulty was experienced with the adsorbent used in that it was too retentive to allow complete elution of the vitamin A. Dr. John R. Foy, Nopco Chemical Company, Harrison, New Jersey, has done some extensive work (6) on conditioning the magnesia used in this vitamin A assay. He suggests that the magnesia be conditioned by allowing a thin layer of the powder to be exposed to air for 60–70 hours (longer or shorter period of exposure, depending on the humidity). The conditioned magnesia thus prepared has absorbed water and carbon dioxide and should lose 25–30 per cent of its weight when heated at 400°C. for 8 hours. The adsorptive characteristics of the magnesia are somewhat changed and its is considerably less retentive for vitamin A. A 50–50 mixture by weight of this magnesia and Hyflo Super Cel should be checked for recovery of vitamin A, by drawing thru a column containing this adsorbent 50 ml of a hexane soln of a known number of units of true vitamin A (25 to 40 units) plus approximately 100 micrograms of beta carotene. Elution and colorimetry follow the prescribed procedure. Recovery should be 90–100 per cent if the magnesia is properly conditioned.

Chromatography.—The complete elution of the colorless vitamin A band from the adsorption column presented some difficulties to a few collaborators. The directions regarding the chromatography were easily followed. However, when either dehydrated alfalfa meal or a carotene soln made from 10% alpha-90% beta crystalline carotene was used as a source of carotene to provide a means of visually following the colorless vitamin A band down the column, elution of some of the first caro-

tene band did not completely elute the vitamin A. Actually the colorless vitamin A band just precedes the *beta* carotene band as it moves down the column, so elution of some of this pigment should indicate complete elution of the vitamin A. The difficulty arises from the fact that, although beta carotene predominates in practically all feed ingredients, particularly alfalfa meal, there is some alpha carotene present and alpha carotene is eluted from a magnesia adsorption column before beta carotene. Investigations indicate that the colorless vitamin A band, although eluted before the beta carotene band, may *not* be all eluted before the alpha carotene band. Therefore, the usually very light alpha carotene band must be all eluted, and a small amount of the heavy beta carotene band must be washed from the column, in order to pass the vitamin A into the eluate.

In the chromatography employed, a possible means of reducing the misleading effect caused by the presence of an alpha carotene band may be to derive the tracer carotene from a soln of crystalline beta carotene (General Biochemicals, Inc., 677 Laboratory Park, Chagrin Falls, Ohio) in hexane instead of from the extract of the added dehydrated alfalfa meal. A heavy band of beta carotene can be produced by mixing approximately 100 micrograms of beta carotene into the aliquot of the feed extract just prior to passing the soln through the adsorption column. The beta carotene band (identifiable by its intensity) may be followed as it moves down the column and is slightly eluted.

TABLE 1.—*Composition of experimental feed mixtures*

	SAMPLES					
	NO. 1	NO. 2	NO. 3	NO. 4	NO. 5	NO. 6
Wheat Bran	15%	15%	5%	5%	15%	5%
Wheat Middlings	10	10	5	5	10	5
Corn Gluten Meal	—	—	5	5	—	5
Ground Corn	20	20	50	50	20	50
Soybean Oil Meal	35	35	25	20	35	20
Dehydrated Alfalfa	10	10	5	5	10	5
Meat Scraps	10	—	—	5	—	5
Fish Meal (Vitamin A-free)	—	10	5	5	10	5
Vitamin A (Calculated Units A per g)	4.0	6.0	9.0	10.5	6.0*	10.5*

* The theoretical or calculated vitamin A potencies of samples No. 5 and No. 6 were divulged to the collaborators, while the vitamin A values for the other four samples were not given.

Colorimetry.—Although the general directions on colorimetry as given in the procedure gave no trouble, six of the collaborators desired more explicit information concerning preparation of a standard colorimetric curve. The official standard to use for development of the colorimetric curve in this work is the U.S.P. Vitamin A Reference Standard obtainable from the United States Pharmacopoeia Reference Standards, 46 Park Avenue, New York 16, New York. This is in the form of capsules each of which contains close to 2500 units of vitamin A (as vitamin A acetate). The potency of the oil in these capsules is 10,000 units of vitamin A per gram. colorimetric curve should be established using the *whole* oil. (See Note 4, under Recommendations, at end of this report.)

DESCRIPTION OF COLLABORATIVE SAMPLES

Six mixed feed samples were submitted to each collaborator. Each sample contained a certain added amount of true vitamin A, which was derived from a fish liver oil having a blue color potency of 12,500 units of vitamin A per gram.

The composition of the experimental feed mixtures is given in Table 1, which shows that Samples 2 and 5 were duplicates, as were Samples 4 and 6.

LIST OF COLLABORATORS

R. N. Allen, Gorton Pew Fisheries Co., Ltd., Gloucester, Massachusetts
R. E. Anderson, Archer Daniels Midland Co., Minneapolis, Minnesota
F. A. Bacher, Merck and Co., Inc., Rahway, New Jersey
L. D. Braitberg, Vitamins, Inc., Chicago, Illinois
W. B. Brew and A. Schulz, Ralston Purina Co., St. Louis, Missouri
A. O. Call and R. E. Parker, Western Condensing Co., Appleton, Wisconsin
J. A. Campbell and T. K. Murray, Food and Drug Division, Ottawa, Canada
A. P. Fischer, General Mills, Inc., Minneapolis, Minnesota
W. R. Flach and C. D. Sander, Eastern States Farmers' Exchange, Buffalo, New York
J. R. Foy, Nopco Chemical Co., Harrison, New Jersey
P. R. Frey, Colorado A. and M. College, Fort Collins, Colorado
J. C. Fritz, Borden Co., Elgin, Illinois
N. L. Hobbs, R. P. Scherer Corp., Detroit, Michigan
H. H. Hoffman, Florida Department of Agriculture, Tallahassee, Florida
H. C. Johnson, California Department of Agriculture, Sacramento, California
C. H. Krieger and Ruth Robbins, Wisconsin Alumni Research Foundation, Madison, Wisconsin
D. H. Leweke and R. Bensing, Hales and Hunter Co., Chicago, Illinois
D. J. Mitchell, South Dakota State Chemical Laboratory, Vermillion, South Dakota
K. Morgareidge, Food Research Laboratories, Inc., Long Island City, New York
M. Narod, Lyle Branchflower Co., Seattle, Washington
I. Olcott and Grace Blumer, Dawe's Products Co., Chicago, Illinois
D. B. Parrish, Kansas State College, Department of Chemistry, Manhattan, Kansas
F. W. Quackenbush, Agricultural Experiment Station, Purdue University, Lafayette, Indiana
F. E. Randall, Cooperative G.L.F. Exchange, Buffalo, New York
M. A. Rust, Industrial Laboratories Co., Denver, Colorado
C. L. Smith, Spartan Grain and Mill Co., Spartanburg, South Carolina
D. M. Stalter, Ohio State Department of Agriculture, Columbus, Ohio
J. B. Wilkie, Food and Drug Administration, Washington, D. C.

DISCUSSION OF COLLABORATIVE DATA

Table 2 presents the average values of all results received for the six feed samples as compared with the calculated value for each sample. As previously stated, Samples 2 and 5 were duplicates and Samples 4 and 6 were duplicates. Included in Table 2 are the mean results for each set of

TABLE 2.—*Average values found for vitamin A in collaborative samples*

COLLABORATOR NO.	(UNITS OF VITAMIN A PER GRAM) SAMPLES						2 AND 5 COMB.*	± VARIATION FROM MEAN	4 AND 6 COMB.*	± VARIATION FROM MEAN
	NO. 1	NO. 2	NO. 3	NO. 4	NO. 5	NO. 6				
								per cent		*per cent*
1	4.8	6.6	9.1	11.6	6.2	11.9	6.40	3.1	11.75	1.3
2	5.2	6.2	8.7	10.6	6.5	10.9	6.35	2.4	10.75	1.4
3	3.8	5.4	7.8	9.7	5.3	9.6	5.35	0.9	9.65	0.5
4	2.7	3.2	4.4	5.7	3.7	6.9	3.45	7.2	6.30	9.5
5	4.4	6.0	8.2	10.2	5.9	10.0	5.95	0.8	10.10	1.0
6	4.3	5.7	7.6	10.6	5.9	9.9	5.80	1.7	10.25	3.4
7	7.4	4.3	9.8	10.6	5.7	9.7	5.00	1.4	10.15	4.4
8	6.4	7.4	10.4	13.3	7.9	12.8	7.65	3.3	13.05	1.9
9	3.6	5.0	6.4	9.3	5.4	9.4	5.20	3.9	9.15	1.6
10	3.7	4.0	7.2	9.8	6.2	8.5	5.10	21.6	9.15	7.1
11	3.6	3.8	5.4	5.8	4.0	8.2	3.90	2.6	7.00	17.2
12	3.6	6.5	7.5	12.3	5.4	10.4	5.95	9.3	11.35	8.4
13	3.4	4.7	8.0	9.6	5.3	8.4	5.00	6.0	9.00	6.7
14	3.3	5.4	6.3	8.6	5.6	6.2	5.50	1.8	7.40	16.2
15	3.3	4.0	6.8	8.4	6.7	8.7	5.35	25.2	8.55	1.8
16	4.6	6.7	7.0	10.0	5.9	9.3	6.30	6.3	9.65	3.6
17	2.9	4.2	6.6	7.6	3.8	8.3	4.00	5.0	7.95	4.4
18	3.7	5.0	8.5	9.6	5.5	9.1	5.25	4.8	9.35	2.7
19	5.1	6.6	8.5	12.7	6.7	11.3	6.65	0.8	12.00	5.8
20	3.6	4.6	7.7	8.4	5.1	9.1	4.85	5.2	8.75	4.0
21	4.0	3.8	11.6	9.5	4.3	11.1	4.05	6.2	10.30	7.8
22	3.4	4.9	6.8	7.6	4.2	8.3	4.55	7.7	7.95	4.4
23	3.9	6.7	9.5	10.4	6.0	9.6	6.35	5.5	10.00	4.0
24	2.9	3.9	7.1	7.5	3.9	7.6	3.90	0.0	7.55	0.7
25	4.5	6.6	8.4	10.5	6.2	10.0	6.40	3.1	10.25	2.4
26	5.0	6.5	8.6	11.6	4.9	10.1	5.70	14.0	10.85	6.9
27	3.0	5.1	8.2	9.4	5.2	9.3	5.15	1.0	9.35	0.5
28	4.6	5.9	9.1	10.7	5.6	11.0	5.75	2.6	10.85	1.4
Calc. Value	4.0	6.0	9.0	10.5	6.0	10.5	6.0		10.5	
Mean	4.10	5.31	7.90	9.70	5.46	9.49	5.38		9.50	
Std. Deviation	1.02	1.13	1.48	1.80	1.00	1.41	.91		1.66	
Coef. of Variation	24.9%	21.3%	18.7%	18.6%	18.3%	14.8%	16.7%		17.5%	

* Duplicate samples are Nos. 2 and 5 and Nos. 4 and 6.

duplicates by collaborators, as well as the percent variation of the two individual samples from that mean for each set of duplicates.

Referring to the last two columns in Table 2, it was found in the Sample 2 and 5 combination that 90 per cent of the collaborators had a variation from the mean of ±10 per cent or less, and that 61 per cent had a variation of ±5 per cent or less. Then, considering the 4 and 6 combination, 93 per cent had a variation from the mean of ±10 per cent or less, and 68 per cent had a variation of ±5 per cent or less. This is a good indication that the method provides reliable results on duplicate samples for this type of assay.

TABLE 3.—*Comparison of average values found for vitamin A in two collaborative studies*

A.O.A.C. Study for 1951

	SAMPLES (UNITS OF VITAMIN A PER GRAM)					
	NO. 1	NO. 2	NO. 3	NO. 4	NO. 5	NO. 6
Calculated Value	4.0	6.0	9.0	10.5	6.0	10.5
Mean (28 Collaborators)	4.10	5.31	7.90	9.70	5.46	9.49
Standard Deviation	1.02	1.13	1.48	1.80	1.00	1.41
Coefficient of Variation	24.9%	21.3%	18.7%	18.6%	18.3%	14.8%

A.O.A.C. Study for 1950

	SAMPLES (UNITS OF VITAMIN A PER GRAM)					
	NO. 1	NO. 2	NO. 3	NO. 4	NO. 5	NO. 6
Calculated Value	7.5	11.5	17.0	11.5	17.0	15.5
Mean (22 Collaborators)	8.47	10.43	16.48	10.44	17.20	16.05
Standard Deviation	3.03	1.95	2.21	2.10	3.59	3.48
Coefficient of Variation	35.7%	18.7%	13.4%	20.1%	20.8%	21.6%

A comparison of the average values for the 1950 and 1951 collaborative studies is given in Table 3. Of the 22 collaborators who participated in the 1950 study, 18 also participated in 1951. Of the 18 laboratories which collaborated both years, two showed poor performance in both studies. The rest were good and showed a general improvement in ability to apply the method. In Table 3 (although the samples in the recent study are different and have a considerably lower potency of vitamin A than those of the previous study) the standard deviations and coefficients of variation are generally reduced.

Further evidence of improvement in application of the method is revealed in Table 4, which compares the essential results of two analyses of variance for both years. Only collaborators who submitted triplicate results were included in these analyses. Approximately 60 per cent of the

TABLE 4.—*Essential results of an analysis of variance*
(Collaborators reporting triplicate results)
A.O.A.C. 1951 Study

SOURCE OF VARIATION	FIRST ANALYSIS (WITH 16 COLLABORATORS)		SECOND ANALYSIS (WITH 12 COLLABORATORS[a])	
	DEGREES OF FREEDOM	MEAN SQUARE	DEGREES OF FREEDOM	MEAN SQUARE
Collaborators	15	21.0*	11	7.7*
Samples	5	269.0*	5	218.0*
Interaction ($C \times S$)	75	1.75	55	1.67
Residual	192	0.23	144	0.17
Total	287		215	
Standard Error of a Single Determination	0.48		0.41	

A.O.A.C. 1950 Study

SOURCE OF VARIATION	FIRST ANALYSIS (WITH 18 COLLABORATORS)		SECOND ANALYSIS (WITH 14 COLLABORATORS[b])	
	DEGREES OF FREEDOM	MEAN SQUARE	DEGREES OF FREEDOM	MEAN SQUARE
Collaborators	17	132.0*	13	18.8*
Samples	5	740.0*	5	646.0*
Interaction ($C \times S$)	85	6.62	65	2.42
Residual	216	0.69	168	0.33
Total	323		251	
Standard Error of a Single Determination	0.83		0.57	

[a] Omitting 1951 erratic collaborators No. 8, 14, 22 and 24.
[b] Omitting 1950 erratic collaborators No. 10, 11, 16 and 21.
* Exceeds 1% level of significance.

collaborators whose results were used in compiling the 1951 data were also represented in the 1950 analysis. In both studies there were a few laboratories whose results were extremely erratic. These were included in the first analysis, but were omitted in the second analysis.

Examination of Table 4 (first analysis) demonstrates: (1) while there is a highly significant variation between laboratories in both studies, there is a great reduction in this variation in 1951 (see 21 for 1951 as against 132 for 1950)—undoubtedly some of this reduction in variation is due to lower potency samples used in the 1951 study; (2) while the samples in both studies vary, as they would be expected to, inasmuch as they were prepared to have different potencies, the range and difference in po-

tencies is not so great in the recent collaboration (see 269 for 1951 as against 740 for 1950); (3) while the highly significant interaction mean square indicates a definite absence of a tendency for each laboratory to obtain consistently high or consistently low results in both studies, the interaction is greatly reduced in 1951, which indicates more consistent results (see 1.75 for 1951 as against 6.62 for 1950); (4) the standard error of a single determination (square root of residual mean square) is considerably reduced in the 1951 study (0.48 for 1951 as against 0.83 for 1950). (The second analysis in Table 4 follows much the same pattern as the first analysis.)

SUMMARY

A proposed procedure for the measurement of vitamin A in mixed feeds was subjected to collaborative assay in 1949 and 1950. As a result of these studies, the method was adopted first action (October, 1950). Collaborative study of the procedure was continued in 1951, demonstrating an improvement in application of the method compared with previous studies. The feed samples used in the recent work had vitamin A potencies within a more practical range. The samples included two sets of duplicates, and results on these indicate that assays of replicate samples were in reasonably close agreement. The method is more accurate for higher potency feed samples (15 or more units of vitamin A per gram) than it is for lower potency feeds (4 or less units of vitamin A per gram), because the ratio of vitamin A content to interfering materials is greater. However, collaborative data on lower potency feed samples showed that three-fourths of the laboratories obtained results which were within ±20 per cent of the mean, and the mean was practically the same as the theoretical potency of the feed. Indications are that the method should be acceptable for the measurement of the vitamin A content of mixed feeds within the limits of accuracy expected for this type of vitamin assay.

ACKNOWLEDGMENT

Appreciation is extended to Mr. Irwin Olcott, Dawe's Products Company, Chicago, Illinois, for furnishing the vitamin A oil (as well as information pertaining thereto) which was used in preparation of the collaborative samples employed in this study.

RECOMMENDATION*

The Associate Referee recommends the status of the method be continued first action subject to further collaborative studies, and that the following corrections and additions be made in the procedure:

Referring to the procedure as given in *This Journal*, **34**, 97 (1951):

(a) Under "Apparatus" on page 97, line 9, delete "Soxhlet" and add "any suitable ground-glass joint refluxing apparatus."

* For report of Subcommittee A and action of the Association, see *This Journal*, **35**, 46 (1952).

(b) Under "Reagents" on page 98, line 2, after description of "Adsorbent" add "(See Note 1)" and append the following to the procedure:

NOTE 1: Check the adsorbent for recovery of vitamin A by drawing thru the described adsorption column with suction 50 ml of a hexane soln containing a known number of units of true vitamin A ester (25–40 units) plus ca 100 micrograms of beta carotene (obtainable from General Bio-Chemicals, Inc., 677 Laboratory Park, Chagrin Falls, Ohio). As to chromatography and colorimetry follow the prescribed procedure. Recovery should be 90–100 per cent. If the adsorbent is too retentive it may be conditioned by allowing a thin layer (2 or 3 mm thick) of the magnesia (prior to mixing with Super-Cel) to be exposed to air for 60–70 hours, or a recovery factor for the adsorbent used may be employed in final calculation of results.

(c) Under "Reagents" page 98, line 8, after description of "Antimony trichloride reagent" add "(See Note 2)" and append the following to the procedure:

NOTE 2: In preparing this reagent, the use of a fresh bottle of antimony trichloride crystals which has not been opened previously is desirable. The crystals should possess a translucent appearance. Crystals which have been stored too long or are kept in a bottle which has been opened, frequently contain an objectionable brown-colored decomposition material. The antimony trichloride plus the prescribed volume of chloroform are merely heated on a hot plate until solution takes place. The solution is cooled and 3 per cent of acetic anhydride is mixed in. The reagent thus prepared usually is clear; if not, it may be easily centrifuged, filtered through glass wool or allowed to settle and decanted. It may be stored in a brown bottle for several months.

(d) Under "Procedure" on page 98, line 9, after description of "extraction" add "(See Note 3)" and append the following to the procedure:

NOTE 3: Although beta carotene predominates in practically all feed ingredients, particularly alfalfa meal, there is some alpha carotene present and alpha carotene is eluted from a magnesia adsorption column before beta carotene. Investigations indicate that the colorless vitamin A band, although eluted before the beta carotene band, may not be all eluted before the alpha carotene band. Therefore, the usually very light alpha carotene band must be all eluted and a small amount of the heavy beta carotene band must be washed from the column in order to pass the vitamin A into the eluate. Instead of using the dehydrated alfalfa meal as source of the tracer carotene, a heavy band of beta carotene can be produced by mixing ca 100 micrograms of beta carotene (from a solution of crystalline beta carotene in hexane) into the aliquot of the feed extract just prior to passing the solution thru the adsorption column. The beta carotene band (identifiable by its intensity) may be followed as it moves down the column and is slightly eluted.

(e) Under "Procedure" on page 98, line 26, after description of "Colorimetry" add "(See Note 4)" and append the following to the procedure:

NOTE 4: For development of a colorimetric curve, use U.S.P. Vitamin A Reference Standard (U.S.P. Reference Standards, 46 Park Avenue, New York 16, New York). Potency of this standard is 10,000 units of vitamin A per gram. Weigh 0.2 g of the *whole* oil on a small watch glass, wash into a 50 ml volumetric flask with chloroform and make up to volume. Each ml of this solution contains 40 units of vitamin A. From this standard solution make a series of dilutions in chloroform so that 1 ml aliquots when placed in individual colorimeter tubes develop intensities of color which spread over the range of the colorimeter. Suggested concentrations in consecutive tubes might be 5, 10, 20, 30, and 40 units of vitamin A. From the resulting color readings a standard colorimetric curve is established.

LITERATURE CITED

(1) BREW, W. B., and SCOTT, M. B., *Ind. Eng. Chem.*, Anal. Ed., **18**, 46 (1946).
(2) COOLEY, M. L., *This Journal*, **34**, 370 (1951).
(3) COOLEY, M. L., CHRISTIANSEN, J. B., and SCHROEDER, C. H., *Ind. Eng. Chem.*, Anal. Ed., **17**, 689 (1945).
(4) COOLEY, M. L., CHRISTIANSEN, J. B., and KOEHN, R. C., *Anal. Chem.*, **21**, 593 (1949).

(5) NAROD, M., and VERHAGEN, D., *Anal. Chem.*, **20**, 627 (1948).
(6) Personal communication.
(7) SCHAEFER, H. C., *This Journal*, **33**, 615 (1950).
(8) *Ibid.*, **34**, 97 (1951).
(9) WALL, M. E., and KELLEY, E. G., *Anal. Chem.*, **20**, 757 (1948).

REPORT ON VITAMIN D IN POULTRY FEED SUPPLEMENTS

By LEO FRIEDMAN (Food and Drug Administration, Federal Security Agency, Washington 25, D. C.), *Associate Referee*

The A.O.A.C. has not conducted a collaborative study of the chick method for vitamin D for about ten years, but the method has received considerable attention in many formal and informal discussions. The results of numerous attempts to improve the method have not provided a basis for any important modification, either of basal ration or of procedure.

In the experience of the Associate Referee, the A.O.A.C. method as it stands today has not shown the large variability so frequently assigned to it. The adoption of the new USP Vitamin D_3 Reference Standard to replace the USP Cod Liver Oil No. 2 has eliminated a potential source of variation. Also the experience and familiarity with the details of the method gained in many laboratories during the past ten years have resulted, undoubtedly, in an increased precision and accuracy. It was deemed desirable, therefore, to evaluate the method, as it is routinely being used at present.

Since the decision to conduct a collaborative study was arrived at rather late in the year, invitations to collaborate were addressed only to laboratories that were known to be conducting A.O.A.C. chick assays routinely. All those invited indicated a willingness to cooperate. Samples and instructions were sent to ten laboratories (including the Referee's laboratory). At the time of preparation of this report, results have been received from seven laboratories.

Three samples of oil, labeled as A.O.A.C. Collaborative Samples No. 1, No. 2, and No. 3, were sent to each collaborator, with instructions indicating that the samples contained 200–300 International Chick Units of Vitamin D per gram. It was suggested that they be assayed at 250 I.C.U. per gram. The detailed instructions to collaborators were as follows:

"It is expected that these samples will be included in your current routine assay program. It is not necessary that they all be included in the same assay. It would be preferable if at least two assay runs were represented. Only one assay of each sample is requested.

"It is presumed that you are following in detail the method described in the A.O.A.C. Book of Methods. These samples are to be assayed by that procedure with the provisions that:

1. The U.S.P. Vitamin D_3 Reference Standard shall be used (*not* the RCLO No. 2).
2. The U.S.P. Vitamin D_3 Reference Standard is fed at three dosage levels.
3. Each sample to be assayed is fed at only two dosage levels.
4. Since the critical range of response varies from one laboratory to another the dosage levels shall be determined by the experience in your own laboratory.

"You are requested to report the per cent ash of the moisture and fat free tibia for each dosage level of the sample and reference oil and the negative control group, the number of chicks in each group, and, in addition, your interpretation of the potency of the samples assayed.

"If you are in a position to determine per cent toe ash and/or the tmt distances by X-ray on these same chicks, this will be valuable additional information. If data by toe ash and X-ray are included a detailed description of the procedures used would be desirable."

The principal objective of this study was to ascertain with some certainty the actual reproducibility and accuracy of the method at the present time. For this purpose the three samples used were identical, being aliquots of a cottonseed oil solution of pure crystalline vitamin D_3 prepared to contain 250 I.C.U. per gram.

RESULTS

The results obtained from seven laboratories are presented in Table 1. Laboratories No. 1 and 4 assayed all the samples at one time; laboratory No. 3 ran each sample twice, three samples with one in duplicate in one assay, and the other two samples duplicated in a succeeding assay; laboratory No. 7 ran each of the samples in two separate assays; and laboratories No. 2, 5 and 6 ran each sample once but divided among two assays. Collaborator No. 4 assayed the samples at three dosage levels; all the others used two levels.

The collaborators submitted the pertinent bone ash data for each assay and an estimate of potency for each sample. The data from each assay have been interpreted in four different ways. The Associate Referee derived the potency from each level of sample fed in any one assay by interpolation on a dose response curve. These values are given in Table 1 under method of calculation a. The individual values so obtained were averaged to give the values shown under method of calculation b. Method of calculation c is the statistical interpretation by means of least squares according to Knudsen and Tolle. Under method d are tabulated the estimates of each collaborator. Since Samples 1, 2 and 3 were identical and the assays therefore constituted triplicate determinations, the values obtained by each method of calculation were averaged to give the average potency, and the standard deviation and coefficient of variation for each laboratory was determined. Since the exact potency of the collaborative samples was known it was possible to tabulate the average potency as the per cent of the true value. For simplicity and ease of comparison some of the averages are retabulated in Tables 2 and 3. In Table 2 the average

TABLE 1.—*Results of collaborative study of the A.O.A.C. test for vitamin D in poultry feed supplements*

LABORATORY NUMBER	METHOD† OF CALCULATION	SAMPLES			AVERAGE POTENCY	STD. DEV.	COEFFICIENT OF VARIATION	AVERAGE POTENCY AS TRUE VALUE
		1	2	3				
		units/gm	*units/gm*	*units/gm*			*per cent*	*per cent*
1	a	244*	258*	219*	236	19	8.0	94.4
		213	229	254				
	b	228	244	237	236	8	3.3	
	c	188	207	218	204	15	7.4	81.6
	d	228	245	237	237	8.5	3.6	94.8
2	a	240*	275**	258**	258	28	10.8	103.2
		295	264	215				
	b	268	270	237	258	18	6.9	
	c	275	278	221	258	32	12.4	103.2
	d	268	270	237	258	18	6.9	103.2
3	a	250* 239**	320* 222**	328* 271*	258	33	12.7	103.2
		250 242	250 244	228 249				
	b	250 241	285 233	278 260	258	21	8.1	
	c	265 280	290 270	260 270	273	11	4.0	109.2
	d	280 233	305 230	275 285	268	30	11.2	107.2
4	a	163*	240*	153*	238	49	20.5	95.2
		250	279	244				
		268	296	250				
	b	227	272	216	238	30	12.6	
	c	237	280	227	248	28	11.2	99.2
	d	239	280	227	249	28	11.2	99.6
5	a	259*	290*	341**	297	27	9.0	118.8
		308	292	292				
	b	284	291	317	297	17	5.7	
	c	275	283	293	284	9	7.2	116.4
	d	274	285	293	284	9	3.2	113.6
6	a	245*	273**	218**	245	29	11.8	98.0
		285	220	225				
	b	265	247	222	245	22	8.9	
	c	265	238	222	242	22	9.0	96.8
	d	258	240	225	241	17	7.1	96.4
7	a	293* 279**	357* 237**	378* 265**	296	64	21.6	118.4
		275 418	323 220	293 210				
	b	284 349	340 229	336 238	296	54	18.2	
	c	257 320	316 223	301 228	274	44	16.0	109.6
	d	266 328	330 227	250 213	269	50	18.6	107.6
8	d	255	254	258	256	2	0.8	102.4

† Methods of Calculation:
a. Potency derived from each level of sample fed in any one assay by interpolation on dose response curve.
b. Potency derived is average of individual values obtained graphically.
c. Statistical calculation according to Knudsen and Tolle.
d. Collaborator's estimate of potency.
* Assay No. 1.
** Assay No. 2.

TABLE 2.—*A.O.A.C. vitamin D_3 collaborative study. Average of all samples for each laboratory*

LAB. NO.	AVERAGE POTENCIES EXPRESSED AS PER CENT OF TRUE VALUE			COEFFICIENT OF VARIATION (PER CENT)		
	METHOD OF CALCULATION					
	B	C	D	B	C	D
1	94.4	81.6	94.8	3.3	7.4	3.6
2	103.2	103.2	103.2	6.9	12.4	6.9
3*	103.2	109.2	107.2	8.1	4.0	11.2
4	95.2	99.2	99.6	12.6	11.2	11.2
5	118.8	113.6	113.6	5.7	3.2	3.2
6	98.0	96.8	96.4	8.9	9.0	7.1
7*	118.4	109.6	107.6	18.2	16.0	18.6
Grand Average (27 values)	106.0	103.6	104.0	14.0	13.1	12.1

* 6 values included in average, all others are averages of 3 values.

potencies, as calculated by the graphic method b, the statistical method c, and the collaborator's estimate d, and expressed as per cent of true value, are tabulated with the corresponding coefficient of variation in per cent. Included also for each of these methods of calculation is the grand average of all 27 values and the coefficient of variation for each. In Table 3 are shown the potencies for each of the 3 samples, expressed as per cent of the true value, obtained by averaging the values from each laboratory.

TABLE 3.—*A.O.A.C. vitamin D_3 collaborative study. Averages of all laboratories for each sample*

METHOD OF CALCULATION	SAMPLES		
	1	2	3
	AVERAGE POTENCY AS PER CENT OF TRUE VALUE		
b	106.4	107.2	104.0
c	104.8	106.0	100.4
d	105.6	107.2	99.6
	COEFFICIENT OF VARIATION (PER CENT)		
b	14.3	13.1	16.2
c	13.6	13.2	13.2
d	11.4	13.2	11.5

b. Potency derived is average of individual values obtained graphically.
c. Statistical calculation according to Knudsen and Tolle.
d. Collaborator's estimate of potency.
Coefficients of variation obtained from analysis of variance using values of method b:
Within laboratory variation..... 12.1 per cent
Between laboratory variation..... 14.3 per cent

The coefficient of variation in this case is an approximate measure of the between-laboratory variability.

It can be seen in Table 1 that, depending upon the laboratory and the method of interpretation of results, the potency values vary from 81.6 per cent of the true value for Laboratory No. 1 to 118.8 per cent for Laboratory No. 5. The coefficient of variation, which is a measure in this case of the within-laboratory variability, ranges from 3.2 for Laboratory No. 5 to 21.6 per cent for Laboratory No. 7.

In Table 2 the coefficients of variation indicate the reproducibility to be expected within each laboratory. With the exception of Laboratory No. 7 the coefficients of variation are less than 12.6 per cent. The variation between laboratories, as expressed by the coefficient of variation in Table 3, ranges from 11.5 to 16.2 per cent. As is to be expected, this between-laboratory variation is slightly greater than the within-laboratory variation.

The largest coefficient of variation for the grand average of 27 values is 14 per cent. If we take the grand average 106 plus or minus 28, which would be the same as 2 standard deviations (giving us a probability of 95%), then we should expect almost all of the values tabulated in Table 1 to fall within these limits. In terms of I.U. these limits are 195 to 335. The values in Table 1 that fall outside these limits are Laboratory 1, method c, Sample 1, value of 188; Laboratory 4, method a, Sample 1, 163 and Sample 3, 153; Laboratory 5, method a, Sample 3, 341. Laboratory 7, method a, Sample 1, 418, Sample 2, 357, and Sample 3, 378; method b, Sample 1, 349, Sample 2, 340, and Sample 3, 336.

