TUESDAY—MORNING SESSION

REPORT ON ENZYMES

By J. W. Cook (Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.), *Referee*

An Associate Refereeship was established last year to develop enzymatic methods for small quantities of insecticides. The use of an enzymatic method for the determination of some organic phosphate and carbamate insecticides appears to be very promising. It seems likely that the method can be made sufficiently versatile to be applicable to food residue amounts of some of the insecticides that are not determinable at that level by other chemical means. As stated last year, it is hoped that at least some specificity can be attained by the inhibition of two different enzyme systems or more, by solubility fractionation in immiscible solvents, or by some other means.

No work was accomplished on methods for testing the activity of the various enzymes used in analytical procedures, nor was any done on the urease–bromthymol blue test paper method for urea.

It is recommended*—

(1) That collaborative results be obtained on the enzymatic method for organic phosphate and carbamate insecticides.

(2) That attempts be made to make the method specific for some insecticides.

(3) That methods for testing the activity of the various enzymes used in analytical procedures be studied for the purpose of inclusion in the chapter on enzymes.

(4) That the first action urease–bromthymol blue test paper method for urea be further studied, preparatory to final action.

REPORT ON DETERMINATION OF INSECTICIDES BY ENZYMATIC METHODS

By J. W. Cook (Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.), *Associate Referee*

The poisonous properties of the organic phosphate and some carbamate insecticides are supposed to be due primarily to their inhibition of the

* For report of Subcommittee C and action of the Association, see *This Journal*, 37, 74, 75 (1954).

cholinesterase (CHE) enzyme system of both insects and mammals. Some of the compounds are such powerful inhibitors that this property can be used as the basis of a quantitative procedure to determine residue amounts in food products.

CHE hydrolyzes acetylcholine, yielding acetic acid and choline. Either of these compounds can be determined to follow the course of the hydrolysis. Most of the current methods for CHE studies measure the acetic acid liberated by the reaction either by a ΔpH measurement or by the carbon dioxide yielded from its reaction with a bicarbonate solution. The latter method requires the use of a Warburg apparatus which is not available in most food control laboratories. Another method is based on the colorimetric determination of the acetylcholine remaining after hydrolysis (1).

Giang and Hall (2) published a method in 1951 for the determination of organic phosphate insecticides which was based on their inhibition of CHE. The amount of action or inhibition was measured by the change in pH caused by the liberation of acetic acid from acetylcholine. The method herein reported is based on the inhibition of cholinesterase, like that of Giang and Hall, but the measurements are made by the colorimetric procedure outlined in the method of Metcalf (1). The acetylcholine remaining after hydrolysis is converted to a hydroxamic acid by treatment with alkaline hydroxylamine. Hydroxamic acid with ferric chloride in acid solution gives a red complex. This method is more sensitive than the ΔpH method, is quite easy to perform, and uses equipment available in most laboratories.

The enzyme reactions involved are:

(I) Cholinesterase (CHE) + inhibitor → inhibited CHE + uninhibited CHE
(CHE must be in excess of inhibitor)

(II) Acetylcholine + uninhibited CHE → acetic acid + choline

The quantity of acetylcholine not hydrolyzed is proportional to the inhibited enzyme and therefore is proportional to the inhibitor.

METHOD

REAGENTS

(a) *Buffer soln.*—Dissolve 16.72 g clear crystals $Na_2HPO_4 \cdot 12\ H_2O$ and 2.72 g KH_2PO_4 in 1 l H_2O. Should be pH 7.2.

(b) *Acetylcholine standard soln.*—1.0 mg/ml. Dissolve 0.111 g acetylcholine chloride in 100 ml buffer soln **(a)**. (This reagent is hygroscopic and must be handled as rapidly as possible.) This soln must be refrigerated. A drop of toluene will aid permanence of standard.

(c) *Hydroxylamine hydrochloride soln.*—Dissolve 13.9 g $NH_2OH \cdot HCl$ in 100 ml H_2O. This soln must be refrigerated.

(d) *Sodium hydroxide soln.*—Dissolve 14.0 g NaOH in 100 ml H_2O.

(e) *Alkaline hydroxylamine soln.*—Mix equal vols solns **(c)** and **(d)**. Make fresh before use.

(f) *Hydrochloric acid soln.*—Dil. 1 vol. concd HCl (sp. gr. 1.18) with 2 vols. H_2O.

(g) *Hydrochloric acid soln.*—0.1 *N*.

(h) *Ferric chloride soln.*—Dissolve 10.0 g $FeCl_3 \cdot 6\ H_2O$ in 100 ml soln (g).

(i) *Cholinesterase enzyme soln.*—Use human plasma obtainable from hospital or Red Cross blood banks. Also available as concentrate from Winthrop-Stearns, Inc., New York. Human plasma that has been sterilized by filtration is not a satisfactory source of cholinesterase.

DETERMINATION

Preparation of acetylcholine standard curves.—Pipet 0 (Control tube A), 0.2, 0.4, 0.6, 0.8, and 1.0 ml aliquots of soln (b) into glass-stoppered test tubes, add buffer soln (a) to give 1.0 ml in each tube, and then add 1 ml H_2O and 1 ml buffer soln (a). Add 2 ml alkaline hydroxylamine soln (e) to each test tube and mix by shaking vigorously. After not less than 1 min., add 1.0 ml HCl soln (f) and 1.0 ml $FeCl_3$ soln (h), and shake vigorously after each addn. (Vigorous shaking as indicated will aid materially in reducing bubble formation in the tube during color absorbance readings.) Measure the absorbance of the color in each tube at 540 mμ, using the reagent blank tube (control tube A) to adjust the instrument to 0. Plot absorbance against mg acetylcholine.

Determination of plasma cholinesterase.—Dil. 0 (Control tube B), 0.75, 1.00, 1.25, and 1.50 ml human blood plasma to 25 ml with buffer soln (a). Pipet 1.0 ml portions of each of these dilns into test tubes and add 1 ml H_2O to each. Place tubes in constant temperature bath at 37°C. When tubes and controls have come to temp., add 1.0 ml acetylcholine standard soln (b) to one tube at a time, at intervals of 1 min. each, and mix. Shake incubating tubes as often as possible and allow enzyme to hydrolyze the acetylcholine for exactly 30 min. Then add 2 ml alkaline hydroxylamine soln (e), again at intervals of 1 min., and shake vigorously. Continue detn as under *Preparation of acetylcholine standard curve* procedure, except filter the colored solns through CS&S No. 589 blue paper or equivalent, before absorbance detn. (It may be necessary to pour first portion of filtrate back onto filter in order to get a clear filtrate.) Run controls with each series because the acetylcholine standard soln changes gradually even under refrigeration. The controls consist of 1 tube with all reagents except enzyme and acetylcholine (control tube A) and one with all reagents except the enzyme (control tube B). In each case the reagent deleted is replaced by 1 ml buffer soln (a). Adjust photometer with control tube A and read acetylcholine tube along with others. From absorbance readings and acetylcholine standard curve, det. quantity acetylcholine remaining in each tube, using the acetylcholine tube (control B) run at the time of the enzyme reactions as the quantity acetylcholine added to each tube. Det. which diln of enzyme hydrolyzed ca 80% of the acetylcholine in 30 min. and use this quantity of plasma in making subsequent dilns. Both the stock plasma and the dilns retain activity for some time in refrigerator. However, they do change gradually; thus it is necessary to run enzyme activity as a control with each experiment or detn (control tube C).

Determination of Systox[1] *standard.*—(Other inhibitors are run similarly, except for modifications which allow for differences in solubility and activity.) *Caution:* Great care must be exercised in handling all of these organic phosphate and carbamate insecticides because they are extremely poisonous. They are readily absorbed through skin, eyes, and respiratory and digestive systems. Never pipet by mouth, and always wash hands thoroly with soap and warm water when any of the solns have been handled.

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[1] Systox is the registered trademark of Chemagro Corp. for a mixture of the demeton isomers O, O, diethyl, O-ethylmercapto ethyl thiophosphate, and O, O, diethyl, S-ethylmercapto ethyl thiophosphate.

Commercial Systox has been used as standard. Pipet (using vacuum) 0.09 ml tech. Systox and dissolve in 100 ml Et ether. Then dil. 1 ml ether soln to 1 l with distd H_2O. This produces soln contg 1 mmg tech. Systox/ml. Pipet 0 (control tube C), 0.2, 0.4, 0.6, 0.8, and 1.0 ml Systox soln into sep. test tubes, and add sufficient H_2O to make total vol. of 1 ml. Put tubes in constant temp. bath at 37°C.; when tubes have attained bath temp., add 1 ml dil. enzyme soln to each tube at 1 min. intervals and allow incubation for 30 min. At end of 30 min., add 1.0 ml acetylcholine standard soln **(b)**, and proceed as under *Determination of plasma cholinesterase*, starting with (line 4): " . . . add 1.0 ml acetylcholine standard soln **(b)** . . . ." Be sure to include reagent blank with no acetylcholine (control tube A), and the tube with acetylcholine (control tube B).

Calculation of % inhibition.—From absorbance reading and acetylcholine standard curve, det. acetylcholine remaining in each tube, including acetylcholine control tube B. (Tube B represents amt acetylcholine added to each tube.) Subtract acetylcholine value of each tube from value of tube B. This represents amt of acetylcholine hydrolyzed in 30 min. by enzyme in each tube. The tube with no Systox (tube C) represents max. amt of hydrolysis, and inhibited tubes will have values between those of tube C and tube B. Calc. % inhibition by following formula, using the figures which represent mg acetylcholine hydrolyzed in 30 min.

$$\text{Per cent inhibition} = 100 - (100)\,\frac{(\text{Unknown})}{(\text{Tube C})}$$

Plot % inhibition against mmg Systox.

Determination of Systox in apples.—Macerate 500 g representative sample in Waring blendor with 100 ml H_2O. Filter, using 6 in. milk filtering pad. Transfer 20 ml aliquot of filtrate to sep. funnel, add 20 ml $CHCl_3$, and shake well. Allow to sep. (Centrifugation may be required.) Transfer 2 ml aliquot of $CHCl_3$ layer to test tube, and evap. $CHCl_3$ by drawing slow current of air (by aspiration) from the tube. Care should be exercised because Systox is volatile. Stop air current as soon as $CHCl_3$ has disappeared. Add 1 ml H_2O, put in 37° bath, add 1 ml enzyme soln, and proceed as under *"Determination of Systox standard."* Calc. % inhibition and compare % inhibition against Systox standard curve to obtain quantity Systox. From this calc. p.p.m. by the following formula:

$$\left(\frac{x}{2}\right)\left(\frac{600}{500}\right) = \text{p.p.m.}$$

where x = mmg Systox in 2 ml $CHCl_3$.

It is recommended* that this method be used to obtain collaborative analyses on food products.

REFERENCES

(1) METCALF, R. L., *J. Econ. Ent.*, **44**, 883 (1951).
(2) GIANG, P. A., and HALL, S. A., *Anal. Chem.*, **23**, 1830 (1951).

* For report of Subcommittee C, and action of the Association, see *This Journal*, 37, 74, 75 (1954).

REPORT ON DECOMPOSITION AND FILTH IN FOODS (CHEMICAL INDICES)

By W. I. PATTERSON (Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D.C.), *Referee*

During investigations carried out in 1953 on chemical indices of decomposition and filth in foods, formic, acetic, lactic, and succinic acids showed no promise as indices of rot in tomatoes. Similarly, no correlation has been shown between the color in strawberries and the rot content.

In contrast to this situation, histamine is a promising index of decomposition in fish. The bioassay method may not appeal to some chemists in spite of its relative simplicity. The chemical method mentioned in this report will probably be submitted next year.

The only addition to the detection of the use of decomposed cream in the manufacture of butter is a fast sorting method.

If progress in this field seems to be slow, it must be remembered that the labor involved in preparing and analyzing one authentic food pack may be eight to ten man-weeks. With fish, this would include only one species of tuna, such as skipjack. An authentic pack of every other species of tuna would require a similar expenditure of time, unless several packs were prepared simultaneously. Before an established, objective index can be used for one species, 4 or 5 authentic packs of this species must be prepared. Thus the time for each proposed application may be one man-year.

Now if there were some reliable way of choosing for study only those indices which are successful, the expenditure of one man-year would be warranted, but for every successful index there may be several unsuccessful choices, and sometimes the year's work may have been almost finished before it is realized that the index chosen has no value.

RECOMMENDATIONS

It is recommended*—

(1) That gluconic acid be studied as an index of decomposition in fruits.

(2) That studies on succinic, lactic, and volatile acids as indices of decomposition in tomatoes be discontinued.

(3) That the relationship of pigment changes in strawberries to decomposition be discontinued.

(4) That the search be continued for acceptable indices of decomposition in fruits and vegetables.

(5) That study of histamine in fish be continued.

* For report of Subcommittee C and action of the Association, see *This Journal*, 37, 70 (1954).

(6) That search for suitable chemical indices in shellfish be continued.

(7) That study be initiated on the correlation of 3,4-dihydroxyphenylacetic acid with insect filth in cereal foods.

(8) That the "Rapid Method for the Estimation of Water Insoluble Fatty Acids in Cream and Butter" be adopted, first action.

REPORT ON DECOMPOSITION IN STRAWBERRIES (PIGMENT CHANGES)

By HOWARD P. BENNETT (Food and Drug Administration, Department of Health, Education, and Welfare, New Orleans 16, La.), *Associate Referee*

Because decomposition in strawberries is usually accompanied by marked changes in the color of the berry, the investigation reported here was undertaken by the Association to ascertain if these color changes could be used to measure decomposition.