Ten out of 135 values fall outside the limits of 95 per cent probability. This is slightly larger than what is expected on the basis of a normal distribution. However, considering that 6 of these 10 values are from one laboratory, it is probable that the values from this laboratory do not belong to the same population as the other values in Table 1.

The results from Laboratory No. 8 were received after summarization of the data was completed. The collaborator's estimates have been included in Table 1 with their coefficients of variation and standard deviation. The accuracy and precision of these results are the best of any of the laboratories that have reported. (In considering the over-all evaluation of this study it should be noted that the statistical summary does not include results of Laboratory No. 8, which have a small degree of variability, but do include those of Laboratory No. 7, which have a variability so great as to be on the borderline of acceptability.)

It can be seen from Table 2 that, on the average, the potencies obtained by the various methods of interpretation show no significant difference. It has been suggested in the past that the use of statistical methods of interpretation for the results of an assay might be of help in reducing variability. It is only logical that all data obtained from an assay be

utilized in arriving at a potency for a sample, and that this be accomplished by the most efficient means. Furthermore, it has been suggested that it is possible to arrive at some measure of the validity of an assay by means of the standard error which can be calculated statistically. However, from the results of this study, and the assay of several hundred samples conducted during the past 3 years in the Referee's laboratory, wherein potencies have been calculated both graphically and statistically, the Associate Referee must conclude that from a practical standpoint there is not much difference between the results obtained by these two methods. Furthermore, the standard errors of the assay for the collaborative study (which have not been tabulated) show no correlation either with the precision of the assay as exemplified by the coefficient of variation, or by the accuracy of the assay as obtained by comparison of the results with the true value. It is apparent that all the causes of variation are not accounted for in calculating the standard error of the assay from group bone ash data.

Considering the potency values for the three samples, given in Table 3, it is not difficult to conclude that the samples are identical in potency. This conclusion has been arrived at, also, from an analysis of variance. Here the differences between potencies for each sample at different levels of dosage were compared with the differences between samples. Since no significant difference between samples was found, and since the data include results from separate assays, it follows that there was no significant difference between results of separate assays.

A measure of the accuracy of the method can be seen from the values in the last column of Table 1, from the grand average of 27 values given in Table 2, and the average potencies given in Table 3. The average values in Table 3 range from 99.6 to 107.2 per cent of the true value of 250 units per gram of sample.

The chick method of assay has been in routine use in each of the collaborating laboratories for a number of years. From this study it appears that experience with the method serves to improve to an important degree the precision that may be expected. Precision of the method has been discussed in detail and is in a range acceptable for biological assays. Furthermore, with the study designed to estimate the accuracy of the method it was found that the range of variability of the over-all potency of 106 per cent includes the true value of 100 per cent.

It appears from the results obtained that a re-evaluation of the precision and accuracy of this method has been justified.

ACKNOWLEDGMENTS

The following collaborators took part in the study:

(1) H. de Bussieres, Curtis and Tomkins, Ltd., San Francisco, Calif.

(2) F. W. Franco, Div. of Nutrition, U. S. Food & Drug Adm., Washington, D. C.

(3) J. C. Fritz, The Borden Co., Elgin, Ill.
(4) R. B. Hubbel, Connecticut Agricultural Experiment Station, New Haven, Conn.
(5) C. H. Krieger, Wisconsin Alumni Research Foundation, Madison, Wisconsin
(6) I. Motzok, Department of Animal Nutrition, Ontario Agricultural College Guelph, Canada
(7) B. L. Oser, Food Research Lab., Inc., Long Island City, N. Y.
(8) L. Rosner, Laboratory of Vitamin Technology, Chicago, Ill.

The Associate Referee wishes to express his appreciation to William Weiss and Lila F. Knudsen for their help in analyzing the collaborative results, and to the collaborators for their interest and cooperation in this work.

REPORT ON A CHEMICAL METHOD FOR NICOTINIC ACID

By J. P. SWEENEY (Food and Drug Administration, Federal Security Agency, Washington 25, D. C.), *Associate Referee*

A report on a collaborative study of methods for the chemical determination of nicotinic acid was given at the 1950 A.O.A.C. meeting. In this study sulfanilic acid and Tobias acid were used as aromatic amines. As the result of the study the sulfanilic acid method was adopted first action.*

It is necessary to extract the nicotinic acid from food and feed products and to convert any nicotinamide present to nicotinic acid. For this purpose the samples were autoclaved for 30 minutes at 15 pounds pressure with 0.25 *N* H_2SO_4. However, as illustrated by Table 1, 0.25 *N* H_2SO_4 is not of sufficient strength for complete conversion of nicotinamide to nicotinic acid.

TABLE 1.—*Nicotinamide (4 micrograms) autoclaved 30 minutes at 15 pounds pressure*

ACID	ABSORBANCY
0.25 *N* H_2SO_4	.268
0.5 *N* H_2SO_4	.288
1.0 *N* H_2SO_4	.312
Conc. HCl	.305

Since food products are occasionally enriched with nicotinamide rather than nicotinic acid, the use of an acid of insufficient strength for complete conversion of the amide to the acid might result in considerable error. In Table 2 are shown nicotinic acid values of flour and corn meal used in the 1950 collaborative study. A comparison of results obtained by hydrolysis with 0.25 *N* H_2SO_4 and 1.0 *N* H_2SO_4 is given, together with the average microbiological value obtained.

* *This Journal*, 34, 99 (1951).

TABLE 2.—*Comparison of results obtained by hydrolysis with varying strength acids*

	SULFANILIC ACID		TOBIAS ACID		MICROBIOLOGICAL
	0.25 *N* H_2SO_4	1.0 *N* H_2SO_4	0.25 *N* H_2SO_4	1.0 *N* H_2SO_4	AVERAGE VALUE
Flour	17.5	18.0	18.1	18.7	17.7
Corn Meal	19.7	20.9	21.0	21.8	22.4

The results on both flour and corn meal show a small, but consistently higher nicotinic acid value when 1.0 *N* H_2SO_4 is used.

It is recommended* that the strength of the H_2SO_4 used for extraction of nicotinic acid and conversion of nicotinamide to nicotinic acid be increased from 0.25 *N* to 1.0 *N*.

REPORT ON PANTOTHENIC ACID, MICROBIOLOGICAL METHOD

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

During the past year the Associate Referee conducted a collaborative study of a microbiological method of assay for added pantothenic acid in feeds and in pharmaceutical products. As the result of an earlier study, reported in 1944, it was recommended that further work be done on the microbiological method (1). The method studied at that time was based on the one described by Strong, Feeney, and Earle (2), as modified by Neal and Strong (3), and it included additional minor changes. The test organism used was *L. casei* (A.T.C.C. No. 7469), and an attempt was made to determine the total pantothenic acid content of samples following enzyme digestion. The results of that study indicated that the determination of pantothenic acid in natural materials is difficult and requires more than simple extraction. Strong and his group (4, 5, 6, and 7) have pointed out the complex nature of the naturally bound forms of pantothenic acid and have suggested the use of a dual enzyme digestion. The coenzyme A of Lipmann and co-workers (8 and 9) and the more recently announced pantethine of Snell and co-workers (10), are indicative of the nature of the extraction problem that is still unsolved.

Pantothenic acid is added in the form of calcium pantothenate to feed supplements and to pharmaceutical preparations. A microbiological method that will measure added calcium pantothenate is desirable, at least until we are able to determine accurately the total pantothenic acid of natural materials.

* For report of Subcommittee A and action of the Association, see *This Journal*, 35, 46 (1952).

During the year, 10 laboratories accepted an invitation to take part in the study of a proposed microbiological method of assay for "free" pantothenic acid. Basically, there is nothing new in this proposed method. It is similar to the nicotinic acid method (11) except for the modifications necessary to adapt it to the assay for pantothenic acid. The test organism, *L. arabinosus 17–5* (A.T.C.C. No. 8014), has been used in a number of laboratories in the assay for pantothenic acid.

Four samples were included in the study and were as follows:

Sample No. 1, a solution of calcium pantothenate, was made to contain 0.394 mg of pantothenic acid per ml. Sample No. 2 was a B-Vitamins capsule preparation purchased in a local pharmacy, labeled to contain 2.0 mg of pantothenic acid per capsule. Sample No. 3 was a finely ground poultry feed mixture to which was added sufficient calcium pantothenate to make 227 mg of added pantothenic acid per pound of feed. Sample No. 4 was prepared in the same manner to contain 80 per cent of the amount of pantothenic acid of Sample No. 3, or 181 mg per pound. The

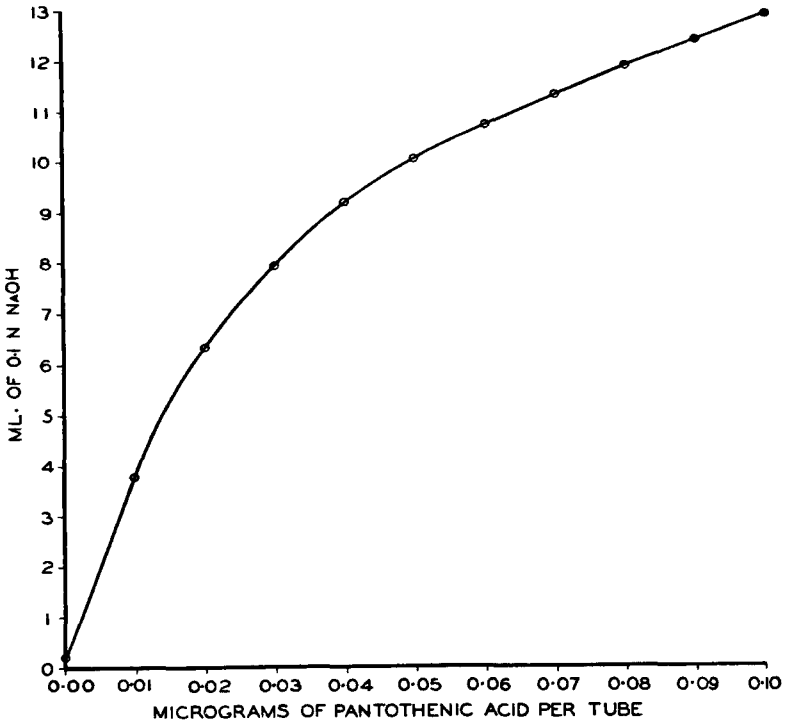

FIG. 1.—Growth Response of *L-arabinosus* (A.T.C.C. No. 8014) to Pantothenic Acid. (72 Hours Incubation at 34°C.)

basal feed mixture used was found by microbiological assay to have a "free" pantothenic acid content of approximately 3.0 mg. per pound. Thus the naturally occurring pantothenic acid was insignificant in comparison to the amount added.

Details of the method are given in *This Journal*, **35**, 103–105 (1952).

This method has been used in the laboratory of the Associate Referee for a number of years for the determination of pantothenic acid in pharmaceutical preparations. The type of response curve obtained routinely in this assay will be of interest and is presented in Fig. 1. It is apparent that with this type of a curve a high degree of precision is obtainable.

TABLE 1.—*Summary of reports*

LABORATORY NUMBER	SAMPLE 1	SAMPLE 2	SAMPLE 3	SAMPLE 4
	mg/ml	*mg/cap.*	*mg/lb.*	*mg/lb.*
1	0.392	2.53	237	181
2	0.363	2.20	211	169
3	0.403	2.39	237	180
4	0.381	2.36	216	166
5	0.364	1.78	202	165
6	0.396	2.14	226	180
7	0.390	1.81	212	176
8	0.410	3.07	239	197
Weighted Average	0.383	2.17	218	172

RESULTS OF COLLABORATORS

Reports from 8 laboratories were received; one additional report was received too late to be included in the summary. The average values reported by collaborators for each of the four samples are presented in Table 1. From the number of assays made in each laboratory, a weighted average was calculated and it also is given in Table 1. The weighted average values for samples 1, 3, and 4 were 3 to 7 per cent lower than the true values. On this basis these results are considered acceptable for a collaborative study of this type. This, however, does not properly reflect the variation within laboratories and between laboratories as shown in Table 2.

The standard deviations are greater than are desirable for this method of assay. It has been shown by studies on microbiological methods of assay for other vitamins that a higher degree of precision is possible. In view of these considerations, further collaborative study is desirable. It is apparent from the data received that differences in results, within and between some of the laboratories, were small and that with further experience greater precision can be acquired with this method. On this basis,

TABLE 2.—*Standard deviation of results given in table 1*

SOURCE OF VARIATION	STANDARD DEVIATION*
	per cent
Within Laboratory—Within Sample	5.63
Within Laboratory—Between Samples	7.76
Between Laboratories	9.56

* These are standard deviations of percentages. They were obtained by the method of variance components.

it is intended that further study will be made during the coming year, but it is the opinion of the Associate Referee that the results of this study are an adequate basis for a recommendation for acceptance of the method as first action, at this time.

COLLABORATORS

E. De Ritter, Hoffmann-La Roche, Inc., Nutley, N. J.

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B. L. Oser, Food Research Laboratories, Inc., Long Island City, N. Y.

R. T. Pierce, Jr., Pennsylvania State College, State College, Pa.

H. C. Schaefer, Ralston Purina Co., St. Louis, Mo.

E. W. Toepfer and E. G. Zook, Bureau of Human Nutrition and Home Economics, U.S.D.A., Washington, D. C.

The Associate Referee wishes to express appreciation to the collaborators and to their organizations for their cooperation in this study.

Appreciation is also expressed to Mr. Howard Edelson of the Food and Drug Administration for statistical analysis of the data.

RECOMMENDATION*

1. It is recommended that the microbiological method for the assay of added pantothenic acid herein described be adopted "first action."

2. It is further recommended that collaborative study of the method be continued during the coming year.

LITERATURE CITED

(1) *This Journal*, **28**, 567 (1945).
(2) *Ind. Eng. Chem.*, Anal. Ed., **13**, 566 (1941).
(3) *Ibid.*, **15**, 654 (1943).
(4) *Arch. Biochem.*, **19**, 287 (1948).
(5) *J. Biol. Chem.*, **185**, 335 (1950).
(6) *J. Nutr.*, **44**, 361 (1951).
(7) Private communications.

* For report of Subcommittee A and action of the Association, see *This Journal*, **35**, 47 (1952).

(8) *J. Biol. Chem.*, **167**, 869 (1947).
(9) *Ibid.*, **177**, 97 (1949).
(10) *J. Am. Chem. Soc.*, **72**, 5349 (1950).
(11) *Methods of Analysis, A.O.A.C.*, 1950, p. 782.

REPORT ON VITAMIN B_6, CHEMICAL METHOD

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

There has been presented at this meeting a paper entitled "A New Chemical Method for the Determination of Vitamin B_6," by J. P. Sweeney and W. L. Hall, and chemists have available now another colorimetric method for vitamin B_6 analysis.

It will be noted in Table 2 of the paper mentioned above that the data represent analytical results obtained from simple and complex pharmaceutical products having pyridoxine or vitamin B_6 present. In light of the results obtained to date, it is recommended that a collaborative study be made during the coming year on the application of the new colorimetric method for vitamin B_6 to pharmaceutical products.

REPORT ON VITAMIN B_{12}, MICROBIOLOGICAL METHOD

By CARL H. KRIEGER (Wisconsin Alumni Research Foundation, Madison, Wisconsin), *Associate Referee*

Vitamin B_{12} has been established as specific therapy in megaloblastic anemias, especially pernicious anemia. In addition it has been found to be an essential factor in stimulating growth of various animal species. Because of the biological significance of vitamin B_{12}, considerable time and effort have been spent in attempting to devise a reliable method of assay. Since the time it was shown that, under specified conditions, *L. lactis Dorner* required vitamin B_{12}, an observation which in turn led to the isolation of this vitamin, a number of methods of assay employing several other microorganisms have been recommended for its determination.

In the fall of 1950 a U.S.P. collaborative study on the microbiological assay for vitamin B_{12} was completed. The method was designed for application to U.S.P. preparations. The results of this study were sufficiently favorable to warrant consideration of the U.S.P. Study Panel to recommend to the U.S.P. Anti-Anemia Preparations Advisory Board that the proposed method be adopted for U.S.P. purposes.

Since the proposed U.S.P. method showed considerable promise, and in an effort to avoid marked differences in methods between the U.S.P and the A.O.A.C., it appeared desirable to carry out the A.O.A.C. collaborative study employing this method, with a minor modification. The proposed U.S.P. method requires a 72-hour incubation period fol-

lowed by a titrimetric evaluation of the growth of the microorganisms. Investigations in a number of laboratories have revealed that equally good results could be obtained employing a shorter incubation period (18 to 24 hours) and a turbidimetric evaluation. The latter procedure, if practicable, would make laboratory results available in a considerably shorter period of time. Consequently, the proposed U.S.P. procedure was modified to this extent in the collaborative study described herein.

It is to be realized that the proposed U.S.P. procedure had been employed in the assay of relatively pure materials containing reasonably substantial quantities of vitamin B_{12}. The purpose of this collaborative study was to determine what inter-laboratory correlation existed on crude materials of relatively low potency. This posed a problem, not so much in the execution of an assay technique, but rather in which manner the sample should be treated to give the most reliable assay values. Water insoluble materials had been found to be the main offender. Autoclaving at an acidic or neutral *p*H, autolysis or steaming in a buffered solution, allowing the sample to stand in distilled water, extraction by various solvents, digestion with enzymes, as well as the aseptic addition of sterile extracts to the autoclaved medium, all have been procedures employed for vitamin B_{12} extraction. In addition, consideration has been given to the stability of the extracted vitamin.

The A.O.A.C. collaborative study reported here was designed to answer in part some of these problems. In view of the fact that vitamin B_{12} and its analogues are water soluble, it is only natural that a water extraction procedure should be included. Autoclaving from 0.1 to 1.0 gram of test material for 15 minutes at 121°C. in 25 ml of distilled water was one of the proposed procedures.

Thompson, et al. (1) reported that water extracts of animal proteins failed to give microbiological vitamin B_{12} values comparable to those obtained by rat assay. Tryptic digestion was suggested to give maximum release. Consequently, the second method of sample treatment recommended was to subject 0.1 to 1.0 gram of sample to 36 hours of incubation at 37°C. with 10 mg of trypsin (1/250) in 25 ml of 0.8 per cent sodium bicarbonate. Both toluene *and* chloroform were recommended as a preservative to minimize the possibility of anaerobic processes under the toluene layer giving rise to vitamin B_{12} or other stimulatory substances.

A water and trypsin digestion of a commercial vitamin B_{12} feed supplement yielded low results. On the other hand, treatment of the test material with an organic solvent prior to assay substantiated the claimed potency. On the basis of this observation a third method of sample treatment was included. This consisted of shaking 0.1 to 1.0 gram of test material with 25% neutral ethyl alcohol for at least one minute.

Fricke (2) reported that bisulfite had a profound stabilizing effect on vitamin B_{12b} in the presence of a variety of materials. This was particu-

larly true in a cup plate assay method using *L. leichmannii 4797*. Application of this technique to samples prepared for the tube method of assay indicated a similar stabilizing effect. Hence, in the fourth method of sample treatment it was suggested that the test material be autoclaved for 15 minutes in 0.1 *M* phosphate-citrate buffer at *p*H 4.5 (4.54 ml of 0.2 *M* disodium phosphate plus 5.46 ml of 0.1 *M* citric acid; T. C. MacIlvaine, Lange's Handbook of Chemistry) containing 0.1 per cent sodium metabisulfite.

Three different crude materials were subjected to assay. The samples were selected so as to afford a wide range of potencies and to illustrate the effectiveness of the sample treatment techniques. Sample A was a commercial vitamin B_{12} antibiotic feed supplement claimed to contain 3 mg. of vitamin B_{12} per pound (6.61 mmg/g). Sample B was a commercial grade 50 per cent condensed fish solubles. Sample C was an all vegetable protein practical chick starter mash, fortified with crystalline vitamin B_{12} to the extent of 5 mmg/kg. The ration prior to supplementation revealed a vitamin B_{12} activity of approximately 2 mmg/kg resulting in a total potency of 7 mmg vitamin B_{12}/kg (.007 mmg/g).

The three samples were subjected to vitamin B_{12} assay by the following method after being treated by each of the four methods described above. In addition, the collaborators also subjected the samples to the method of treatment currently employed in their laboratory. One collaborator assayed the samples biologically by a modification of the rat growth test of Hartman, Dryden, and Cary (3, 4).

VITAMIN B_{12} ASSAY PROCEDURE

THE MEDIUM

Basal Medium Stock Solution—

Acid-hydrolyzed Casein Solution	25 ml
Cystine-Tryptophane Solution	25 ml
Tomato Juice Solution	50 ml
Asparagine Solution	5.0 ml
Adenine-Guanine-Uracil Solution	5.0 ml
Xanthine Solution	5.0 ml
Riboflavin-Thiamine-Biotin-Niacin Solution	10 ml
p-Aminobenzoic acid-Calcium pantothenate-Pyridoxine-Pyridoxal-Pyridoxamine-Pteroylglutamic acid Solution	10 ml
Salt Solution A	5.0 ml
Salt Solution B	5.0 ml
Polyoxyethylene Sorbitan Monooleate (Tween 80) Solution	5.0 ml
Dextrose, Anhydrous	10 g
Sodium Acetate, Anhydrous	5.0 g
Ascorbic Acid	1.0 g

Dissolve the anhydrous dextrose, anhydrous sodium acetate, and ascorbic acid in the previously mixed solns, add about 50 ml of water, adjust to a *p*H of 6.0 with sodium hydroxide soln, and finally add water to make 250 ml. Prepare the various solns as follows:

Acid-hydrolyzed casein soln.—Mix 100 g of vitamin-free casein with 500 ml of

constant-boiling HCl (approximately 20 per cent HCl), and reflux the mixture for 8 to 12 hours. Remove the HCl from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the soln to a *p*H of 3.5 (plus or minus 0.1) with sodium hydroxide soln, and add water to make 1000 ml. Add 20 g of activated charcoal, stir for 1 hour, and filter. Repeat the treatment with activated charcoal. Store under toluene in a refrigerator at a temperature not below 10°. Filter the soln if a precipitate forms upon storage.

Cystine-tryptophane soln.—Suspend 4.0 g of l-cystine and 2.0 g of l-tryptophane (or 4.0 g of d, l-tryptophane) in 700 to 800 ml of water, heat to 70° to 80° and add 20 per cent HCl, dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 ml. Store under toluene in a refrigerator at a temperature not below 10°.

Tomato juice soln.—Centrifuge 3000 ml of fresh or canned tomato juice. Suspend 10 to 20 g of analytical filter-aid in the supernatant liquid and filter, with the aid of reduced pressure, through a layer of analytical filter-aid of sufficient thickness so that a clear, straw-colored filtrate is obtained. Adjust the soln to a *p*H of 3.5 (plus or minus 0.1) with HCl soln, add 40 to 50 g of activated charcoal, stir for 20 to 30 minutes and centrifuge. Suspend 10 to 20 g of analytical filter-aid in the supernatant liquid and filter, with the aid of reduced pressure, through a layer of analytical filter-aid of sufficient thickness so that a clear filtrate is obtained. Store under toluene in a refrigerator.

Asparagine soln.—Dissolve 2.0 g of l-asparagine monohydrate in water to make 200 ml. Store under toluene in a refrigerator.

Adenine-guanine-uracil soln.—Dissolve 0.2 g each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 ml of 20 per cent HCl, cool, and add water to make 200 ml. Store under toluene in a refrigerator.

Xanthine soln.—Suspend 0.2 g of xanthine in 30 to 40 ml of water, heat to 60° or 70°, add 6.0 ml of ammonia T.S., and stir until the solid is dissolved. Cool, and add water to make 200 ml. Store under toluene in a refrigerator.

Riboflavin-thiamine-biotin-niacin soln.—Prepare a soln containing, in each ml, 25 mmg of riboflavin, 25 mmg of thiamine hydrochloride, 0.2 mmg of biotin, and 50 mmg of niacin, by dissolving crystalline riboflavin, crystalline thiamine hydrochloride, crystalline biotin (free acid), and crystalline niacin in 0.02 *N* acetic acid. Store, protected from light, under toluene in a refrigerator.

p-Aminobenzoic acid-calcium pantothenate-pyridoxine-pyridoxal-pyridoxamine-pteroylglutamic acid soln.—Prepare a soln in neutral 25 per cent alcohol to contain 50 mmg of p-aminobenzoic acid, 25 mmg of d-calcium pantothenate, 100 mmg of pyridoxine hydrochloride, 100 mmg of pyridoxal hydrochloride, 20 mmg of pyridoxamine dihydrochloride and 5.0 mmg of pteroylglutamic acid in each ml. Store in a refrigerator.

Salt soln A.—Dissolve 10 g of monobasic potassium phosphate and 10 g of dibasic potassium phosphate in water to make 200 ml. Add 2 drops of HCl and store under toluene.

Salt soln B.—Dissolve 4.0 g of magnesium sulfate, 0.2 g of sodium chloride, 0.2 g of ferrous sulfate, and 0.2 g of manganese sulfate in water to make 200 ml. Add 2 drops of HCl and store under toluene.

Polyoxyethylene sorbitan monooleate (Tween 80) soln.—Dissolve 20 g of polyoxyethylene sorbitan monooleate (Tween 80) in sufficient alcohol to make 200 ml. Store in a refrigerator.

Culture Medium

Dissolve 0.75 g of water-soluble yeast extract, 0.75 g of peptone, 1.0 g of anhydrous dextrose, and 0.2 g of monobasic potassium phosphate in 60 to 70 ml of

water. Add 10 ml of tomato juice soln (without activated charcoal adsorption) and 1.0 ml of polyoxyethylene sorbitan monooleate soln. Adjust the soln to a *p*H of 6.8 with sodium hydroxide soln and add water to make 100 ml. Add 10-ml portions of the soln to test tubes. Plug the tubes with cotton, sterilize in an autoclave at 121°C. to 123°C., and cool.

Suspension Medium

To 100 ml of basal medium stock soln, add water to make 200 ml. Add 10-ml portions of the soln to test tubes, plug the tubes with cotton, sterilize in an autoclave at 121°C. to 123°C., and cool.

PREPARATION OF THE INOCULUM

Stock Culture of Lactobacillus Leichmannii

To 100 ml of culture medium, before addition to tubes, add 1.0 to 1.5 g of agar, and heat the mixture, with stirring, on a steam bath, until the agar dissolves. Add approximately 10-ml portions of the hot soln to test tubes, plug the tubes with cotton, sterilize in an autoclave at 121°C. to 123°C., and allow tubes to cool in an upright position. Prepare stab cultures in 3 or more of the tubes, using a pure culture of *Lactobacillus leichmannii*,* incubating 16 to 24 hours at any selected temperature between 30°C. and 37°C. but held constant to within plus or minus 0.5°C., and finally store in a refrigerator.

Prepare a fresh stab of the stock culture thrice weekly and do not use for inoculum if the cuture is more than 4 days old.†

Inoculum

Make a transfer of cells from the stock culture of *Lactobacillus leichmannii* to a sterile tube containing 10 ml of the culture medium. Incubate this culture for 16 to 24 hours at any selected temperature between 30°C. and 37°C. but held constant to within plus or minus 0.5°C. Under aseptic conditions, centrifuge the culture, and decant the supernatant liquid. Suspend the cells from the culture in 10 ml of sterile suspension medium, centrifuge, and decant the supernatant liquid. Again, suspend the cells in 10 ml of sterile suspension medium, centrifuge, and decant the supernatant liquid. Repeat this process a third time. Finally, resuspend the cells in 10 ml of sterile suspension medium, add 1.0 ml of this suspension to 10 ml of sterile suspension medium, and mix. The cell suspension so obtained is the inoculum.

THE ASSAY PROCEDURE

Prepare standard Vitamin B_{12} tubes as follows: To duplicate test tubes (20×150 mm), add 0.0 ml, 0.5 ml, 1.0 ml, 1.5 ml, 2.0 ml, 2.5 ml, 3.0 ml, 3.5 ml, 4.0 ml, 4.5 ml, and 5.0 ml, respectively, of standard vitamin B_{12} soln containing 0.02 millimicrograms vitamin B_{12} per ml. To each tube add 5.0 ml of basal medium stock soln and water to make 10 ml.

Prepare tubes containing the material to be assayed as follows: To duplicate text tubes (20×150 mm) add, respectively, 0.5 ml, 1.0 ml, 1.5 ml, 2.0 ml, 3.0 ml, and 5.0 ml of the test soln of the material to be assayed. To each tube add 5.0 ml of basal medium stock soln and water to make 10 ml.‡

* Pure cultures of *Lactobacillus leichmannii* may be obtained from the American Type Culture Collection, 2029 M St., N.W., Washington 6, D.C., as number 7830.

† To activate a culture for use in this assay, make at least ten successive transfers of the culture in a two-week period.

‡ Owing to the high sensitivity of the organism to small amounts of vitamin B_{12}, great precaution should be taken in using scrupuously clean glassware throughout this assay. (Loy, Haggerty, and Kline, *Archives of Biochemistry*, **29**, 451 (1950).)

After mixing, plug the tubes with cotton, or cover with caps, and sterilize in an autoclave at 121° to 123°.* Cool, aseptically inoculate each tube with 1 drop of inoculum, and incubate for 18 to 24 hours at any selected temperature between 30°C. and 37°C. but held constant to within plus or minus 0.5°C. Contamination of the assay tubes with any foreign organism invalidates the assay. Read the turbidity of the tubes in a suitable instrument after thorough shaking.

THE CALCULATION

Prepare a standard curve of the vitamin B_{12} standard galvanometer readings by plotting the average of the galvanometer readings for each level of the vitamin B_{12} standard soln used, against millimicrograms of vitamin B_{12} contained in the respective tubes. From this standard curve, determine by interpolation the vitamin B_{12} content of the test soln in each tube. Calculate the vitamin B_{12} content in each ml of the test soln for each of the tubes. The vitamin B_{12} content of the test material is calculated from the average of the values obtained from not less than 6 of these tubes that do not vary more than plus or minus 10 per cent from the average.

LIST OF COLLABORATORS

1. A. Baker, U. S. Industrial Chemicals Co., Baltimore, Md.
2. Miss M. Burton and A. G. Lochhead, Division of Bacteriology and Dairy Research, Science Service, Department of Agriculture, Ottawa, Canada.
3. P. H. Derse and B. E. Kline, Wisconsin Alumni Research Foundation, Madison, Wis.
4. J. C. Fritz, The Borden Co., Elgin, Ill.
5. N. L. Hobbs, R. P. Scherer Corp., Gelatin Products Div., Detroit, Mich.
6. H. W. Loy, Jr., and J. F. Haggerty, Division of Nutrition, Food and Drug Administration, Washington, D. C.
7. F. E. Randall, Cooperative G. L. F. Exchange, Inc., Buffalo, N. Y.
8. R. M. Stern, Pabst Laboratories, Milwaukee, Wis.

COMMENTS AND SUGGESTIONS BY COLLABORATORS

Collaborator No. 2.—Treatments currently used by collaborator: (1) Heat in water bath to 40°C. in the presence of 5 ml of chloroform and 5 ml of Tween 80 (1–10) then place on a mechanical shaker for 30 minutes to 1 hour (these were values reported in tables); (2) Dissolve by means of a Waring Blender a mixture of the sample in a soln of 13 parts of acetone, 6 parts of distilled water, and 1 part 4 *N* HCl, adding also 5 ml of Tween 80 (1–10). The volume employed is usually 35 per cent of the first dilution with further dilutions being made with distilled water. Sample is adjusted to *p*H 6.0 before completion of first dilution (values obtained by this method were 7.53, 0.22, and 0.0066 for samples A, B, and C, respectively).

Collaborator No. 3.—*Treatment*—add water, adjust to *pH* 4.0, autoclave 10 minutes at 15 lbs., shake with glass beads (values reported in the tables). *Assay*—medium of Capps, et al., (*J. Biol. Chem.*, **178**, 517 (1949)) plus thiomalic and guanylic acids; *L. leichmannii 4797*, incubation 72 hours, 37°C., titration. (Values obtained by this method were 5.64, 0.151, and 0.0075 for samples A, B, and C respectively.)