A comprehensive review of the literature relating to color pigments was made, and a limited amount of laboratory work was done on the problem. It is indicated that more than 90 per cent of the color in strawberries is due to pelargonidin 3-galactoside, an anthocyanin. Work done by the author disclosed that there was little or no loss in the pigment, as measured by the modified method of Sondheimer and Kertesz (1), during short-term decomposition of strawberries (two to three days), even though much souring, mold growth, and great changes in color took place. The destruction of pelargonidin 3-galactoside in strawberry products over long periods of time is well known and has been widely investigated by such workers as Beattie, Wheeler, and Pederson (2); Kertesz and Sondheimer (3), and others. Pelargonidin 3-galactoside apparently breaks down into compounds of non-specific nature which have not as yet been characterized. An absorption curve for an extract of rotten strawberries differed little from the curve for good berries (from 240 to 900 mμ). It has been suggested that flavonoid compounds, such as kaempferol and quercetin, may also be used in an approach to this problem. Because these compounds occur in strawberries in such small amounts and must be extracted from very large samples by laborious methods, it is doubted that a useful method could be based on the changes affecting these substances.

Since the measurement of the loss of natural pigments is a negative approach to the problem (overlapping into the aging process not connected with decomposition) and since no specific new pigments which form when

decomposition takes place in strawberries have as yet been discovered, it is recommended* that this subject be discontinued.

REFERENCES

(1) SONDHEIMER, E., and KERTESZ, Z. I., *Anal. Chem.*, **20**, 245 (1948).
(2) BEATTIE, H. G., WHEELER, K. A., and PEDERSON, C. S., *Food Research*, **8**, 395 (1943).
(3) KERTESZ, Z. I., and SONDHEIMER, E., *Food Industries*, **20**, 1300 (1948).

REPORT ON CHEMICAL INDICES OF DECOMPOSITION IN FISH (HISTAMINE)

By DAVID W. WILLIAMS (Food and Drug Administration, Department of Health, Education, and Welfare, San Francisco 2, Calif.), *Associate Referee*

Several indices of decomposition such as volatile acids, succinic acid, water-insoluble acids, and indole have found application in the detection of decomposition in canned fish. Chemical indices of decomposition should correlate well with the organoleptic classification of the fish before processing. Because of the different physiological systems of breakdown whereby different end products are formed, no single chemical index has proved successful for detecting all types of decomposition. Therefore, the search is continued for end products which will serve as indices of the different types of decomposition.

Suzuki (1) reported histamine in tuna extracts. Igarasi (2) affirmed that histamine was found in large quantities in spoiled fish. He also attributed certain poisoning, resulting from eating fish, to the presence of histamine. Histamine has been blamed for many fish poisonings resulting from eating decomposed fish (3–5). Guggenheim (6) found that histamine was readily formed from the histidine of proteins by putrefactive microorganisms. Van Veen and Latuasan (5) isolated from bonito two strains of bacteria capable of producing histamine from the histidine of fish muscle under aerobic and anaerobic conditions. Geiger, *et al.*, (7, 8) found that the histamine content of fresh fish was quite low but increased *post mortem*. Assays using the isolated guinea pig ileum were made on a limited number of samples of the mackerel-like fishes. The data showed that the presence of substantial amounts of histamine in canned mackerel-like fish was indicative of decomposition in this fish prior to processing.

It would be fortunate if histamine could be used as an index of decomposition since it is practically non-volatile and should remain unchanged in the mackerel-like fishes when they are precooked and canned in normal

* For report of Subcommittee C and action of the Association, see *This Journal*, **37**, 70 (1954).

factory practice. Histamine is a very stable compound and loses none of its physiological activity after being heated to 100° in air or *in vacuo* (9). It is not affected by boiling for long periods with concentrated hydrochloric acid and is affected only slightly by boiling for extended periods with strong sodium hydroxide solution.

EXPERIMENTAL SAMPLES

A series of experiments using the bioassay method was designed to investigate further the correlations between decomposition in the raw fish and histamine content of canned fish. Experimental packs of decomposed and good tuna, Pacific mackerel, California sardines, salmon, codfish, and crabmeat were used in these experiments.

Tuna.—Three experimental packs were studied, namely, a spoilage pack in which the fish in each of three stages of decomposition were ground, mixed, and canned (13); and one pack each of progressively spoiled yellowfin and skipjack tunas canned as in normal factory practice; each pack consisted of tuna from each of three stages of spoilage (13). In addition, several miscellaneous tuna samples were assayed to determine relationship of honeycombing to histamine content as well as the amount of histamine present in sound fish.

Pacific Mackerel.—Two experimental packs were studied. One contained cans of stage 1 and cans of stage 3, while the other contained cans of stage 1 and cans of a mixture of stages 3 and 4.

California Sardines.—One experimental decomposition pack was studied, consisting of cans of stage 1 fish and cans of a mixture of stages 3 and 4.

Salmon.—One experimental pack was studied, which consisted of cans of stage 1 and cans of stage 3.

Crabmeat.—One experimental pack was studied; it included cans of stages 1, 2, 3, and 4.

Codfish Flakes.—A few cans of one experimental pack were assayed, including cans of stage 1 or stage 4.

DETERMINATION OF HISTAMINE-LIKE SUBSTANCE

The procedure for sample preparation and assay used is based on the principles outlined by Geiger (7), Minard (10), and others (11, 12), but which have been adapted to the problem at hand.

APPARATUS

(a) *Kymograph.*—Kymograph with horizontal muscle lever arm having friction or gravity writing point.

(b) *Muscle bath.*—At least 50 ml capacity, surrounded by 37° constant temp. bath.

REAGENTS

(a) *Sodium chloride stock soln.*—180 g/l.

(b) *Potassium chloride stock soln.*—42 g/500 ml.

(c) *Sodium bicarbonate stock soln.*—15 g/500 ml.

(d) *Atropine sulfate stock soln.*—1.0 g/500 ml.

(e) *Calcium chloride stock soln.*—24 g anhyd. salt/500 ml.

(f) *Dextrose.*—Anhyd. Use as solid.

(g) *Ringer-Locke soln.*—NaCl, 0.9%; KCl, 0.042%; $CaCl_2$, 0.024%; $NaHCO_3$, 0.015%; dextrose, 0.1%; and atropine sulfate, 0.001%. Add 100 ml (a) and 10 ml (b), (c), and (d) to 2 l volumetric flask. Add H_2O to vol. of ca 1800 ml and add 10 ml (e) while swirling to mix well. Before using add 2 g (f). Make to vol. with H_2O. This dextrose-contg soln must be kept in the refrigerator when not in use and may become moldy after 2 days.

(h) *Standard histamine diphosphate master soln.*—0.1 mg/ml histamine diphosphate in boiled H_2O. This soln should be kept in the refrigerator when not in use and will keep for at least 3 months. Dild standards of 0.01 mg/ml and 0.005 mg/ml may be made up as required. 50 ml (g) was used in the muscle bath. If a smaller vol. bath is used, it is recommended that dilns of (h) be made with (g) to prevent significant diln of the bath when addns of the standards are made.

(i) *Rosolic acid indicator.*—0.1 g in 100 ml 50% alcohol.

(j) *Guinea pig intestine.*—Guinea pigs weighing from 300–400 g should be used. The pig should be starved for 24 hrs, killed by a blow on the head, and the intestine removed and severed at a point proximal to the ileocecal junction with ca 12 cm of the terminal ileum retained. Wash this section with soln (g) and use ca the first 2 cm for the first series of assays. Place the remainder of the intestine on cotton in a petri dish just below the surface of soln (g). Prop the lid to admit air and store in a refrigerator at ca 40–45°F. If the intestine is stored below 40°F, it loses its activity. Use addnl portions of the intestine as required as long as it shows sufficient response to histamine stimulus (usually 8 days). These addnl portions of the intestine are not as sensitive as the ileum but give uniform response with barely noticeable pendulum movements after storage.

SAMPLE PREPARATION

Drain canned fish for 2 min. to remove excess liquid, grind the entire contents in a food chopper, mix well, and weigh 10 g into small mortar. Add sufficient H_2O to make smooth paste while grinding with a pestle. Transfer paste with little addnl H_2O to 100 ml Kohlrausch flask and add 1 ml (1+1) HCl. Add H_2O to total vol. of ca 70 ml, mix well, and heat flasks in boiling H_2O bath for ca 20 min. Remove from bath, cool, make to vol. with H_2O, mix, and filter on Whatman No. 12 folded filter. (The ext. filters slowly but only ca 5 ml need be collected for analysis.) These filtrates may be stored in a refrigerator for 10 days without diminished activity. Before assay add 1–2 drops rosolic acid indicator to filtered ext. and neutralize to pink with solid Na_2CO_3.

ASSAY

Attach intestine to muscle lever and allow to rest for at least 0.5 hr in 50 ml soln (g) in constant temp. bath at 37°. With a fresh ileum, non-rhythmic contractions and relaxations may be encountered for ca 2–3 hrs with extreme and not always uniform responses to histamine stimuli. Detns may be carried out during this period, but it is necessary to add small and increasing amounts of dild standards to rest the intestine, and to check responses several times.

Add known amount of dil. standard to bath, record response, and remove writing lever from contact with drum. Drain inner bath, add fresh 37° soln (g) to wash chamber and intestine, remove, and refill with fresh soln (g). Allow intestine to rest for 3 min. Estimate amount and diln of neutralized fish ext. which will give approximately an equal response, and add to the bath. Repeat recording of response,

wash muscle chamber, and allow 3-min. rest. During this 3 min. interval, measure step heights with mm scale and calc. amt standard necessary to match fish ext. step height. Continue using ext. and standard alternately until exact match is obtained.

EXPERIMENTAL DATA

Values for histamine-like substances calculated as mg of histamine per 100 g are shown in Table 1. In addition to the figures shown in this table, numerous determinations were made on commercial packs of known good tuna. All of these gave values of less than 5 mg/100 g calculated as histamine. Geiger (7) adjusted the *p*H of the digestion mixture to *p*H 4–5 before digestion. No difference in histamine content was noted at a lower *p*H, so 1 ml of HCl (1+1) was added to each digestion flask with a resulting *p*H of 3–5. In neutralizing the extract prior to assay, higher values than *p*H 7.2 will be reached before a definite pink color is obtained. Experiments were conducted up to *p*H 10 and no difference in histamine values were found when small volumes of the extract were used. (The kymograph may be sprayed with liquid plastic to preserve the recordings of analysis on the smoked drum.)

SUMMARY

(1) When determined by the bioassay method, the amount of histamine-like substances in canned fish correlates well with the organoleptic classification of the raw material in the tunas and mackerel-like fishes. Limited data indicate that the bioassay for histamine will also be helpful in detecting spoilage in canned salmon and possibly in canned codfish. Results on one crabmeat pack were inconclusive in that no histamine-like substances were detected.

(2) The amount of histamine present in good canned fish in all the experimental packs was of low order.

(3) Badly honeycombed fish, regardless of species, showed much higher histamine values than those of the same stage without honeycomb.

(4) Small fish appear to have greater histamine content than large fish.

In addition to the work reported above, work was carried out on new qualitative and quantitative chemical methods for histamine in canned fish. A quantitative chemical method was studied. Further study on the application of chemical methods for histamine to the problem of decomposition in canned fish is indicated.

RECOMMENDATIONS

It is recommended*—

(1) That the bioassay method for the determination of histamine-like substances be subjected to collaborative study.

* For report of Subcommittee C and action of the Association, see *This Journal*, **37**, 70 (1954).

TABLE 1.—*Histamine-like substances present in experimental packs of canned fish*

PACK	MG/100 G AS HISTAMINE			
	STAGE 1	STAGE 2	STAGE 3	STAGE 4
Tuna	2.6, 2.4	1.8, 2.0	36.2, 34.3, 37.9, 37.6	—
Yellowfin Tuna	0.4, 0.5, 0.3, 0.9, 1.3, 0.3, 2.6, 0.8, 0.3, 1.7, 3.9	0.5, 1.4, 1.6, 1.8, 0.8, 3.3, 0.6, 4.0	8, 10, 29, 40, 5, 5, 10, 8	7, 14, 29, 10, 9, 39, 55
Av.	1.2	1.7	14	23
Skipjack Tuna	3, 3, 0, 4, 2, 1, 1, 1	2, 2, 8, 2, 2, 3, 9, 9	17, 15, 26, 15, 8, 16, 9, 16	211, 12, 11, 133, 12, 48, 181, 181
Av.	2	4	15	98
Yellowfin Tuna	—	2.9, 7	—	39, 26, 43
Eastern Little Tuna	0.6	—	—	60
Honeycomb Yellowfin Tuna	No Detectable Odor After Precooking			99
Pacific Mackerel	0.2, 0.6, 1.1	—	26, 58, 10, 32, 47, 14	—
Av.	0.6		31	
Pacific Mackerel	0.3, 0.3, 0.2, 0.2, 0.1, 0.1, 0.5	—	124, 234, 181, 145, 204, 210, 159, 279	
Av.	0.2		Stages 3 and 4-192	
California Sardines	3, 6, 2, 1, 1, 2.1	—	289, 278, 243, 296, 268, 262, 230, 362	
Av.	2		Stages 3 and 4-291	
Silver Salmon	Trace, 0, 2, 0, 0, 0, 0	—	38, 15, 18, 4, 19	
Av.			19	
Crabmeat	None found in any stage of this pack			

(2) That the chemical method for histamine be studied further.

(3) That further experimental decomposition packs of canned fish be prepared and analyzed by the bioassay method.

REFERENCES

(1) SUZUKI, U., YONEYAMA, C., and ODAKI, S., *J. Coll. Agr. Tokyo Imp. Univ.*, **1**, 5 (1912); thru *Chem. Zentr.*, I, 1042 (1913).
(2) IGARASI, H., *J. Chem. Soc. Japan*, **59**, 1258 (1938)
(3) LEGROUX, R., and LEVADITE, J., *Compt. rend. soc. biol.*, **141**, 998 (1947)
(4) ———, LEVADITE, J. C., BOUDIN, G., and BOVET, D., *Presse med.*, **54**, 545 (1946).
(5) VAN VEEN, A. G., and LATUASAN, H. E., *Documenta Neerl. et Indones. Morbis Trop. II*, **1**, 18 (1950).
(6) GUGGENHEIM, M., *Biochem. Z.*, **65**, 189 (1914).
(7) GEIGER, E., *Food Research*, **9**, 293 (1944).
(8) ———, COURTNEY, F., and SCHNAKENBERG, G., *Arch. Biochem.*, **3**, 311 (1944).
(9) HANKE, M. T., and KOESSLER, K. K., *J. Biol. Chem.*, **43**, 557 (1920).
(10) MINARD, D., *Am. J. Physiol.*, **132**, 327 (1941).
(11) SCHILD, H. O., *ibid.*, **101**, 115 (1942).
(12) CODE, C. F., *ibid.*, **90**, 485 (1937).
(13) HILLIG, F., PATTERSON, W. I., and MCLEAN, M., *This Journal*, **33**, 834 (1950).