Collaborator No. 4.—Samples are allowed to extract in distilled water for one hour at room temperature before being diluted and placed in assay tubes (values reported in tables).

Collaborator No. 5.—Bisulfite treatment same as described in the procedure—modified Skeggs, et al., (*J. Biol. Chem.*, **184**, 211 (1950)) procedure (values reported

* Overheating (oversterilizing) of the assay tubes may produce unsatisfactory results. Sterilization for not more than 10 minutes is recommended.

in the tables). In general we found the assay satisfactory, although we found it necessary to work in a little higher range of B_{12} than suggested in the directions. We found that a range from 0.025 to 0.03 millimicrograms per tube was more satisfactory. Our technique is quite similar to that of the collaborative method. We do not use tomato juice and can see no reason why it is included in the medium. The only other differences between the two methods is the inclusion of fumaric acid and the substitution of cysteine for cystine in the medium. We also employ *strain 4797* and find it to be—at least the transfer we have—a little more sensitive than the *7830* which you sent us. Since we use the bisulfite treatment, we also convert our standard to sulfito-cobalamin, a step which seems logical.

Collaborator No. 6.—Sample treatment similar to the alcohol treatment except as follows: sample plus ethyl alcohol plus 0.1 per cent bisulfite plus phosphate-citrate buffer to *p*H 4.5, taken to boiling point, cooled, diluted, and filtered (values reported in the tables). We are convinced, however, that conversion of B_{12} to the stable bisulfite form is desirable and, therefore, have combined the virtues of the bisulfite and alcohol treatments. Results support our contention. The data reported here indicate to us that the hydroxo form of the vitamin is quite unstable, and must be converted during the first step in the extraction to a more stable form. Enzyme hydrolysis does not appear necessary, and may lead to low values if provision is not made to stabilize the vitamin before enzyme treatment.

In our opinion, higher values for sample A are obtained where alcohol is used because of the eluting effect. This does not hold to the same degree for feed samples where adsorptive materials are absent, or present in smaller amounts. Again, it is clear that our most important problem is the stabilizing of the naturally occurring form or forms of vitamin B_{12}.

Collaborator No. 8.—Biological rat assay. You will note that on this assay Sample A seemed to be slightly below the range that you indicated, while Sample C is considerably above. We feel reasonably confident of the results on Samples A and B, but have less confidence in the results on Sample C.

DISCUSSION OF COLLABORATIVE DATA

The vitamin B_{12} values obtained after the four different sample treatments as well as the biological value for samples A, B, and C are given in Tables 1, 2, and 3, respectively.

The bisulfite or alcohol treatment of sample A gave the highest and most consistent values, as borne out by the calculated mean, the standard deviation, and coefficient of variation. Larger differences between laboratories and lower values were obtained with the water or enzyme treatment. The latter treatment gave a slightly higher value (although not in all cases) than the water treatment. Previous experience with this test material indicated that these observations were to be expected. Collaborator No. 6 obtained the highest value (9.74 mmg/g) with a combined bisulfite and alcohol treatment. The rat assay value fell below the bisulfite or alcohol treatment values as well as the claimed value.

The best inter-laboratory correlation was obtained with sample B. With this sample the bisulfite treatment gave the highest value. Little or no difference was observed between the water and alcohol treatments. Obviously elution of the vitamin B_{12} was not a factor with this test material. As with sample A, the rat assay value was slightly less than

TABLE 1.—*Vitamin B_{12} values* for sample A*

COLLABORATOR	SAMPLE TREATMENT				
	WATER	TRYPSIN	BISULFITE	ALCOHOL	OTHER SAMPLE TREATMENTS
1	5.17	4.46	7.30	6.92	—
2	5.40	3.80	7.40	6.80	7.14
	4.80	5.10	8.20	8.00	—
3	3.39	3.86	7.38	5.93	5.05
4	4.68	8.31	9.34	8.33	8.25
5	1.25	3.43	8.01	6.08	7.84
6	4.58	5.64	8.00	8.40	9.74
7	2.80	4.30	7.00	7.00	7.00
Mean	4.01	4.61	7.83	7.18	7.50
Standard Deviation	1.37	1.02	0.74	0.96	1.42
Coefficient of Variation	34.1%	22.1%	9.4%	13.4%	18.9%
8	Rat Assay Value	5.6			
Claimed Value		6.61			

* Values expressed in terms of micrograms vitamin B_{12}/g.

the mean value (in this case, regardless of the manner in which the sample was treated).

The largest variations were obtained with sample C. Because of its very low potency, this was to be expected. With the exception of two unusually high values obtained with trypsin digestion by collaborators 4 and 5 (which in turn markedly raised the calculated mean, the standard

TABLE 2.—*Vitamin B_{12} values* for sample B*

COLLABORATOR	SAMPLE TREATMENT				
	WATER	TRYPSIN	BISULFITE	ALCOHOL	OTHER SAMPLE TREATMENTS
1	.204	.136	.204	.209	—
2	.180	.170	.210	.190	.160
	.170	.150	.170	.190	—
3	.182	.192	.257	.178	.194
4	.280	.300	.320	.260	.270
5	.188	.210	.215	.189	.193
6	.185	.183	.196	.197	.198
7	.160	.130	.190	.160	.190
Mean	.194	.184	.220	.197	.201
Standard Deviation	0.011	0.031	0.035	0.027	0.033
Coefficient of Variation	5.8%	16.8%	15.9%	13.9%	16.6%
8	Rat Assay Value	0.137			

* Values expressed in terms of micrograms vitamin B_{12}/g.

TABLE 3.—*Vitamin B_{12} values* for sample C*

COLLABORATOR	SAMPLE TREATMENT				
	WATER	TRYPSIN	BISULFITE	ALCOHOL	OTHER SAMPLE TREATMENTS
1	.0079	.0163	.0087	.0074	—
2	.0060	—	.0070	.0060	.0050
	.0040	—	.0070	.0050	—
3	.0082	.0059	.0144	.0097	.0106
4	.0110	.0270	.0170	.0160	.0200
5	.0055	.0266	.0071	.0078	.0071
6	.0048	.0054	.0065	.0067	.0067
7	.0030	.0085	.0075	.0075	.0075
Mean	.0063	.0149	.0094	.0083	.0091
Standard Deviation	0.0024	0.0091	0.0037	0.0032	0.0023
Coefficient of Variation	38.1%	61.1%	39.4%	38.6%	25.3%

8 Rat Assay Value 0.031
Calculated Value .007

* Values expressed in terms of micrograms vitamin B_{12}/g.

deviation, and the coefficient of variation), the bisulfite treatment again gave the highest values. There is also an indication that with this sample the alcohol treatment appeared superior to the water treatment. Contrary to the results obtained on samples A and B, the rat assay showed a markedly higher value than the microbiological assay.

In general, reasonably good inter-laboratory agreement was obtained. This was better than expected in view of the nature and potencies of the test materials. However, it is obvious that further work must be done to provide for still more reliable results. This contention is supported in part by the intra-laboratory results reported by collaborator No. 2. The samples were assayed on two occasions three weeks apart. Although this is a report from only one laboratory it indicates that additional work is warranted to investigate the significance of such intra-laboratory variation.

It must also be kept in mind that all possibilities of sample treatment to elute and/or stabilize vitamin B_{12} and its analogues were not exhausted by this study. It may well be that better methods will be forthcoming. Furthermore, the samples tested were rather limited in number and, hence, could not represent all types of materials containing crude vitamin B_{12}.

The data reveal that with crude, water-insoluble test materials the method of sample treatment prior to assay is very important. Apparently a simple water treatment does not lend itself to maximum elution and stabilization of the vitamin B_{12}. Enzyme digestion (trypsin) may in some cases afford values higher than those obtained by the water treatment, but at best does not yield results equal to those obtained by the bisulfite

or alcohol treatment. It may be argued that the highest values are not necessarily the right values. However, in this study two entirely different treatments (bisulfite or alcohol) in general gave consistently higher results, with the bisulfite treatment tending to be the higher. If the high value is the correct value, it appears that the bisulfite treatment does have a stabilizing effect, and in addition, possibly aids in the extraction of the vitamin. A solvent treatment such as alcohol apparently is of value with certain samples. However, the data seem to indicate that its stabilizing effect is not quite equal to that of the bisulfite. Although only one report was given (collaborator No. 6) wherein a combination of the bisulfite and alcohol treatment was employed, the results obtained indicate that such a treatment is worthy of further consideration.

SUMMARY

A proposed procedure for the microbiological assay of vitamin B_{12} in crude materials was subjected to collaborative study in 1951. The assay technique employed was the proposed U.S.P. method modified to evaluate the growth of the microorganism turbidimetrically (after 18–24 hours) rather than titrimetrically (after 72 hours). The three samples subjected to test were so selected to afford a wide range of potencies and to permit the evaluation of the effectiveness of certain sample treatment techniques. In general, reasonably good inter-laboratory agreement was obtained. The method of sample treatment was found to be an important factor. Water or enzyme (trypsin) treatments gave variable and low results in most cases. A bisulfite or alcohol treatment yielded the highest values on the three test materials studied. These higher values are attributed to the eluting and/or stabilizing properties of the alcohol or bisulfite.

ACKNOWLEDGEMENT

The author gratefully acknowledges the supply of Bi-Con 3 plus 1, kindly supplied by Dr. Herbert Luther of Chas. Pfizer and Co., Inc., Brooklyn, N.Y. and the condensed fish solubles supplied by Mr. James C. Fritz of The Borden Co., Elgin, Ill.

RECOMMENDATIONS*

It is recommended—

(1) That work on the microbiological assay for vitamin B_{12} be continued.

(2) That further consideration be given to sample treatment techniques.

(3) That consideration should be given to possible intra-laboratory variations.

(4) That the test materials should continue to be crude materials of a wide potency range.

* For report of Subcommittee A and action of the Association, see *This Journal*, 35, 47 (1952).

REFERENCES

(1) THOMPSON, H. T., DIETRICH, L. S., and ELVEHJEM, C. A., *J. Biol. Chem.*, **184**, 175 (1950).
(2) FRICKE, H. H., *Presented at the Meeting of the Association of Vitamin Chemists, Chicago, Ill., March 8, 1951.*
(3) HARTMAN, A. M. *Fed. Proc.*, **5**, 137 (1946).
(4) HARTMAN, A. M., DRYDEN, L. P., and CARY, C. A. *Arch. Biochem.*, **23**, 165 (1949)

REPORT ON CAROTENE

By F. W. QUACKENBUSH (Agricultural Experiment Station, Lafayette, Ind.), *Associate Referee*

The two principal lines of activity during the past year were: (a) monthly check samples and (b) collaborative study to compare the official method with the Thompson and Bickoff "short method" (*This Journal*, **34**, 219, 1951).

MONTHLY CHECK SAMPLE

John Kephart of National Alfalfa Dehydrators and Milling Company, Lamar, Colorado, has kindly continued to send out monthly alfalfa meal samples to all who wished to participate. A total of 92 laboratories is currently receiving these check samples. While there seems to have been a continued slight improvement in agreement between laboratories during the past year, the relatively wide range of reported values on all samples still leaves something to be desired.

COLLABORATIVE STUDY

Alfalfa meal Sample No. 23 was a blend of approximately equal parts of freshly dehydrated meal and old meal which had lost much of its carotene during storage. Instructions to collaborators were as follows:

The objective is to determine whether precision of the carotene method can be improved by (1) use of a shorter adsorption column and (2) doubling the amount of eluant for removing carotene from the column. All laboratories can complete part A of report form, and all who have precise spectrophotometers, e.g. Beckman, are requested to complete part B.

Weigh out six 2-gram samples of alfalfa meal No. 23 and extract each in the usual manner (simultaneously, if enough equipment is available).

Transfer the total extract and residue of samples No. 1 and No. 2 to 100 ml volumetric flasks and add hexane to the mark. After thorough mixing of flask contents pipet 5 ml of the supernatant extract onto a column 12 mm in diameter packed to 40 mm in height with magnesia: diatomacious earth mixture, 1+1 by weight. (Proposed method of Thompson and Bickoff, *This Journal*, **34**, 219, 1951). Elute the carotene with a 1+9 mixture of acetone in hexane until 25 ml is collected in the volumetric flask. Measure the pigment photometrically, calculate as β-carotene and report in part A of report form.

Chromatograph samples 3 to 6 as usual in columns packed 10 cm deep with magnesia: diatomacious earth, 1+1 by weight. Perform the elution in two steps:

TABLE 1.—*Summary of carotene results obtained with two methods*

COLLABORATOR NUMBER	SHORT METHOD	CAROTENE MG/LB AOAC METHOD	AOAC VALUE PLUS 2ND ELUATE
1	42.8	32.1	32.6
2	42.5	33.6	36.0
3	41.7	34.3	38.8
4	38.1	36.2	38.0
5	51.0	37.9	40.1
6	41.5	37.0	41.2
7	41.4	39.0	41.0
8	39.8	39.6	40.2
9	42.0	40.0	40.5
10	45.8	40.0	41.4
11	50.1	40.1	44.7
12	46.8	40.2	42.3
13	43.1	40.2	41.6
14	43.4	40.4	42.9
15	45.7	40.7	47.7
16	47.6	40.7	42.5
17	43.9	40.8	42.3
18	47.0	41.7	44.8
19	45.0	41.7	42.9
20	45.6	41.8	43.9
21	45.1	42.1	43.3
22	42.3	42.2	42.5
23	45.9	42.4	43.1
24	46.1	42.9	45.4
25	46.6	43.0	44.9
26	47.5	43.1	44.6
27	43.0	43.2	43.2
28	45.9	43.4	44.3
29	49.4	43.7	50.9
30	49.1	43.7	45.4
31	47.6	43.9	45.5
32	44.5	43.9	46.2
33	44.4	44.3	45.8
34	48.6	44.4	46.9
35	48.0	44.7	46.4
36	46.9	45.3	49.1
37	62.4	48.3	62.7
38	55.3	48.8	56.3
39	74.8	60.2	61.9 (Omitted from average)
Average	45.8	41.3	42.8
Standard Deviation	4.32	3.54	
40		60.5	(Omitted from average)
41	42.2		
42	41.7		
43	50.3		
44	53.1		
45		45.6	
46		37.0	
47		45.5	
48		44.0	
49		42.0	
50	46.5	45.7	
51		49.1	
52	44.8	40.7	
53	50.1	38.4	
54	49.7	51.3	(Omitted from average)
55		54.9	(Omitted from average)
56	48.0	43.7	
57	57.3	48.7	
58	84.2	16.7	(Omitted from average)
Over-All Average	46.4	41.9	42.8

First, elute the carotene band with 50 ml of eluant, or slightly more if necessary. Just when the upper edge of the broad yellow band leaves the column, break the vacuum and remove the first eluate from the suction flask. Save these solutions for photometry and report on part A of form. Second, immediately reattach the chromatogram and pass another 100 ml of eluate (1+9 mixture of acetone in hexane) through the column. Combine the second eluates from all four columns (total 300 to 400 ml) in a 1-liter round-bottom flask or distilling flask and, using a water pump and water bath at 40°C., evaporate the solution to 10–15 ml under reduced pressure. (Some manual agitation may be necessary to prevent bumping). Clamp off the vacuum line and swish the contents of the flask to get all pigment in solution before opening flask to air. Immediately transfer the pigment to a 25 ml volumetric flask, make to final volume with 1+9 acetone-hexane and read at 436 millimicrons. Optical density reading should be between .100 and .800. If it is outside this range, readjust final volume to bring reading into this range. Make readings at 330 and 340 and at 5 mμ intervals between 400 mμ and 500 mμ and enter values in part B on report form attached. Make similar readings on the first eluate from sample No. 5.

RESULTS OF COLLABORATORS

Thirty-nine collaborators performed the complete analysis on the sample. Nineteen others completed part of the work (Table 1).

DISCUSSION OF RESULTS

The short method gave higher carotene values in almost all laboratories. The average value was 11 per cent higher than that for the official method and 7 per cent higher even after the pigment obtained in the second eluate was added to the latter. This is consistent with the higher eluate:absorbent ratio used in the short method.

Representative spectral absorption curves are shown in Figure 1. Curves plotted from the data reported on the first eluate (Sample 5) in most cases agreed well with published curves for β-carotene. However, positions of maxima clearly showed that in some laboratories instruments should be readjusted. Spectral absorption curves plotted from the data reported on second eluates deviated substantially from the typical curve for β-carotene (Fig. 1). While in some cases the curves could be rationalized as representing mixtures of β-carotene and its known neoisomers, many showed exceedingly high general absorption in the region between 400 and 450 mμ. Marked differences of absorption in the region of the cis peak (330–340 mμ) were also observed. Cis peaks were comparatively low when extractions were performed overnight at room temperature; however, similarly low values were also reported by some who extracted under reflux. High absorption values in this region suggested that in some laboratories excessive heating or other drastic treatment had caused an undue amount of isomerization and perhaps also some destruction of carotene.

It is apparent that the second eluate contained substantial although varying amounts of pigment substance other than β-carotene, and it is

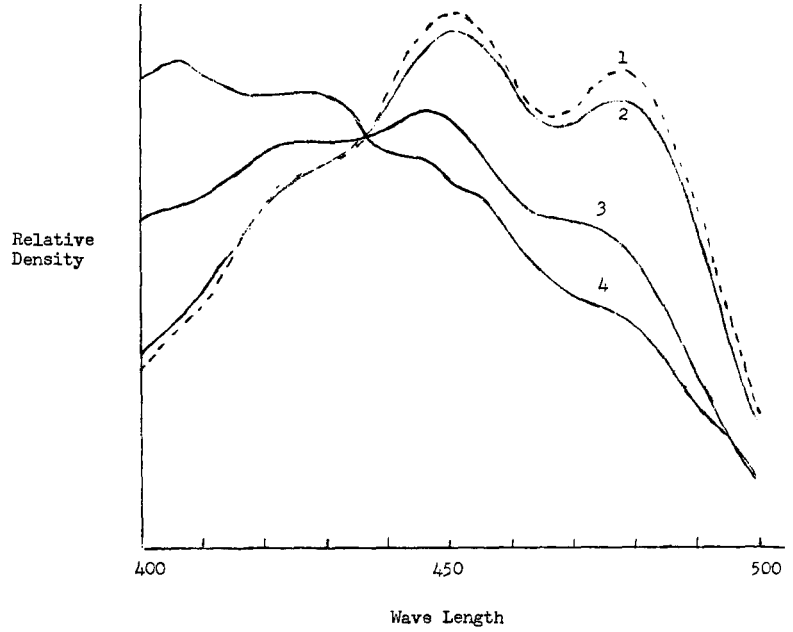

FIG. 1.—Spectral absorption curves for: 1. *β-carotene* (all-trans) (Beadle and Zscheile, J.B.C. **144,** 21, '42). 2. *First eluate,* collaborator No. 21. 3. *Combined second eluates,* collaborator No. 21. 4. *Combined second eluates,* collaborator No. 23.

assumed that the higher "carotene" value obtained by the short method is attributable, at least in part, to this contaminant. Accordingly, the short method should not be adopted for general use until the purity of carotene pigment in the eluates from all types of samples can be established.

Some laboratories have from time to time stated that 50 ml of solvent was insufficient to completely elute the visible carotene band from the column. During the past year we have for the first time observed this in the Associate Referee's laboratory with certain samples of adsorbent. A recommendation is included to correct this in the procedure.

RECOMMENDATIONS*

It is recommended that—

(1) The statement in the procedure (Methods of Analysis, Seventh Ed., 1950, **40.7**, page 769) " . . . pour extract into chromatographic column and use 50 ml. of acetone-hexane (1+9) to wash carotene into adsorbent and develop chromatogram." be changed to read " . . . pour extract into

* For report of Subcommittee A and action of the Association, see *This Journal,* **35,** 46 (1952).

chromatographic column and use 50 ml., or slightly more if necessary, of acetone-hexane (1+9) to develop chromatogram and wash the visible carotene band through the adsorbent."

(2) Studies on carotene analysis be continued.

No report was given on vitamin A in oleomargarine or on folic acid (micro-biological method).

The contributed paper "A New Chemical Approach to the Determination of Vitamin B_6" by J. P. Sweeney and W. L. Hall, appeared in *This Journal*, **35,** 479 (1952).

The contributed paper entitled "Studies on a Microbiological Method for Vitamin B_{12} Activity," by H. W. Loy, Jr., J. F. Haggerty, and O. L. Kline, appeared in *This Journal*, **35,** 161 (February, 1952). The contributed paper "Stability of Vitamins B_{12} and B_{12b}," by the same authors appeared on page 169 of the same issue.

The contributed paper, "Absorption Spectra of Dehydrated Alfalfa Meal Extracts and Standard Carotene Solutions," by M. L. Cooley appeared in *This Journal*, **35,** 487 (1952).

ANNOUNCEMENTS

Referee Assignments, Changes, Appointments

COSMETICS:

John F. Clements, Division of Cosmetics, Food and Drug Administration, Washington 25, D. C., has been appointed Associate Referee on Cold Permanent Waves and on Deodorants and Anti-Perspirants, to succeed Henry Kramer.

DAIRY PRODUCTS:

James H. Cannon, Food and Drug Administration, St. Louis 1, Missouri, has been appointed Associate Referee on Foreign Fats in Dairy Products.

ECONOMIC POISONS:

H. A. Rooney, Department of Agriculture, Sacramento 14, California, has resigned as Associate Referee for Organic Thiocyanates.

FISH AND OTHER MARINE PRODUCTS:

Henry M. Risley, Food and Drug Administration, Seattle, Washington, has been appointed Associate Referee on Total Solids and Ether Extract in Fish.

METALS, OTHER ELEMENTS, AND RESIDUES IN FOODS:

Paul A. Clifford, Division of Food, Food and Drug Administration, Washington 25, D. C., has been appointed Associate Referee on sodium in foods.

CONTRIBUTED PAPERS

A PROCEDURE FOR THE BIOLOGICAL ASSAY OF INSECTICIDES BY ORAL ADMINISTRATION TO FLIES

By JOHN P. FRAWLEY, EDWIN P. LAUG, and O. GARTH FITZHUGH
(Division of Pharmacology, Food and Drug Administration Federal Security Agency, Washington, 25, D. C.)

The rapid advance in insect control during the last decade has placed an enormous burden upon chemists in the regulatory field. Because of the high order of toxicity of some of the newer insecticides, methods for their analysis must be able to detect fractions of a part per million. Sensitive chemical methods have proven most difficult to develop and apply, and suggested methods frequently require instruments not readily available to most chemists.

Where adequate chemical methods are lacking, various forms of biological assay can be used. Their drawback is non-specificity, but they can usually measure the quantity of toxic residues to a degree of sensitivity unattainable by chemical methods.

One of the principal objections to existing biological assay procedures is the difficulty of obtaining the spray residue in a solution free of background interference. The most common technic (1) for biological assay with flies consists of depositing an aliquot of an extract on a glass surface, evaporating the solvent, and allowing the insects to walk on the contaminated surface. The extract must be essentially free of fat, waxes, and other materials which could increase mortality by direct toxicity or by physical immobilization of the insect, or which could decrease mortality by preventing absorption due to physical occlusion of the insecticide. Other biological procedures using mosquito larvae (2) are subject to the same background interference.

The method described here avoids these complications by taking advantage of the natural habit of the fly to ingest a large variety of food products. It consists in making an ether extract of the sample, evaporating it to dryness over sugar, dissolving the sugar in an aqueous medium and allowing the flies to feed *ad libitum* on the solution for 24 hours.

METHOD

APPARATUS

(1) *Specially constructed wire containers*, conical shape, diameters 4 and 12 cm, height 15 cm (Figure 1).

(2) *5 ml pipets.*

(3) *Glass stoppered 10 ml graduated cylinders.*

(4) *Balance*, triple beam, sensitivity, 10 mg.

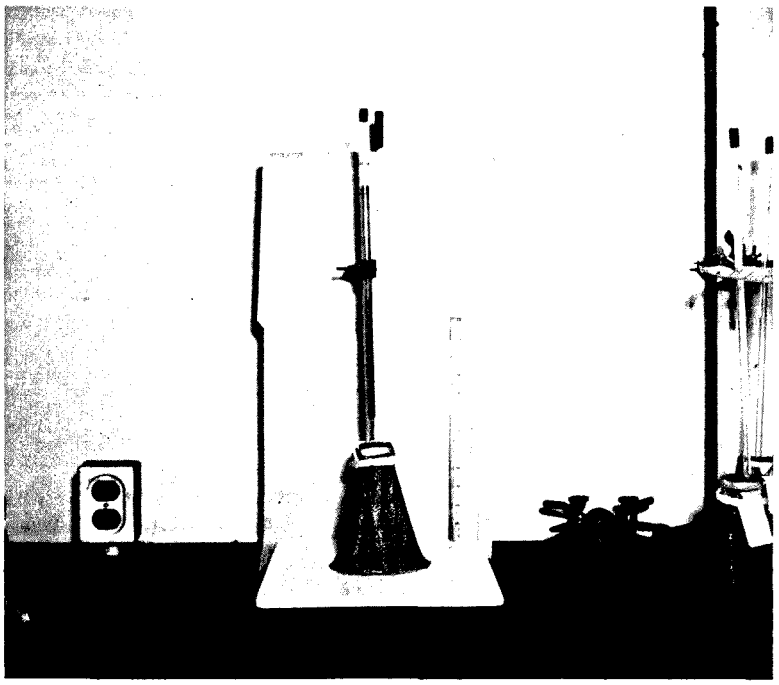

FIG. 1.—Set-up for oral administration of toxicants to flies.

EXPERIMENTAL ANIMAL

(1) *Musca domestica* (house fly), *strain: NAIDM 1948* (Nat. Assoc. Insecticide and Disinfectant Manufacturers).

REAGENTS

(1) *Sodium sulfate*—anhydrous powder.
(2) *Ether, diethyl*—ACS.
(3) *Sucrose.*
(4) Tween 60.[1]

DETERMINATION

A. *Preparation of Test Extracts:*

(1) Dry sample, if necessary, by grinding with anhydrous sodium sulfate. (Sample size is dependent upon insecticide present and degree of sensitivity desired.)

(2) Extract dry, powdered sample in a Soxhlet with ether, allowing a minimum of 25 siphonings to occur.

(3) Transfer ether extract to a beaker of suitable size and evaporate the ether to approximately 40 ml with aid of a gentle stream of air.

[1] Manufactured by Atlas Powder Co., Wilmington, 99, Del.

(4) Transfer the ether soln and washings to a 50 ml beaker containing 2 g sucrose and allow ether to evaporate to dryness.

(5) Carefully transfer sugar to a 10 ml glass-stoppered graduated cylinder.

(6) Wash beaker with two 4 ml portions of a 1 per cent aqueous Tween 60 soln and add washings to graduated cylinder.

(7) Shake vigorously to dissolve and adjust volume to 10 ml. (Further dilutions may be made, if a preliminary assay so indicates.)

B. *Preparation of Standard Extracts:*

(1) Extract control samples as directed in 1 and 2 under *Preparation of Test Extracts.* (Samples must be of similar material known to be untreated with the particular (or interfering) insecticide sought.)

(2) Add 4 times the quantity of the test insecticide necessary to kill 100 flies.

(3) Carry out the preparation as with *Test Extract,* from 3 to 7.

(4) Transfer several aliquots (ranging from 5 ml to 0.1 ml) of the aqueous soln to 10 ml graduated cylinders and make to volume with 20 per cent sucrose—1 per cent Tween 60 water soln.

C. *Assay Procedure:*

(1) Weigh empty wire flask with top to two decimal places on a triple beam balance.

(2) Place approximately 100 flies (fasted for 24 hours) in the wire flask, and cover with wire top. (1)

(3) Place container with flies in a desiccator and blow in carbon dioxide until the flies are anaesthetized.

(4) Weigh flask again and note the weight of flies.

(5) Fill a 5 ml pipet with the test or control soln and seal the tip with a rubber plug.

(6) Invert the filled pipet and allow it to rest on the wire top of the containers filled with flies (Fig. 1).

(7) Note initial meniscus level. Allow the flies to feed *ad libitum* for 24 hours. Note final meniscus level.

(8) Count the number of dead and living flies.

(9) Construct a standard curve, plotting the log dose in micrograms per gram against probits of the per cent mortality (2).

(10) Determine the quantity of insecticide in test samples by comparing the probits of the per cent mortality of test and standard solutions.

DISCUSSION

The technic for raising and handling the flies is essentially the same as that recommended by Laug (1). The culture matrix consists of Purina laboratory diet for flies* supplemented with ground horsemeat. The flies are used for assay purpose between 2 and 5 days after emergence.

During the actual assay, the fly is able to insert its proboscis through the wire mesh and drink *ad libitum.* Flies, fasted for 24 hours prior to the assay, consume more of the test solution (total of 4 to 5 ml in 24 hours for 100 flies) than non-fasted flies, and consequently are more sensitive experimental animals. As demonstrated by Laug, temperature, light, sex, length of assay period, and strain of fly all affect the sensitivity of the

* Ralston Purina Co., St. Louis, Mo.

TABLE 1.—*Sensitivity of flies to the oral administration of insecticides. NAIDM 1948 strain*

INSECTICIDE	50% MORTALITY
	micrograms
DDT	15
Parathion	3
EPN	4
Lindane	5

bioassay. Therefore, as with any biological assay procedure, controls must be run simultaneously with each set of unknowns.

The calculations employed for the procedure are similar to those commonly used. Plotting mg/kg against per cent mortality gives rise to a sigmoid curve. But as Bliss (2) has demonstrated, this curve can be converted to a straight line by plotting the logarithm of the dose (mg/kg) against per cent mortality expressed in "probits." Unknown quantities of an insecticide can be derived from the standard assay curve.

The method has been applied to several types of food products, which include apples, cucumbers, strawberries, peas, and yeast. No background toxicity has been encountered with these foods. Recoveries with lindane and parathion have been complete within biological variation. The relative sensitivity of the procedure is shown in Table 1, where micro-

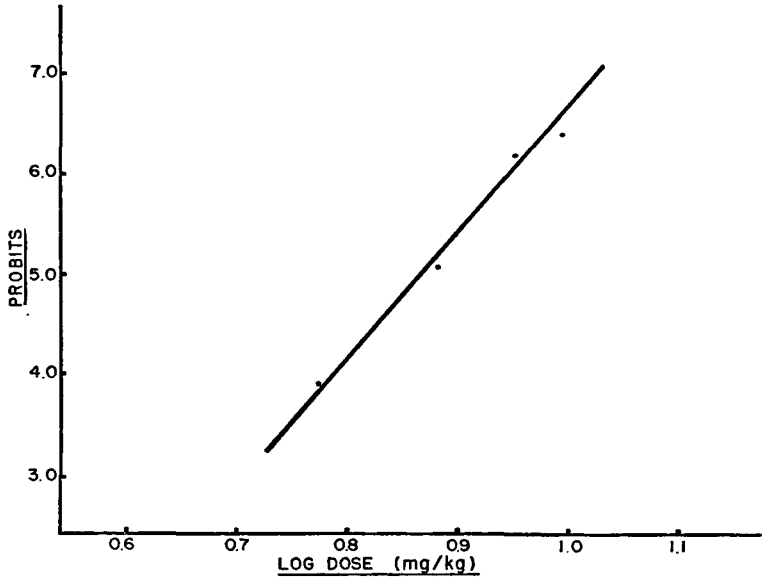

FIG. 2.—Standard curve for DDT.

gram figures represent the approximate 24-hour oral LD_{50}. A typical standard curve, obtained with DDT, is shown in Figure 2. With some insecticides, the sensitivity of the oral assay is somewhat less than that of the contact assay. However, the simplicity of sample preparation and lack of background interference seem to make application of the oral assay more extensive.

SUMMARY

A technic is described for the biological assay of insecticides by oral administration to flies. This technic obviates the meticulous purification of sample extracts frequently necessary for other bioassay procedures. The method is sensitive to 3 micrograms of parathion, 4 micrograms of EPN, 5 micrograms of lindane and 15 micrograms of DDT.

BIBLIOGRAPHY

(1) LAUG, E. P., *J. Pharm. & Exper. Therap.*, **84**, 324 (1946).
(2) BLISS, C. I., *Science*, **69**, 409 (1934).

THE IN VIVO INHIBITION OF FLY CHOLINESTERASE AS A MEASURE OF MICROGRAM QUANTITIES OF ORGANIC PHOSPHATE INSECTICIDES*

By JOHN P. FRAWLEY, EDWIN P. LAUG, and O. GARTH FITZHUGH
(Division of Pharmacology, Food and Drug Administration, Federal Security Agency, Washington 25, D. C.)

One of the most toxic groups of insecticides consists of a series of organic phosphate compounds that may have an oral LD_{50} in mammals as low as 1 mg/kg (1). This group of compounds owes most of its toxicity to its ability to inhibit the cholinesterase enzyme in the body (1, 2). Chemical methods for the detection of these compounds are based either on the organic phosphorus content of the plant or vegetable (3) or on the measurement of reactive groups present in the specific compound (4). Both methods suffer from a lack of sensitivity and varying background interference. A biochemical procedure has been suggested by Giang (5) using the *in vitro* inhibition of hog serum cholinesterase as a measurement of organic phosphate insecticides. Involved sample purification and enzyme isolation make the procedure almost too laborious for routine use.

An alternate procedure, using the inhibition of cholinesterase in the fly, is reported here. This procedure eliminates extensive sample purification and permits multiple determinations to be carried out simultaneously. The procedure consists in allowing approximately 100 weighed

* Presented at the Annual Meeting of the Association of Official Agricultural Chemists held at Washington, D. C., Oct. 1–3, 1951.

flies to feed *ad libitum* on the test solution, blending the flies with water in a Waring blendor, and measuring the cholinesterase activity of the extract by the change in *p*H during a one hour incubation with acetylcholine. The *ad libitum* feeding technic used is that reported by Frawley *et al.* (6) (see preceding paper), and the cholinesterase measurements are made by a modification of the method of Michel (7).

METHOD

APPARATUS

To the apparatus specified in the preceding paper, add:

(1) Waring blendor

(2) Constant temperature bath (35°C.).

(3) *p*H meter.

EXPERIMENTAL ANIMAL

(1) *Musca domestica* (house fly), *strain NAIDM 1948.* (Nat. Assoc. Insecticide and Disinfectant Manufacturers.)

REAGENTS

To the reagents specified in the preceding paper, add:

(1) *Buffer Soln*—0.01*M* sodium barbital, U.S.P.; 0.002 *M* potassium phosphate, monobasic, ACS; 0.60 *M* potassium chloride, c.p.

(2) *Substrate*—3% acetyl choline bromide.

DETERMINATION

In the preparation of test and standard extracts follow the directions given in the preceding paper under A and B.

C. *Assay Procedure:*

Follow the directions of the preceding paper (p. 741) through *step 7*. Additional steps are:

(1) Place container in a desiccator and blow carbon dioxide into the desiccator until the flies are anaesthetized.

(2) Pour anaesthetized flies into a Waring blendor which contains 50 ml of distilled water per gram of initial weight of flies. Grind for 1 minute.

(3) Filter the homogenate through glass wool.

(4) Mix 2 ml of filtrate with 2 ml of buffer and 0.4 ml of acetyl choline solution in a small test tube.

(5) Measure initial *p*H by means of a *p*H meter.

(6) Incubate for 1 hour at 35°C. Determine final *p*H.

(7) Note the change in *p*H for test flies and for control or standard flies (ΔpH).

(8) Calculate the per cent inhibition by dividing by the ΔpH for selected groups of control flies, and subtracting from 100.

(9) Construct a standard curve by plotting per cent inhibition against the logarithm of the dose in millimicrograms insecticide per gram of flies.

(10) Estimate the insecticide content of test solution from the standard curve.

DISCUSSION

The procedure has been employed for the analysis of the organic phosphate insecticide content of cucumbers, peas, apples, and straw-

berries. No background interference was encountered with any of these products. The insecticides which have been studied by this technic have been parathion (diethyl *p*-nitrophenyl thiophosphate) and EPN (ethyl *p*-nitrophenyl thionobenzene phosphonate). The procedure will detect between 0.5 and 1.0 micrograms of parathion and between 1.0 and 2.0 micrograms of EPN when 24 hour fasted flies, *strain NAIDM 1948*, are used. A typical standard curve for parathion is presented in Figure 1.

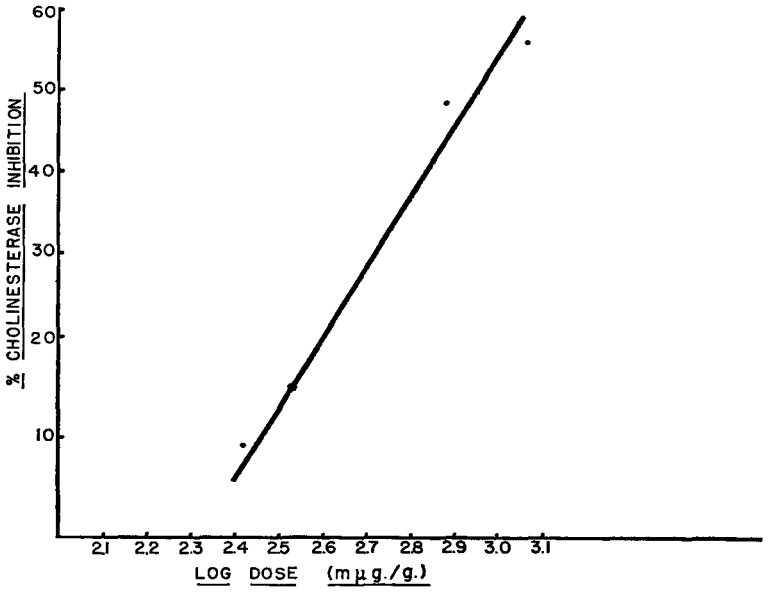

FIG. 1.—Typical standard curve for parathion.

The procedure will not differentiate between various organic phosphate insecticides, but measures the active anticholinesterase potency of the plant extract. Since the toxicity of many of the various compounds is closely related to the anticholinesterase activity, the potential hazard of a spray residue can be estimated by this procedure, regardless of which insecticide is used for the standard curve. Cholinesterase inhibition in the fly can readily be used to supplement other bioassay procedures by indicating whether observed mortality was caused by organic phosphates or by other insecticides.

SUMMARY

A technic is reported for the measurement of organic phosphate insecticides by cholinesterase inhibition of flies. The procedure is sensitive to between 0.5 and 1.0 micrograms of parathion and 1.0 and 2.0 micrograms

of EPN. Analyses of several food materials have demonstrated a lack of background interference and the applicability of the method.

BIBLIOGRAPHY

(1) HAGAN, E. C., and FRAWLEY, J. P., *Fed. Proc.*, **10**, 304 (1951).
(2) FRAWLEY, J. P., and HAGAN, E. C., *Fed. Proc.*, **10**, 297 (1951).
(3) ALLEN, R. J. L., *Biochem. J.*, **34**, 858 (1940).
(4) AVERELL, P. R., and NORRIS, M. V., *Anal Chem.*, **20**, 753 (1948).
(5) GIANG, P. A., and HALL, S. A., *Ibid.*, **23**, 1830 (1951.
(6) FRAWLEY, J. P., LAUG, E. P., and FITZHUGH, O. G., *This Journal*, **35**, 741 (1952).
(7) MICHEL, H. O., *J. Lab. Clin. Med.*, **34**, 1564 (1949).

WATER INSOLUBLE FATTY ACIDS IN BUTTER

"SORTING" METHOD AND STORAGE EXPERIMENT

By FRED HILLIG (Food and Drug Administration, Federal Security Agency, Washington 25, D. C.)

During the past several years intensive investigations of chemical methods designed to establish the relative fitness of the cream from which butter was churned have been carried out. Water insoluble fatty acids (WIA)* have been found to be one satisfactory index of the condition of the cream.

The method* devised for the determination of WIA involved the following steps:

(1) Alkaline extraction of the fatty acids from an ether solution of the butter into the water-curd phase.

(2) Ether extraction of the fatty acids from the acidified water-curd phase.

(3) Alkaline extraction of the fatty acids from their ether solution into a 50 per cent alcohol solution.

(4) Separation of the water insoluble fatty acids by precipitation and filtration.

(5) Solution of WIA in ether, evaporation of ether, drying and weighing the acids.

(6) Solution of dried acids in neutral alcohol or benzene, titration with standard alkali, and calculation of mean molecular weight.

Obviously a method involving these several steps does not lend itself to routine analysis of numerous samples within limited periods of time. Often an exact measurement of the WIA is not essential, as, for example, in a routine series in an investigation, or as a means of quality control.

* *This Journal*, **30**, 575 (1947); **31**, 739 (1948); **31**, 750 (1948); **32**, 731 (1949); **34**, 777 (1951); **34**, 782 (1951).

As with gravimetric methods in general, the preparation and weighing of the constituent being determined is the time consuming operation. Approximately 1½ hours per determination can be saved by dissolving the residual acids from the ether in neutral alcohol or benzene (fifth step) and titrating with standard alkali. By use of an average mean molecular weight the quantity present can be approximated by calculation. The time gained makes the method more adaptable to routine use. When results indicate that greater accuracy is desired, the alcoholic solution of the titrated acid can be evaporated to dryness and the acids again precipitated and weighed as provided in the original method.

I. "SORTING" PROCEDURE FOR ESTIMATION OF WIA

Proceed as directed in **15.118**, page 260, 7th Ed., *Methods of Analysis*, to line 14, page 261. Evaporate ether, dissolve acids in neutral alcohol, and titrate with

TABLE 1.—*Comparison of WIA determined by official and "sorting" methods*

SAMPLE NO.	OFFICIAL METHOD		"SORTING" METHOD		
	WIA GRAVIMETRIC (COL. 1)	MEAN MOLECULAR WEIGHT	WIA BY TITRATION, USING AVERAGE MEAN MOL. WEIGHT OF 270. (COL .2)	BY WEIGHT	
				WIA GRAVIMETRIC (COL. 3)	MEAN MOL. WEIGHT
	mg/100g fat		*mg/100g fat*	*mg/100g fat*	
1	206	263	215	201	261
	202	262	223	204	263
2	181	258	189	179	260
	184	261	189	177	263
3	205	262	223	203	260
	205	260	220	198	260
4	236	260	253	233	263
	230	262	256	235	258
5	164	262	188	176	265
	174	263	179	166	261
6	289	269	310	290	276
	299	277	317	285	276
7	186	276	188	180	276
	194	267	194	189	269
8	215	265	234	217	262
	209	278	235	215	261
9	324	274	331	321	268
	317	264	340	334	270

0.1 *N* NaOH (phenolphthalein indicator). Compute WIA in the sample using an average mean molecular weight of 270. To determine WIA gravimetrically, evaporate the neutralized alcoholic solution of the acids to dryness and reprecipitate and weigh the acids as directed in **15.118.**

The data presented in Table 1 show that the quantities of WIA found by the official method (column 1) in which the acids were dried and

TABLE 2.—*Mean molecular weights of WIA from butter*

NO. OF SAMPLES	MEAN MOLECULAR WEIGHTS BETWEEN:
1	250–260
40	260–270
31	270–280
1	280–290
Total 73	Av. 269

TABLE 3.—*Storage experiment at 0°F., "continuous churn" butter*

SAMPLE NO.	WIA—MG/100 G FAT	
	IN ORIGINAL BUTTER	AFTER 4 MONTHS STORAGE
1	197	206
	203	202
2	204	181
	208	184
3	223	205
	236	205
4	225	236
	223	230
5	177	164
	172	174
6	294	289
	298	299
7	173	186
	163	194
8	209	215
	225	209
9	293	324
	284	317

weighed; (column 2) in which the "sorting" method was employed using an average mean molecular weight of 270, and (column 3) in which the titrated acids in the "sorting" method were reprecipitated and weighed, agree very well.

A study of the data from previous investigations shows that the mean molecular weights of the acids from butter lie within a restricted range. The results are summarized in Table 2.

The actual average of the mean molecular weights is 269. However, since 71 of the 73 samples have mean molecular weights between 260 and 280, for the sake of simplicity a figure of 270 has been chosen.

II. STORAGE EXPERIMENT AT 0°F., "CONTINUOUS CHURN" BUTTER

During the month of June 1951, the investigation of WIA in cream and butter was extended to butter produced by the "continuous" process.* WIA were determined in these butters at the time of preparation and again after 4 months of storage at 0°F. The data in Table 3 indicate that there was no significant change in WIA in these butters during this storage period.

SUMMARY

A time saving "sorting" procedure is proposed for the estimation of WIA in cream and butter. Results obtained by its use closely approximate those found with the official method.

Four months storage of "continuous churn" butter at 0°F. did not cause any significant change in WIA.

THE DETERMINATION OF 1,4-DIHYDROXY-ANTHRAQUINONE IN D&C VIOLET NO. 2 AND D&C GREEN NO. 6

By LEE S. HARROW and KEITH S. HEINE, JR. (Division of Cosmetics, Food and Drug Administration, Federal Security Agency, Washington 25, D. C.)

D&C Violet No. 2 and D&C Green No. 6 are colors listed as certifiable for use in drugs and cosmetics in the regulations promulgated by the Administrator of the Federal Security Agency (1). There is no specification governing the permissible amount of free 1,4-dihydroxy-anthraquinone in these colors. The presence of an excessive amount of this intermediate in a batch of either of the colors must, however, be considered an indication of poor manufacturing practice in producing the color.

The proposed method for 1,4-dihydroxy-anthraquinone in D&C Violet No. 2 and D&C Green No. 6 is a modification of Clark's methods (2)

* *This Journal*, 34, 777 (1951)

for the determination of this intermediate in D&C Green No. 5. The intermediate is extracted with dilute sodium hydroxide from an ether suspension of the color and determined spectrophotometrically.

METHOD

APPARATUS

A spectrophotometer capable of isolating a 5 mμ band width in the region 220–400 mμ.

REAGENTS

Standard 1,4-dihydroxy-anthraquinone solution.—Weigh 100 mg of 1,4-dihydroxy-anthraquinone (recrystallized twice from toluene, M.P. 194–195°C.), and dissolve in one liter of 0.1 *N* sodium hydroxide. Dilute a 10 ml aliquot to 100 ml with 0.1 *N* sodium hydroxide to give a solution containing 10 mg per liter.

PROCEDURE

Weigh 100 mg of color into a 250 ml beaker, suspend in 150 ml of ethyl ether and transfer the mixture to a 500 ml separatory funnel. Rinse the beaker with two 50 ml portions of ether and add these to the main suspension. Extract the ether suspention with successive 25 ml portions of ca 0.2 *N* sodium hydroxide solution until no more color is extracted. Wash the combined alkaline extracts with 50 ml portions of

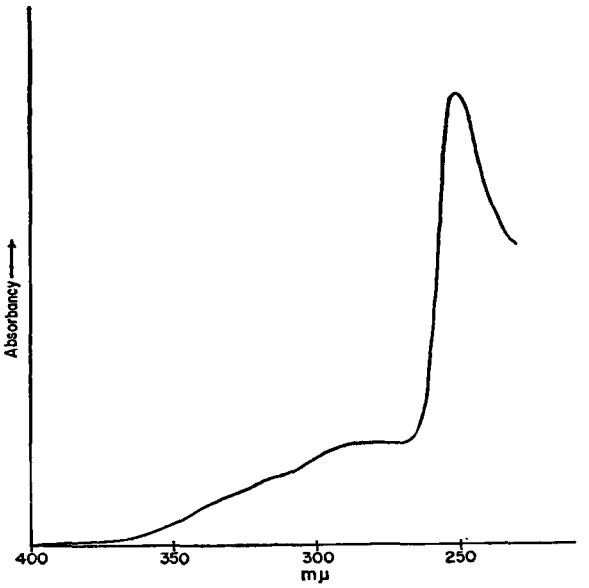

FIG. 1—Ultraviolet spectra of 1,4-dihydroxy-anthraquinone.

Conc: 10.0 mg/liter
Solvent: 0.1 Sodium Hydroxide
Cells: 1 cm.

ether until a colorless ether layer is obtained; discard the ether layers. Transfer the alkaline solution to a 250 ml beaker and heat on a steam bath for one hour to remove the last traces of ether. Cool the solution and transfer to a 200 ml volumetric flask. Dilute to volume with water, and determine the absorbancies of the unknown and standard solution at 230, 255 and 280 mμ.

%1,4-dihydroxy-anthraquinone

$$= \frac{A_{255}\ \text{Unknown} - \left(\frac{A_{230}+A_{280}}{2}\right)}{A_{255}\ \text{Standard} - \left(\frac{A_{230}+A_{280}}{2}\right)} \times \text{concn. Standard (mg/l.)} \times 20.$$

EXPERIMENTAL

A sample of 1,4-dihydroxy-anthraquinone was purified by repeated recrystallization from toluene until the melting point of the material was constant. The ultraviolet spectrum of the compound is shown in Figure 1. Solutions of the intermediate in 0.1 *N* sodium hydroxide follow Beer's law to within $\pm 1\%$.

A sample of D&C Violet No. 2 was extracted with dilute sodium hydroxide solution until very little intermediate was shown by the proposed method. Known amounts of recrystallized 1,4-dihydroxy-anthraquinone were added to the purified color sample and recovery experiments were conducted by the proposed method. The results of these experiments are given in Table 1.

TABLE 1.—*Recovery of 1,4-dihydroxy-anthraquinone from D&C Violet No. 2*

WEIGHT PER DYE	1,4-DIHYDROXY-ANTHRAQUINONE			
	ADDED	RECOVERY (GROSS)	RECOVERY (NET)	RECOVERY
mg	*mg*	*mg*	*mg*	*per cent*
100	—	0.56	—	—
100	—	0.71	—	—
100	1.0	1.47	0.83	83
100	1.0	1.63	0.99	99
100	1.0	1.43	0.79	79
100	2.0	2.28	1.64	82
100	2.0	2.47	1.83	92
100	3.0	3.20	2.56	85
100	3.0	3.33	2.69	90
				Av. 87

A sample of D&C Green No. 6 was suspended in dilute sodium hydroxide solution and then filtered with suction. This operation was repeated until the filtrate was colorless. Known amounts of recrystallized

1,4-dihydroxy-anthraquinone were added to the purified color sample and recovery experiments were conducted by the proposed method. The results of these experiments are given in Table 2.

TABLE 2.—*Recovery of 1,4-dihydroxy-anthraquinone from D&C Green No. 6*

WEIGHT PER DYE	1,4-DIHYDROXY-ANTHRAQUINONE			
	ADDED	RECOVERY (GROSS)	RECOVERY (NET)	RECOVERY
mg	*mg*	*mg*	*mg*	*per cent*
100	—	0.01		
100	—	0.01		
100	1.0	0.94	0.93	93
100	1.0	0.93	0.92	92
100	1.0	0.83	0.82	82
100	2.0	1.90	1.89	94
100	2.0	1.87	1.86	93
100	2.0	1.96	1.95	98
100	3.0	3.06	3.05	101
100	3.0	3.01	3.00	100
100	3.0	3.00	2.99	100
				Av. 95

The method appears to give satisfactory results when applied to the determination of one to three per cent of the intermediate in samples of D&C Violet No. 2 or D&C Green No. 6.

REFERENCES

(1) S.R.A., F.D.C. 3. Coal-Tar Color Regulations, Food and Drug Adm.
(2) CLARK, G. R., *This Journal*, 22, 785 (1939).

DETERMINATION OF 1-(4-METHYLPHENYLAZO)-2-NAPHTHOL IN D&C RED NO. 35

By CHARLES GRAICHEN and LEE S. HARROW (Division of Cosmetics, Food and Drug Administration, Federal Security Agency, Washington, D. C.)

1-(2-nitro-4-methylphenylazo)-2-naphthol is a color listed as certifiable for use in drugs and cosmetics as D&C Red No. 35 (1). A subsidiary color which may be present in commercial batches of D&C Red No. 35 is 1-(4-methylphenylazo)-2-naphthol. A large amount of the subsidiary color would cause erroneous analytical results in the determination of pure dye content, and might be considered to be an indication of poor manufacturing practice. Accordingly, a method has been developed for the

determination of this subsidiary color in samples of D&C Red. No. 35.

Since the subsidiary color is more soluble in alcohol than is D&C Red No. 35, a partial separation of the two compounds is made by dissolving the sample in chloroform, then diluting the solution with alcohol. The precipitated D&C Red No. 35 is filtered, then redissolved in chloroform and again precipitated with alcohol. The combined filtrate, now relatively rich in the subsidiary color, is analyzed spectrophotometrically as a two-component mixture.

METHOD

APPARATUS

Any spectrophotometer capable of isolating a 10 mμ band width while operating between 400 and 600 mμ.

REAGENTS

Standard 1-(4-methylphenylazo)-2-naphthol.—Dissolve 100 mg of recrystallized 1-(4-methylphenylazo)-2-naphthol in 100 ml of chloroform. Make appropriate dilutions to give a final solution containing 10 mg/liter in chloroform.

Standard D&C Red No. 35 solution.—Dissolve 10 mg of recrystallized D&C Red No. 35 in 250 ml of chloroform. Dilute a 25 ml aliquot to 100 ml with chloroform.

DETERMINATION

Weigh 200 mg of D&C Red No. 35 into a 500-ml wide-mouth Erlenmeyer flask, add 75 ml of chloroform, and heat on a steam bath. When all the material is in solution add 200 ml of 95% alcohol., remove the flask from the steam bath, and cool the resulting mixture to 0–5°C. in an ice bath. Filter the precipitated color mixture through a retentive fluted filter paper and reserve the filtrate in a one-liter container. Place the drained filter paper in the original flask, macerate with a strong glass rod, add 75 ml of chloroform, and repeat the precipitation and filtration procedure. Evaporate the combined filtrates to ca 50 ml on a steam bath using a gentle air stream to hasten the process. Cool to room temperature and transfer the concentrate to a 100-ml volumetric flask. Rinse the container with several small portions of 95% alcohol and add the washings to the volumetric flask. Dilute to volume with 95% alcohol and filter the mixture through a retentive filter paper. Evaporate 50 ml of the filtrate to dryness, dissolve the residue in chloroform, and dilute to exactly 100 ml. Determine the absorbancy of the standard and unknown solutions at 420 and 512 mμ. If the unknown solution is too concentrated for accurate measurement, make an appropriate dilution and determine the absorbancies of the resulting solution.

CALCULATIONS

a = The absorbancy per mg per liter of subsidiary color at 420 mμ.
b = The absorbancy per mg per liter of D&C Red No. 35 at 420 mμ.
c = The absorbancy per mg per liter of subsidiary color at 512 mμ.
d = The absorbancy per mg per liter of D&C Red No. 35 at 512 mμ.
$As_{(420)}$ = The absorbancy of the unknown at 420 mμ.
$As_{(512)}$ = The absorbancy of the unknown at 512 mμ.
X = The concentration of subsidiary color in mg per liter.
Y = The concentration of D&C Red No. 35 in mg per liter.

$$aX + bY = As_{(420)}$$
$$cX + dY = As_{(512)}$$

Solve the equations for X; % Subsidiary color $= (X)(F)/10$, where F is a dilution factor if additional dilutions were made.

EXPERIMENTAL

A purified sample of D&C Red No. 35 was used as a standard (m.p. 276.5°C. uncorr.) (2). A purified sample of the subsidiary color, 1-(4-methylphenylazo)-2-naphthol (m.p. 131–2°C), was supplied Louis Koch, Associate Referee on subsidiary dyes in D&C colors.

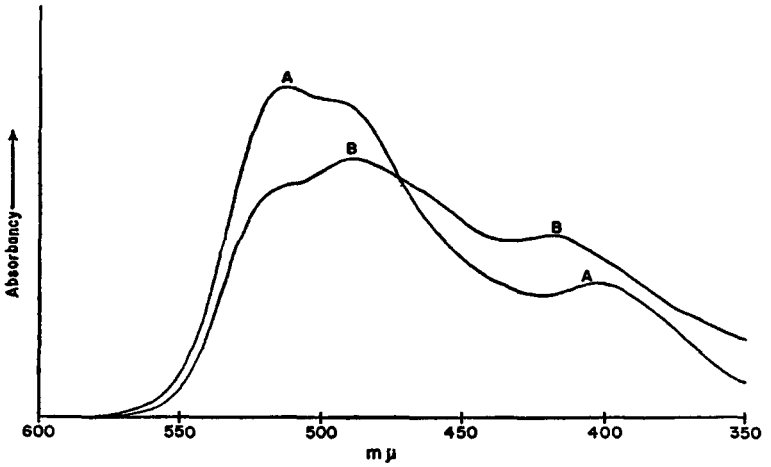

FIG. 1.—Absorbancy spectrum of D&C Red No. 35 and 1-(4-methylphenylazo)-2-naphthol.

Conc: 13.0 mg/liter
Solvent: $CHCl_3$
Cells: 1 cm
CURVE A—D&C Red No. 35
CURVE B—1-(4-methylphenylazo)-2-naphthol

TABLE 1.—*Recovery of 1-(4-methylphenylazo)-2-naphthol*

ADDED	FOUND (TOTAL)	RECOVERY	
mg	*mg*	*mg*	*per cent*
0.0	0.62	0.0	—
0.0	0.62	0.0	—
0.50	1.08	0.46	92
1.00	1.50	0.88	88
1.50	1.85	1.23	82
2.00	2.33	1.71	86
5.00	4.91	4.29	86
10.00	9.82	9.20	92
			Av. 88

Figure 1 shows the absorption curves of these two colors in chloroform as determined on a Cary Model 11 spectrophotometer. The concentration of each color is 13 mg per liter.

Known amounts of the subsidiary color were added to 200 mg portions of a composite sample of commercial D&C Red No. 35 and the content of subsidiary color was determined by the proposed procedure. The recoveries are listed in Table 1. The average recovery was 88 per cent.

Several samples of D&C Red No. 35 were examined for subsidiary color content by the proposed procedure. The results of these determinations are given in Table 2.

TABLE 2.—*Subsidiary dye content of samples of D&C Red No. 35*

SAMPLE	SUBSIDIARY COLOR
	per cent
1	0.61
2	1.55
3	2.78
4	4.69

SUMMARY

A spectrophotometric method for the determination of 1-(4-methylphenylazo)-2-naphthol in D&C Red No. 35 is presented. The average recovery of added intermediate was 88 per cent.

REFERENCES

(1) S.R.A., F.D.C. 3. Coal-Tar Color Regulations, Food and Drug Adm.
(2) SCLAR, R. N., *This Journal*, **30**, 522 (1947).

APPLICATION OF FLAME PHOTOMETRY TO THE DETERMINATION OF POTASH IN FERTILIZERS*†

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Flame photometry offers a rapid and convenient means of determining several of the elements. It is particularly adaptable to the determination of the alkali metals, since they have a relatively high emission intensity. Recently several papers have reported the use of this new analytical tool for the determination of one or more of these metals in plasma and urine (1), water (2), soils (3), and in milk (4), with an accuracy comparable to that obtained with conventional chemical methods. The

* Journal Paper No. 577 of the Purdue University Agricultural Experiment Station.
† Presented at the Annual Meeting of the Association of Official Agricultural Chemists, held in Washington, D. C., Oct. 1–3, 1951.
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flame photometer appeared to be an ideal instrument for the routine determination of potash in fertilizers, and this report concerns a study made to determine its adaptability for this purpose.

APPARATUS

A Beckman flame spectrophotometer, consisting of a model DU spectrophotometer and a No. 10,300 Beckman flame attachment, was employed in this investigation. The red sensitive photocell regularly furnished with the spectrophotometer (No. 156) was replaced by a No. 157 photocell (available from manufacturer). The No. 157 was designed particularly for flame photometry and has extremely low dark current characteristics. The .00025 microfarad condenser, recommended by the manufacturer to reduce galvanometer fluctuations, was also installed.

Air pressure for the unit was supplied by a laboratory size rotary compressor through a ballast tank. This was necessary because the air line in the laboratory was subject to pressure variations. Commercial bottled gas and U.S.P. tank oxygen were used to obtain a steady flame.

STANDARD SOLUTIONS

A stock solution containing 5000 p.p.m. of potassium was prepared by dissolving reagent grade potassium chloride in distilled water. Working standards containing 10, 20, 25, 30, 40 and 50 p.p.m. of potassium were then prepared by appropriate dilution.

PREPARATION OF SAMPLE SOLUTIONS

Solutions of the fertilizer samples to be analyzed were prepared by weighing 2.5100 grams into a 250 ml volumetric flask and adding 50 ml of saturated ammonium oxalate solution and 125 ml of water. One ml of of 2 per cent diglycol stearate in equal parts of benzene and alcohol was also added to prevent foaming. After boiling for 30 minutes a slight excess of ammonium hydroxide was added, and the solutions, when cool, were diluted to volume with distilled water according to the following scale: Samples containing up to 20 per cent potash were diluted to 250 ml directly in the flask used for the boiling. Samples containing 20 to 30 per cent potash were quantitatively transferred to a 500 ml volumetric flask and diluted to volume. Those in the 30 to 60 per cent potash range were quantitatively transferred to two liter volumetric flasks and diluted to volume. The solutions were then well mixed and filtered. Except for the slight difference in sample weight and the variation in the final volume of the solution, this procedure is identical with the Lindo-Gladding method outlined under section **2.40a** in the Official Methods of Analysis (5).

Aliquots from the filtered solutions above were diluted with distilled water as follows: If the expected potash content of the original sample

TABLE 1.—*Preparation of sample for flame photometric analysis*

EXPECTED K_2O CONTENT OF SAMPLE	SAMPLE TO VOL. OF	ALIQUOT	ALIQUOT TO VOL. OF	DILUTION FACTOR
(*per cent*)	(*ml*)	(*ml*)	(*ml*)	(*D*)
0–10	250	15	250	1
10–20	250	7½	250	2
20–30	500	7½	250	4
30–60	2000	15	250	8

was less than 10 or greater than 30 per cent, 15 ml. was diluted to 250 ml. If between 10 and 30 per cent, 7½ ml was diluted to 250 ml. Following this dilution technique, summarized in Table 1, the potassium (K) concentration in the diluted samples ready for flame analysis was 50 p.p.m. or less in all cases.

The above dilution system was selected over several alternate possibilities because it appeared to offer less chance of error and to be more adaptable to routine work. It avoids transferring small amounts of concentrated solutions by diluting the higher potash samples before withdrawing the aliquot, and permits the use of flasks of uniform size for the final dilution. This is convenient where a large number of samples are handled.

EXPERIMENTAL

Calibration of Instrument.—The emission intensity of potassium is greatest in a relatively cool flame. The optimum operating conditions at 767 mμ were found to be approximately 8 to 12 inches of water oxygen pressure, 0.5 to 1.0 cm gas pressure, and 15 to 20 p.s.i. air pressure. After an initial warm up period of at least 15 minutes, during which distilled water was atomized into the heated spray chamber to establish temperature equilibrium, the instrument was adjusted with the standard solutions so that a linear response was obtained over the range of 0 to 50 p.p.m. of potassium, with 50 p.p.m. set to read 100 on the transmission scale. Once established, the standard curve remained quite constant but was checked frequently during the analysis of a number of samples in order to detect and correct any slight drift that might occur.

After the standard curve was established the potassium concentration in the fertilizer sample was determined by aspirating the diluted solution and noting the reading on the transmission scale necessary to return the galvanometer to zero. With an initial sample weight of 2.51 grams and the dilution technique described above one division (1%T) on the transmission scale equals 0.1% potash (Fig. 1). The potash content of the sample was then determined by the formula $R \times D$, where R equals the K_2O value read from the scale and D is the dilution factor as shown in Table 1. Thus, the final value for samples in the 0–10 per cent range was read directly, as $D = 1$ in this case.