REPORT ON DECOMPOSITION IN TOMATO PRODUCTS

DETERMINATION OF ACETIC, FORMIC, SUCCINIC, AND LACTIC ACIDS

By HALVER C. VAN DAME (Food and Drug Administration, Department of Health, Education, and Welfare, Kansas City 6, Mo.), *Associate Referee*

In the 1952 report on decomposition in tomato products (1), it was recommended that the method for acetic, formic, succinic, and lactic acids be studied further, since the results on the collaborative sample were not in good agreement. The recovery of formic acid was quite low in several cases.

After some experimenting this year, it was found that if too much strong (1+4) sulfuric acid was used in regenerating the acids before addition to the column, low recoveries of formic acid were obtained. By careful regenerating, addition of a measured excess of acid, and changing of the immobile phase from 0.5 *N* to 0.1 *N* sulfuric acid, this difficulty appeared to have been eliminated.

With these modifications, two samples, each containing an added 10.30 mg of propionic, 11.27 mg of acetic, 11.37 mg of formic, 9.23 mg of succinic, and 11.62 mg of lactic acids, were analyzed by the method used last year (1). Table 1 gives the results.

TABLE 1.—*Analysis of tomato products for five acids*

ACID	SAMPLE 1			SAMPLE 2		
	MG ADDED	MG FOUND	RECOVERY	MG ADDED	MG FOUND	RECOVERY
			per cent			*per cent*
Propionic	10.30	9.92	96.3	10.30	9.92	96.3
Acetic	11.27	11.24	99.7	11.27	11.21	99.5
Formic	11.37	11.10	97.6	11.37	11.05	97.2
Succinic	9.23	9.23	100.0	9.23	9.23	100.0
Lactic	11.62	11.85	102.0	11.62	11.80	101.5

The results show that the method will quantitatively separate acetic, formic, succinic, and lactic acids.

It was also recommended that more information be obtained as to the correlation of the amounts of these acids with the mold counts on various samples of tomato products. Four samples of tomato juice and four samples of tomato puree packed in commercial plants in Ohio and Indiana during 1952 were used for this study.

Two samples of juice, numbers 3A and 4A, and two samples of puree, numbers 1A and 2A, were packed in plants in which the raw stock was examined by Mr. Frank R. Smith of the Division of Microbiology, Food and Drug Administration, Washington, D.C., and by the author. The juice and puree made from adequately trimmed and sorted tomatoes, samples 1A and 3A, showed low mold counts. The juice and puree made from poorly sorted and trimmed stock, samples 2A and 4A, showed a high mold count. The raw stock going into samples 1B, 2B, 3B, and 4B was not examined. Table 2 gives the results of the mold counts and the determination of acetic, formic, succinic, and lactic acids in milligrams per 100 grams on these samples.

TABLE 2.—*Mold and acids in tomato puree and juice*

SAMPLE NO.	MOLD COUNT	ACETIC, MG/100 G	FORMIC, MG/100 G	SUCCINIC, MG/100 G	LACTIC, MG/100 G
		Tomato puree			
1 A	10	37.8	23.1	3.3	5.0
1 B	6	23.6	21.9	2.4	1.9
2 A	39	19.3	22.0	5.8	5.2
2 B	36	20.0	16.2	3.2	2.6
		Tomato juice			
3 A	2	28.7	18.9	2.6	2.1
3 B	24	7.7	5.2	2.7	5.3
4 A	38	16.9	14.8	3.8	4.0
4 B	28	5.9	6.0	2.1	3.6

It is difficult to draw definite conclusions on the basis of so few analyses; yet the above figures indicate that none of the four acids listed would be

a good index of decomposition in tomato products.

In the case of samples 1A and 3A, the product was made from adequately sorted and trimmed tomatoes. However, the amounts of acetic and formic acids found in these samples were greater than those found in the case of samples 2A and 4A, which were made from tomatoes which were not adequately sorted and trimmed. The amounts of succinic and lactic acids found in samples 2A and 4A do not seem to be enough greater than the amounts found in 1A and 3A to be of significance.

SUMMARY

(1) The method (1) for acetic, formic, succinic, and lactic acids, as modified in this report, is an adequate method for the quantitative separation and determination of these acids.

(2) The amounts of acetic, formic, succinic, or lactic acids found do not seem to be indices of the amounts of rot due to molds present in the product.

It is recommended* that the study of acetic, formic, succinic, and lactic acids as indices of decomposition in tomato products be discontinued.

REFERENCE

(1) VAN DAME, H. C., *This Journal*, **36**, 580 (1953).

No reports were given on animal fecal matter, apples, fish products, (acids), pineapple, shellfish, spinach, strawberries, uric acid in cereal products, or uric acid in nuts.

REPORT ON METALS, OTHER ELEMENTS, AND RESIDUES IN FOODS

By L. L. RAMSEY (Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.), *Referee*

During the past year progress has been made on several topics in Chapter 24. The qualitative and quantitative methods for 1080 were studied collaboratively and, in general, the results were acceptable. A report of the Associate Referee on benzene hexachloride indicates that the Schechter–Hornstein method gives reliable and reproducible results in the hands of various analysts. The Associate Referee on methoxychlor has worked on a specific method and believes that the method is ready for collaborative study. The Associate Referee on enzymatic methods reports that a cholinesterase method for certain insecticides appears to be quite satisfactory. The report on sodium indicates that both the chemical and

* For report of Subcommittee C and action of the Association, see *This Journal*, **37**, 70 (1954).

flame photometer methods look promising and that a further study of the two methods is desirable. No reports were received from the Associate Referees on fluorine, zinc, DDT, parathion, aldrin, or insecticides in canned foods. It is urged that the work on these topics go forward next year.

Within the past several years, partly as a result of the impetus to research given by World War II, a large number of new organic insecticides have been introduced and many of them are highly toxic to man as well as to insects. Associate Refereeships should be established for those insecticides which, by their great toxicity, by their high degree of persistence, or by the large volume used, appear to create a residue hazard. This year an Associate Referee for aldrin was appointed. Associate Referees should also be appointed for dieldrin, heptachlor, and chlordane.

RECOMMENDATIONS

It is recommended*—

(1) That an Associate Referee be appointed for copper and that the copper methods be studied further.

(2) That the study of methods for zinc be continued.

(3) That the extent of the interference of other insecticides (specifically including dilan, rhothane, and dimite) with the colorimetric determination of DDT be investigated.

(4) That the qualitative method for 1080 as revised in the report on this topic be adopted, first action.

(5) That the quantitative method for 1080 as revised in the report on this topic be adopted as official.

(6) That the method developed by the Associate Referee for methoxychlor be subjected to collaborative study.

(7) That the Schechter–Hornstein method for benzene hexachloride be further studied.

(8) That the fluorine method for products high in silica be studied and that the typographical error in the fluorine method (see report on 1080) be corrected.†

(9) That chemical and enzymatic methods for the determination of trace amounts of organic phosphate insecticides, including parathion, be studied.

(10) That methods for the determination of aldrin, dieldrin, heptachlor, and chlordane be studied.

(11) That because of the close relationship of heptachlor and chlordane, a single Associate Referee be appointed for these two topics.

(12) That an Associate Referee be appointed for dieldrin.

(13) That the study of methods for the determination of sodium be continued.

* For report of Subcommittee C and action of the Association, see *This Journal*, **37**, 71 (1954).
† See *This Journal*, **37**, 100 (1954).

REPORT ON BENZENE HEXACHLORIDE IN FOODS

By ALFRED K. KLEIN (Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.),
Associate Referee

The Schechter–Hornstein colorimetric method of determining benzene hexachloride (1) was submitted for collaborative study during 1953. Briefly, the method consists of (*a*) conversion of BHC to benzene by refluxing with zinc and acetic acid; (*b*) nitration of benzene, as evolved, to *m*-dinitrobenzene; and (*c*) evaluation of the magenta-colored complex formed by reacting the dinitrobenzene with butanone-2 and 40 per cent potassium hydroxide. The dehalogenation and nitration are conducted in the same special apparatus.

Five collaborators volunteered for the collaborative study which consisted of the determination of BHC added to refined peanut oil and to carbon tetrachloride extracts of apples. (Both the original peanut oil and the untreated apple extracts were tested and found to be free of BHC or any interfering aromatic material.) The "unknowns" consisted of two peanut oil samples to which *gamma* BHC had been added in the amounts of 2.0 and 4.0 p.p.m., and two carbon tetrachloride extracts of apples containing added *gamma* BHC in the proportions of 3.0 and 7.0 p.p.m. Untreated samples serving as material for recovery runs were also supplied, together with a standard solution of *gamma* BHC in glacial acetic acid. Collaborators were instructed to develop a standard working curve in the 0–50 microgram range (which should obey Beer's Law) and to obtain satisfactory recovery runs with both 10 grams of untreated oil and 10 ml of apple extract before undertaking the analyses of like amounts of the collaborative samples. Reprints of the Schechter–Hornstein procedure (1) and a later publication of Hornstein (2) accompanied the instructions. The results are given in Table 1.

The recoveries submitted by collaborators A, B, and C are entirely satisfactory and average 97.6 per cent. (Average values on oil samples Nos. 1 and 2 are 2.01 and 3.94 p.p.m.; average deviations from the mean are 0.05 and 0.22 p.p.m., respectively. Similarly, the average results of the analyses of extracts Nos. 1 and 2 are 2.85 and 6.97 p.p.m.; average deviations from the mean are 0.11 and 0.20 p.p.m., respectively.) However, the results submitted by collaborators D and E are much too low. Subsequent investigation revealed that the rather consistent 75 per cent recoveries obtained by collaborator D were caused by contamination or concentration of the standard BHC solution used in developing the standard curve. When the absorbance data originally obtained from the unknowns were compared to a standard working curve derived from a freshly prepared

TABLE 1.—*Collaborative results for BHC*

COLLABORATOR	OIL SAMPLES		APPLE EXTRACTS	
	NO. 1	NO. 2	NO. 1	NO. 2
	2.0 P.P.M. BHC ADDED	4.0 P.P.M. BHC ADDED	3.0 P.P.M. BHC ADDED	7.0 P.P.M. BHC ADDED
A	2.02	4.00	2.73	6.46
	2.04	4.06	2.73	6.51
B	2.10	4.10	3.10	6.80
	2.00	3.30	2.90	6.80
		4.20		
C	1.88	4.00	2.85	6.95
			2.76	7.20
D	1.52	3.16	2.19	5.40
	1.56	3.11	2.17	5.38
E	1.00	2.10	1.60	5.60
	1.25	1.60	1.60	5.90

solution of *gamma* BHC, the results were calculated as follows: Oil sample No. 1, 1.98 and 1.98 p.p.m. BHC; sample No. 2, 4.01 and 3.89 p.p.m. BHC. Similarly, apple extracts were calculated as follows: sample No. 1, 2.88 and 2.87 p.p.m. BHC; sample No. 2, 7.20 and 7.10 p.p.m. BHC.

In his report to the Associate Referee, collaborator E stated that erratic results were encountered in developing the standard curve as well as in recovery runs. The difficulty was subsequently traced to two causes. Inconsistent nitration was encountered because water was not completely removed from the apparatus between runs. (The apparatus, especially the nitration column, must be initially dry.) The second source of inconsistency was the use of different batches of acetic acid. Almost all brands of reagent grade acetic acid contain varying amounts of aromatic compounds which measure eventually as BHC. Some contain as much as 15 micrograms of apparent BHC per 10 ml of reagent, the volume used in the determinations. The need for a rigid control and measurement of the blank is therefore evident. In evaluating small quantities of BHC the analyst should distil the acid and discard the first and last one-fifth portions (1).

When these precautions were observed, collaborator E readily obtained a standard working curve which followed Beer's Law. Furthermore, in working up carbon tetrachloride extracts of apple and string bean slurries to which 5.0 p.p.m. *gamma* BHC had been added, he obtained 4.5 p.p.m. in both instances.

As a result of the collaborative study the Associate Referee recom-

mends* that the Schechter-Hornstein procedure be subjected to further collaborative trial.

REFERENCES

(1) SCHECHTER, M. S., and HORNSTEIN, I., *Anal. Chem.*, **24**, 544 (1952).
(2) HORNSTEIN, I., *ibid.*, **24**, 1036 (1952).

REPORT ON METHOXYCHLOR

SIMPLIFIED PROCEDURE FOR THE ANALYSIS OF METHOXYCHLOR IN FRUITS AND VEGETABLES, DAIRY PRODUCTS, AND BIOLOGICAL MATERIALS

By FRIEDA M. KUNZE (Division of Pharmacology, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.), *Associate Referee*

Numerous and accurate methods exist for the determination of methoxychlor in microgram quantities (1–7), but no one method, *per se*, is readily applicable to fruits and vegetables, dairy products, and biological materials. The procedure described in this report incorporates the principles of several methods (1–4). It is specific for methoxychlor, sensitive to 1 p.p.m. (sample >10 g), and applicable to any of these materials.

The isolation of methoxychlor from fats and plant waxes is achieved by acetonitrile extraction in the manner described by Jones and Riddick (1). The acetonitrile extract is concentrated; methoxychlor is dehydrohalogenated and converted to the dehydrochloride by alkaline hydrolysis (3). Carotenes and residues which produce char and turbidity are removed by chromatographing through a column of a 1:1 mixture of absorptive magnesia and Celite (4). Final color development is in 85 per cent sulfuric acid by the Fairing method (2).

METHOD

APPARATUS

(a) *Chromatographic tubes.*—10 mm O.D.×130 mm long. For convenience, fuse a 100 ml standard taper boiling flask to the top of the tube to allow addn of larger amounts of solvent and application of air pressure. Any reservoir system, e.g., a separatory funnel, will suffice.

(b) *Other apparatus.*—All other apparatus is mentioned below.

REAGENTS

(a) *Acetonitrile.*—C.P. (Matheson Coleman & Bell Inc.), b.p. 80–82°. Sat. with petr. ether.

(b) *Petroleum ether.*—Reagent grade, b.p. 30–60°.

* For report of Subcommittee C and action of the Association, see *This Journal*, **37**, 71 (1954).

(c) *Ethanolic KOH.*—Dissolve 20 g of reagent KOH in 95% ethanol; make to one l.

(d) *Aqueous alcohol.*—50%.

(e) *Celite 545.*—Johns-Manville Co.

(f) *Absorptive powdered magnesia.*—Westvaco #2641.

(g) *Sulfuric acid.*—Reagent grade, 85%. Store in a Pyrex bottle stoppered with an automatic buret.

(h) *Diethyl ether.*—Reagent grade.

(i) *Anhydrous sodium sulfate.*—Reagent grade.

(j) *Benzene.*—Reagent grade.

SAMPLE PREPARATION

Fruits, vegetables, dairy products: Follow the procedure prescribed in *Official Methods of Analysis*, 7th Ed., **24.28.**

Animal tissues: To ext. 1–20 g of tissue, proceed in one of the two following ways:

Fatty tissue.—Use a mortar and pestle. Grind the tissue and ext. by repeated diethyl ether washes. Continue the extn until the fat is reduced to a fine powder. Use a minimum of 50 ml of diethyl ether to ext. each g of tissue.

All other tissues.—Using a mortar and pestle, macerate the tissue with sand. Dehydrate with anhyd. Na_2SO_4, grind until the mixture is dry and powdered, and ext. with repeated diethyl ether washes. Grind thoroly; allow the particulate matter to settle, and then decant each time. Use a minimum of 20 ml of diethyl ether to ext. each g of tissue.

To ext. larger amounts of tissue: Use the procedure outlined by Jones and Riddick (1).

DETERMINATION

Acetonitrile extraction.—Use an aliquot of the ether exts equivalent to 10 g of fruits or vegetables or to 4 g of fat. Evap. the solvent and dissolve the residue in 45 ml of petr. ether (Note A). Transfer to a separatory funnel and ext. with 15 ml of acetonitrile by shaking vigorously for 2–3 min. (Note B). Allow the phases to sep. and drain the lower (acetonitrile) layer into a clean separatory funnel. Ext. the petr. ether with 3 addl 15 ml portions of acetonitrile and combine these exts, discarding the petr. ether. For further purification, shake the combined acetonitrile exts with 20 ml of petr. ether (Note C). Drain the acetonitrile into a boiling flask, and wash the petr. ether with two 5 ml portions of acetonitrile, combining the acetonitrile washes with that in the flask and discarding the petr. ether. Place the flask on a steam bath, introduce a gentle current of air, and conc. the acetonitrile to ca 5 ml, avoiding overheating (Note D).

Hydrolysis.—Add 50 ml of ethanolic KOH to the acetonitrile concentrate; attach a condenser and reflux for 30 min. (Note E). Dil. the sample with 25 ml of distd H_2O, ext. once with 50 ml of petr. ether, and shake vigorously for 2–3 min. Discard the alkaline layer and wash the petr. ether once with 20 ml of 50% aq. alcohol; discard the alcohol and carefully conc. the petr. ether at room temp. with a gentle current of air to ca 5 ml. Loss will be incurred if the flask is heated (Note F).

Chromatography (Note G).—Place a small plug of glass wool in the bottom of the chromatographic tube. Add 5 g of a 1:1 Celite and absorptive powd. magnesia mixt. to the tube and pack by tamping (Note H). Moisten the column with 5–10 ml of petr. ether, discarding the rinsings. Pour the petr. ether concentrate containing the dehydrohalogenated methoxychlor over the column and chromatograph the concentrate, using air pressure. Rinse the flask several times with petr. ether. Collect 100 ml of eluate, transfer to a glass-stoppered flask, and evap. the petr. ether to

dryness with a current of air, avoiding excess drying (Note I) or heat (Note F).

Color development.—Develop the color with 10 ml of 85% H_2SO_4 (2). Stopper the flasks, shake frequently, and allow a minimum of 30 min. before measuring absorbance on a suitable instrument. (The color is stable for ca 2 hrs. Routinely, all samples were read after 45 min.)

Preparation of standards.—Methoxychlor, *p,p*-isomer, m.p. 88–89°. Recrystallize commercial methoxychlor 3 times from ethyl alcohol.

Weigh 10 mg of pure methoxychlor into 100 ml volumetric flask, dissolve in benzene, and make to vol. For a convenient work standard, transfer a 10 ml aliquot to a second volumetric flask and make to vol. with benzene (1 ml = 10 mmg).

Run a blank and standards at a min. of 3 points (0–50 mmg range) with each series of color detns. Hydrolyze and process the standards and blank in the manner described (the acetonitrile isolation and chromatography steps are unnecessary). Read at 550 mμ, using 85% H_2SO_4 as an instrument blank.

NOTES ON THE METHOD

(A) If a blank is run with each standard series, no distillation of reagent grade petroleum ether is necessary.

(B) In a 1:3 volume ratio of acetonitrile to petroleum ether, 81% of the methoxychlor is in the acetonitrile phase after one extraction. The acetonitrile contribution to the blank is reduced to zero by chromatography. No distillation of the brand of acetonitrile used was necessary; however, the blanks produced by various other brands were much higher, and purification of such solvents would be obligatory.

(C) This step was followed routinely to insure complete removal of waxes under all circumstances.

(D) Overheating may result in loss. Traces of acetonitrile do not interfere with subsequent hydrolysis.

(E) The time is not critical; optimum fat hydrolysis is completed within 20 to 30 minutes. No losses were noted after heating 2 hours, but prolonged hydrolysis does not reduce the unsaponifiable residue.

(F) Dehydrohalogenated methoxychlor must not be heated. Even mild heating resulted in a 25% loss.

(G) To facilitate acetonitrile extraction, chromatography would logically be the first step in the method, rather than the last. The order followed is necessary for two reasons: (1) Small quantities of methoxychlor are adsorbed by wax and held on the column; (2) chromatography eliminates the acetonitrile contribution to the blank.

(H) The grade of magnesia used is critical. Magnesia #2642, for instance, is much more absorptive than #2641. When magnesia #2642 was used, it was necessary to increase the volume of wash three-fold to elute the methoxychlor.

(I) Excessive drying decreases the solubility of the residue in 85% sulfuric acid.

DISCUSSION

The acetonitrile extraction technique is extremely efficient for isolating methoxychlor from fats and waxes; however, if small quantities of animal tissue are analyzed, e.g., 4 grams of fat, 5 grams of kidney, or 10 grams of liver, hydrolysis and chromatography will suffice, and the extraction step is unnecessary.

Measured amounts of standard methoxychlor were added to alfalfa, apple, cabbage, and butter extracts. The results obtained, expressed as averages, are given in Table 1.

A convenient standard range is 0 to 50 micrograms; however, the deter-

TABLE 1.—*Determination of methoxychlor*

PRODUCT	SAMPLE	ADDED	AV. RECOVERY	AV. RECOVERY	RANGE	NO. OF ANALYSES
	g	*p.p.m.*	*p.p.m.*	*per cent*	*per cent*	
Alfalfa	10.0	2.0	1.97	98	95–105	3
	10.0	3.0	2.85	95	90–105	15
Apple	10.0	2.0	1.98	99	95–100	3
	10.0	3.0	2.92	97	95–100	2
Cabbage	10.0	2.0	1.98	99	95–100	3
	10.0	3.0	2.88	96	94– 99	2
Butter	4.0	2.5	2.45	98	95–100	5

mination of less than 10 micrograms is not recommended. For fruits and vegetables, analysis at a level of 2 to 3 p.p.m. is desirable. In three analyses of alfalfa made at a level of 0.75 p.p.m., recovery averaged 95 per cent. However, the concentration of the sample increased both emulsion formation and the time required for acetonitrile extraction. Assuming that 4 grams of butter represents fat extracted from 100 grams of milk, recovery at 0.1 p.p.m. is feasible.

It is recommended* that the method for the determination of methoxychlor, described in this report, be submitted to collaborative study.

REFERENCES

(1) JONES, L. R., and RIDDICK, J. A., *Anal. Chem.*, **24**, 569 (1952).
(2) FAIRING, J. D., and WARRINGTON, H. P., JR., *Advances in Chemistry Series*, No. 1, American Chemical Society, Washington, D. C., 1950, p. 260.
(3) PRICKETT, C. S., KUNZE, F. M., and LAUG, E. P., *This Journal*, **33**, 880 (1950).
(4) KUNZE, F. M., and LAUG, E. P., *ibid.*, **34**, 537 (1951).
(5) CLABORN, H. V., and BECKMAN, H. F., *Anal. Chem.*, **24**, 220 (1952).
(6) DOBLE, J., and THORNBURG, W., Paper presented before the Division of Agricultural and Food Chemistry of the American Chemical Society, Atlantic City, N. J., September 1949.
(7) *Methoxychlor, A Summary of Analytical Methods*, E. I. duPont de Nemours & Co., Inc., Wilmington, Del., February 1951.

REPORT ON SODIUM FLUOROACETATE (1080)

By L. L. RAMSEY (Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.), *Associate Referee*

The quantitative method for 1080 (1) was adopted, first action, on the basis of a collaborative study made in 1949 (2). The method was again

* For report of Subcommittee C and action of the Association, see *This Journal*, **37**, 71 (1954).

studied collaboratively in 1952, but the results in general were not satisfactory. The qualitative method of Ramsey and Patterson (3) was first studied collaboratively in 1952, but one collaborator failed to obtain satisfactory results. Both the quantitative method and the qualitative method, each slightly modified on the basis of previous studies, were again subjected to collaborative study this year.

QUANTITATIVE METHOD

For the study of the quantitative method, four samples were prepared by adding varying amounts of 1080, as the potassium salt in aqueous solution, to 100 g samples of the foods. The flour and sugar samples were allowed to dry before closing the sample jars, and the hamburger samples were held under refrigeration until analyzed. However, each collaborator (except No. 3) was sent only three of these four samples for analysis in duplicate; the samples sent were selected according to a staggered pattern (Table 1). The collaborators were supplied with the following reagents: Standard 1080 solution, standard potassium fluosilicate solution, tertiary amyl alcohol, lime-suspension, and silicic acid. The collaborators were instructed to follow the official method except for the following changes: (1) Centrifugation of the phosphotungstic acid protein precipitate was directed in the case of flour in order to speed up the analysis; (2) the protein-free filtrate in the case of the flour and hamburger was not to be

TABLE 1.—*Collaborative results obtained with the quantitative method*

	RECOVERY ON CONTROL	FLOUR		SUGAR	HAMBURGER
		1	2	3	4
Added, 1080	1.0 mg	*p.p.m.* 0	*p.p.m.* 10.0	*p.p.m.* 5.0	*p.p.m.* 15.0
Found, 1080	*per cent*				
Analyst 1	91	—	9.2	4.6	13.7
	93	—	9.3	4.6	14.1
Analyst 2	97	0	—	4.6	14.1
		0	—	4.7	14.5
Analyst 3	96	0.1	9.0	4.4	13.9
		0.2	9.6	4.4	14.4
Analyst 4	73[a]	0.4[a]	8.4[a]	—	12.9[a]
	110	0.7	9.8	—	14.6
Analyst 5	91	0.6	4.8	4.7	—
	93	0.7	5.3	4.9	—
Associate Referee	90	0.2	9.7	4.1	14.8
	91	0.2	10.3	4.3	15.7
Minimum	90	0	4.8	4.1	12.9
Maximum	110	0.7	10.3	4.9	15.7
Average	94.7	0.3	8.5	4.5	14.3

[a] Obtained with "slow" extractor: 73% recovery excluded from average and range.

concentrated prior to ether extraction; and (3) the samples were to be extracted for three hours in the continuous extractors. The collaborators were also instructed not to proceed with the unknowns until they had adequately familiarized themselves with the method by running 1.0 mg of 1080 through the entire procedure and obtaining a recovery of at least 90 per cent.

The collaborative results obtained with the quantitative method as shown in Table 1 are satisfactory on the whole. Analyst 5 explained that his failure to obtain good checks was due to the use of two extractors, one of which gave a good ether recycling rate and the other of which gave an extremely slow recycling rate. The three hour extraction period was not adequate with the slow extractor for obtaining all of the 1080. In the official method, the analyst is directed to extract with ether until all the fluoroacetic acid, as determined by a preliminary experiment, has been extracted. In this study it was not the intention of the Associate Referee to substitute for this general direction an arbitrary time limit on the extraction, but on the basis of previous experience it was thought that, in general, a three hour period would be adequate with most extractors. With sample 2, analyst 5 obtained rather low recoveries, 48 and 53 per cent, although his recoveries on sample 3 and on the 1.0 mg 1080 control were quite satisfactory.