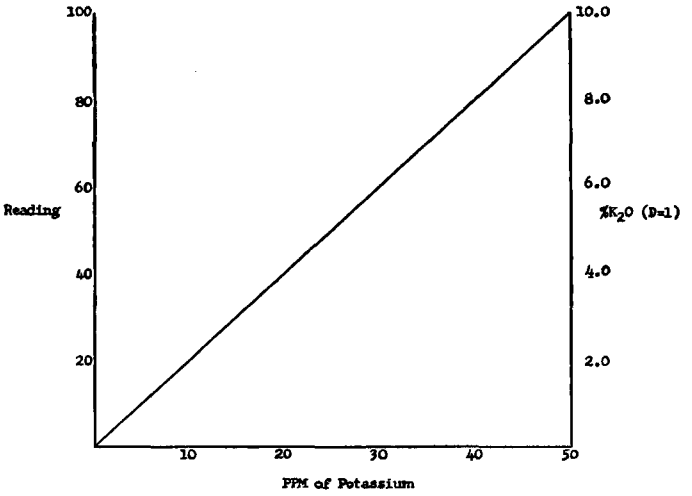

FIG. 1.—Standard curve.

Effects of Other Ions.—It is well known that the presence of other ions in the solution subjected to flame analysis may alter the radiation intensity of the element being determined and result in erratic values unless the operator takes necessary corrective measures. Anions as well as cations have been reported to exhibit interference effects (2), with some ions decreasing the reading from the true value and others increasing it. Since fertilizers are mixtures of several salts, the effect of extraneous

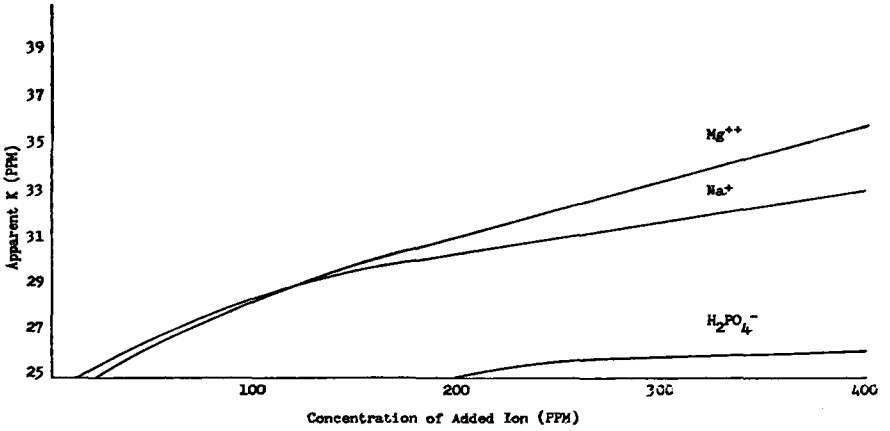

FIG. 2.—Effect of interfering ions.

ions on the emission intensity of potassium under normal operating conditions was investigated.

Sodium, magnesium, ammonium, and calcium were the cations considered most likely to occur with potassium in mixed fertilizers. The effect of each of these in concentrations up to 400 p.p.m. was determined by subjecting a series of test solutions containing 25 p.p.m. of potassium and graded amounts of the cation in question to flame analysis, and noting whether the reading departed from the true value. Calcium and ammonium ions had no detectable effect, but sodium (added as the chloride, sulfate and nitrate) and magnesium (as chloride) increased the reading when their concentration approached or exceeded that of the potassium (Fig. 2). The effect of anions was determined in a similar manner by adding the ammonium salt in each case. Chloride, oxalate, nitrate, sulfate, and monohydrogen phosphate, in anion concentrations up to 400 p.p.m., failed to produce any change in the radiation intensity. Dihydrogen phosphate was also without effect in concentrations below 200 p.p.m. but gave a slight increase above this concentration.

Analysis of fertilizers prepared from salts of high purity.—The fertilizer samples analyzed in this study were mixed in the laboratory from high purity salts, so that the true potash content would be known within narrow limits. Thus, by using samples of definite composition, the precision of the chemical as well as the flame photometric method could be studied.

100 gram samples of seven grades of fertilizer (2-12-6, 3-12-12, 3-9-18, 3-18-9, 8-8-8, 4-12-8 and 3-9-27) were prepared by mixing reagent grade ammonium nitrate, ammonium sulfate, sodium nitrate, mono-, di- and tri-calcium phosphate, potassium chloride, and potassium sulfate. Only one of each nitrogen, phosphorous and potassium carrier was used in the preparation of an individual sample, but all possible combinations of the carriers were made in preparing samples of each grade. Calculated amounts of the ground, oven dry salts and ground sand, which was used as a filler, were mixed by rolling for 24 hours in 500 ml glass, screw cap jars equipped with spiral vanes. Analysis of the sand by both methods showed this material to be free of potassium. After mixing, the samples were stored in tightly stoppered bottles.

Analyses were made employing the flame photometric procedure, and, for comparative purposes, the samples were also analyzed following the official A.O.A.C. method.

RESULTS AND DISCUSSION

The results (Table 2) are average values of duplicate determinations and show very close agreement, with the average values for the flame photometer slightly lower than those obtained by the official method. Duplicates by the flame method showed less variation, and the average

TABLE 2.—*Comparison of flame photometer and A.O.A.C. methods for the determination of potash in fertilizers*

COMPOSITION OF FERTILIZER	3-12-12		2-12-6		3-9-18		3-18-9		8-8-8		4-12-8		3-9-27	
	FLAME	A.O.A.C.	FLAME	A.O.A.C.	FLAME	A.O.A.C.	FLAME	A.O.A.C.	FLAME	A.O.A C	FLAME	A.O.A.C.	FLAME	A.O.A.C.
NH_4NO_3, $Ca(H_2PO_4)_2$, KCl	12.00	12.01	5.94	5.97	18.03	18.07	8.91	9.03	8.00	8.08	7.95	7.92	27.10	27.25
NH_4NO_3, $CaHPO_4$, KCl	12.25	12.23	6.02	6.06	18.24	18.14	8.98	9.04	8.09	8.16	8.08	8.15	27.30	27.33
NH_4NO_3, $Ca_3(PO_4)_2$, KCl	11.98	12.00	5.90	5.94	18.02	17.99	9.02	9.13	8.00	7.96	8.03	8.11	27.06	27.19
NH_4NO_3, $Ca(H_2PO_4)_2$, K_2SO_4	11.98	11.99	5.98	5.90	18.02	17.99	8.94	9.02	8.00	8.01	8.09	8.17	27.12	27.15
NH_4NO_3, $CaHPO_4$, K_2SO_4	12.32	12.18	6.05	6.09	18.15	18.23	9.07	9.01	8.04	8.01	8.04	8.15	27.00	27.06
NH_4NO_3, $Ca_3(PO_4)_2$, K_2SO_4	12.07	12.12	6.05	6.10	17.95	18.06	9.04	9.05	7.98	8.05	8.00	8.08	27.30	27.14
$NaNO_3$, $Ca(H_2PO_4)_2$, KCl	12.08	12.04	6.05	5.95	17.88	17.98	9.09	9.35	8.10	8.12	8.11	8.24	26.94	27.13
$NaNO_3$, $CaHPO_4$, KCl	12.18	12.31	6.05	5.99	18.15	18.20	9.04	9.04	8.18	8.32	8.13	8.28	27.06	27.11
$NaNO_3$, $Ca_3(PO_4)_2$, KCl	12.18	12.31	6.13	6.17	18.20	18.25	9.11	9.23	8.06	8.11	8.00	8.04	26.88	27.17
$NaNO_3$, $Ca(H_2PO_4)_2$, K_2SO_4	11.97	12.06	6.04	6.10	18.14	18.21	8.98	9.23	8.15	8.24	8.02	7.92	27.16	27.29
$NaNO_3$, $CaHPO_4$, K_2SO_4	12.10	12.07	5.99	6.11	18.12	18.14	9.23	9.13	8.19	8.14	8.08	8.12	27.06	27.40
$NaNO_3$, $Ca_3(PO_4)_2$, K_2SO_4	12.27	12.29	6.05	6.11	17.93	18.01	9.28	9.25	8.14	8.10	8.15	8.31	27.04	27.27
$(NH_4)_2SO_4$, $Ca(H_2PO_4)_2$, KCl	12.00	12.15	5.97	6.03	18.03	18.00	8.74	8.94	8.03	7.95	7.88	7.96	26.96	27.11
$(NH_4)_2SO_4$, $CaHPO_4$, KCl	11.97	12.01	6.08	6.11	18.13	18.06	8.95	9.03	8.01	8.05	7.88	8.00	27.16	27.18
$(NH_4)_2SO_4$, $Ca_3(PO_4)_2$, KCl	12.03	12.06	5.95	6.12	18.07	18.08	8.94	9.04	7.98	8.02	8.29	8.11	26.92	27.19
$(NH_4)_2SO_4$, $Ca(H_2PO_4)_2$, K_2SO_4	11.93	11.99	5.98	6.00	18.35	18.40	9.07	9.08	8.03	8.07	7.93	8.18	27.10	27.16
$(NH_4)_2SO_4$, $CaHPO_4$, K_2SO_4	12.10	12.17	6.13	5.98	18.16	18.22	9.02	9.17	8.06	8.12	7.93	8.19	26.90	27.05
$(NH_4)_2SO_4$, $Ca_3(PO_4)_2$, K_2SO_4	11.97	12.01	5.94	6.11	17.98	18.09	9.01	9.11	8.00	8.15	7.99	8.07	27.04	27.11
Average	12.07	12.11	6.01	6.05	18.08	18.12	9.02	9.10	8.06	8.09	8.03	8.11	27.06	27.18
Average difference between duplicates	.06	.05	.05	.08	.07	.08	.04	.08	.02	.05	.03	.07	.11	.08

Average of all samples—flame photometer 12.62%
Average of all samples—A.O.A.C. 12.68%
Theory 12.57%

values were generally nearer to the theoretical potash content of the samples than values obtained with the A.O.A.C. procedure. The results of this study are in agreement with other work in this laboratory in which the average flame photometer values for approximately 1000 commercial fertilizer samples were also slightly lower than the average values obtained by the official method.

The flame photometer reduces the time required for the determination of potash in fertilizers to a fraction of that required by conventional chemical methods. The analysis of individual samples can be completed in approximately 75 minutes including the thirty minute digestion period with ammonium oxalate. When working with a large number of samples the flame procedure also enables the analyst to handle many more determinations per unit time. Since the instrument can handle 75 to 100 determinations per hour, the limiting factor in time saving is the rate at which solutions can be prepared for analysis.

The nature and magnitude of interference effects depend upon a number of factors, including the flame temperature, concentration of potassium, and the ratio of extraneous ions to potassium in the solution subjected to flame analysis. Since the optimum operating conditions may vary from one laboratory to another, depending upon the type of instrument used, it is essential that the effect of extraneous ions be thoroughly investigated under conditions identical with those used for the potash determination. With the conditions and instrument employed in this study, sodium and magnesium increased the intensity of the potassium radiation if their concentration approached or exceeded that of the potassium in the solution analyzed. Although these salts may be present in fertilizers, very few cases have been found where their concentration approached the interference level. In addition, magnesium is precipitated in the preparation of the sample solution in the presence of soluble phosphates, so that interference errors from this source are negligible. By observing the flame color the presence of sodium is easily detected, because the intense yellow color, even at relatively low concentrations, completely masks the potassium color. Where this condition is observed corrections may be necessary.

The "radiation buffer" method of obviating interference effects (2) appears to have merit in cases where the interference is due to a combination of several ions. In this procedure, relatively large amounts of the diverse ions are added in equal amounts to the standards and to the sample, so that small variations of the ion concentrations in the sample themselves are without effect. The internal standard method has also been widely used but is not readily adaptable to all instruments. Gilbert, Hawes, and Beckman (6) discuss the theoretical aspects of interferences in detail and evaluate some of the means of decreasing them.

The results of this study indicate that the flame photometer can be

used advantageously in the potash analysis of mixed fertilizers. The method is rapid and convenient, and the accuracy is comparable to that obtained with conventional chemical methods. Operation of the flame photometer is simple and in this laboratory several analysts have used it without difficulty.

SUMMARY

Representative grades of fertilizers, in which the true analysis was known within narrow limits, were prepared in the laboratory from reagent grade ingredients. Analysis of these samples demonstrated that potash can be determined in fertilizers by flame photometry with an accuracy comparable to that of the chemical procedure. In general the flame values were slightly lower and nearer the theoretical potash values than the results obtained by the chemical method.

ACKNOWLEDGMENT

The assistance of F. W. Quackenbush and W. J. Rabourn is gratefully acknowledged.

BIBLIOGRAPHY

(1) Mosher, R. E., Boyle, A. J., Bird, E. J., Jacobson, S. D., Batchelor, T. M., Iseri, L. T., and Myers, G. B., *Am. J. Clin. Path.*, **19**, 461 (1949).
(2) West, P. W., Folse, P., and Montgomery, D., *Anal. Chem.*, **22**, 667 (1950).
(3) Toth, S. J., and Prince, A. L., *Soil Sci.*, **67**, 439 (1949).
(4) Keirs, R. J., and Speck, S. J., *J. Dairy Sci.*, **33**, 413 (1950).
(5) *Methods of Analysis, A.O.A.C.*, 7th Ed. (1950).
(6) Gilbert, P. T., Jr., Hawes, R. C., and Beckman, A. O., *Anal. Chem.*, **22**, 772 (1950).

DIRECT DETERMINATION OF AVAILABLE PHOSPHORIC ACID BY TITRATION OF AMMONIUM PHOSPHOMOLYBDATE*†

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MacIntire, Shaw, and Hardin (5) published a direct method for available phosphoric acid in fertilizers in which the digestant solution differed from that in the A.O.A.C. method (1). Barton (2) determined phosphoric acid in rock phosphate photometrically, using ammonium vanadate and ammonium molybdate solutions to develop the color complex. Kitson and Mellon (4) stated that the phosphorus compound was molybdivanadophosphoric acid. Epps (3) combined water-soluble and citrate-soluble

* This investigation, made in connection with a project of the Kentucky Agricultural Experiment Station, is published by permission of the Director.
† Presented at the Annual Meeting of the Association of Official Agricultural Chemists held at Washington, D. C., Oct. 1–3, 1951.

portions from a 1-gram sample of fertilizer and applied the photometric phosphovanadomolybdate method to this solution. He thus obtained a direct method for available phosphoric acid without materially changing the A.O.A.C. directions in regard to digestion in ammonium citrate solution.

Using a Coleman model 14 Spectrophotometer, the writer tried the Epps method with some modifications, but without entire success. At times very good checks with the A.O.A.C. method were obtained but other results were erratic.

Precipitation of the phosphorus in the mixed water-soluble and citrate-soluble portions as ammonium phosphomolybdate was investigated. It has been stated (3, 5) that this could not be done with the usual sample aliquots because of interference by the citrate. This was found to be true when an aliquot equivalent to 0.1 gram of sample was used. However, when the sample aliquot was reduced to 0.05 gram, thus reducing the concentration of the citrate ion, quantitative precipitation as ammonium phosphomolybdate was obtained and the phosphorus could be titrated with standard alkali as in the A.O.A.C. method. Since the phosphorus must be present as orthophosphate, the method is not applicable to calcium metaphosphate or to organic materials.

PROCEDURE FOR MIXED FERTILIZER AND SUPERPHOSPHATE

(Reagents are the same as in the A.O.A.C. method except that extra HNO_3 is not added to the molybdate soln.) Weigh 1 g into a 250 ml Erlenmeyer flask, add 50 ml water and shake for 10 minutes on Ross-Kershaw shaker (or stir by other method). Add 5 ml conc. HNO_3 to a 1000 ml filter flask and filter with suction through 9 cm filter paper on Hirsh funnel attached to filter flask. Wash with water to volume of about 125 ml. Proceed with the insoluble portion as in the A.O.A.C. method for citrate-insoluble phosphoric acid by digesting in neutral ammonium citrate soln at 65°C. (1). (In this study a water bath equipped with a continuous agitation device was used.) After digestion for one hour, filter the soln into the flask containing the water-soluble portion and wash with water at 65°C. to a total volume of 450 ml as indicated by a mark on side of flask. Transfer contents of filter flask to a 500 ml volumetric flask, cool to room temperature and adjust to volume. Mix and transfer 25 ml to a 250 ml Erlenmeyer flask, add 30 ml molybdate soln (40 ml for 45 per cent P_2O_5) and 15 grams of NH_4NO_3 in soln, and adjust temperature to 30–35°C. Shake for 30 minutes on a Ross-Kershaw shaker. Filter and titrate as in the A.O.A.C. method, using NaOH and HNO_3 of 0.3240 normality. Use 25 ml burets in the titration.

DISCUSSION

When using the smaller aliquot samples, more care is needed in titration. The standard NaOH solution should be rechecked by titration with a standard such as KH_2PO_4 using a sample comparable to the amount of P_2O_5 in the aliquot taken for the determination. It is possible that NaOH and HNO_3 standard solutions of one-half the normality specified in the A.O.A.C. method would be preferable and this is being investigated.

At times a fine ammonium phosphomolybdate precipitate was obtained which was not retained on the filter paper. Adding the NH_4NO_3 after the molybdate solution causes a precipitate of larger particle size. The solution should be 30°C. or higher during precipitation. In this laboratory, filtration through a Whatman No. 2 paper on a Hirsh funnel is used. Some preliminary analyses indicate that it maybe advantageous to precipitate the phosphorus at 40°C. in a continuous agitation bath.

If this method is applied to a sample of calcium metaphosphate, only about one-fourth of the phosphorus is obtained. However if the precipitation takes place at 40°C. with constant agitation and the solution stands over night before filtering, nearly all the phosphorus is returned.

This direct method has been applied to about 1000 samples in this laboratory. All results which were below guarantee and results one unit or more above guarantee were rechecked by the A.O.A.C. method. In 224 determinations by both methods, 79 per cent of the results agreed within 0.25 per cent. For the last 94 determinations 86 per cent agreed within 0.25 per cent.

Results of analyses, in triplicate, comparing the direct method and the A.O.A.C. method are given in Table 1.

TABLE 1.—*Comparison of A.O.A.C. and direct methods for available phosphoric acid (results in per cent P_2O_5)*

GRADE	A.O.A.C. METHOD AVERAGE*	DIRECT METHOD AVERAGE*	DIFFERENCE	A.O.A.C. METHOD RANGE†	DIRECT METHOD RANGE†
3-12-12	12.22	12.10	−0.10	0.13	0.20
0-14- 7	14.27	14.17	−0.10	0.05	0.10
6- 8- 6	8.34	8.27	−0.07	0.10	0.10
0-20-10	20.30	20.27	−0.03	0.05	0.10
6- 8- 6	8.22	8.00	−0.22	0.05	0.00
0-14- 7	14.34	14.17	−0.17	0.10	0.10
0-46- 0	45.89	45.87	−0.02	0.23	0.10
4-12- 8	12.82	12.57	−0.15	0.05	0.10
5-10-10	12.48	12.53	+0.05	0.15	0.10
0-12-12	12.13	12.30	+0.17	0.05	0.20
0-14-14	14.25	14.23	−0.02	0.15	0.10
0-20- 0	20.91	20.87	−0.04	0.07	0.10

* Average of triplicate analyses.
† Range between high and low analyses of triplicates.

SUMMARY

Available phosphoric acid in mixed fertilizers and superphosphate can be determined directly from the combined water-soluble and citrate-soluble extracts. This is done by the usual precipitation as ammonium phosphomolybdate. The aliquot taken for analysis is reduced to 0.05 gram to eliminate interference of the citrate.

All the phosphorus present in the fertilizer as calcium metaphosphate, or phosphorus of organic origin is not obtained by this method.

This method is considerably shorter that the official A.O.A.C. method and it is suggested as a screening method. All results below the guaranteed analysis should be re-checked by the A.O.A.C. method.

REFERENCES

(1) *Methods of Analysis, A.O.A.C.*, 7th Ed., 9–11.
(2) BARTON, C. J., *Anal. Chem.*, **20**, 1068 (1948).
(3) EPPS, ERNEST, JR., *Ibid.*, **22**, 1062 (1950).
(4) KITSON, R. E., and MELLON, M. G., *Ibid.*, **16**, 379 (1944).
(5) MACINTIRE, W. H., SHAW, W. M., and HARDIN, L. J., *Ibid.*, **10**, 143 (1938).

A STUDY OF THE DETERMINATION OF HORSE FAT IN THE PRESENCE OF PORK AND BEEF FATS*

By L. R. DUGAN, JR., and MARJORIE PETHERAM (The American Meat Institute Foundation, The University of Chicago)

It has been known for a number of years (7, 11) that the fatty acid composition of horse fat differs from that for beef or pork fat in that it contains appreciably more linolenic acid. Schuette, *et al.* (11) reported a value of 4.48 per cent linolenic acid for horse fat. Paschke (10) developed a method for the identification of horse fat in admixture with pork, beef, and mutton fat based on the formation of hexabromides insoluble in ethyl ether. Fiedler (5) described a similar method. Crowell (3) offered a modified procedure for the Paschke determination which was claimed to give more reliable results. Dalley (4) has described a bromination procedure which yielded 191 mg hexabromides per gram of horse fat, compared to the 41.2 mg obtained by Paschke, and 56.8 mg obtained by Crowell. None of the methods based on this empirical polybromide method give a quantitative estimation of the total linolenic acid present; White and Brown (12) point out values of linolenic acid determined by the polybromide method are only one-third to one-fourth the true value. These authors proposed the determination of linolenic acid in fats by interpolation from a curve in which hexabromide yields are plotted against amounts of linolenic acid brominated. Work in this laboratory, and recent publications by Brooker and Shorland (2) and Cupta and Hilditch (6) indicate that horse fat contains considerably more linolenic acid than would be shown by hexabromide values unless one could carefully establish a working curve as was done by White and Brown. The report by Hynds (8), based on collaborative studies in five laboratories, revealed a marked difference between hexabromide numbers reported on fat extracted from ground samples of horse meat, beef, and mixtures of beef

* Journal Paper No. 48, American Meat Institute Foundation.

and horse meat. Work in this laboratory has included both spectrophotometric (9) and bromination methods. The spectrophotometric method is deemed to be more desirable, since it is rapid, relatively simple, and reproducible. The purpose of this study is to point out its value for the estimation of horse fat in admixture with pork or beef fats.

EXPERIMENTAL

A number of horse fat samples were obtained. They were, respectively, composite horse oils, abdominal fats, and trimmings from around muscle tissue. These fats were rendered by standard laboratory procedures. Representative composites of pork and beef fat were also obtained. These fats were analyzed separately and in various combinations by the spectrophotometric and bromination methods. The mixtures of fat samples were made up in the following weight per cent ratios:

Beef to horse—90/10, 75/25, 50/50, 25/75 and 10/90
Pork to horse—75/25, 50/50, and 25/75

The spectrophotometric method of Mitchell, Kraybill, and Zscheile (9) was used in the analysis of the polyunsaturated fatty acids. The procedures of Dalley (4) (referred to as Method 1) and of White and Brown (12) (referred to as Method 2) were employed in the bromination of the beef fat-horse fat mixtures. Only Method 1 was used on the pork fat-horse fat mixtures. A sintered glass crucible was used in preference to the Gooch crucible employed by Dalley.

RESULTS AND DISCUSSION

The composition of a number of horse fats, as determined by spectrophotometric analysis, is given in Table 1. The degree of unsaturation of horse fat is evidenced by iodine values ranging from 78.34 to 91.33. This unsaturation is further established by the fatty acid composition. The arachidonic acid values are within the ranges commonly found in pork and beef fats but the trienoic acid content expressed as linolenic acid is much greater than would normally be found in pork and beef fats. (It should be noted here that Beadle, Wilder and Kraybill (1) found trienoic acid values of this range in yellow-fat from swine which had been condemned as unfit for human use. They interpreted the high trienoic acid values to be caused by an abnormal diet which involved a high percentage of linolenic acid which was deposited in the depot fats of the swine concerned.) The remaining polyunsaturated fatty acid, linoleic acid, is present in amounts not greatly different from the amounts found in fat from swine, but greater than the amount normally found in beef fat.

When a typical horse fat was mixed with beef fat which was representative of the composite fat from a large number of beef animals, a

TABLE 1.—*Fatty acid composition of several horse fats*

DESCRIPTION	IODINE* VALUE	PERCENTAGE COMPOSITION OF FATTY ACIDS				
		ARACHIDONIC	LINOLENIC	LINOLEIC	OLEIC	SATURATED
Horse Oil (composite)	86.91	0.73	11.07	11.45	41.57	35.18
	90.61	1.14	11.52	12.62	40.63	34.09
Abdominal Fat	86.40	0.74	12.75	8.66	40.53	37.32
	85.91	1.02	12.57	9.59	38.50	38.32
	—	0.45	13.72	11.40	37.34	37.09
Trim from Muscle	78.34	1.09	10.55	8.86	37.06	42.44
	91.33	0.92	11.39	12.12	43.61	31.96
	79.96	1.22	8.34	10.44	41.99	38.01
	89.80	0.95	10.87	15.09	37.36	35.73
Average		.92	11.42	11.14	39.84	36.68

* Glyceride basis.

range of analytical values was obtained as shown in Table 2. The values obtained from mixtures of horse fat with lard are shown in Table 3.

The iodine value would serve more adequately to indicate the presence of horse fat in admixture with beef fat than to indicate its presence in lard. (The difference in iodine value of beef fat and horse fat is much greater than the difference in iodine value for lard and horse fat.) If the iodine values shown in Tables 2 and 3 were plotted against the fat compositions, there would be a straight line for each set of mixtures, with that for the beef/horse combinations having the steeper slope.

In the bromination of the fatty acids it was possible to obtain higher yields of hexabromides by Method 1. This is shown in Table 2. By this

TABLE 2.—*Analytical and linolenic acid values from mixtures of beef and horse fats*

COMPOSITION OF FAT SAMPLES (BEEF/HORSE)	IODINE VALUES (WIJS)	HEXABROMIDES MG/G FAT		LINOLENIC ACID FROM HEXABROMIDE DETN. (METHOD 1)	LINOLENIC ACID FROM SPECTRO. DETN.	THEORETICAL LINOLENIC ACID MEASURED BY HEXABROMIDES*
		METHOD 1	METHOD 2			
				per cent	*per cent*	*per cent*
100/0	41.95	0	0	0	0.50	0
90/10	46.7	4.95	2.95	0.18	1.69	10.8
75/25	53.1	18.2	5.35	0.67	3.72	18.0
50/50	64.1	37.0	25.0	1.36	6.46	21.0
25/75	74.1	71.3	61.2	2.62	9.71	27.0
10/90	81.5	77.4	68.1	2.84	11.63	24.4
0/100	86.4	89.7	65.4	3.30	12.75	25.8

* Based on assumption that Spectrophotometric values = theoretical.

method the yield of hexabromides was more consistent with the amount of linolenic acid in the sample. However, the yields of hexabromide are erratic and subject to uncorrected variations and are not in proportion to the amount of linolenic acid present in the samples of fat. This is more apparent when the percentages of total linolenic acid measured by hexabromides are compared with the calculated values shown in the last column of Table 2. These are based on the linolenic acid values as determined by the spectrophotometric method, and are consistent with the change in horse fat content of the samples. Note in the last column of Table 2 that at the lower values of linolenic acid in the fats, the percentage of total linolenic acid as determined by the hexabromides is very low. As the amount of linolenic acid in the fats increases, the percentage of total linolenic acid determined by the hexabromides increases up to the 25 per cent range. This is consistent with the observation of White and Brown noted above.

Comparable observations may be made from a study of the analytical values obtained from the pork fat-horse fat mixtures. (Table 3.)

TABLE 3.—*Analytical and linolenic acid values from mixtures of lard and horse fat*

COMPOSITION OF FAT SAMPLES LARD/HORSE FAT	IODINE VALUES (WIJS)	HEXABROMIDE MG/G FAT	LINOLENIC ACID FROM HEXABROMIDE DETN. (METHOD 1)	LINOLENIC ACID FROM SPECTRO. DETN.	THEORETICAL LINOLENIC ACID MEASURED BY HEXABROMIDES*
			per cent	*per cent*	*per cent*
100/0	63.4	0	0	0.67	0
75/25	68.7	15.7	0.58	3.96	14.6
50/50	74.5	43.7	1.60	6.71	23.9
25/75	80.1	72.8	2.68	9.59	27.9
0/100	85.9	87.2	3.21	12.57	25.5

* Based on assumption that Spectrophotometric values = theoretical.

The spectrophotometric method (9) appears to be superior to the hexabromide method for the estimation of horse fat in admixture with pork and beef fats. Not only does this method allow a quantitative determination of the amount of linolenic acid in the fat (the criterion for differentiation of horse fat from beef and/or pork fat) but its precise nature permits the qualitative estimation of the presence of horse fat at much lower levels than could be derived from hexabromide determinations. It should be noted that in cases where a fat analysis indicates a level of linolenic acid only slightly in excess of that to be expected in a normal beef or pork animal, results should be substantiated by serological tests.

SUMMARY

1. The unsaturated fatty acid composition of a number of horse fats has been determined. The trienoic fatty acid composition expressed as linolenic acid was found to average 11.4 per cent.

2. The trienoic fatty acid content and the hexabromide number of mixtures of horse fat with beef fat and with pork fat have been determined.

3. The hexabromide method was found to give variable and inconsistent results for determining horse fat in the presence of beef or pork fat.

4. The spectrophotometric method of analysis for trienoic fatty acids is recommended for the estimation of horse fat in the presence of beef or pork fats.

BIBLIOGRAPHY

(1) BEADLE, B. W., WILDER, O. H. M., and KRAYBILL, H. R., *J. Biol. Chem.*, **175**, 221 (1948).
(2) BROOKER, E. G., and SHORLAND, F. B., *Biochem. J.*, **46**, 80 (1950).
(3) CROWELL, G. K., *This Journal*, **27**, 448 (1944).
(4) DALLEY, R. A., *Analyst*, **75**, 336 (1950).
(5) FIEDLER, H., *Fette u. Seifen*, **46**, 34 (1939).
(6) GUPTA, S. S., and HILDITCH, T. P., *Biochem. J.*, **48**, 137 (1951).
(7) HEIDUSCHKA, A., and STEINRUCK, A., *J. prakt. Chem.*, **102**, 241 (1921).
(8) HYNDS, CLAUDE E., *This Journal*, **34**, 355 (1951).
(9) MITCHELL, J. H., JR., KRAYBILL, H. R., and ZSCHEILE, F. P., *Ind. Eng. Chem. Anal. Ed.*, **15**, 1 (1943).
(10) PASCHKE, B., *Z. Untersuch. Lebensm.*, **76**, 476 (1938)
(11) SCHUETTE, H. A., GARWIN, THELMA M., and SCHWAEGLER, E. J., *J. Biol. Chem.*, **107**, 635 (1934).
(12) WHITE, MARY F., and BROWN, H. B., *J. Am. Oil Chemists Soc.*, **26**, 133 (1949).

THE COLORIMETRIC DETERMINATION OF PIPERONYL BUTOXIDE[1]

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Within the last few years the combination of piperonyl butoxide and pyrethrins[3] has come into widespread use for the control of insects in the household, in food processing plants, in warehouses, and on domestic animals, as well as for the protection of grains and other foodstuffs. The marked synergism of insecticidal effect exhibited by this combination (1, 2), its very low order of toxicity to warm-blooded animals (3, 4),

[1] Presented at the Annual Meeting of the Association of Official Agricultural Chemists, held in Washington, D.C., Oct. 1–3, 1951.
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[3] Combinations of technical piperonyl butoxide and pyrethrins are marketed by U. S. Industrial Chemicals Company under the trade name "Pyrenone."

and its stability (5) have permitted a more economical and much broader range of application than would have been possible with the pyrethrins alone.

Piperonyl butoxide is (3,4-methylenedioxy-6-propylbenzyl) (butyl diethylene glycol) ether (6). The technical product contains a minimum of 80 per cent of this compound; the remainder is related insecticidally active materials.