COMMENTS OF COLLABORATORS

Comments were received from only two collaborators. Analyst 2 stated: "For distillations silver sulfate was used to precipitate free chlorine since no silver perchlorate was on hand. During titration of distillates, I used 100 ml aliquots twice, but used same volumes in sample and known tubes." Analyst 4 commented: "As set up, extractor No. 1 would recycle ether in a virtually uninterrupted stream, whereas No. 2 would distil only at a fast drip, or at best, a quite discontinuous stream. With the exception of one quantitative determination, all extractions were discontinued at the end of three hours, in accordance with the specific instructions for this series. . . . Recovery experiments were done in duplicate, the '73%' recovery being made from the slow extractor. . . . Extract from the fast extractor had quite measurably more acidity. . . . It would appear that my lack of checks is due in part to the relative efficiency of the extractors. I feel that if a time limit is to be set on extraction, some workable measure of extraction rate must be specified. In refreshing my memory on the titration procedure, I noted the example involving the amount of 0.05 *N* KCl to be added to a specified aliquot, as set forth in *Official Methods of Analysis*, 7th Ed., **24.48**, top of p. 396. Since a 75/150 or ½ aliquot is taken, I would have added ½ of 1.50 ml of 0.05 *N* KCl, or 0.75 ml."

QUALITATIVE METHOD

The same four samples were sent to the collaborators for the study of the qualitative method, but they were identified with the letters A, B, C, and D, which corresponded to the numbers 1, 2, 3, and 4, respectively. The collaborators were supplied with the thiosalicylic acid and with carbon, purified according to the directions for the carbon purification in the

TABLE 2.—*Collaborative results obtained with the qualitative test*[a]

ANALYST	METHOD OF PURIFICATION	FLOUR		SUGAR	HAMBURGER
		A, 1	B, 2	C, 3	D, 4
		p.p.m. 1080 Added			
		0	10.0	5.0	15.0
1	Chromatographic	0 yellow	+ red	+ red	+ orange
2	Carbon	0	+ faintly pos.	− integrity of sample in question	+ strongly pos.
3	Carbon	0	+	+	+
	Chromatographic	0	+	+	+
4	Carbon	0	+	+	+
5	Carbon	0	+	+	+
Assoc.	Carbon	0	+	+	+
Referee	Chromatographic	0	+	+	+

[a] + is positive; 0 is negative.

lactic acid method (4). In the hands of the Associate Referee this purified carbon was found to be quite satisfactory and, in fact, superior to all others tested. The collaborators were instructed to make a single test on each sample by the method, with these slight modifications: (1) In the carbon treatment of the ether extract the carbon was decreased from 1.0 to 0.5 g/100 ml ether; and (2) in the carbon treatment of the aqueous solution the carbon was decreased from 1.0 to 0.5 g and the acidity was adjusted to *p*H 4–6. The collaborators were also instructed not to proceed with the unknown until they had adequately familiarized themselves with the method by running 0.5 mg of 1080 through the entire procedure and obtaining a positive test.

As indicated in Table 2, the Associate Referee and Analyst 3 used both the carbon and chromatographic methods of purification (3); Analyst 1 used the chromatographic method and all others used the carbon method. The results (Table 1) are quite satisfactory. No one obtained a positive test on sample 1, which contained no 1080; and all the analysts obtained positive tests on samples 2, 3, and 4, except in the case of Analyst 2 who failed to obtain a positive test on sample 3. However, the integrity of this particular sample is questionable and the negative result is excluded from consideration. Analyst 2 stated that this particular sample did not contain a lump when he examined it at the time of analysis. Since the 1080 was added in aqueous solution to the sugar samples and the water was allowed to evaporate prior to closing the sample jar, it appears that the 1080 was

inadvertently omitted from this sample. Moreover, Analyst 2 did obtain a positive qualitative test on a similar sample last year.

COMMENTS OF COLLABORATORS

Analyst 3 stated: "In general, the chromatography tests were appreciably stronger. By the carbon treatment there was appreciable orange color in D; however, a distinct change was noted upon addition of $K_3Fe(CN)_6$ and a ppt formed on standing." Analyst 4 commented as follows, for *Sample A:* "No immediate change in the ferricyanide color was noted within one hour; only a colorless solution with a small white sediment remained. No insoluble color was noted in the filtered residue." For *Sample B:* "An immediate red color was noted, and within an hour considerable insoluble red precipitate was observable." For *Sample C:* "The immediate color of the solution was very slightly orange with respect to the color of subsample A at this stage. At one hour, when subsample A's color had faded completely, subsample C bore a yellow color weaker than the color of the pure ferricyanide reagent. After three hours, no insoluble color was visible, but upon filtration onto 5 cm S&S 589 white ribbon paper, a quite evident red precipitate was demonstrated." For *Sample D:* "An immediate red color was noted, and in a short while a satisfactory amount of insoluble red matter was demonstrable."

RECOMMENDATIONS

It is recommended*—

(1) That the quantitative method (*Official Methods of Analysis*, 7th Ed., 1950) be revised as follows:

In Sec. **24.77 (b)**, on p. 414, line 4, after the word "convenient" insert: ", or more quickly, centrifuge and decant the supernatant liquid. Use at least 375 g of the filtrate or supernatant liquid. (Since sp. gr. of filtrate is very close to 1, measuring out aliquot in graduated cylinder is satisfactory.)" Delete the rest of the paragraph down to "**(c)** *Wheat.*"

(2) That the revised quantitative method be adopted as official, and that work on the method be discontinued.

(3) That the qualitative method be revised as follows:

In *This Journal*, **34**, 828 (1951) under METHOD, change line 6 to read "anhyd. Na_2SO_4 and 0.5 g decolorizing carbon per 100 ml ether, and shake." For the parenthetical statement in line 7 of the same paragraph, substitute this statement: "(Use carbon prepared as directed in *Official Methods of Analysis*, 7th Ed., 1950, Sec. **15.9 (b)**)." Also in this same paragraph, change line 13 to read: "and NaOH, adjust to *p*H 4–6. Add 0.5 g carbon and place on the steam bath for 15 min."

(4) That the revised qualitative method be adopted, first action.

(5) That in *Official Methods of Analysis*, 7th Ed., Sec. **24.48**, p. 396, line 5, the amount of 0.05 *N* KCl be corrected by changing "0.5 ml" to "0.75 ml."

ACKNOWLEDGMENT

The Associate Referee gratefully extends his appreciation to the following collaborators who participated in both the qualitative and quantitative studies:

H. W. Conroy, Food and Drug Administration, Kansas City, Mo.

* For report of Subcommittee C and action of the Association, see *This Journal*, **37**, 71 (1954).

L. G. Ensminger, Food and Drug Administration, Cincinnati, Ohio
S. H. Judd, State Department of Public Health, Berkeley, Calif.
F. Sabatino, Jr., Food and Drug Administration, Washington, D. C.
J. F. Weeks, Jr., Food and Drug Administration, New Orleans, La.

REFERENCES

(1) *Official Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Box 540, Benjamin Franklin Station, Washington 4, D. C., 1950, **24.74–24.80.**
(2) RAMSEY, L. L., and CLIFFORD, P. A., *This Journal*, **33**, 608 (1950).
(3) ———, and PATTERSON, W. I., *ibid.*, **34**, 827 (1951).
(4) *Official Methods of Analysis*, 7th Ed., **15.9 (b).**

REPORT ON THE DETERMINATION OF SODIUM IN FOODS

GRAVIMETRIC AND FLAME PHOTOMETRIC METHODS

By P. A. CLIFFORD, *Associate Referee*, and W. O. WINKLER
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In last year's report of the Associate Referee (1) it was pointed out that the increasing use of low-sodium therapy in certain disease states has produced an unexpected need for sensitive and accurate methods for the determination of sodium in a variety of foods. This is particularly true of the many specially labeled preparations recommended for use in sodium-restricted diets.

Official Methods of Analysis, 7th Ed., A.O.A.C., describes methods for the determination of sodium in various materials (baking powder, chloramine T, waters, plants, soils, etc.). Most of these methods are based upon the classical procedure of Fresenius in which sodium and potassium are separated as the mixed chlorides. Potassium is then determined as the chloroplatinate and the sodium is calculated by difference. Bills, *et al.*, (2) point out that because of a solubility effect the results for potassium tend to be low; the net result is a too-high sodium value, especially for materials containing much potassium and very little sodium.

Many of the older data on the sodium content of foods are based on this method. In a special report of the National Research Council (3), it is noted that frequently a re-evaluation of the natural sodium content of many foods by means of newer and more specific methods indicates a much lower sodium content than has heretofore been assumed. In the opinion of the Council these lower values are more nearly correct.

An exception with respect to specificity and sensitivity is the magnesium

uranyl acetate procedure for sodium in plants (par. **6.20–6.21**). This method applies well to the determination of sodium in most foods but is apt to fail when very low quantities of sodium, accompanied by much potassium (as in the case of legumes), are encountered.

Development of methods has been along two lines: *A*—refinement and application of the gravimetric sodium-zinc-uranyl acetate method; and *B*—application of flame-photometric techniques.

A. GRAVIMETRIC METHOD

During the year, Mr. W. O. Winkler of this laboratory has adapted the sodium-zinc-uranyl acetate method (4–6) to the determination of sodium in foods. The method has several advantages over the magnesium uranyl acetate procedure for sodium in plants, mentioned above; it is faster, and provision is made for the removal of potassium if it should appear in interfering amounts. A simple qualitative test warns the analyst if excessive potassium is present in the food sample; in such a case, sodium is isolated by means of a butanol–ethyl acetate extraction of the sodium from the mixed perchlorates. The method has adequate sensitivity for routine work because of the small factor for sodium in the bulky, heavy precipitate.

METHOD

(All glassware must be thoroly rinsed with hot tap water, then distd water, to remove all traces of soap and cleaning solns. Pipets require special care in cleaning. Ordinary volumetric ware may be used if solns are not held for long; Pyrex, or other resistant, low-alkali glass is preferable. It is permissible to use the fingers in such manipulations as folding filter papers, preparing samples, etc., if they are rinsed often with distd water. Soak filter papers (S&S No. 589, or equivalent) overnight in distd water, wash and drain on a clean Büchner and allow to air-dry. Most reagents used are specially purified; however, it is still necessary to conduct careful blank detns with the exact quantities of reagents used and to correct results accordingly.)

REAGENTS

(a) *Hydrochloric acid, constant boiling.*—Dil. ca 540 ml of concd HCl to ca 1 l and distil from an all-glass Pyrex system. Discard first 100 ml of distillate. Preserve the next 700–800 ml in Pyrex. (The distn is primarily to reduce the quantity of Na, and exact strength of acid is not particularly important.)

(b) *Ammonia, redistd.*—Place 1 l of concd reagent NH_4OH into still of all-glass Pyrex system. Adjust condenser outlet below surface of 500 ml of ice-cold distd H_2O contained in an ice-cold receiver. Using H_2O bath, gradually bring temp. of still from room temp. to near the b.p. Preserve distillate in a heavily paraffined bottle. (Again, exact strength of the ammonia distillate is not critical, but it is well to have it near that of original reagent. Strength will depend upon temp. of receiver, rate of heating, etc. Vol. of receiving H_2O will expand notably during the ammonia evolution; if vol. of the 500 ml of receiving H_2O is allowed to expand to 750–800 ml (mark on receiver), the redistd reagent is suitable for use. Exact strength can be detd by gravity or titration.)

(c) *Calcium chloride, 10% w/v.*—Slurry a definite wt (100 g or more) of purest $CaCO_3$ (Merck's low-alkali and -chloride, or equivalent) in H_2O, and slowly stir in the constant boiling HCl until soln clears. Filter, and add a calcd slight excess of

strong oxalic acid soln. Bring to ca *p*H 4.5 with the redistd ammonia (bromcresol green indicator and outside spot-plate; most indicators are Na salts). Digest on steam bath, with frequent stirring, for ca 1 hr; filter thru a large Büchner, and wash with copious quantities of hot distd H_2O. Dry the Ca oxalate ppt thoroly in a large tared silica dish and ignite to the oxide (muffle at 700°C.; progress of the conversion can be followed roughly by periodic weighing of the dish). When conversion appears to be complete, slake with H_2O and cautiously dissolve with the constant boiling HCl (the amount can be approximately calcd). Cool and dil. to ca 10% w/v strength. (Final soln should be acid.)

(d) *Zinc-uranyl acetate soln.*—

(1) To 40 g of $UO_2(C_2H_3O_2)_2 \cdot 2H_2O$ in a 500 ml Erlenmeyer flask, add 23 ml of 30% v/v acetic acid and 200 ml of H_2O.

(2) To 110 g of $Zn(C_2H_3O_2)_2 \cdot 2H_2O$ in a 500 ml Erlenmeyer flask, add 12 ml of 30% acetic acid and 163 ml of H_2O.

Heat (1) and (2) separately, with frequent stirring, until all salts are dissolved (a temp. of ca 70°C. may be required). Mix the two solns, while they are hot, by pouring back and forth. Cool the mixt. and allow to stand overnight. Filter immediately before use thru a washed filter paper.

(e) *Wash soln.*—Sat. 95% alcohol contg 3% v/v glac. acetic acid with solid sodium zinc uranyl acetate (obtained by pptn of Na with reagent (d)). Filter immediately before use thru washed filter paper.

(f) *Butanol–ethyl acetate mixt.*—Mix equal vols of anhyd., redistd *n*-butanol and anhyd., redistd ethyl acetate.

PREPARATION OF SAMPLE

Weigh a well-mixed sample of suitable size (usually 20–30 g of moist or liquid products; 2–10 g of dry products) into a Pt (or silica or Vycor) dish. (The weight of sample taken for analysis will depend upon the expected Na content; consult tables; (2–3).) Dry, and char without ignition under an overhead radiant heater. (Excessively fatty or oily products require considerable time.) Ash at 525°C. until little or no carbon remains. When ashing is complete, take up with ca 10 ml of distd H_2O, cover dish, and add 2.0 ml of the constant boiling HCl. Rinse cover, evap. to dryness on the steam bath, take up with H_2O plus 2 drops of HCl, and rinse into a 100 ml volumetric flask. (Some products ash with difficulty at this temp. In such cases leach the char with hot H_2O, break up carbonaceous lumps with a flat-tipped stirring rod, and filter into a 100 ml volumetric flask thru a washed filter paper. Wash filter with a few small portions of hot H_2O, return it to the dish, dry, and re-ash. Proceed as above, beginning " . . . when ashing is complete . . . " etc.)

Dil. to ca 60–70 ml, add 2 ml of the 10% $CaCl_2$ soln, and make slightly ammoniacal with 5–6 drops of the redistd ammonia. Phosphates are thrown down as a gelatinous ppt. Allow the ppt. to settle, and test with a further drop of the $CaCl_2$ soln. If there is further pptn, add $CaCl_2$ soln to complete pptn of phosphates. Make to mark, mix, and filter through a washed, dry filter.

DETERMINATION

Measure a 25 ml aliquot of the filtrate into a 100 ml beaker. Evap. nearly to dryness and make to 6 ml in a graduated cylinder or marked test tube. Add, with shaking, 0.25 ml of 60–70% $HClO_4$, measured closely from a Mohr pipet. If no ppt or only a very slight ppt forms within 1–2 min., proceed with the detn of Na as in (*A*), below. (The perchlorate test on the 25 ml aliquot will detect 25 mg of K or 1 mg per ml, and up to 50 mg of K can be tolerated in the sodium-zinc-uranyl acetate pptn. Hence, if K is below the 1 mg per ml level, an aliquot as large as 50

ml may be taken for the detn.) If the quantity of K is shown to be excessive, proceed as in (*B*).

(*A*) *Procedure when K is not excessive.*—Depending upon the amount of Na expected, evap. an aliquot (usually 50 ml) on the steam-bath. When about 5 ml remains, add 4–5 drops of the HCl and evap. to dryness. Cool the beaker and add exactly 2.0 ml of H_2O to dissolve residual salts. Filter a portion of the zinc uranyl acetate reagent thru a washed filter (20 ml is required per detn) and add 5 ml, measured from a rapid pipet or rapid buret, to the beaker. Mix, and immediately decant into a 125 ml glass-stoppered Erlenmeyer flask with the aid of a glass rod, taking care not to wet the ground-glass neck of the flask. Complete the transfer with three more 5 ml portions of reagent **(d)**, mixing contents of the flask after each transfer.

If a ppt of the Na triple salt appears within a few minutes, stopper flask and let stand for 1.5–2.0 hrs. If there is no perceptible pptn within a few min., stopper the flask and place in an ice bath for 30–45 min., with occasional swirling. Remove, and let stand at room temp. for about an hour; then shake 15–20 min. in a shaking machine.

Filter the soln by suction thru a tared fritted glass crucible (Pyrex medium or equivalent) with the aid of a rubber-tipped glass rod. Transfer as much of the ppt as possible in the process, drain flask well, then rinse flask and wash ppt several times with ca 2 ml portions of the acid-alcohol wash soln (Soln **e**) from a wash bottle. Finally scrub flask, and rinse ppt and the filter, with several addnl 5–10 ml portions of the wash soln, allowing the crucible to suck dry each time. Wash down the sides of the crucible, suck dry, and rinse with 10–15 ml of ethyl ether. Suck dry, wipe the outside of the crucible, and in particular wipe out any condensate which may have formed below the fritted glass filter pad. Warm the crucible for 5–10 min. in a 100° oven, cool in an efficient desiccator, and weigh. Multipy the wt of the ppt by 0.01495 to obtain the wt of Na. The quantity of uranyl reagent specified (20 ml) will handle at least 16 mg of Na (over 1.0 g of ppt).

(*B*) *Procedure when K is excessive.*—If the qualitative test shows K in excess of 50 mg in the aliquot taken for analysis, proceed with a smaller aliquot if feasible, or remove excessive K as follows (7):

Evap. the aliquot to dryness on the steam-bath, add 1–2 ml of 60–70% $HClO_4$ and evap. to dryness on a hot-plate at not over 350°C., or under an overhead radiant heater. Expel all acid from the beaker (brush with free flame, if necessary), cool, take up with ca 5 ml of H_2O, and again evap. to dryness on steam-bath and hot-plate. (Caution: soln has tendency to spatter as it approaches dryness.) Cool, add 10–20 ml of the butanol-ethyl acetate mixt., digest near the b.p. for 2–3 min., and cool. Filter with suction thru a fritted glass crucible and collect the solvent in a small filter flask or side-arm test tube of adequate size. Wash the insol. residue in beaker and on filter 3 times with 5 ml portions of the solvent mixt.; then decant filtrate and washings into a 150 ml beaker. Dissolve the residue in the crucible in a minimum of hot H_2O and transfer soln to the original beaker. Again evap. to dryness, digest with 10 ml of the solvent mixt., and filter through the same crucible (which meanwhile has been dried, conveniently with acetone).

Wash residue in beaker and on filter with at least ten 1–2 ml portions of the mixed solvent, combine in the beaker with the first extracts, and evap. to dryness on the steam-bath. Take up residue with 1–2 ml of the constant boiling HCl and again evap. to dryness. Cool, and take up with 2 ml of H_2O to dissolve residual salts. Proceed as under (*A*) above. " . . . Filter a portion of the zinc uranyl acetate reagent," etc.

In all cases, correct the results by means of a blank determination.

B. FLAME PHOTOMETRIC METHODS

Flame photometry is emission spectroscopy in its simplest form, and current flame photometers are simple, direct-reading spectrographs. Their operation involves the spraying, successively, of unknown and standard solutions into a stable flame at the same definite rate, under the same physical and chemical conditions, and a comparison of the intensities of selected spectral lines or bands. The physical conditions involve the temperature of the flame, viscosity of the unknown and standard solutions, stability of the detector, etc.; the chemical conditions involve the absence (or presence of the same concentrations) of interfering cations, anions, or organic materials. The technique thus involves comparative and not absolute measurements of light intensity; flame methods are strictly comparison methods. Their advantage over arc methods of emission lies in the fact that it is relatively easy to control the temperature, shape, and position of a gas flame; a disadvantage is that the relatively low temperature of the gas flame excites only a few elements. Thus, utility of most commercial flame photometers is limited to the determination of the alkalies and alkaline earths.

However, the flame lines or bands of these are so few in number, and usually so intense, that simple equipment can be used for their isolation and detection. Thus, the Barclay, Baird, Janke, and Fox instruments employ monochromatic filters for isolation of the desired lines and use barrier-layer photocells and sensitive galvanometers for intensity measurement. The more elaborate flame photometers employ prisms for light dispersion, and phototubes and amplifiers (Perkin-Elmer 52 C; Beckman). By the use of a hotter flame and special detection equipment such as multiplier phototubes, many more elements may be detected.

Most progress in flame methods has been made within the last ten years and many articles have appeared during this time. The titles of some of them (8–14) suggest an over-enthusiastic acceptance of flame methods at first, followed by a gradual recognition of their limitations. Flame photometers are usually calibrated with aqueous solutions of some pure salt of the metal sought, e.g., sodium chloride. With a good instrument, the fine precision which can be attained in calibration may give a false impression of the over-all accuracy of the determination. It is hardly possible by any feasible means of sample preparation to isolate the sodium constituent of a food as a pure aqueous solution of a sodium salt. Usually it is necessary to compensate in some manner for the effect of unavoidable extraneous materials and this fact is now fairly well recognized.

However, commercial flame photometers vary greatly in design and details of operation, and the effects of extraneous ions and of organic materials likewise vary. Further confusion is added by the constant introduction of new models of the commercial instruments; careful studies of interference effects made with previous models tend to become obsolete. The

analyst must recognize that flame methods are subject to the effects of numerous interferences and that these must be appraised for his *particular* instrument (regardless of make or model). The type of interferences will, of course, depend upon the character of the material he analyzes routinely. Helpful information is given in the instruction manuals of the various commercial flame photometers and in the literature (8–15).

Some publications describe the operation and interference effects of specially constructed "home-made" flame instruments, burners, and atomizers. Such information is interesting but may be of little value to the technician who must rely upon the commercially available designs. There have been complaints, likewise, that directions for calibration and operation of the various commercial instruments are usually not "spelled out" in sufficient detail for the ordinary technician. There appears to be no unanimity as to proper methods of instrument standardization, interference correction, and evaluation of results.

In the routine analysis for sodium of many foods the Associate Referee has used two commercial flame photometers: the Beckman DU with the No. 9200 accessory, acetylene-oxygen burner, and regular phototubes; and the Perkin-Elmer Model 52 C, operated with a lithium internal standard. It is proposed to detail procedures of calibration, operation, interference appraisal, etc., which the Associate Referee has found workable for both instruments. It is not claimed that the following procedures are best, and it is emphasized that the two instruments employed happened to be the ones available for use; for the determination of sodium in foods, other instruments may be equal or superior in performance.

For the flame photometric determination of sodium the sample preparation, which follows the procedure outlined above for the gravimetric determination, is designed to eliminate the effect of phosphate, an emission depressant, and of calcium, a band, and stray light interference. It is hardly possible to eliminate the effect of potassium, and methods of correcting for its interference are presented; these involve separate flame determinations of potassium. (As will be seen, the K correction is applied in entirely different ways with the two instruments studied.)

FLAME METHODS

ADDITIONAL REAGENTS

Calcium (used to ppt phosphates) does not interfere in the gravimetric procedure, above, but for the successful application of flame methods it is necessary to insure its complete removal from the sample soln. This is done by an oxalate pptn and filtration. It is well to check the strength of reagent **c**, above, by pptng a small, measured portion with oxalate in order to be sure that the later addn of the prescribed amount of oxalate will insure complete removal of Ca. The oxalate reagent is described below. Use of a lithium internal standard with Perkin-Elmer instrument (not with the Beckman) requires description of another reagent, *viz.*, lithium chloride. Because of its extreme hygroscopicity the chloride is hard to handle but its use avoids the interfering effect of, for instance, the sulfate ion.

(g) *Ammonium oxalate, satd soln.*—Prep. from the re-crystd salt and store in a polyethylene or heavily paraffined bottle.

(h) *Lithium chloride soln.*—Re-cryst. the reagent grade salt by boiling down its satd soln on the hot-plate until a copious ppt results. Filter on a Büchner and suck as dry as possible (reserve liquor for another crop). Dry overnight in an oven at 135°C. and finally to roughly constant wt in the muffle at 250–275°C. in a glass vessel fitted with tight cover. Prep. a strong stock soln (by wt diff. from the covered vessel) of ca 1% Li strength (factor, LiCl:Li = 6.1091). From the stock soln, prep. a soln contg 2 mg Li/ml. Check this final strength by drying down a measured portion, as above, in a large glass-stoppered weighing bottle. Preserve Li solns in polyethylene or paraffined bottles.

(Exact strength of the 2 mg/ml soln is not critical if it is remembered that in operations with the Perkin-Elmer instrument, equal vols of *the same* Li soln must be added to both standards and unknowns. This re-crystn procedure will reduce, but not eliminate, traces of Na and K. If prepd in vol. and carefully preserved, a batch of LiCl soln will last a long time; if a new batch is made, new standard curves for the Perkin-Elmer instrument must be made.)

(i) *Standard NaCl solns.*—Prep. a soln contg 2 mg Na/ml from the reagent grade salt and preserve in a polyethylene bottle. (Pulverize, and dry several hours at ca 110°C. before weighing out.) Prep. dilns as needed.

(j) *Standard* KCl *solns.*—Prep. from the thrice re-crystd salt. (Na content can be reduced to less than 0.2 p.p.m.) Pulverize, and dry the salt for several hours at ca 110°C. before weighing out. Prep. a stock soln contg 2 mg K/ml, and dilns as needed. Preserve in polyethylene bottles.

PREPARATION OF SAMPLE

Proceed as in *A*, above, to " . . . make slightly alkaline with 5–6 drops of the redistd ammonia." Make to vol. at this point, without further addn of $CaCl_2$, and filter off the phosphate ppt. Place a 50 ml aliquot of the filtrate into another 100 ml volumetric flask, and add 2.5 ml of reagent g. Dil. nearly to vol. and digest on the steam-bath for an hr. (preferably in a warm place overnight). Adjust temp., make to vol., and filter through a washed filter. Filtrate must be clear; if not, return thru filter. (Excess oxalate has no appreciable effect upon the emission intensities of either Na or K with the Beckman or Perkin-Elmer instruments.)

DETERMINATION (BECKMAN)

Two sodium ranges are employed, 0–10 p.p.m. and 0–100 p.p.m. Na. In addition, a 0–100 p.p.m. K range is used. The calibration curves and correction curves for K are illustrative only and, of course, will not hold for all Beckman instruments. Curves must be prepd for each instrument and burner. Other Na ranges may be more convenient for particular products. Satisfactory operation of the Beckman instrument requires a perfectly clean burner and perhaps more than usual attention to the condition of the batteries. The following operating conditions were employed by the Associate Referee (Table 1).

Preparation of standard curves.—Allow instrument to warm up thoroly and aspirate a standard Na soln representing the top of the range (e.g., 10 p.p.m. Na). With the T dial set at 100, adjust wavelength dial to max. deflection; then zero the needle with the sensitivity knob. Then aspirate intermediate concns of Na: 1.0, 2.0, 3.0 p.p.m., etc., and balance with the dial. Between readings for each intermediate concn, check the 100% adjustment. Repeat readings, working down the range (from 9.0 to 1.0 p.p.m.). Readings should check within 0.5 division on the T scale. Finally, aspirate distd H_2O and det. the flame background. It should be about

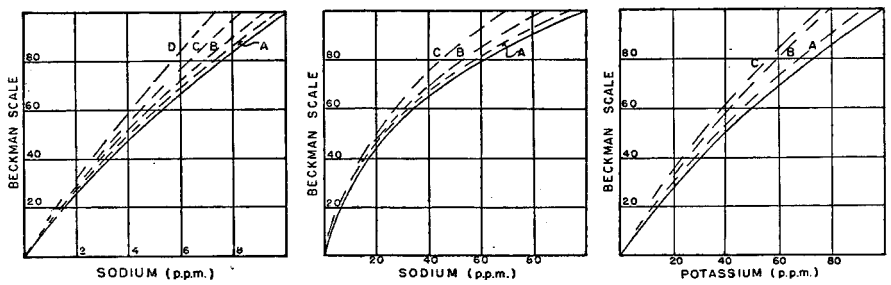
TABLE 1.—*Operating conditions for Beckman instrument*

	SODIUM		POTASSIUM
Range (p.p.m.)	0–10	0–100	0–100
Selector	0.1	0.1	0.1
Resistor (10,000 megohm)	Position 2	Position 2	Position 2
Sensitivity	About mid-way	About mid-way	About mid-way
Slit (mm)	0.05	0.02	0.15
Wavelength (mμ)	589[a]	589[a]	768[a]
Phototube	Blue-sensitive	Blue-sensitive	Red-sensitive
Filter slide	In	In	Out[b]
O_2 pressure (tank)	30	30	30
O_2 pressure (dial)	19	19	19
C_2H_2 pressure (tank)	20	20	20
C_2H_2 pressure (dial)	6	6	6

[a] With atomizer in operation, adjust wavelength dial to maximum deflection at about this wavelength.
[b] 4.0 mm Corning didymium glass was used to mask stray Na light.