The increasing use of piperonyl butoxide made it necessary to develop a method for its quantitative analysis. A qualitative test for compounds containing the methylenedioxyphenyl group, described some years ago by Labat (7), was first tried. The basis of this test is a green color, obtained when the substance is heated with concentrated sulfuric and gallic acids. However, the reaction could not be utilized as the basis for a quantitative procedure for piperonyl butoxide because of the charring effect of the sulfuric acid and the rapid fading of the green color.

Other acids and phenolic materials were then tested, and as a result of this study it was found that a clear blue color of good stability was obtained when a solution of piperonyl butoxide in deodorized base oil (purified kerosene) was heated with a solution of tannic acid in a mixture of phosphoric and glacial acetic acids. Further study showed that this revised test was quite specific for piperonyl butoxide and compounds closely related to it; the color was not obtained with many other compounds containing the methylenedioxyphenyl group. The test was reasonably sensitive, and the relationship between concentration and intensity of color followed Beer's law within a suitable range. The quantitative method developed from this test has proven very useful, not only for control analyses, but in research studies on new applications of piperonyl butoxide.

METHOD

APPARATUS

Water bath.
Test-tube basket to fit water bath.
Photoelectric colorimeter (Klett-Summerson or equivalent).
Special color filter with transmission range of about 625 to 635 millimicrons.[4]

MATERIALS FOR REAGENT

1. *Tannic acid:* The tannic acid must be specially purified as follows: To 20 g of tannic acid (pure, reagent, or U.S.P. grade) add 100 ml of ethyl acetate (99 per cent) and agitate the mixture with a mechanical stirrer for about one hour. Filter by suction through a sintered glass funnel, and wash the residue on the filter with three 5-ml portions of ethyl acetate. To the combined filtrate and washings add 2 g of finely powdered Darco G-60 (or equivalent decolorizing carbon), and agitate with a mechanical stirrer for about one-half hour. Filter by gravity through a double thickness of Whatman No. 1 (or equivalent) filter paper into a graduated dropping funnel. Wash residue several times with ethyl acetate until the volume of the filtrate

[4] The filter used in this work was obtained from the Farrand Optical Company, New York 66, New York.

and washings is about 125 ml. Place the dropping funnel over a 1 liter, 3-neck, round bottom flask, equipped with a mechanical stirrer, and with vigorous agitation in the flask add the filtrate dropwise to five times its volume of dry toluene. The purified tannic acid is precipitated immediately in the toluene.

Filter by suction through a sintered glass funnel, and wash the product thoroughly with dry toluene, stirring the solids and toluene to assure complete removal of ethyl acetate. Continue the suction until practically all the toluene has been removed. Dry this purified tannic acid in a vacuum oven at about 40°C., and place in a tightly stoppered bottle.

2. *Acetic acid*, glacial, c.p.
3. *Phosphoric acid*, 85 per cent, c.p.

PROCEDURE

Preparation of Reagent.—Dissolve completely exactly 0.025 g of specially purified (as above) tannic acid in 20 ml of glacial acetic acid by shaking at room temperature. When the tannic acid is all in solution, add 80 ml of 85 per cent phosphoric acid and mix the solution thoroughly. Prepare fresh daily, and keep tightly stoppered since it is hygroscopic.

Preparation of Samples.—Dissolve in or dilute a weighed amount of the sample to be tested with deodorized base oil (Deobase, Ultrasene, or equivalent) to contain preferably from 25 to 75 mg of piperonyl butoxide per 100 ml of solution. At the same time prepare a standard containing 50 mg of a known sample of piperonyl butoxide per 100 ml of deodorized base oil solution.[5]

Development and Reading of Color.—Place exactly 0.1 ml (from 1 ml pipet graduated in 0.1 ml) of solution of sample to be tested in an 18×150 mm test tube. To this add exactly 5 ml of the above reagent and shake the tube vigorously for one minute. Treat the standard, and a blank consisting of 0.1 ml of deodorized base oil, simultaneously in the same manner. Make all determinations in duplicate.

Place the test tubes in a test-tube basket and place the basket in a vigorously boiling water bath for 5 minutes while the blue color develops. At the end of this period remove the basket and allow the tubes to cool to room temperature. Then transfer the samples to Klett-Summerson colorimeter tubes and read in the usual manner using the 625 to 635 millimicrons filter. (After cooling to room temperature there is no appreciable change in color value for several hours.)

Calculation of Results.—Subtract the reading for the blank of deodorized base oil from the readings of both the unknown sample and the standard. Then calculate the results by a direct proportion between the unknown and the standard.

A typical result on the Klett-Summerson colorimeter using the 625 to 635 millimicron filter would be a reading of 150 (after blank correction) for a 0.1 ml aliquot of a standard containing 50 mg of butoxide per 100 ml. Assuming the solution under test gave a reading of 141 (after blank correction) the calculation is simply: $x = 50(141)/150$ or 47 mg piperonyl butoxide per 100 ml in the solution of sample. From the amount of the original sample taken to prepare the test solution, calculate the per cent by weight of piperonyl butoxide in the sample in the usual manner.

DISCUSSION OF THE METHOD

Reagent.—Early work on the present method was done with a single lot of "pure" tannic acid which gave excellent results. It later developed that many other samples of tannic acid gave little or no blue color, and in some cases gave a turbid reagent. The method described above for the

[5] For samples containing pyrethrins see section on "Interfering Substances," this paper, page 778.

purification of the tannic acid to be used in the reagent, when applied to about 20 samples of U.S.P., pure, or reagent grade tannic acid, gave purified products which resulted in clear reagents and reasonably uniform color values in the test. Various lots of purified tannic acid differed somewhat in the intensity of color obtained in the test, but all were within a usable range. Technical grades of tannic acid should not be used, as they did not give entirely satisfactory products when submitted to the purification procedure.

Various proportions of tannic, acetic, and phosphoric acids have been tried in the reagent. For some time a reagent containing 0.1 g pure tannic acid (not specially purified) in a mixture of 30 ml of glacial acetic acid and 70 ml of 85 per cent phosphoric acid was used. With the specially purified tannic acid, however, it was found that the proportion given in the foregoing method gave a reagent of maximum clarity and produced a color of maximum intensity.

Preparation of Sample and Standard.—Deodorized base oil, recommended as the solvent for the sample, originally was used so that the method would be readily applicable to the usual types of oil-base insecticides. This type of solvent has been found satisfactory also for the analysis of other types of products. Dusts, surface deposits, paper treatments, and grains and other foodstuffs have been extracted with a volatile solvent such as hexane, and the solvent-free extract dissolved in deodorized base oil for the color test. (As will be mentioned later, extracts of certain materials, such as those containing vegetable oils, may require special treatment before application of the color test.)

Pure piperonyl butoxide, which may be obtained by efficient low-pressure fractional distillation of the technical product, may be used as a primary standard in the color test. A comparison of the color values obtained on distillation fractions (8) of, and isolated by-products from, technical piperonyl butoxide has shown that pure piperonyl butoxide is the major color-producing constituent of the technical product.

Technical piperonyl butoxide, with a minimum purity of 80 percent, as now manufactured may have a somewhat higher range of purity. For control analyses in which the exact content of the technical product is required, the same lot of technical butoxide as used in the sample, or a lot of equal purity, has been used as a standard. For most purposes, when the exact purity of the butoxide is not known, comparisons may be made with a pure piperonyl butoxide standard and the results reported in terms of content of the pure material.

Optical Measurements.—To select the proper light filter for the color measurement, the absorption spectrum of the blue color obtained with pure piperonyl butoxide was determined on the Beckman DU spectrophotometer in the wave length range from 500 to 675 millimicrons. Figure 1 shows the results obtained with 50 micrograms (0.1 ml aliquot

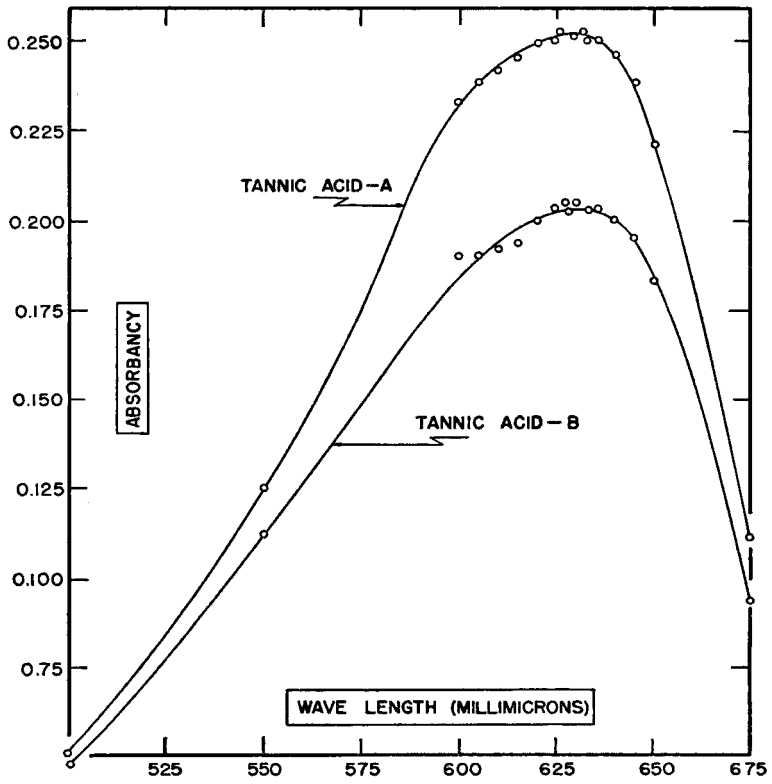

FIG. 1.—Absorbancy—Wave length curves (50 micrograms butoxide per 0.1 ml).

of a solution containing 50 mg per 100 ml) of pure butoxide using two different lots of specially purified tannic acid. These two lots of tannic acid were selected from the 20 samples tested, since they represented approximately the maximum and minimum values obtained; both were within a usable range. It will be noted that maximum absorbancy occurs in the region from 625 to 635 millimicrons. Similar measurements with technical butoxide have given slightly lower values, but curves were parallel and maxima fell within the same wave length range.

The No. 54 and No. 66 filters supplied with the Klett-Summerson instrument have been used with moderately satisfactory results, but with these filters the method was less sensitive, required a higher concentration range of butoxide, and was subject to greater errors from interfering colors present in certain samples.

Spectrophotometers and photoelectric colorimeters other than the Klett-Summerson have been used for the color comparison with satisfac-

tory results provided measurements were made in the range of maximum absorbancy.

Time and Method of Shaking.—The method used for shaking the tubes containing the reagent and sample, and the time of shaking do not appear to be critical. Identical samples shaken by hand for one, two, and three minutes gave the same color values. Mechanical shaking was tried, but it seemed to present no advantage over the manual method of shaking. The principal requirement is that the sample and reagent be thoroughly and uniformly mixed. In the present studies the manual method of shaking for 1 minute was used.

Time of Heating.—During the heating in the boiling water bath, the color increases in intensity during the first 5 minutes and then remains substantially constant. Figure 2 shows the relationship between time of heating and intensity of color for two concentrations of butoxide.

The time of heating is sufficiently critical so that all samples in a comparative series must be heated at the same time. For the same reason it is also advisable to use standards with each set of samples, rather than to use a calibration curve obtained from previous measurements.

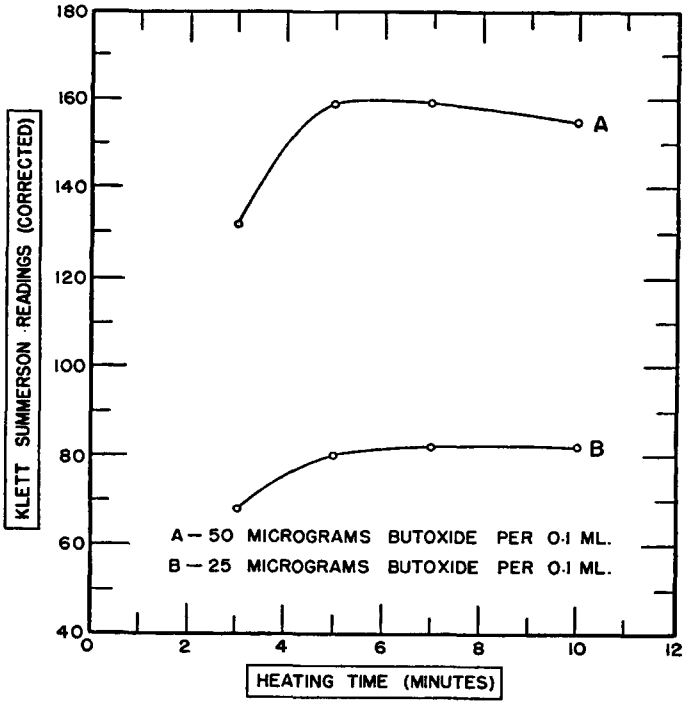

FIG. 2.—Effect of heating time on intensity of color.

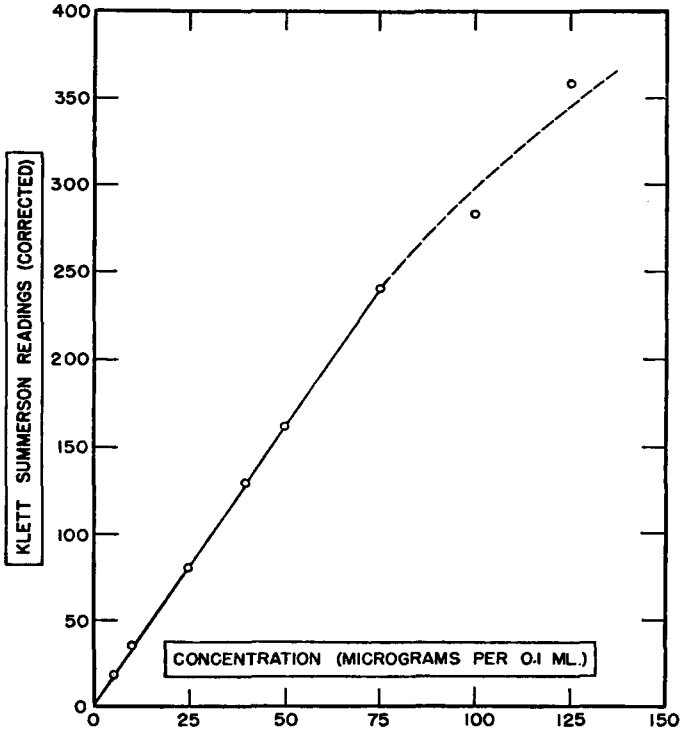

FIG. 3.—Standard curve.

After heating for 5 minutes and cooling to room temperature the intensity of the color remains practically constant for at least 4 hours.

With the usual type of oil-base samples the blue color obtained is entirely clear. In the analysis of some samples, however, there may be a slight turbidity. It has been found that this turbidity can usually be removed by centrifuging.

Concentration Range and Precision.—As shown in Figure 3, the relationship between concentration and color intensity follows Beer's law in the range from about 5 mg to 75 mg of pure piperonyl butoxide per 100 ml (5 to 75 micrograms per 0.1 ml aliquot). Above the latter concentration there is a gradual deviation from the straight line function. For optimum precision the range from 25 to 75 mg per 100 ml is suggested, but reasonably precise measurements may be made at concentrations below 25 mg per 100 ml.

The precision of the method is comparable to that of other colorimetric procedures. Using the method as described, and operating within the range of 25 to 75 mg of butoxide per 100 ml, the standard deviation of

results by a single analyst is about ±3 per cent. The precision of the method could probably be increased by the use of aliquots larger than 0.1 ml and aliquots of 0.2 ml have been used with good results. As larger aliquots are used, however, it becomes increasingly difficult to obtain thorough mixing with the reagent during the shaking procedure.

Specificity of Test.—As mentioned earlier, the color reaction appears to be specific for piperonyl butoxide and certain closely related compounds. The color reaction is given by 3,4-methylenedioxy-6-propylbenzyl alcohol, as well as other ethers and certain esters of this alcohol. The test, however, is not given by many other compounds having the methylenedioxyphenyl group, such as safrole, isosafrole, dihydrosafrole, piperonal, piperonyl cyclonene, sesamin, and others. Most of the by-products present in technical piperonyl butoxide do not give the test.

It appears that, in addition to the 3,4-methylenedioxy group, a substituent in the 6-position, with either $-CH_2OH$ (or a derivative of it) in the 1-position of the benzene ring, are required for the color reaction. The mechanism of the reaction is not known at present.

Interfering Substances.—It has been found that pyrethrum extract causes a reduction in the intensity of the blue color. The spectrum of the color obtained with mixtures of piperonyl butoxide and pyrethrins is the same as that for butoxide alone, but the values for absorbancy are reduced as the proportion of pyrethrins is increased.

Samples containing various ratios of pyrethrins (from commercial, purified pyrethrum extract) to butoxide were analyzed in comparison with butoxide alone. At a 1–10 ratio of pyrethrins to butoxide there was a 3 per cent reduction in the color value, at the 1–5 ratio the reduction was 7 per cent, and at the 1–1 ratio the value was reduced 15 per cent in comparison with the straight butoxide standard. At ratios greater than 1–10, such as 1–15 or 1–20, the interference was so slight that no correction was necessary.

At least two methods for overcoming this interference from pyrethrum extract may be used. If the ratio of pyrethrins to butoxide in the sample under test is known, the simplest method is to incorporate pyrethrins with butoxide in the standard at the same ratio as in the sample. This has been done in this laboratory with entirely satisfactory results.

Another method has been proposed by Davidson and Terrell (9). This procedure involves saponification of the pyrethrins in the sample before colorimetric analysis. The standard butoxide alone must be similarly treated, since the saponification method employed causes some reduction in the color value. This method, although time-consuming, appears to be satisfactory for samples in which the ratio of pyrethrins to butoxide is not known.

Samuel has found that piperonyl butoxide may be separated from certain other insecticides by partition chromatography (10) and, in

preliminary work, has shown that butoxide may possibly be separated from the pyrethrins by this method. Further work may show that partition chromatography is a satisfactory means of separating butoxide not only from pyrethrins, but also from other interfering materials such as those present in certain vegetable and animal products.

In recent preliminary work allethrin has shown about the same degree of interference as the pyrethrins. From this and other evidence it would appear that the pyrethrins themselves in pyrethrum extract are the cause of the reduction in color value.

No extensive study has been made of possible interference from other materials that may be present in finished insecticide formulations. From the limited amount of work that has been done it appears that DDT, chlordane, crude benzene hexachloride, and chlorinated camphene do not of themselves interfere with the test. Some of the solvents commonly used with these materials, however, do interfere. For example alkylated naphthalenes, cyclohexanone, and acetone do cause interference. When the nature and amount of the interfering material are known, correction can usually be made by using a standard in which this material is incorporated in the same proportion as in the sample.

Certain vegetable oils, such as wheat germ oil, contain substances which interfere with the development of the blue color. In the analysis of milling fractions of grain for piperonyl butoxide, wheat germ was extracted with hexane, the extract freed of solvent, and the butoxide separated from interfering materials by gentle but complete saponification of the oil with alcoholic alkali, followed by dilution with water and extraction of the butoxide with hexane.

It has been found that flour, to which butoxide has been added for test purposes, may be analyzed by extraction at room temperature with redistilled benzene and treatment of the extract with a small proportion of activated carbon and fullers earth. This method of extraction and treatment has separated the butoxide from certain materials which interfere with the test. As little as 2 p.p.m. of butoxide in flour has been determined with reasonable accuracy by this method.

Piperonyl butoxide has in some cases been used in paraffin wax. Although the wax does not interfere with the color development in a chemical sense, the butoxide must be separated from it in order to conduct the test. Small amounts of butoxide have been separated from large amounts of paraffin wax by dissolving the sample in hexane, thoroughly extracting this solution with aqueous methanol (85–90 per cent), and, after dilution with water, extracting the butoxide from the aqueous methanol with fresh hexane.

Applications of the Method.—The colorimetric procedure has been highly satisfactory in its application to oil-base materials and dusts containing piperonyl butoxide and pyrethrins. It has also been applied

with good results to paper coatings and other deposits of this combination.

The procedure has been used for the analysis of feces and urine from experimental animals fed piperonyl butoxide (4) and is now being applied to grains treated with the butoxide-pyrethrins combination, and to milling fractions of these grains, such as bran, germ, and flour.

As shown by the examples given above, special methods for the extraction and separation of butoxide have been required in certain cases, particularly for the handling of fractions of grains. The details of these procedures are not within the scope of this paper; furthermore, additional work which is now being done may show that these methods can be improved and simplified.

SUMMARY

A quantitative colorimetric procedure is described for the determination of piperonyl butoxide. The relationship between concentration and intensity of color follows Beer's law within a suitable range. The test is sensitive to small amounts of the compound and is reasonably precise. The color reaction appears to be specific for piperonyl butoxide and closely related compounds. At certain proportions pyrethrins cause a reduction in the intensity of the color, but at least two methods are available for overcoming this interference. The method has been applied successfully to oil solutions of piperonyl butoxide with pyrethrins, as well as to dusts, paper coatings, and other materials containing this combination.

ACKNOWLEDGMENT

The authors gratefully acknowledge the assistance of Mr. W. L. Johnson and Mr. N. W. Matthews on certain phases of this problem, particularly the investigation of a procedure to purify tannic acid for use in the reagent.

LITERATURE CITED

(1) DOVE, W. E., *Amer. J. Trop. Med.*, **27**, 339 (1947).
(2) WACHS, H., JONES, H. A., and BASS, L. W., *Advances in Chemistry Series*, No. **1**, 43 (1950).
(3) SARLES, M. P., DOVE, W. E., and MOORE, D. H., *Amer. J. Trop. Med.*, **29**, 151 (1949).
(4) SARLES, M. P., and VANDEGRIFT, W. B., *Amer. J. Trop. Med.* (In Press.)
(5) CHAMBERLAIN, R. W., *Amer. J. Hygiene*, **52**, 153 (1950).
(6) WACHS, H., *Science*, **105**, 530 (1947).
(7) LABAT, J. A., *Bull. Soc. Chim. Biol.*, **15**, 1344 (1933).
(8) MILLER, A. C., PELLEGRINI, J. P., JR., POZEFSKY, A., and TOMLINSON, J. R., *J. Econ. Entom.* **45**, 94 (1952).
(9) DAVIDSON, J. C., and TERRELL, H. D., Paper presented at Amer. Chem. Soc., Meeting-in-Miniature, Philadelphia, Pa., Jan. 20, 1949.
(10) SAMUEL, B. L., *This Journal*, **35**, 391 (1952).

UREA AND AMMONIACAL NITROGEN IN MISCELLANEOUS FEEDING STUFF PRODUCTS*†

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The present authors (1) have published data showing the amount of nitrogen normally occurring as urea and ammonia in packing house products. Similar data, showing the amount of nitrogen normally occurring in miscellaneous feeding stuffs and mixed feeds, are now presented. With such information at hand, it is possible to check the amount of added urea more accurately.

The data in Table 1 show the amount of total nitrogen, ammoniacal and urea nitrogen, and ammoniacal nitrogen in a number of feeding stuffs and feeds. The methods used to determine the nitrogen have been outlined by Perkins, Merrill, and Rogers (1). Changes have been made in the A.O.A.C. method (2) for urea, as it was found to be unreliable under certain conditions of acidity or alkalinity. The primary change is in the addition of a buffer.

With the exclusion of three samples showing an extremely high urea and ammoniacal nitrogen content it may be seen that none of the feeds contained more than 0.20 per cent of such nitrogen, and the average is 0.07 per cent. The three samples containing the highest percentages of urea and ammoniacal nitrogen were eliminated from the average, as it is probable that they contained added urea. In determining the amount of urea added to mixed feeds it is suggested that a tolerance of 0.20 per cent is sufficient for the amount of such nitrogen normally appearing in such feeds.

The present A.O.A.C. method for the determination of urea and ammoniacal nitrogen in feeding stuffs has failed to give accurate recoveries of known additions of urea. This failure has been found to result from not using a buffer to control the *p*H of the suspension during the urease digestion.

The method used by Perkins, Merrill, and Rogers (1) in reporting the urea and ammoniacal nitrogen content of packing house products and in reporting the data in Table 1 of the present paper has presented some difficulties in obtaining check analyses. In a few cases, multiple analyses (up to 8) were made before the average of all analyses would agree with theoretical figures. Accordingly the method was modified by the use of a two gram (instead of a one gram) sample, and by the use of 2 ml of buffer.

The proposed method for urea determination can be varied in many

* Contribution No. 461, Department of Chemistry, Kansas Agricultural Experiment Station, in cooperation with the Kansas State Board of Agriculture.
† Presented at the Annual Meeting of the Association of Official Agricultural Chemists held at Washington, D.C., Oct. 1–3, 1951.

TABLE 1.—*Nitrogen content of miscellaneous feeds*

LAB. NO.	TOTAL NITROGEN*	UREA AND AMMONIACAL NITROGEN*	AMMONIACAL NITROGEN*	DESCRIPTION
	per cent	*per cent*	*per cent*	
29208	3.15	0.05	0.01	Dehydrated alfalfa
29210	3.33	0.03	0.02	Dehydrated alfalfa
29226	2.97	0.06	0.02	Dehydrated alfalfa
29409	2.86	0.05	0.01	Dehydrated alflafa
29118	2.51	0.11	0.03	Sun cured alfalfa
29410	2.63	0.15	0.05	Sun cured alfalfa
29456	2.51	0.12	0.06	Sun cured alfalfa
29477	2.40	0.17	0.06	Sun cured alfalfa
28848	0.54	0.04	0.03	Cottonseed Hulls
29205	6.68	0.05	0.06	Cottonseed Meal
29216	6.86	0.08	0.05	Cottonseed Meal
29316	6.54	0.11	0.07	Cottonseed Meal
29317	6.62	0.07	0.06	Cottonseed Meal
29413	6.53	0.11	0.08	Cottonseed Meal
28853	4.48	0.06	0.05	Corn distilling grains
29537	7.11	0.09	0.04	Corn gluten feed
28684	5.36	0.03	0.03	Linseed Meal
28935	5.56	0.06	0.04	Linseed Meal
29584	5.47	0.06	0.03	Linseed Meal
28780	0.11	0.08	0.06	Mineral mix
28932	1.36	0.07	0.02	Bone meal
29091	4.95	0.11	0.06	Mineral mix
29458	2.71	0.06	0.04	Bone meal
29514	0.37	0.31	0.21	Mineral mix
29595	1.44	0.03	0.03	Bone meal
29605	0.68	0.03	0.03	Yeast and mineral
29404	1.75	0.10	0.02	Oats pulverized
29606	2.42	0.08	0.03	Oat meal
29356	6.85	0.08	0.06	Soybean meal
29440	7.00	0.07	0.05	Soybean meal
29535	6.99	0.09	0.05	Soybean meal
29536	6.96	0.07	0.05	Soybean meal
28691	2.76	0.02	0.02	Wheat bran & screenings
29209	2.89	0.05	0.01	Wheat shorts
29225	2.81	0.04	0.02	Wheat shorts & screenings
29227	2.89	0.09	0.02	Wheat shorts
29252	2.72	0.03	0.01	Wheat shorts & screenings
29397	2.83	0.07	0.02	Wheat bran
29457	2.77	0.04	0.02	Wheat bran & screenings

TABLE 1.—(*continued*)

LAB. NO.	TOTAL NITROGEN*	UREA AND AMMONIACAL NITROGEN*	AMMONIACAL NITROGEN*	DESCRIPTION
	per cent	*per cent*	*per cent*	
28945	2.97	0.03	0.02	Sweetened feed
28961	2.00	0.05	0.03	Sweetened feed
29531	0.64	0.01	0.01	Sweetened feed
28646	2.69	0.04	0.02	Poultry feed
28647	2.35	0.02	0.02	Poultry feed
28650	3.22	0.03	0.03	Poultry feed
28756	3.09	0.03	0.02	Poultry feed
29290	3.36	0.02	0.02	Poultry feed
29435	3.40	0.04	0.03	Poultry feed
29574	3.56	0.07	0.02	Poultry feed
28652	6.06	0.04	0.04	Hog feed
28680	6.43	0.07	0.07	Hog feed
28728	7.03	0.15	0.09	Hog feed
28662	2.76	0.46	0.03	Dairy & cattle feed
28664	2.61	0.03	0.03	Dairy & cattle feed
28889	2.09	0.02	0.02	Dairy & cattle feed
28897	5.38	0.06	0.06	Dairy & cattle feed
29219	1.81	0.20	0.03	Dairy & cattle feed
29671	2.84	0.06	0.05	Dairy & cattle feed
28656	4.52	0.02	0.02	All purpose mash
28660	3.84	1.20	0.04	All purpose mash
28942	4.50	0.10	0.02	Concentrate
29652	2.45	0.03	0.03	Rabbit feed
29760	1.95	0.04	0.04	Horse feed

* Average of two or more determinations.

ways. Among the variations that have been found satisfactory are: The addition of calcium chloride to the Kjeldahl flask during distillation to precipitate the carbon dioxide instead of removing it by boiling the receiving flask; careful adjustment of *p*H value and temperature for the enzyme digestion (which will result in complete digestion of the urea in less than an hour); use of other buffers; removal of urea from the samples by leaching with water; and an increase in size of sample to improve checkability.

The results presented in Table 2 show the recovery of known amounts of urea nitrogen added to selected samples. The samples were selected from those listed in Table 1 on the basis of variety, and uniformity of results obtained. Results were corrected for the amount of urea and ammoniacal nitrogen shown in Table 1. The total nitrogen figures in

Table 2 were obtained by the regular Kjeldahl method, using tablets prepared by the Mill Creek Products Company of Kansas City as a catalyst.

TABLE 2.—*Determination of known amounts of urea nitrogen added to feeds*

SAMPLE	TOTAL NITROGEN*	GRAMS NITROGEN ADDED AS UREA		
		0.050	0.020	0.010
			grams recovered	
28650 Poultry feed	3.21	0.050	0.020	0.010
28656 All purpose mash	4.54	0.050	0.020	0.010
28756 Poultry feed	3.10	0.050	0.020	0.010
28897 Dairy & cattle feed	5.36	0.051	0.021	0.011
28935 Linseed meal	5.54	0.050	0.020	0.011
29205 Cottonseed meal	6.66	0.050	0.020	0.011
29208 Alfalfa	3.14	0.050	0.020	0.011
29209 Wheat shorts	2.89	0.050	—	—
29216 Cottonseed meal	6.84	0.050	0.021	0.011
29226 Alfalfa	2.99	0.050	0.021	0.010
29252 Wheat shorts & scrgs.	2.70	0.050	0.020	0.010
29356 Soybean meal	6.85	0.050	0.021	0.010
29440 Soybean meal	7.03	0.050	0.021	0.011
29456 Alfalfa	2.49	0.051	0.021	0.011
29458 Bone meal	2.71	0.050	0.020	0.010
29477 Alfalfa	2.40	0.051	0.021	0.010
29537 Corn gluten feed	7.10	0.050	0.021	0.011
29574 Poultry feed	3.58	0.050	0.020	0.010

* Average of two or four determinations.

SUMMARY AND CONCLUSIONS

Miscellaneous mixed feeding stuffs have been found to yield an average of 0.07 per cent nitrogen as determined by a method previously proposed for urea and ammoniacal nitrogen. It is proposed that a tolerance of 0.20 per cent nitrogen in miscellaneous feed samples is sufficient when testing for additions of urea to feeding stuffs.

A revision of the proposed method is presented and has been found to give excellent results in determining the addition of known amounts of urea to 18 feeds. It is recommended that the proposed method be studied by the A.O.A.C. with the possibility of its adoption as an official method.

REFERENCES

(1) *This Journal*, **34**, 450 (1951).
(2) *Methods of Analysis*, 7th Ed., 1950, p. 345 (22.22). *This Journal*, **27**, 89 (1944).

ACIDITY AND TOTAL SOLIDS MEASUREMENTS IN CONDENSED MILK BY-PRODUCT FEEDS*

By ARA O. CALL and R. F. VAN POUCKE (Western Condensing Company, Appleton, Wis.)

The determination of water is one of the most frequent analyses made by the chemist. Whether the material being analyzed is relatively dry and the moisture content is expressed as such or whether the material is fluid in nature and the results are expressed as solids, the analyst is in both cases measuring water content. It would seem relatively simple to measure the water content of a sample, but many materials present problems, and unless the method is properly adapted the results obtained are purely empirical. The concept that moisture is the loss in weight occurring when a material is heated to the temperature of boiling water was set forth by this organization at one of its earliest meetings more than 65 years ago. With very little modification, this method is still official for many products, although it is recognized that in many cases the results obtained are not the true measurement of water. Such factors as lack of volatilization of all the moisture, volatilization of substances other than water, and the thermal decomposition and volatilization of some samples, can be mentioned as only a few of the sources of error.

The literature is replete with suggested moisture methods. Many of them are merely modifications of the oven drying method, employing different temperatures, vacuum or forced air, etc. Others differ radically, employing entirely different approaches. Of these, one could mention the distillation methods, the Karl Fischer method, electrical techniques, and still others.

In *Methods of Analysis*, 7th Ed. (1950); section on Grain and Stock Feeds (1), four official moisture methods are recognized: I. **22.3**, Drying in Vacuo at 95–100°; II. **22.4**, By distillation with Toluene; III. **22.6**, Drying without heat over Sulfuric Acid; and IV. **22.8**, Drying at 135°. The first method specifies drying to constant weight and reporting the loss in weight as moisture, while the last method arbitrarily calls for a two-hour drying period. All four methods are essentially the same now as when they appeared in the sixth (2) and fifth (3) editions.

In the Dairy Products section, the official total solids method for milk (1), **15.14**, specifies a solids dish of at least 5 cm diameter, a preliminary drying on a steam bath, and three hours drying at 98–100° in an air oven. The residue remaining is reported as total solids. This is the same method given in the sixth edition (2); however, in the fifth edition (3) dry sand could be placed in the bottom of the solids dish before adding the sample. It was then dried for 30 minutes on a steam

* Presented at the Annual Meeting of the Association of Official Agricultural Chemists held in Washington, D.C., Oct. 1–3, 1951.

bath and finally dried to constant weight in a 100° oven, preferably vacuum. The total solids method for sweetened condensed milk, **15.84**,—in physical characteristics somewhat similar to some of the condensed milk by-product feeds—specifies that dry sand or asbestos fiber is to be placed in the bottom of the solids dish and, after preliminary drying, the sample is heated at 100°, with vacuum, until constant in weight. In the sixth edition (2), the solids method for sweetened condensed milk was the same as for milk or evaporated milk, and required no sand or asbestos nor a vacuum. In the fifth edition (3) the method called again for added sand or asbestos and drying to constant weight at 100°, preferably in vacuum.

In body and in moisture content some condensed milk by-product feeds may be compared to cheese. The official moisture method for cheese, **15.124**, (1) is more specific, in that it describes the dish to be used, calls for a minimum vacuum for drying, and also specifies that dried air is to be admitted during the drying process. Drying is to be carried on until constant weight is achieved (ca 4 hours).

The use of condensed milk by-product feeds has increased considerably during the past few years. As marketed today, they vary widely in solids content. Some may be composed of only one milk by-product, others may be mixtures of two or more, and still others may have added grains or starches. In general, they may be divided into two major classes: (1) Feeds that are to be readily dispersed in water for slop feeding of swine or in the preparation of wet mashes for poultry. These may vary from 17 to 55 per cent solids. (2) Feeds that are intended to be fed "as is" or free choice. They have a plastic, rather hard body. The solids content may be as high as 75 per cent. Considering that there may be a four-fold variation in solids content in these feeds, it seems especially important that the consumer should be protected with proper labels giving the amount of solids he is buying. This assumes that reliable, recognized methods of analysis are used in measuring the solids.

The selection of the method to be used for determining solids or moisture in these products presents a problem. It could apparently be any one of the four official methods under Grain and Stock Feeds mentioned above—for these products are stock feeds; or it could be one of the methods for determining total solids or moisture in Dairy Products—for most of them could be classified as dairy products or mixtures of dairy products. The reason for so many different methods must be because each has demonstrated its superiority for certain products. All are not equally suitable for every product, and it would be unlikely that the results obtained on condensed milk by-product feeds would be the same with all methods.

In many of these products, especially those high in solids, much of the lactose present is in a crystalline state. In this state it is commonly in the

monohydrate form and the water of crystallization amounts to 5 per cent of its weight. This hydrate moisture is lost rather slowly at 100°, and if the heating time is extended long enough to drive it all off, there may be excessive browning or burning of the sample which would indicate decomposition. Thus we see that the usually accepted moisture methods have their limitations for these products. In making solids determinations as given in the Dairy Products section, the sample is first diluted, and any crystallized lactose is dissolved. If the diluted sample is dried at near 100° the dissolved lactose will crystallize in the beta form, which is anhydrous. Such a sample will come to constant weight much more readily than when lactose monohydrate is present. If the preliminary drying of the diluted sample is done at about 90° or lower the lactose will crystallize as the usual alpha hydrate, and the hydrated moisture will cause trouble again.

In the "Report on Sampling and Analysis of Condensed Buttermilk," given at the 1947 meeting (4), the Associate Referee quoted from a letter written to the Referee on Feeding Stuffs when it was suggested the study be undertaken. The difficulties in obtaining a representative sample were pointed out. Regarding methods, the Associate Referee stated: "The methods provided for solids in milk products should all be studied to find which one is most applicable to condensed buttermilk. We know that there is a variation in results depending upon the method chosen."

The Associate Referee, recognizing that little could be done on sampling until a suitable solids method was available, devoted his time to this phase of the problem. In commenting on the results, he stated that two points were established: "(1) On drying of buttermilk products, a charring of the sample is noted; (2) Lactic acid of buttermilk products decomposes when heated." A sample was submitted to eleven collaborators, who were requested to test the acidity and then determine the solids content at 100° and 70°, both with and without vacuum, and also to test the acidity of the dried residue in each case. The oven moisture determinations were then to be repeated, adding 2 g of zinc oxide to each sample. The collaborators were also asked to determine the solids by the toluene distillation method. The solids values reported varied considerably and the acidities on the dried residues showed wide variation. In most cases higher solids values were obtained when zinc oxide was added. Comments from collaborators indicated that charring of the sample and difficulty in reading endpoints made the analysis difficult; in fact, one collaborator stated: "It seems impractical to dry the type of material submitted to constant weight, in the usually accepted sense of the term, by any of the methods used. . . . " Acidity determinations on the samples to which zinc oxide had been added, made by three of the collaborators, showed that more than half of the original acidity was still present. In summarizing, it was stated: " . . . because of the charring, indicating an oxidation possibility,

and the incomplete neutralization of the lactic acid in the zinc oxide experiment, further work on this problem is contemplated."

The annual reports of Subcommittee A on recommendations of referees for the years 1946 to 1949 each recommended: "That collaborative work on the sampling and analysis of condensed buttermilk be continued." A year ago, at the 1950 meeting, the recommendation was that the studies be discontinued. Apparently nothing was published on the problem after the 1947 report.

Because of the recognized fact that some of the acid in these feeds may be lost in a moisture or solids determination, at the October 1946 meeting of the Association of American Feed Control Officials it was proposed and accepted to preface the Milk Products definitions with a clarifying statement. This appeared, in the 1947 Official Publication (5) of that organization as follows:

"In all official and tentative definitions and standards of this Association for Milk Products, lactic acid (naturally present or formed in the product by a culture or ferment) shall be regarded as part of the total solids, and in methods used for the determination of moisture or solids, this fact shall be taken into consideration."

This same statement appeared in the 1948 (6) and 1949 (7) Official Publications; however, in the 1950 (8) issue it was clarified somewhat, by inserting in place of "lactic acid" the phrase "volatile acids, expressed as lactic, driven off in determining moisture." At their 1950 meeting, the Association of American Feed Control Officials dropped the whole prefatory statement.

Despite the fact that the 1950 Official Publication hoped to clarify the interpretation of the parenthetical statement by substituting the phrase "volatile acids expressed as lactic" instead of "lactic acid," and furthermore, that the 1951 Official Publication (9) dropped the whole statement, many of the manufacturers of Condensed Milk By-Product Feeds have continued to qualify their minimum total solids guarantees with the parenthetical legend "(including lactic acid)."

In the dairy industry in the United States, it is common practice to express the alkali neutralizing powers of dairy products as though the neutralization came from lactic acid. This has some basis, because in most dairy products where acidity has developed, lactic acid is the principal acid present. However, the alkali neutralizing power of the sample may come entirely from other sources. It has become common practice to speak of acidities in dairy products as "——% lactic acid," while technically the expression should be "——% acidity, expressed as lactic acid." Freshly drawn normal milk contains no free lactic acid, and yet the alkali neutralizing powers of the sample show a titratable acidity of approximately 0.16 per cent (as lactic acid). This apparent acidity is due to a number of factors, and unless fermentation has taken place, lactic acid is not one of these. Chiefly among them may be mentioned the ash

constituents of the milk. The phosphates (and to a lesser degree citrates) may be responsible for a major portion of this apparent acidity. It should also be pointed out that phenolphthalein, the indicator used in acidity titrations, shows an endpoint which is considerably on the alkaline side. Such factors as the concentration of the ash constituents, which in turn affect their solubilities, will also affect the apparent acidity. It is widely recognized that the dilution of milk samples with water will result in a decrease in the apparent titratable acidity. Relatively insoluble tricalcium phosphate is not precipitated out at the same rate when its concentration is lessened by dilution, and more of the trivalent phosphates and citrates will be measured in the di-basic, instead of the tri-basic state. Obviously the original ash content, as well as the dilution will affect the determination of titratable acidity of condensed milk by-product feeds. Some of these are high in ash, and in turn, when they are partially delactosed either by fermentation or lactose removal, the non-lactose constituents, which include the mineral content, are concentrated. Thus we see that what may be a small factor in measuring the titratable acidity of milk becomes much more of a factor when applied to highly concentrated, high ash, materials.

Apart from the fact that factors other than lactic acid contribute to the titratable acidity, it is common practice deliberately to develop relatively high acidities in condensed milk by-product feeds. This developed acidity adds to the palatability of such feed products, and also lends much to their keeping qualities. In most cases the acid produced from these fermentations is lactic, however, in cultured products there may be appreciable amounts of acetic and propionic acids as well. The total alkali neutralizing capacity of such products is the sum of all these combined factors, although for convenience it is expressed as though it came entirely from lactic acid. Of these alkali neutralizing constituents, the minerals are non-volatile in the solids determination, the acetic and propionic acids are very volatile, and the lactic acid is intermediate. The sour odor common to cultured milk products is usually due to acetic and/or propionic acids. There may also be traces of other fermentation products. For a more complete treatment on the topic of acidity of milk and dairy products, reference is made to the chapter and bibliography on this subject in the book, "Market Milk and Related Products" by Sommer (10).

There is an official method for the determination of lactic acid *per se;* **15.8,** (1). It is, however, a laborious procedure and may require several days before trustworthy results can be obtained. Furthermore, it must be recognized that although the method is for "lactic acid" it will measure any lactic acid salts which may be present. There is no provision for distinguishing between lactic acid *per se* and lactic acid which may have been neutralized in the original sample.

It is recognized that there may be significant losses of volatile, non-wa-

ter substances when high acid condensed milk by-products are tested for solids. It appears that modifications should be made to reduce such non-water losses to a minimum.

Thus, in preliminary trials in our laboratories, three different condensed milk by-product feeds, as they appear on the market, were tested for solids by the accepted vacuum oven method. They were then neutralized and tested. The procedure was to weigh the sample into a solids dish, add 2 drops of phenolpthalein indicator (1 per cent) and titrate to the pink endpoint with 0.1 *N* NaOH. A correction was then applied for the sodium added, by subtracting .0023 g for each ml of 0.1 *N* NaOH required. Table 1 gives the solids results found (average of triplicate determinations). It is

TABLE 1.—*Comparison of unneutralized and neutralized values for solids*

	UNNEUTRALIZED	NEUTRALIZED
	per cent	*per cent*
Sample A	34.9	36.2
Sample B	56.4	58.4
Sample C	70.5	72.3

interesting to note the increased solids values when the neutralization step was used.

These same three samples were tested for solids by the toluene distillation method. In the bottom of each flask 50 to 75 g dry sand was first added to minimize burning. A 25 g sample and a 25 ml modified Dean-Stark distilling tube receiver (Kimble 22011) was used. Titratable acidities, expressed as lactic, were made on all samples, and the acidity of the condensed, trapped moisture was also determined. The results are given in Table 2.

TABLE 2.—*Solids by toluene distillation*

	TITRATABLE ACIDITY (AS LACTIC)	SOLIDS 1 HR. TOLUENE DISTILLATION	ACIDITY OF DISTILLATE*
	per cent	*per cent*	
Sample A	4.0	36.0	7.9
Sample B	4.2	56.0	14.6
Sample C	7.2	69.6	8.4

* Expressed as ml of 0.1 *N* NaOH per 25 g sample.

It is obvious that some acids present in the samples are volatilized, condensed, and dissolved in the trapped water. Variations in distillate acidity are probably caused by varying proportions of the different acids. The buffer capacities of the samples could also vary and this would influence the titratable acidities. The solids values are only in fair agreement with those obtained by the oven method.

These results are only preliminary. The neutralization method mentioned above, in our opinion, merits further study. Recognizing that the solids values obtained may vary considerably, depending on the method used for the determination, it seems imperative that an officially recognized method for these products should be developed. A collaborative study on the determination of solids in condensed milk by-product feeds by this Association is highly desirable and any effort to this end will be welcomed by manufacturers and control officials alike.

REFERENCES

(1) *Methods of Analysis*, 7th Ed., (1950).
(2) *Methods of Analysis*, 6th Ed. (1945), p. 307.
(3) *Methods of Analysis*, 5th Ed. (1940), p. 270.
(4) *This Journal*, **30**, 613–616 (1947).
(5) Official Publication, Amer. Feed Control Officials, Inc., p. 28 (1947).
(6) *Ibid.*, p. 26 (1948).
(7) *Ibid.*, p. 26 (1949).
(8) *Ibid.*, p. 29 (1950).
(9) *Ibid.*, p. 34 (1951).
(10) *Market Milk and Related Products*, Hugo H. Sommer, 2nd Ed., 643–660 (1946), Published by the author, Madison, Wis.

DETERMINATION OF COPPER AND ZINC IN FERTILIZER WITH THE POLAROGRAPH*

By NOAH J. HALBROOK (Chemical Division, Florida State Department of Agriculture, Tallahassee, Florida.)

In the last several years the increasing number of secondary materials added to commercial fertilizer has led to a search for methods of analysis which would be as reliable as those in present use, and less time consuming. Within certain limitations, polarography, which is a micro electroanalysis, lends itself very well to such determinations. The polarographic waves must be separable and the solution must be made free of interfering substances, either by formation of complexes or by previous removal. Waves are reproducible within ± 0.5 mm; this error is mostly due to the geometry of the determination of wave height. The analysis does not destroy the sample; after one hundred successive determinations, less than one per cent of the sample is lost.

The copper method presented here is both rapid and accurate. No interference was experienced from materials ordinarily found in fertilizer. The method for zinc is equally reliable except in the presence of cobalt, which is not likely to be encountered, and therefore should not prove a serious limitation to the method. A rapid qualitative test, the Jones-Tasher reaction (1) is made to determine the presence or absence of cobalt. If

* Presented at the Annual Meeting of the Association of Official Agricultural Chemists held at Washington, D.C., Oct. 1–3, 1951.

cobalt is present, it must be removed by standard chemical means, or determined in the final solution and the proper corrections made to the height of the zinc wave.

Reed and Cummings (2, 3) used the polarograph for the determination of copper and zinc in plant materials. They tested their method by adding large concentrations of materials ordinarily found in plants, but no difficulty was encountered except in certain special cases. Cobalt interfered if its concentration was 1×10^{-5} grams per ml. or greater.

SPECIAL APPARATUS

The voltage curves were obtained with the Sargent-Heyrovsky Model XII polarograph. The galvanometer calibration factor as furnished by the manufacturer was 0.0049 microampere per millimeter. To insure accuracy, samples and standards were brought to room temperature, and the wave heights of the standards were determined at the same time the samples were run. The maximum deviation with increase in zinc and copper concentration and change in sensitivity settings from 5 to 10, 10 to 20, and 20 to 50 was 0.5 mm. This is considered within the geometrical error of measuring wave height. It was found that the best results were obtained with a drop time of from 2 to 2.5 seconds as the curves were neater and the accuracy in measurement was greatly improved. The cell used was similar to that designed by Heyrovsky.

PROCEDURE

A 2-gram sample of fertilizer is digested in a 200 ml wide mouth volumetric flask with 10 ml of concentrated sulfuric acid and 10 ml of concentrated nitric acid to dense white fumes. If the solution becomes dark from organic matter, cool, add a little more nitric acid and digest again to dense white fumes, repeating the operation if necessary until the organic matter is destroyed. The solution is cooled, 50 ml of water is added and boiled for one minute. The solution is then made to volume and allowed to clear by settling. From it, aliquots may be taken for manganese, magnesium, copper, and zinc. For the copper and zinc determinations, a 25 ml portion is pipetted into a 50 ml volumetric flask, made to volume with concentrated ammonium hydroxide, mixed and allowed to settle. A portion of this second dilution, 10 or 20 ml, depending on the copper or zinc content, is pipetted into a scond 50 ml volumetric flask. To this flask, a pea-sized lump of sodium sulfite, 5 ml of normal potassium chloride, and one ml of 0.2 per cent gelatin are added; it is then made to volume and mixed. The polarograms for both copper and zinc are recorded from this solution. Nitrogen is used to sweep the cell free of oxygen just prior to recording the polarograph.

EXPERIMENTAL RESULTS AND DISCUSSION

The wide percentage range over which copper and zinc are incorporated into fertilizer necessitated that an examination first be made of the relation between limiting current and concentration. Walkley (4) made such a study, using a basal solution of 0.1 molar ammonium chloride, 0.02 molar potassium thiocyanate and 0.0002 per cent methyl red. He found

that the relation between current and zinc concentration was linear to within about ±1.5 per cent over a 100-fold concentration range.

For ease in calculating per cent guarantee, solutions were standardized so that a 10 p.p.m. final dilution was equivalent to a 1 per cent guarantee of copper and zinc oxide. On this basis, solutions varying from 0.1 per cent to 10 per cent held to a linear relationship. The deviation from linearity was approximately ±2 per cent of the total, which falls within the probable error in measuring wave height.

Ferric iron also interferes with the copper determination; therefore it must be eliminated quantitatively. This is accomplished without appreciable loss of copper or zinc by adding an equal volume of concentrated ammonium hydroxide to the acid solution of the digested sample. To test the suitability of this reaction, determinations were made on standards with and without addition of a compensating solution containing all the materials commonly found in fertilizer. Wave heights were apparently the same for both solutions.

The addition of sodium sulfite to the ammoniacal solution removes dissolved oxygen and eliminates the necessity of bubbling with hydrogen or nitrogen. Once the sample solutions are prepared a polarographic wave can be recorded in approximately two minutes.

TABLE 1.—*Comparison of A.O.A.C. and polarographic methods for the determination of copper and zinc oxide*

SAMPLE NO.	COPPER OXIDE			ZINC OXIDE		
	FOUND A.O.A.C.	FOUND POLAROGRAPH	DIFFERENCE	FOUND A.O.A.C.	FOUND POLAROGRAPH	DIFFERENCE
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	0.54	0.58	+0.04	0.70	0.71	+0.01
2	0.60	0.60	0	0.64	0.66	+0.02
3	0.66	0.66	0	0.55	0.53	−0.02
4	0.35	0.35	0	0.30	0.29	−0.01
5	0.42	0.42	0	—	—	—*
6	0.42	0.36	−0.06	0.32	0.20	−0.03
7	0.71	0.68	−0.03	1.01	0.98	−0.03
8	0.26	0.28	+0.02	0.18	0.19	+0.01
9	0.26	0.28	+0.02	0.29	0.27	−0.02
10	0.30	0.29	−0.01	0.44	0.44	0
11	0.31	0.31	0	0.52	0.50	−0.02
12	—	—	—*	5.78	5.80	+0.02

* *Not guaranteed—not determined.*

The final ammonium hydroxide concentration in these experiments varied from about 1.6 to 3.2 molar without apparent effect on the wave height. Such a wide tolerance indicates that the method should be adaptable to other types of material.

In order to test the accuracy of the method presented, nineteen samples

chosen at random were analyzed by the official A.O.A.C. methods (5) and the polarographic method. Results on twelve of the samples, including those of greatest deviation, are shown in Table 1. The average deviation was found to be 0.02 per cent for copper and 0.02 per cent for zinc. This close agreement was found in samples having widely varying guarantees of iron, manganese, magnesium and boron.

TABLE 2.—*Polarographic determination of copper and zinc oxide by addition of a known percentage to fertilizer*

SAMPLE NO.	COPPER OXIDE			ZINC OXIDE		
	CuO FOUND	FOUND (0.42% ADDED)	DIFFERENCE	ZnO FOUND	FOUND (0.42% ADDED)	DIFFERENCE
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	0.57	0.97	−0.02	0.71	1.13	0
2	0.56	0.98	0	0.67	1.11	+0.02
3	0.36	0.77	−0.01	0.25	0.70	−0.03
4	0.37	0.78	−0.01	0.34	0.74	−0.02
5	0.29	0.70	−0.01	0.17	0.59	0
6	0.29	0.71	0	0.27	0.67	−0.02
		(0.90% added)			(1.00% added)	
7	0.60	1.60	0*	0.56	1.56	0
8	0.36	1.26	0	0.35	1.34	−0.01
9	0.50	1.41	+0.01	0.86	1.84	−0.02
10	0.53	1.44	+0.01	0.48	1.48	0
11	—	—	—	5.80	6.80	0
12	0.33	1.23	0	0.50	1.48	−0.02
13	0.27	1.20	+0.03	0.38	1.36	−0.02

* 1.00% CuO added.

The data for Table 2 were obtained by taking aliquots from the digested samples and to one adding 5 ml of a solution containing known amounts of copper and zinc. The samples were then analyzed according to the procedure, beginning with "made to volume with concentrated ammonium hydroxide." The average deviation between the amount added and found was about 0.01 per cent for both copper oxide and zinc oxide.

Figure 1 illustrates typical curves obtained with fertilizer material. The wave heights for the zinc are evaluated by measuring at the half wave potential the vertical distance between a straight line extension of the residual current and a parallel line constructed through the limiting current. The slope lines are constructed through the center of the oscillation amplitudes. Copper oxide is calculated from the wave height of the second copper wave. This height is determined by a straight line extension of the limiting current line and a perpendicular to the mid-point between the first and second copper wave.

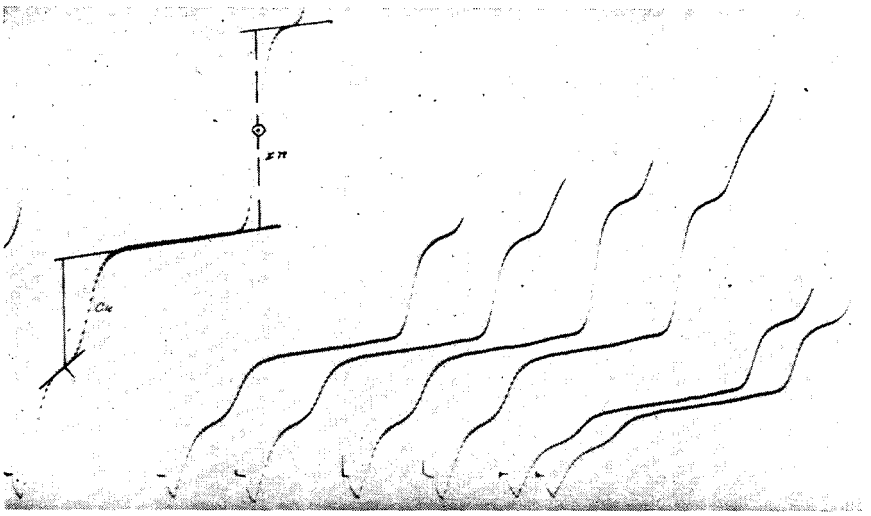

FIG. 1.—Typical copper and zinc waves in commercial fertilizer material illustrating the method of measuring wave height.

SUMMARY

A procedure has been proposed for the polarographic determination of copper and zinc in fertilizer. Aliquot portions of the sample are made strongly alkaline with ammonium hydroxide to remove iron. This also brings the copper and zinc ammonium complexes into solution. Sodium sulfite is added to the final dilution to remove oxygen and this eliminates long periods of bubbling with an inert gas.

No interference is offered by any of the anions or cations likely to be found other than cobalt, the absence or presence of which is determined by a simple qualitative check.

The proposed procedure gives excellent results, and, because of the time-saving factor, is especially valuable in routine analysis.

ACKNOWLEDGMENT

The author wishes to acknowledge his indebtedness to A. H. Gropp, University of Florida, for his continued advice and assistance in making this study possible.

REFERENCES

(1) JONES, H. O. and TASHER, H. S., *J. Chem. Soc.*, **95**, 1904 (1909).
(2) REED, J. F., and CUMMINGS, R. W., *Ind. Eng. Chem. Anal. Ed.*, **12**, 489 (1940).
(3) ——, and ——, *Ibid.*, **13**, 124 (1941).
(4) WALKLEY, A. J., *J. Am. Chem. Soc.*, **63**, 2278 (1941).
(5) *Official Methods of Analysis, A.O.A.C.*, 7th Ed. (1950), pp. 24–26.

CHROMATOGRAPHIC DETECTION OF GRAPE WINES IN THE PRESENCE OF OTHER WINES

By PAUL W. SIMONDS and M. J. PRO (Alcohol and Tobacco Tax Division, Laboratory Branch, Bureau of Internal Revenue)

The object of this work was to continue the study of the chromatographic detection of grape wines. Hamill and Simonds (1) demonstrated that when grape, apple, and cherry wines are adsorbed on alumina a yellow fluorescence which distinguishes them from blackberry wine is produced. However, these authors encountered difficulties in the elution and purification of these fluorescent compounds for identification purposes. Therefore, by means of paper chromatography, efforts were directed toward isolating the yellow fluorescent component of these wines in a relatively pure state. In the course of experiments leading to partial accomplishment of these objectives, a new type of paper strip and method for depositing samples was developed.

EXPERIMENTAL

Paper strips of varied shapes were tried and the shape illustrated in Figure 1 was found the most practical for this type of work. Attainment of maximum area for sample deposit necessitated fabrication of a paper strip with a broad base as shown in Figure 1. Construction of a narrow shaft facilitated concentration of the components being developed, and prevented contact of the paper with the cylinder walls. Deposition of the wine material on a specific area of the paper strip was accomplished by floating normal butanol on the aqueous phase. The butanol also performed the function of moving the fluorescence characteristic of the paper to a designated mark on the shaft.

Development of the deposited material required a solvent or solvent system that would move the anthrocyanins and fluorescent components up the strip at different rates. It was also important to keep the sugars and pectins behind as completely as possible. It was found that a definite mixture of aqueous saturated alum solution and absolute ethanol was sufficient to attain separations. The fluorescent component was found to be immobile in absolute ethanol, and the anthrocyanins were mobile in water and hydroxylic solvents. The alum-alcohol system moved the anthrocyanins at a faster rate than the fluorescent component and enabled the bulk of each to separate fairly well. Processing of the developed strip in ammonia vapors indicated that the fluorescent component had formed a yellow band at the lower portion of the shaft.

METHOD

REAGENTS

(1) *Normal butyl alcohol.*

(2) *Saturated aqueous potassium aluminum sulfate.*

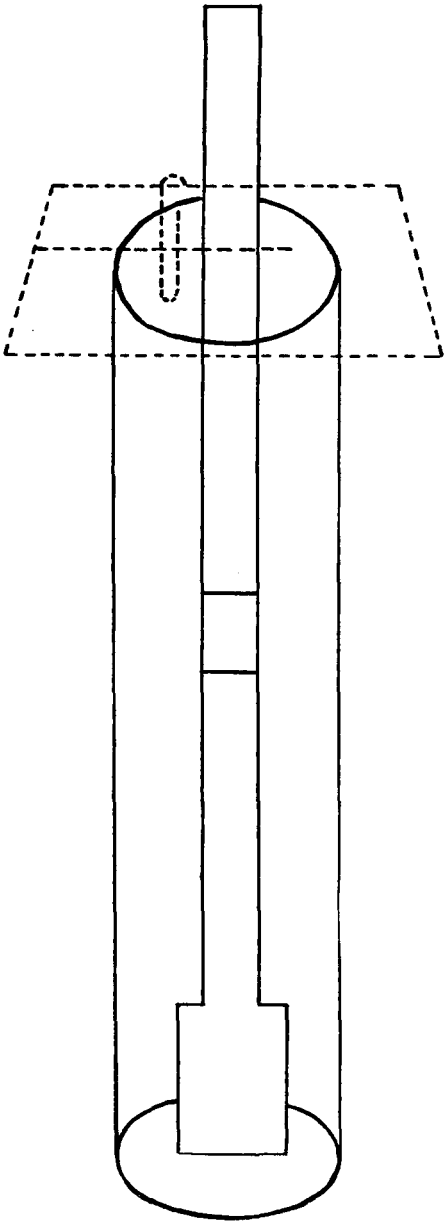

FIG. 1.—Chromatographic Set-up.

(3) *Absolute ethanol.*
(4) *Concentrated ammonium hydroxide.*

APPARATUS

(1) *Ultraviolet equipment.*—Lamp made by Hanovia Chemical and Manufacturing Company.* 500 watt quartz-mercury tube. Red-purple glass filter No. Sc. 5024.

(2) *Glass Cylinders.*—About 19.5 cm high and 2.7 cm in diameter.

(3) *Glass Container.*—Wide mouth type, about 500 ml capacity.

(4) *Paper Strips.*—Spade-shaped strips of Whatman No. 1 filter paper with shaft 1.0×19.5 cm and blade 2.5 cm long by 2.5 cm wide. Make a pencil mark on the shaft at 9.5 cm and 11.0 cm from the lower edge of the blade.

(5) *Paper Card.*—A stiff card 7.5×3.0 cm; in it cut a 5.0 cm slit lengthwise from the center of one edge.

PROCEDURE

Dilute a sample of wine with an equal volume of water, and place 10 ml into a glass cylinder. Float 5 ml of normal butanol on the diluted sample. Proceed similarly with a standard blackberry wine as a blank. Insert a paper strip into the slit of the paper card and adjust until the lower edge of the blade barely touches the bottom of the tube when the card is flat across the mouth. The slit of the paper card should be kept closed with a paper clip. It is important that no part of the strip touches the sides of the glass cylinder.

Allow to stand until the butanol rises to the 11.0 cm pencil mark. At this time the sample will have formed a deposit at the lower portion of the butanol phase. Remove the strip and quickly blot the section below the deposit, if visible, with paper toweling. Dry the strip under a light bulb, or by other means but do not exceed a temperature of 80°C.

In a dry glass cylinder place 2 ml of saturated aqueous potassium aluminum sulfate solution and 3 ml of absolute ethanol. Mix thoroughly, and insert the strip as previously directed. Allow absorption to the 9.5 cm pencil mark, remove, blot, and dry under the same conditions.

Suspend the strip for three minutes in a wide mouth type bottle which contains 15 ml of concentrated ammonium hydroxide. Do not allow the strip to touch the liquid. Observe under ultraviolet light. If grape wine is present a yellow fluorescent band will be seen just above the enlargement of the strip. (In some cases the band location and size will exhibit a slight variation.)

CONFIRMATORY TEST FOR GRAPE WINE

Repeat the above procedure, using 3.5 ml of standard blackberry wine and 1.5 ml of suspected wine. A yellow fluorescent band confirms the presence of grape wine.