1% for the 0–10 p.p.m. range, and negligible for the 0–100 Na and K ranges. Subtract this background intensity from all readings, including the top-of-range, 100% reading; average, and plot to obtain standard curves (Figs. 1–3, solid lines).

In routine operation, check the permanance of the standard curves weekly by aspirating an intermediate Na standard (e.g., 5 p.p.m.) If the T value is significantly



1 2 3

FIG. 1—Standard curve for 0–10 p.p.m. Na (solid line)
Curve A: Potassium-sodium ratio, 1:1
Curve B: Potassium-sodium ratio, 10:1
Curve C: Potassium-sodium ratio, 100:1
Curve D: Potassium-sodium ratio, 1000:1

FIG. 2—Standard curve for 0–100 p.p.m. Na (solid line)
Curve A: Potassium-sodium ratio, 1:1
Curve B: Potassium-sodium ratio, 10:1
Curve C: Potassium-sodium ratio, 100:1

FIG. 3—Standard curve for 0–100 p.p.m. K (solid line)
Curve A: Sodium-potassium ratio, 1:1
Curve B: Sodium-potassium ratio, 5:1
Curve C: Sodium-potassium ratio, 10:1

above or below the line, run other standards such as 2.0, 3.0, 4.0, and 6.0, 7.0, 8.0, both above and below the range mid-point. If the curvature of the standard curve has changed, plot these values to form a new curve. It is especially important to re-check curves after adjustment, or repairs such as replacement of batteries or tubes, of the electrical circuits of the photometer.

Evaluation of K *interference.*—With the Beckman instrument, Na and K exert a pronounced mutual enhancing effect (Figs. 1–3). It was noted that this positive error, expressed as per cent, was roughly proportional to the *ratio* of interfering metal to the metal sought; thus a soln contg 1 p.p.m. of Na plus 10 p.p.m. of K and a soln contg 8 p.p.m. of Na plus 80 p.p.m. of K (ratios of 1:10 in each case) would

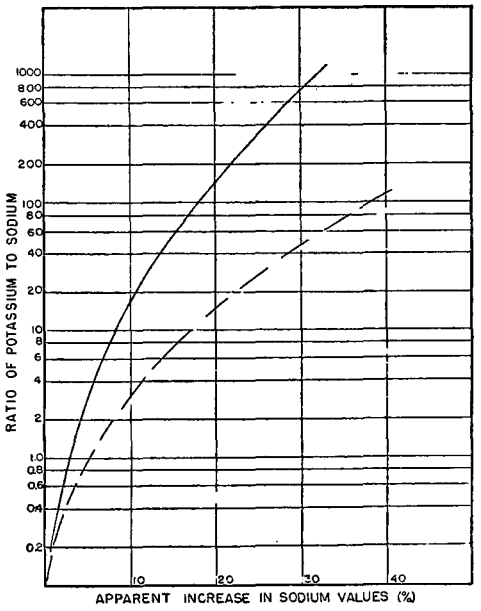


FIG. 4—Correction curves
Solid line: 0–10 p.p.m. Na range
Dashed line: 0–100 p.p.m. Na range

both read too high by about 8%, or about 0.08, and 0.6–0.7 p.p.m. too high, respectively. This fact rendered possible the construction of correction curves (Fig. 4) for both Na ranges. A similar correction curve (not shown), involving the effect of Na upon the K detn, was made for the 0–100 p.p.m. K range.

To evaluate the effect of K upon the flame analysis of Na, first prep. 1:1 solns of Na plus K (e.g., 2.0 p.p.m. Na plus 2.0 p.p.m. K, 4.0 plus 4.0, 6.0 plus 6.0, etc.) Flame these and det. the average positive error, as per cent, from the standard curve. Repeat with 1:10, 1:100, and 1:1000 ratios (1:1000 for 0–10 p.p.m. Na range only). Plot av. positive errors for each ratio on poly-phase, semi-log paper, and connect with a smooth curve similar to those shown in Fig. 4. For the high K ratios, it may be necessary to correct for a slight amount of Na in the re-crystd KCl. Derive this correction by direct flaming of a soln of 10,000 p.p.m. (1%) K strength. (Ratios of

K to Na as high as 1000:1 or higher may be encountered in certain foods.)

Flame photometry of sample solution.—Flame a portion of the Ca-free filtrate obtained under PREPARATION OF SAMPLE, above. The appearance of the flame will usually indicate what range and diln, if any, to employ. (Best accuracy is obtained from the upper portions of the standard curves.) Obtain average Na readings, switch to the K settings, and obtain a reading for K. (In food work this usually necessitates a separate, weaker diln of the sample soln.) Correct readings for background with distd H_2O and derive the indicated p.p.m. of both Na and K (taking into account the diln factors) in the undild sample soln, which, of course, represents only half the wt of sample originally ashed.

Calc. the Na:K ratio. In food work, this ratio, and hence the effect of Na upon the K detn, is usually low enough to be ignored. If not, correct the K figure for the influence of Na by means of a correction curve, as described above, similar to the ones shown in Fig. 4. The corrected value, X, is derived from the relationship: $Y - aX = X$, where Y is the uncorrected or indicated value, and a is the apparent *per cent* increase in the true value ($\times 10^{-2}$) read from the ratio correction curve. From the (corrected) K value, derive the ratio of K to the indicated value for Na in the sample soln, and read the correction factor for Na from a curve similar to those of Fig. 4. As above, Y, the uncorrected value for Na, is related to the corrected value thru the equation, $Y - aX = X$.

Experience has shown that these corrected values are not significantly altered by carrying them thru a second series of adjustments. With the Beckman instrument the Associate Referee has found it convenient to record analytical data in a table with the headings: (1) wt of sample ashed, (2) Na range employed, (3) dilution, (4) reading (av.), (5) reading (corr.), (6) indicated p.p.m. Na from curve, (7) p.p.m. Na times dilution factor; (8) dilution for K, (9) reading for K, (10) indicated p.p.m. K from curve, (11) p.p.m. K times dilution factor; (12) Na/K ratio, (13) correction to K value, (14) corrected K value, (15) K (corr.)/Na ratio, (16) correction to Na value, (17) corrected Na value in p.p.m., (18) corrected Na value minus reagent blank, (19) wt sample in 100 ml final sample soln (one-half of amount originally ashed), (20) results for Na in terms of mg/100 g of sample, (21) results for K expressed in the same manner.

The reagent blank should not be more than 0.3–0.4 p.p.m. in the 100 ml of Ca-free sample soln.

If desired, check results for Na by comparing flame readings with a synthetic mixt. contg pure Na and K in the indicated amount. Readings should check closely (within 1–2 scale divisions.)

DETERMINATION (PERKIN-ELMER)

A Perkin-Elmer, Model 52 C instrument, designed for convenient internal standard operation with lithium, was employed by the Associate Referee. Theoretically, use of the lithium internal standard should compensate for fluctuations in air and fuel pressures, in line voltage, and in temp. It should also act as a matrix in the masking of certain interferences. One hundred p.p.m. of Li (5 ml of a soln contg 2 mg Li/ml, per 100 ml; reagent **h**), added to both standards and unknowns, provided adequate sensitivity and good galvanometer stability. Propane, plus filtered compressed air from the laboratory line, was used to feed the burner. The atomizer funnel emptied in ca 65 sec.

As with the Beckman, Na ranges of 0–10 and 0–100 p.p.m. and one K range of 0–100 p.p.m. were employed. It is again pointed out that standard curves are characteristic of only one instrument and atomizer. Operating conditions employed for the Perkin-Elmer instrument are given in Table 2.

TABLE 2.—*Operating conditions for the Perkin-Elmer flame photometer*

	SODIUM		POTASSIUM
Range (p.p.m.)	0–10	0–100	0–100
Li (p.p.m.)	100	100	100
Wavelength	589[a]	589[a]	768[a]
Coarse gain	Position 9	Position 5	Position 3
Air pressure	10 lbs	10 lbs	10 lbs
Propane pressure	5 lbs	5 lbs	5 lbs

[a] Adjust to max. deflection at marked positions on scale. The movable slit automatically admits light to proper phototube.

Preparation of standard curves.—Allow the instrument to warm up thoroly, and adjust the needle to near the mid-point of the scale (50) by means of the galvanometer adjustment knobs. Set the internal standard dial to 100, and aspirate a soln contg the top of the Na range and contg in addition 100 p.p.m. Li (reagent h); e.g., 10 p.p.m. Na plus 100 Li. Balance to near the mid-point with the course and fine gain controls and adjust the slit to maximum deflection. Center the needle on the mid-point (the 49 or 51 scale marks furnish a finer reference line) by readjusting the fine gain control. Then turn the internal standard dial to zero, aspirate a soln contg 100 p.p.m. of Li only, and adjust needle to the same reference mid-point by means of the fine galvanometer adjustment. Repeat with top-of-range soln, and the 100 p.p.m. Li soln, until no further adjustments are necessary (usually 2–3 funnelfuls of each). Then aspirate intermediate Na concns, e.g., 1.0, 2.0 3.0, etc., p.p.m., all of which contain 100 p.p.m. Li, and record readings of the internal standard dial. Check the zero and 100 per cent points repeatedly. Plot dial readings against p.p.m. of metal to obtain curves similar to those of Fig. 5. (The

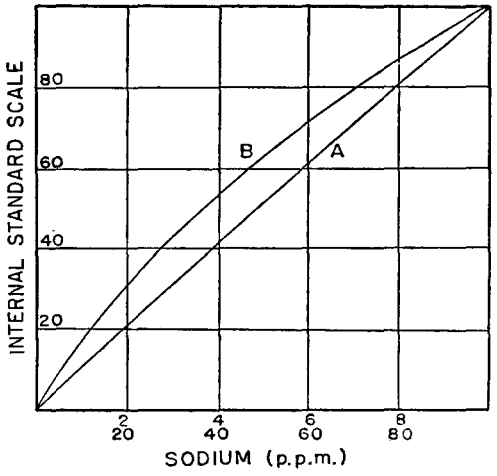


FIG. 5—Standard curves with Perkin-Elmer flame photometer
Curve A: 0–10 p.p.m. Na range
Curve B: 0–100 p.p.m. Na range
(100 p.p.m. Li internal standard)

curve for the 0–10 p.p.m. Na range was found to be very nearly linear; that for the 0–100 range showed some curvature. The curve for 0–100 p.p.m. K (not shown) was similar to that for 0–100 Na, but was somewhat more linear.)

Dial readings will fall off as the funnel empties, and should not be taken unless the funnel is at least ¾ full. A suction flask fitted with an inverted U-shaped glass tube may be used to suck excess soln from the funnel, and thus save time between readings. As with the Beckman instrument, check standard curves frequently and construct new ones if they are observed to change significantly.

Evaluation of K *interference.*—While not so marked as with the Beckman instrument, the effect of K upon the Na detn with the Perkin-Elmer is variable and difficult to evaluate. Due to the relatively low-temperature flame of the latter instrument, enhancement effects might be expected to be smaller. However, with internal standard operation, actual repression effects are often noted. These plus or minus errors vary with the Na range employed. Error compensation, perhaps due to leakage of stray K light to the internal standard (Li) phototube, coupled with decreased rate of aspiration due to increased viscosity at the higher K levels, may be responsible. These are only speculative explanations. In the Associate Referee's experience with only one Perkin-Elmer instrument, it was not possible to derive simple ratio corrections for K, and corrections had to be set up in tabular form (see Tables 3 and 4).

To evaluate errors due to K, proceed as follows (0–10 p.p.m. Na range): For each step in the Na range, prep. two solns, one contg only Na, and the other contg 5, 10, 25, 50, etc., p.p.m. K, in addn. (Each soln, of course, contains 100 p.p.m. Li as internal standard.) By means of the fine gain control, exactly balance each K-free soln to its proper point on the standard curve, then aspirate, alternately, the K-free soln and the solns contg the same amount of Na but increasing amounts of K. Depending upon the range and the amount of K, the needle may show a definite shift to left or right. Adjust to mid-point with the internal standard dial, note deviations from the standard reading, and translate these into positive or minus errors from the respective standard curves for Na. Construct correction tables similar to Tables 3 and 4.

TABLE 3.—*Corrections*[a] *to Na values (0–10 p.p.m. Na range; Perkin-Elmer instrument) for various concns of K*

SODIUM (P.P.M.)	POTASSIUM (P.P.M.)							
	5	10	25	50	100	500[b]	1000[b]	10,000[b]
0.5	—	.01	.02	.02	.03	.07	.11	.22
1.0	.02	.03	.03	.04	.06	.08	.12	.20
2.0	.04	.05	.06	.06	.10	.12	.13	.07
3.0	.05	.07	.10	.11	.11	.10	.10	—
4.0	.05	.09	.13	.15	.12	.08	.06	*.12*
5.0	.06	.10	.16	.18	.13	.06	—	*.24*
6.0	.06	.11	.19	.22	.15	.04	*.05*	*.35*
7.0	.06	.11	.23	.25	.20	.03	*.05*	*.45*
8.0	.07	.12	.26	.29	.25	.02	*.06*	*.63*
9.0	.07	.12	.29	.33	.29	—	*.10*	*.80*
10.0	.08	.12	.32	.36	.34	—	*.17*	*1.00*

[a] Figures in ordinary type mean *subtract* from indicated Na value. Figures in italics mean *add* to indicated Na value.
[b] Corrected for Na in the KCl soln used: 10,000 p.p.m. K = .18 p.p.m. Na; 500 p.p.m. K = .01 p.p.m. Na.