DISCUSSION

Numerous samples of standard wines prepared in the laboratory by Peter Valaer, Jr., were evaluated for yellow fluorescence by the method. The wines giving no yellow fluorescence were raspberry, blackberry, nectarberry, youngberry, gooseberry, prune, muscadine, pomegranate, papaya, fig, currant, and strawberry. Peach, cherry and apple wines developed a characteristic yellow fluorescent band, which was, however, much less intense than those exhibited by numerous types of grape wines.

* (or equivalent ultraviolet source).

The fluorescent bands exhibited by the grape wines ranged from chrome to cadmium yellow, while those of peach, cherry, and apple were faint brown-yellow. Since the alum-alcohol developer was not entirely adequate for the exclusive detection of grape wines, it was hoped that adjustment of the *p*H before depositing might eliminate trace fluorescence specific to peach, cherry, and apple wines.

Adjustment of the *p*H from highly acid to highly basic conditions with hydrochloric acid and ammonium hydroxide offered no significant improvement. A *p*H below 3.5 accentuated the fluorescence of peach, cherry, and apple wines, while a decidedly alkaline condition accentuated that of grape.

Since the smallest detectable amount of grape wine in very dark blackberry wine was found to be about 3 per cent, and peach, cherry, and apple wines of the same dilution were non-detectable, it was reasoned that the fluorescence of the latter wines could be eliminated by dilution with the blackberry wine used for the blank. Three parts of standard peach, cherry, or apple wines that exhibit a positive test by the Hamill-Simonds method (1) were diluted with seven parts of light blackberry wine; all failed to develop yellow fluorescent bands. The loss of their fluorescence due to dilution introduced a desirable confirmatory identification of grape wine. However, the lower limit of detection for grape wine was decreased three times.

SUMMARY

1. A new form of paper strips and method of preparing samples for development was devised.

2. Concentrations of about 3 per cent grape wine in the presence of dark blackberry wine could be detected.

3. By dilution, grape wine can be distinguished from peach, cherry and apple wines when at least 10 per cent grape wine is present.

REFERENCE

(1) HAMILL, G. K., and SIMONDS, P. W., *This Journal*, **25**, 220 (1942).

THE DETERMINATION OF FAT IN BAKED BISCUIT TYPE OF DOG FOODS*

By ERNEST F. BUDDE (The Quaker Oats Company, Research Laboratories, Chicago, Ill.)

The official methods of analysis for grain and stock feeds are found in chapter 22 of *Methods of Analysis*, 7th Ed., 1950. Dog foods, although not specifically mentioned there, are generally considered to be subject to

* Presented at the Annual Meeting of the Association of Official Agricultural Chemists held at Washington, D. C., Oct. 1–3, 1951.

these same methods. The only method for the determination of fat given in this chapter, with the exception of one specified for use on fish meal, is the direct ether extraction procedure, sometimes also referred to as the Bailey-Walker method (1). If the latter method is employed on some types of commercial dog foods, very low results are obtained.

Generally speaking, dog foods may be classified into three types:

1. The cooked canned type of product, containing cereal products, fresh meat, etc. This product, after being packed into tin cans or glass jars, is sealed and cooked in the container by autoclaving. It usually contains somewhere around 70 per cent moisture.

2. The dry mixture type of product, containing cereal products, meat meal, etc. The various ingredients are dry mixed, without further processing. Sometimes, however, the mix is cubed or pelletted and this results in a change of physical but not chemical properties.

3. The baked biscuit type of product, containing ingredients similar to the dry mix type. Water is added to the mix, forming a dough which is rolled into sheets which are baked in gas fired ovens at temperatures of around 550°F.

The difficulty in fat determination occurs primarily in the baked type of dog food. Similar products for human consumption have already been recognized to yield excessively low fat values when subjected to direct ether extraction. For this reason the Association of Official Agricultural Chemists adopted as official, for baked cereal products, procedures involving hydrolyzing with acid prior to solvent extraction (2).

The development of an acid hydrolysis method was described in 1923 by Hertwig (3), who pointed out that direct ether extraction of alimentary pastes and bread gives fat values considerably lower than are obtained by extracting the combined ingredients in these products. He postulated that ether fails to penetrate the hard glutinous particles and unbroken plant cells sufficiently to extract all the fat. Hydrolysis of the proteins and starch resulted in an easier extraction of the fat with ether.

What is true for bread, alimentary pastes, and other baked cereal products for human food is apparently also true for the baked type of dog

TABLE 1.—*Comparison of fat content of different types of dog food by two methods*

TYPE OF DOG FOOD	NO. OF SAMPLES	THEORETICAL FAT CONTENT CALCULATED FROM *E.E.* FAT OF INGREDIENTS	AVERAGE PER CENT FAT FOUND IN PRODUCTS BY		PER CENT OF THEORETICAL FAT VALUE	
		per cent	*E.E.*	*A.H.*	*E.E.*	*A.H.*
Cooked	14	6.2	5.6	6.6	90.4	106.3
Dry mixed	117	4.1	4.0	5.4	97.5	131.8
Baked	153	4.0	2.2	5.0	55.0	125.0

food. Table 1 gives the summarized results of fat determinations by the two methods, ether extraction (*E.E.*) and acid hydrolysis (*A.H.*), on the three types of dog food products.

The figures show that while ether extraction gives fat values as good or better than acid hydrolysis for the first two types of dog food, it falls far short of accounting for the fat present in the baked biscuit type of product. In this respect the acid hydrolysis values come closer to giving the true fat content, although here the results are decidedly on the high side, being 125 per cent of the calculated value. Ether extraction values were only 55 per cent of the calculated fat content.

Randle has reported earlier on "Fat in Cooked Animal Feed Containing Cereals" (4–7). His last report recommended "that the acid hydrolysis method be not adopted for the determination of fat in cooked animal feeds containing cereals." Our results confirm his conclusion in regard to the cooked canned type of product. Furthermore, we would include the dry mix type in that recommendation. For the baked type of dog food, however, the ether extraction procedure is unsuitable (Table 1). A method that would give results closer to the true fat value than those obtained with either of the methods would be desirable.

Schall and Thornton (8) calculated the true fat content in both the ether extract and the acid hydrolyzate extract of a canned (cooked) dog food on the basis of the glycerol content. The ether extract was found to be 98.8% true fat, the acid hydrolyzate extract only 68.5%. Approximately 30% of extract by acid hydrolysis in this case appeared to consist of materials other than fat. However, the authors concluded that their study did not necessarily prove that all the fat present in the sample was recovered by ether extraction.

The same acid hydrolysis method was employed by Randle and his collaborators, by Schall and Thornton, and by us for the data in Table 1. The procedure is found in Section **13.19** of *Methods of Analysis*, 7th Ed., 1950. Randle experienced some difficulty in adapting this method to dog foods. He found that the hydrolyzed material did not settle below the level of the Röhrig tube side arm after the ethers had been added and this resulted in some non-fatty material draining from the tube. He suggested that this difficulty might be minimized if Mojonnier tubes were used and the material centrifuged. These tubes along with the Mojonnier hand centrifuge have been used in our laboratory over the past 15 years with good results. Schall and Thornton found it "difficult to duplicate results unless a standard method of shaking was adopted. The instructions given in the method are not specific on this point. When the samples were shaken vigorously for a three minute period after each addition of solvent, it was found that consistent results could be obtained." Our experience confirms these observations.

In an effort to further improve on the results obtained from the use of

TABLE 2.—*Analytical results on dog food mixes before and after laboratory baking*

SAMPLE	PER CENT FAT						PER CENT MOISTURE
	ETHER EXTRACT		ACID HYDROLYSIS		ACID HYDROLYSIS RE-EXTRACTED EXTRACT		
Mix A, raw before baking	3.18		3.90		3.43		
	3.16		3.76		3.42		
	3.15		3.99		3.25		10.4
	3.16		4.01		3.53		
Av.		3.16		3.91		3.41	
Mix A, laboratory baked	1.49		4.34		3.85		
	1.37		4.31		4.17		
	1.14		4.48		4.09		0.4
	1.20		4.40		4.05		
Av.		1.30		4.38		4.04	
Mix B, raw before baking	3.32		4.11		3.82		
	3.39		3.98		3.42		
	3.37		4.06		3.55		10.2
	3.21		4.01		3.54		
Av.		3.32		4.04		3.58	
Mix B, laboratory baked	1.85		4.37		4.13		
	1.86		4.32		4.12		
	1.46		4.37		3.89		1.5
	1.60		4.60		4.17		
Av.		1.69		4.41		4.08	
Mix C, raw before baking	2.51		3.48		2.49		
	2.43		3.60		2.27		
	2.49		3.51		2.69		10.4
	2.57		3.57		2.55		
Av.		2.50		3.54		2.50	
Mix C, laboratory baked	1.42		3.51		3.22		
	1.28		3.54		3.06		
	1.15		3.65		3.18		2.2
	1.38		3.55		3.26		
Av.		1.31		3.56		3.18	
Mix D, raw laboratory mixed before baking	3.01		4.03		3.61		
	3.13		4.15		3.49		
	2.89		3.96		3.54		11.0
	3.09		4.22		3.36		
Av.		3.03		4.09		3.50	
Mix D, laboratory mixed and baked	1.61		4.43		3.86		
	1.49		4.08		3.71		
	1.39		4.14		3.63		2.0
	1.51		4.19		3.76		
Av.		1.50		4.21		3.74	

the acid hydrolysis procedure on baked dog foods, it was thought that re-extraction of the original extract might bring the fat values more in line by removing some of the non-fat materials. This was done by dissolving the dried extract in 10 ml of ethyl ether and filtering and washing on filter paper with four additional 10 ml portions of the solvent. The filtrate was evaporated and the fat dried and weighed as before. This method is hereafter referred to as the acid hydrolysis re-extracted extract procedure. The official fat method for fig bars and raisin-filled crackers in section **13.96** (1950), makes use of a similar procedure of re-extraction.

A study of the three methods, direct ether extraction, acid hydrolysis, and the acid hydrolysis re-extracted extract procedure, was conducted on samples of formula mixes representative of commercial baked dog food production. Mixes A, B, and C (in Table 2), obtained from three different manufacturers, were taken from their regular production lines at the dough mixer, before the water was added. Mix D was carefully composited in the laboratory from ingredients previously analyzed for fat by direct ether extraction. Its calculated fat content based on these ingredient fat values was 3.10%. Each of the four raw mixes was analyzed for moisture and for fat by the three methods. To portions of each raw mix, water was then added in customary amounts. The resulting mass of each was kneaded into a dough, then rolled into sheets and baked for 20 minutes in an oven at 525°F. The baked products were broken up, ground, and again analyzed as before. All determinations were made in quadruplicate for each fat method. Agreement between replicate results was

TABLE 3.—*Average fat results, corrected to original moisture basis, on raw and laboratory baked dog food mixes by three methods*

SAMPLE	PER CENT FAT BY:		
	ETHER EXTRACTION	ACID HYDROLYSIS	ACID HYDROLYSIS RE-EXTRACTED EXTRACT
Mix A			
Raw	3.16	3.91	3.41
Baked	1.17	3.94	3.64
Mix B			
Raw	3.32	4.04	3.58
Baked	1.54	4.02	3.72
Mix C			
Raw	2.50	3.54	2.50
Baked	1.20	3.26	2.91
Mix D			
Raw	3.03	4.09	3.50
Baked	1.36	3.82	3.40

fairly good and left little choice as to the more precise method. (The results of this investigation are given in Table 2.)

Since the laboratory baked products from these mixes had a very low moisture content, fat results for each baked sample must be adjusted to the moisture basis of its mix before results can be evaluated. Corrected average fat values are given in Table 3.

Since the ether extraction fat result of 3.03 per cent on the laboratory prepared raw mix, Sample D, is in good agreement with its calculated fat content of 3.10 per cent, we may assume that the ether extraction fat values obtained on all the dry mixes represent true fat content. We can then calculate the per cent of fat recovered or accounted for by each of the three procedures on the finished baked dog food. These figures are shown in Table 4.

TABLE 4.—*Per cent of true fat content by each of three methods for fat determination on baked type of dog food*

SAMPLE	PER CENT RECOVERY BY:		
	ETHER EXTRACTION	ACID HYDROLYSIS	ACID HYDROLYSIS RE-EXTRACTED EXTRACT
A	37.0	124.7	115.2
B	46.4	121.1	112.0
C	48.0	130.4	116.4
D	44.9	126.1	112.2

Again the results obtained by ether extraction are much too low, averaging only 44.1 per cent of the true fat content in the baked dog food. Both of the other two methods give results which, although too high, more nearly approach the true fat value; those obtained by the acid hydrolysis method average 125.5 per cent and those by the acid hydrolysis re-extracted extract method 114.0 per cent of the true fat content.

Since it is recognized that laboratory baking may not simulate commercial baking practice, another investigation similar to the one described was conducted on the finished product of one manufacturer. Twelve five-pound bags of baked dog food product were taken at random from packaging lines over a period of one week. These were separately analyzed for fat by the three methods, and for moisture.

Samples of the ingredients were composited, in ratios according to the formula, and analyzed for fat by direct ether extraction and for moisture. Unfortunately, this composite was taken only during the first day of the week, however there is no good reason to believe that it would not satisfactorily represent all ingredients used during the week. The results of this study are shown in Table 5.

Direct ether extraction fat in the ingredients composite was 3.60 per

TABLE 5.—*Per cent fat by three methods on commercially baked dog food* (true fat content based on *E.E.* fat value of ingredients is 3.60%)

SAMPLE NUMBER	PER CENT FAT			
	ETHER EXTRACT	ACID HYDROLYSIS	ACID HYDROLYSIS RE-EXTRACTED EXTRACT	MOISTURE PER CENT
1	1.6	4.6	3.5	7.2
2	1.6	4.2	3.7	7.0
3	1.7	4.6	4.0	6.3
4	1.5	4.6	3.8	6.7
5	1.7	4.5	3.8	6.9
6	1.9	4.6	4.1	6.2
7	1.6	4.2	3.5	7.0
8	1.8	4.3	3.8	6.8
9	1.8	4.2	3.9	6.8
10	1.6	4.3	3.5	7.0
11	1.7	4.5	3.6	6.6
12	1.8	4.2	3.9	6.4
Average (as is)	1.70	4.40	3.76	6.7
Average (corrected to 10.0% moisture basis)	1.64	4.24	3.63	10.0

cent, moisture 10.0 per cent. If the average fat results on the baked dog foods are now corrected to the moisture basis of the ingredients composite, we may calculate the per cent fat accounted for by each procedure. Here we find that direct ether extraction again gives the poorest results, accounting for only 45.6 per cent of the true fat content, whereas acid hydrolysis and acid hydrolysis re-extracted extract procedure account for 118 and 101 per cent respectively.

On the basis of these investigations, as well as upon the basis of the experience gained by others working with similar baked products of a cereal base composition, there can be no doubt that direct ether extraction does not give correct fat values on the baked biscuit type of dog food. While results obtained by acid hydrolysis are high, they are nevertheless much nearer to being correct. Re-extraction of the dried acid hydrolysis extract results in further improvement of fat values.

LITERATURE CITED

(1) *Methods of Analysis*, 7th Ed., **22.25**,, p. 346 (1950).
(2) *Ibid.*, **13.72, 13.89, 13.96**, pp. 209, 212 (1950).
(3) HERTWIG, R., *This Journal*, **6**, 508 (1923).
(4) RANDLE, S. B., *Ibid.*, **25**, 864 (1942).
(5) ———, *Ibid.*, **26**, 340 (1943).
(6) ———, *Ibid.*, **28**, 768 (1945).
(7) ———, *Ibid.*, **31**, 610 (1948).
(8) SCHALL, E. D., and THORNTON, M. H., *Ibid.*, **26**, 404 (1943).

BOOK REVIEWS

Insect Resistance in Crop Plants. By REGINALD H. PAINTER. 8 vo., cloth, 520 pp., 67 illus., 25 tabs., Bibliog. Macmillan Co., 60 Fifth Ave., New York 11, N. Y. 1951. $8.50.

The spectacular successes of the newer types of insecticides developed during and after World War II have tended to obscure the fact that there are other ways by which insects may be controlled. Further, there still remain large areas where, because of insects, certain crops can not be grown profitably without the use of biological or cultural control measures.

One of the author's purposes in the preparation of this volume is to be of aid in keeping proper balance between various methods so that insect control may have greatest effectiveness for the least cost to both grower and consumer. Another purpose is to bring together and summarize the hitherto widely scattered literature on the resistance of crop plants to insect attack, as an indication of what has been and what can be accomplished by such means.

Fortunately the author has had a background of some twenty-five years of practical experience in the work as project leader at the Kansas Agricultural Experiment Station. In 1926, when the project was started, it was the only one of its kind at any experiment station. While the major work involved wheat, resistance of corn, sorghums, and alfalfa to a variety of insects was also studied. As a result of personal experience and a study of the literature it has been possible for the author to present in the book some general conclusions regarding insect resistance by plants, some of which are of wide biological interest in connection with the evaluation of the relationship between insect and host plant.

Some idea of the general scope of this work may be gained by a survey of its contents and arrangement. In addition to the introduction (pp. 1–22), there are chapters on the mechanisms of resistance (pp. 23–83), factors that affect the expression or permanence of resistance (pp. 84–120), resistance to insects in wheat (pp. 121–192), resistance to insects in corn (pp. 193–274), resistance to insects in cotton (pp. 275–325), resistance to insects in sorghums (pp. 326–356), resistance to insects in potato (pp. 357–399), methods and problems in breeding for resistance to insects in crop plants (pp. 400–424). In addition to a subject bibliography at the end of each chapter, a selected supplementary bibliography, also arranged by subjects, is given (pp. 425–488), making a total of perhaps several thousand references which form a very comprehensive survey of the literature.

The evidence presented in this book indicates that in a number of cases control by host plant resistance, as measured by yield, compared favorably with control by insecticides even where the latter were satisfactory. Such insects as the Hessian fly on wheat, the grape phylloxera, and the woolly aphid may be satisfactorily controlled by host plant resistance but, so far, not by insecticides. In most problems involving phytophagous insects, the use of insecticides will remain an emergency control measure and, as such, emphatically necessary. It is equally necessary that attempts be made to use more permanent and less costly control methods. It is pointed out that use of insect resistance belongs to such a control program, and has proven especially valuable where the unit of value or margin of profit of a crop is small and the acreage large, particularly in those parts of the world where the individual land holdings are too small to permit economical use of insecticides, or where growers are not familiar with their use.

It is the hope of its author that the ideas presented will stimulate further research along these lines, and if so, they will have served their purpose. The book should have a wide usefulness.

J. S. WADE

Chemical Control of Insects. By T. F. WEST, J. ELIOT HARDY, and J. H. FORD. Frontiers of Science Series. General Editor, Bernard Lovell. Small 12 mo., Cloth, 211 pp., 44 illus., 16 tables. Chapman & Hall, Ltd., 37 Essex Street, London, 1951, 15 shillings.

The authors of this little book have attempted to provide in compact form a survey of the main insecticides now in common use, with especial emphasis on the most modern developments in insect control. They have pointed out that in these days of intensive research a worker in a given field may receive a somewhat blurred and inaccurate impression of the scope of advances in other fields. They have cited as an example the development of the use of DDT during the war. It is to fill a clear cut and definite need that they have prepared this volume, since they feel that real facts regarding these matters should be given more and wider consideration.

As would be expected, their story is indeed an interesting one: some of the insecticides widely utilized today were discovered as possessing insecticidal properties many years ago. For instance, the capacity of pyrethrum flowers to kill insects was recognized back at the beginning of the nineteenth century, and, while accounts of this circumstance may vary, it is almost certain that the first observation was an accidental one. On the other hand, the recent discovery of the insecticidal properties of DDT followed prolonged and well-planned research effort. In addition to detailed consideration of the chemistry of the various substances, the authors compare the methods of using the main insecticides, and they take pains to indicate again and again that the possibilities for still other new useful synthetic compounds are by no means exhausted.

The author's preface, the introduction, and a somewhat general discussion of the problem of insect pest control, are followed by chapters that deal with such subjects as fumigation (pp. 37–47), nicotine (pp. 48–59), rotenone and related compounds (pp. 60–67), arsenical compounds (pp. 68–74), petroleum oils (pp. 75–87), coal tar derivatives (pp. 88–95), miscellaneous insecticides (pp. 96–113), soil insecticides and amendments (pp. 114–125), pyrethrum (pp. 126–138), lethane and thanite (pp. 139–143), chlorinated persistent insecticides (pp. 144–185), repellents and attractants (pp. 186–193), and weed control (pp. 194–199). References at the end of each chapter are made to most useful, recent literature on the respective subjects, and an author index (pp. 200–204), and subject index (pp. 205–211), are provided at the end of the volume. No attempt is made to deal with the biology of insects. However, sufficient has been said to emphasize the diversity of development and reactions to be found within the insect group and to show that only broad generalizations can be made.

(It is the purpose of the Frontiers of Science series, of which this volume is part, to fill the gap in scientific publications between comparatively elementary expositions and the specialist text books. To the person with a background of scientific knowledge these books should make clear the most important, growing points in contemporary science, and should prove of inestimable value to scientists who are trying to find out what is happening in subjects other than their own specialty. Since each book is written by one or more authors who are actively engaged in research in the subject on which it deals, the readers are thus taken to the forefront of scientific progress by authors who live with their subjects and who are in position to give to the text a clarity and accuracy not otherwise obtainable.)

J. S. WADE

The Manufacture of Intermediates and Dyes. By G. H. FRANK. 177 pp. Chemical Publishing Co. Inc., 236 King Street, Brooklyn, N. Y., 1952, $4.00.

This is a very brief discussion of dye manufacturing as done in Britain. The plant and equipment are described in outline. The author describes the processes from the isolation of coal tar products, through the preparation of intermediates to the finished dyes. Emphasis is placed on preparation of intermediates.

Azo dyes, anthraquinone dyes, and sulfur dyes are described. The author does not mention that most of the more complex dyes are actually mixtures of the several components produced in the reactions of the intermediates. Although this fact is well known in the industry, it is rarely mentioned in any publications.

From the standpoint of the chemist in the food, drug, or cosmetic field, the book does not tell very much about the production of the purified dyes of the type certified for use in this country. No mention is made of the fluorescein or triphenylmethane dyes that represent a large portion of the certified colors used. The analytical methods given do not reflect the practices of modern, well-equipped analytical laboratories. The chapter on health hazards in the industry is so brief and incomplete that it might serve to minimize a serious problem.

The book will be of little value to the chemist in a dye plant. It will, however, be of considerable assistance to the student who wishes to obtain an idea of the procedures followed in commercial production of some types of dyes.

The author states in his preface that the book was intended for the student or junior chemist. He has produced a well-written text that can be recommended to those groups.

G. ROBERT CLARK

Advances in Agronomy, Vol. III. Edited by A. G. Norman. x+361 pages. Academic Press, Inc., 125 East 23rd Street, New York 10, N. Y., 1951, $7.80.

Advances in Agronomy, Volume III, follows the general pattern of the two preceding volumes, the first published in 1949 and the second in 1950. Discussions of soil and plant sciences dominate the book, but some attention is given to general agriculture.

The chapter on subsoils is a splendid review of a phase of soil science that deserves more attention that it has had in the past. The authors treat of such varied topics as pan layers, exposed and unexposed subsoils, subsoil fertility, and utilization of subsoil nutrients. "Soil Potassium" deals essentially with chemical problems, but plant relationships are not neglected. The brief review of specific feeding powers of plants for the potassium of soil minerals should be of wide interest. A chapter on management of alfalfa meadows covers soil factors as well as hay production and quality of the crop. The volume contains a chapter on agriculture in England and Wales that emphasizes changes in the countryside of Britain. Soil conservation in the United States is treated on a nation-wide basis.

"Irrigation Agriculture and Soil Research in the United States" is a discussion of advances in this great phase of agronomy. The same may be said of the chapter on the breeding of grasses. Control of nematodes by chemical treatment of soils is fully discussed. Materials, methods, the effectiveness of the various treatments, plants particularly affected by nematodes, and effects of nematodes on other organisms are included.

Several of the chapters are essentially miniature monographs, and provide authoritative information that should be of interest to many agronomists.

M. S. ANDERSON

Fundamentals of Soil Science. C. E. MILLAR and L. M. TURK. x+510 pages. John Wiley and Sons, Inc., 440 Fourth Avenue, New York 16, N. Y., 1951, $5.50.

It is a pleasure to read "Fundamentals of Soil Science" and the title of the book is a fitting one. Many readers will probably agree with the reviewer that this is one of the world's great textbooks on soils. It is more than a textbook too; it is a valuable reference book for investigators in varied lines of the natural sciences. County agricultural agents and high school agricultural teachers should find much helpful material in it. The authors have drawn upon their long and successful experience as teachers, and have made extensive use of the findings of many agricultural institutions. No bibliography is included, but the sources of many items of interest are cited.

The book covers the important themes normally included in a soils course, such as: classification, chemistry, physics, biology, and soil-plant relationships, including the use of fertilizers and lime. The great subjects of soil moisture (including irrigation), soil fertility in its broader aspects, and the nutrition of crops are well handled. A discussion of proper ownership of land is a useful feature that writers of older textbooks on soils have avoided.

It is fitting for a reviewer to point out a few minor features that could perhaps be improved. A reader gets the impression that the tonnage of raw rock phosphate used in the United States is much smaller than is actually the case. The treatment of irrigation in the Eastern States could be improved by inclusion of some additional information.

This is a splendid book, well edited and printed. It should appeal to many readers.

M. S. ANDERSON

The Origin, Variation, Immunity, and Breeding of Cultivated Plants: Selected Writings of N. I. Vavilov. Translated from the Russian by K. STARR CHESTER Crown 8vo., paper, 366 pp., 42 illus., bibliog. Chronica Botanica Co., Waltham, Mass., Stechert-Hafner, Inc., 31 East 10th Street, New York, N. Y. 1951, $7.50.

This book is issued as Volume 13 of the well-known Chronica Botanica series, the International Collection of Studies in Method and History of Biology and Agriculture, edited by Dr. Frans Verdoorn, of the Arnold Arboretus of Harvard University. Fortunately for brevity of citation, this work also can be indicated by its back title, simply, as "Cultivated Plants," by Vavilov.

In addition to an introduction entitled "Plant Breeding as a Science" (pp. 1–13), there are also included in the volume five other sections as follows: "Phytogeographic Basis of Plant Breeding" (pp. 14–54); "The Law of Homologous Series in the Inheritance of Variability" (pp. 56–92); "Study of Immunity of Plants from Infectious Diseases" (pp. 96–168); "Scientific Bases of Wheat Breeding" (pp. 170–313); and "Selected Bibliography of the Basic World Literature on Breeding and Genetics of Wheat" (pp. 315–334). There are also included an author index (pp. 353–357), and an index of plant names (pp. 359–364).

It was the purpose of the author in preparation of these papers to furnish resumes and evaluations of world progress to date, as recorded in the literature, of scientific investigations within the respective subject subdivisions. It must be noted that these are of necessity of varying length, scope and comprehensiveness. However, definite separations and designations have been made according to specific countries over the world of the work performed therein by the respective groups of specialists, and all have been carefully and fully documented by means of the bibliographical sections which follow each paper and by the larger general bibliography. Unfortunately, due to space limitations, the brevity of this notice forbids discussion or even mere enumeration of all the principal subdivisions within the

respective divisions of the subjects covered, however, it can be stated that all have been treated with fullness proportionate to data available and to relative importance.

J. S. WADE

Biological Antagonism. The Theory of Biological Relativity. By GUSTAV J. MARTIN, 64 Figures, 44 Tables; 516 Pages, The Blakiston Company, 1012 Walnut St., Philadelphia—1951, $8.50.

The purpose of this book is to present, in support of the theory of biological relativity, the impressive accumulation of examples of biological antagonism in the fields of biochemistry, immunology, pharmacology, and chemotherapy. This is accomplished in a manner to offer stimulating information and ideas to all biologists, particularly to biochemists and pharmacologists. The reader may be intrigued by the theory presented by the author to explain why proteins show species specificity. A biologist might find how he should proceed to create a new chemotherapeutic agent.

In the introductory chapter on enzymatic inhibition we learn that the effects of biological antagonists depend on concentration and affinity for the enzyme or substrate relative to the concentration and affinity of the natural substrate, enzyme, or coenzyme and that "the effect of a given inhibitor in vivo will be greatest for the tissue containing the inhibited enzyme in the lowest concentration." The scope of application of the principles of enzyme inhibition is very broad; for instance we read, "Toxicology in its broadest aspects is probably no more than a reflection of substrate displacement and enzyme inactivation."

The specific examples of biological antagonism are presented in chapters concerned with pharmacological aspects, the sulfonamides, amino acids and proteins (7 chapters), vitamins and related nutritional factors (6 chapters), purines, lipids, inorganic ions, and miscellaneous antagonists. There is also an interesting chapter on drug resistance and a summary chapter where we find, "The theory of biological relativity states that no single molecular structure possesses a function not shared in some degree by structurally related molecules."

The facts presented are well documented by extensive bibliographies. Structural relationships are illustrated by pages of formulae. For instance, the chapter "Pharmacological Aspects" consists of 56 pages, of which 7 are bibliography and about 11 structural formulae and tables.

The book is well printed except for minor errors in the formulae on pages 427 and 444. The reviewer also noted an incorrect reference on page 457 where the paper of Lightbody and Kleinman on liver arginase is given as authority for the statement that the kidney has increased ability to oxidize alcohol following prior treatment of the animal with alcohol.

R. LORIMER GRANT

Communication of Technical Information. By ROBERT M. DEDERICH. Chemonomics, Inc., 400 Madison Ave., New York 17, N. Y. 1952, $5.00.

The Foreword indicates that this book is written for technical students in universities and colleges. The introduction states that the book is an attempt to orient standard texts on report writing in the business framework. It is not the purpose of this review to state whether these primary goals have been accomplished. This review was made to determine whether the publication is of real value to technical people and administrators in the agricultural-chemical, and related fields.

The last two chapters, devoted respectively to "Writing" and "Conferences,"

give helpful summaries on these subjects. The information presented is not original nor novel. The summaries are similar to those available elsewhere.

The preceding chapters of the text are elementary discussions of communication, technical information, and types of reporting.

It appears unlikely that the publication is of material value to the operating agricultural chemist or his supervisor.

W. B. RANKIN

Baking Science and Technology. By E. J. PYLER. Two volumes. Siebel Publishing Co., 747 W. Jackson Blvd., Chicago, Ill. (1952) xvi+803 pages. Price $15.00.

This comprehensive treatise on the scientific and technological aspects of baking, with emphasis on bread, follows in the foot steps of the preceding editions of "Siebel's Manual for Bakers and Millers." Volume I consists of two principal divisions. Part I, consisting of 188 pages, covers the theoretical considerations of the basic sciences relating to baking, and includes chapters on the carbohydrates, fats and oils, proteins, enzymes, vitamins, yeasts, molds and bacteria. As the author points out, a knowledge of these basic scientific facts is essential for the most profitable application of the practical material. Part II, of 183 pages, deals with the materials used in baking and gives information on the composition of wheat flour, miscellaneous flours, sugars and syrups, plastic baking fats, milk and milk products, egg and egg products, water, salt and yeast foods.

Volume II is composed of three main divisions on practical application. Part III, of 173 pages, deals with baking technology and contains chapters on dough mixing, fermentation, dough make-up, the baking process, rye bread production, bread staling, and physical and chemical testing methods. Aspects of cake baking, part IV, includes 82 pages under chapters on cake ingredients, flavor, cake baking technology, and miscellaneous baked products (doughnuts, pie, and sweet yeast doughs). Descriptions of the highly specialized machines used in commercial bakeries today complete the volume, in part V, of 109 pages on bakery equipment. The coverage is indicated by the chapter headings: flour handling equipment, dough handling equipment, oven equipment, cooling, slicing and wrapping equipment, and bakery sanitation.

This treatise has been prepared with the assistance and review of other authorities. It is well indexed, has good photographs supplementing the text, and an extensive bibliography of 568 references. This should be a good source of reference for the bakery production man, baking chemist, cereal chemist, and members of allied trades.

V. E. MUNSEY