TABLE 4.—*Corrections[a] to Na values (0–100 p.p.m. Na range; Perkin-Elmer instrument) for various concns of K*

SODIUM (P.P.M.)	POTASSIUM (P.P.M.)						
	20	50	100	250	500	1000	10,000
10	.2	.3	.2	—	—	*.2*	*1.0*
20	—	.1	—	—	*.2*	*.4*	*2.2*
30	—	—	—	—	*.3*	*.7*	*3.5*
40	—	—	—	—	*.5*	*1.0*	*4.7*
50	—	—	—	*.3*	*1.0*	*2.0*	*6.0*
60	—	—	—	*.6*	*1.5*	*3.0*	*7.4*
70	—	—	—	*.8*	*2.0*	*3.5*	*8.6*
80	—	—	—	*1.0*	*2.5*	*3.8*	*9.7*
90	—	—	—	*1.2*	*3.0*	*4.4*	*10.0*
100	—	—	—	*1.3*	*3.5*	*5.0*	*10.2*

[a] Figures in ordinary type mean *subtract* from indicated Na value. Figures in italics mean *add* to indicated Na value.

(Na may be expected to exert a similar interference in the detn of K with the Perkin-Elmer instrument but this has not been investigated by the Associate Referee.)

Flame photometry of sample solution.—Flame a portion of the Ca-free filtrate obtained under PREPARATION OF SAMPLE, above. It is, of course, possible to add the 100 p.p.m. of Li by adding 5 ml of reagent **h** to the 100 ml volumetric flask before making to vol. and filtering off the calcium oxalate ppt. However, the analyst usually does not know the proper range and/or diln to employ. If Li is added at this stage, further necessary dilns (sometimes as high as 1:20, or 1:50) must be made with 100 p.p.m. Li soln, and this is wasteful of reagent **h**. It is usually better not to add the Li directly, but to make to vol., filter off Ca, aspirate a small portion, and observe the flame; a little experience can tell what Na range and diln to employ. If sodium content of the sample is seen to be low (within the 0–10 p.p.m. range), place 2.5 ml of reagant **h**, measured accurately from a small buret or Mohr pipet, in a 50 ml volumetric flask and make to vol. with the filtrate. (Dilution factor = 0.95.)

Using the proper diln and range, derive the Na content, in p.p.m., of the sample soln. Switch to K operation and likewise derive the K content in terms of the undild sample soln. Calc. the K content *of the diln employed in the Na estimation* (ratio corrections do not hold with the Perkin-Elmer instrument) and correct the indicated Na value from tables such as Tables 3 and 4, interpolating between Na values, if necessary. Correct for reagent blank and calc. Na (and K) content of the sample in terms of mg/100 g. Convenient tabular headings with Perkin-Elmer operation are: (1) wt of sample ashed, (2) K range employed, (3) dilution for K, (4) reading for K, (5) p.p.m. K, (6) p.p.m. K times dilution factor, (7) Na range employed, (8) dilution for Na, (9) reading for Na, (10) indicated p.p.m. Na, (11) p.p.m. *K* in Na soln *as read*, (12) corrected Na figure, (13) corrected Na figure times dilution factor, (14) corrected Na figure minus reagent blank, (15) wt sample in 100 ml final sample soln (one-half of amount originally ashed), (16) results for Na in mg/100 g, (17) results for K expressed likewise.

If desired, check Na readings by flaming a soln contg pure Na and K in the indicated amounts.

DISCUSSION

Flame results on the same sample solutions with the Beckman and Perkin-Elmer instruments, and, where applicable, with the A.O.A.C. method for sodium in plants (par. **6.21**) have checked quite well. Table 5 gives typical results.

TABLE 5.—*Comparison of results (mg/100 g) for sodium in various foods*

FOOD	METHOD		
	BECKMAN	PERKIN-ELMER	A.O.A.C. 6.21
Beets, fresh	30.3	27.8	28.2
Brussels sprouts, fresh	12.2	11.5	12.3
Carrots, fresh	55.5	51.7	51.3
Celery, fresh	94.3	90.3	90.0
Mushrooms, fresh	3.7	3.7	3.9
Tomato, fresh	2.5	2.5	2.3
Honey	4.4	4.8	5.2
Yeast	2.5	2.7	1.9
Milk, fresh	53.0	49.2	48.9
Butter, unsalted	22.4	20.5	22.1
Beefsteak, raw	69.1	68.2	—
Chicken, light, raw	54.1	54.3	—
Chicken, dark, raw	80.6	79.0	—
Chicken, liver, raw	83.7	84.7	—

The gravimetric zinc-uranyl acetate method described above has, at present, been applied to only a limited number of foods, but it is expected to be an improvement over A.O.A.C. **6.21.** In a limited collaboration by this method, two analysts obtained the following results upon orange juices to which had been added 2.0, 4.0, 6.0, 8.0, and 10.0 mg/100 g quantities of sodium. It should be pointed out that the samples were not identical but were prepared at different times from different batches of orange juice (Table 6).

TABLE 6.—*Recoveries of sodium by gravimetric method*

SODIUM ADDED (MG/100 G)	SODIUM FOUND (MG/100 G)			
	ANALYST 1		ANALYST 2	
	TOTAL	NET	TOTAL	NET
none	1.50[a]	—	.90[a]	—
2.00	3.55	2.05	3.05	2.15
4.00	5.50	4.00	5.10	4.20
6.00	7.28	5.77	7.28	6.38
8.00	9.18	7.68	8.90	8.00
10.00	11.43	9.93	11.10	10.20

[a] Includes reagent blank plus natural Na in juice.

SUMMARY AND RECOMMENDATION

A gravimetric method for the determination of sodium in foods, which provides for the removal of interfering potassium, is described. Flame photometric methods with two typical flame instruments are detailed.

It is recommended* that work on the determination of sodium in foods be continued.

REFERENCES

(1) CLIFFORD, P. A., *This Journal*, **36**, 594 (1953).
(2) BILLS, C. E., MCDONALD, F. G. NIEDERMEIER, W., and SCHWARTZ, M. C., *J. Am. Dietet. Assoc.*, **25**, 304 (1949).
(3) *Sodium Restricted Diets*, prepared by a committee of the Food and Nutrition Board, National Academy of Sciences, National Research Council, Washington, D. C. (In press).
(4) KOLTHOFF, I. M. and SANDELL, E. B., *Textbook of Quantitative Inorganic Analysis*, The Macmillan Co., New York, 1943.
(5) KIRK, P. L., *Quantitative Ultramicroanalysis*, John Wiley and Sons, New York, 1950.
(6) GLICK, D., *Techniques of Histo- and Cytochemistry*, Interscience Publishers, Inc., New York, 1949.
(7) HILLEBRAND, W. F., LUNDELL, G. E. F., BRIGHT, H. A., and HOFFMAN, J. I., *Applied Inorganic Analysis*, John Wiley and Sons, New York, 1953.
(8) BERRY, J. W., CHAPPELL, D. G., and BARNES, R. B., *Ind. Eng. Chem., Anal. Ed.*, **18**, 19 (1946).
(9) PARKS, T. D., JOHNSON, H. D., and LYKKEN, L., *Anal. Chem.*, **20**, 822 (1948).
(10) BILLS, C. E., MCDONALD, F. G., NIEDERMEIER, W., and SCHWARTZ, M. C., *ibid.*, **21**, 1076 (1949).
(11) SOMMER, A. J., *Am. J. Med. Technol.*, **17**, 276 (1951).
(12) ELLIOTT, F. H., *Can. J. Technol.*, **29**, 111 (1951).
(13) BERNSTEIN, R. E., *Biochim. et Biophys. Acta*, **9**, 576 (1952).
(14) COLLINS, G. C., and POLKINHORNE, H., *Analyst*, **77**, 430 (1952).
(15) SCOTT, R. K., MARCY, V. M., and HRONAS, J. J., *A.S.T.M. Symposium on Flame Photometry*, Spec. Tech. Publ. No. 116, 1951, pp. 105–114.

No reports were given on DDT as spray residue on foods, fluorine, insecticides in canned foods, mercury, parathion, or zinc.

REPORT ON GUMS IN FOOD

By M. J. GNAGY (Food and Drug Administration, Department of Health, Education, and Welfare, Los Angeles 15, Calif.), *Referee*

No formal reports were received from the Associate Referees this year, although some of them accomplished a great deal of work. The Associate Referee on Gums in Cacao Products and the Associate Referee on Gums

* For report of Subcommittee C and action of the Association, see *This Journal*, **37**, 71 (1954).

in Frozen Desserts are no longer in government service and new Associate Referees should be appointed.

The Associate Referee on Gums in Frozen Desserts has worked out a method which is now ready to be submitted to collaborators.

The Referee has been working on a spectrophotometric method for the detection of stabilizers in soft curd cheeses. The method will detect as little as 0.05 per cent of many of the stabilizers permitted in soft curd cheese.

RECOMMENDATIONS

It is recommended*—

(1) That work be continued on detection of gums in catsup and related products.

(2) That work be continued on the detection of gums and other stabilizing agents in frozen desserts.

(3) That work be done on the detection of alginates in cheese.

(4) That work be continued on detection of algin and algin derivatives in dressings for foods.

(5) That work be continued on detection of the other stabilizing agents which are permitted by the Federal Standard for salad dressing.

(6) That work be continued on gums in cheese spreads.

REPORT ON FISH AND OTHER MARINE PRODUCTS

By MENNO D. VOTH (Food and Drug Administration, Department of Health, Education, and Welfare, Seattle 4, Wash.), *Referee*

The Associate Referee on Total Solids in Oysters continued his investigations during the past year. A detailed study was made of the use of the forced draft oven, and other related phases of the work were also investigated. The findings of the Associate Referee on the use of the forced draft oven were very encouraging. This Referee recommended in his report that the use of the forced draft oven should be authorized for oyster work, since it results in a substantial saving of time.

The Associate Referee on Total Solids and Ether Extract in Fish and Other Marine Products made collaborative studies comparing the forced draft oven and the vacuum oven with the standard air oven in the determination of total solids in fish. Results obtained show that the forced draft oven is equally as good as the air oven and that its use results in a substantial saving in time. It is suggested that additional work be done which will indicate whether this method is applicable to marine products other than fish or oysters.

* For report of Subcommittee C and action of the Association, see *This Journal*, 37, 72 (1954).

The Associate Referee also submitted to collaborative study a rapid method for ether extract in fish, as suggested last year. The results were not conclusive and further work is planned.

RECOMMENDATIONS

It is recommended*—

(1) That the method for determining total solids in fish, as given in the Associate Referee's report and *This Journal*, **35**, 216 (1952) be changed to authorize the use of the forced draft oven by inserting ahead of the last sentence the following "(or preheated forced draft oven set for full draft for 1 hr. at 100°C.)."

(2) That the method thus revised be adopted, first action.

(3) That collaborative work be continued on the suggested rapid method for ether extract in canned fish, extending it to fish other than canned salmon.

(4) That the use of the forced draft oven for determining total solids in oysters be authorized by adopting, first action, the method suggested by the Associate Referee, worded as follows:

"Make duplicate detns. Weigh quickly 10 g of meats, liquid, or mixed meats and liquid, in flat-bottom metal dish ca 9 cm in diam. Spread sample evenly over bottom of dish, insert directly in preheated forced draft oven set at full draft, and dry 1.5 hrs at 100°C. Cool in desiccator and weigh promptly."

(5) That the use of chemical and viscosimetric methods for the determination of solids in oysters be investigated.

REPORT ON TOTAL SOLIDS AND ETHER EXTRACT IN FISH

By H. M. Risley (Food and Drug Administration, Department of Health, Education, and Welfare, Seattle 4, Wash.),
Associate Referee

TOTAL SOLIDS

A method in which an air oven is used has already been suggested for total solids in fish and other marine products (except oysters)[1] but until this year the Associate Referee has been unable to submit it to collaborative study or to compare it with other oven methods. During the past year, five different samples of fish were submitted to collaborators for determination of total solids. All collaborators used the air oven method, and in addition, some used both a forced draft oven and a vacuum oven.

* For report of Subcommittee C and action of the Association, see *This Journal*, **37**, 70, 71 (1954).
[1] *This Journal*, **35**, 216 (1952).

Use of the forced draft oven, when it is available, was suggested because it might save considerable time (preliminary results obtained at this laboratory indicated complete drying in one hour). The vacuum oven was used as a reference method.

The first sample, sent to eight collaborators, was canned pink salmon. Ten one-pound cans were ground, one at a time, in a Waring blendor; the entire amount was then thoroughly mixed and placed in pint jars. Ten ml of a 12.8 per cent Zephiran chloride solution was added as a preservative to each 100 g of ground fish.

The next three samples consisted of fresh skinless and boneless fillets of halibut, Chinook salmon, and rockfish. These samples were ground three times in a food grinder, and were mixed thoroughly between grindings. One-pint portions were frozen and sent to six collaborators.

The last sample, canned Chinook salmon, was prepared like the first, except that no preservative was added and the pint jars were frozen for shipment. Collaborators were asked to determine total solids in duplicate by the following method, then to repeat the determinations, using first a forced draft oven at 100°C. for one hour, and then a vacuum oven at 100°C. for four hours.

TOTAL SOLIDS IN FISH AND OTHER MARINE PRODUCTS (EXCEPT OYSTERS)

Prepare a 9 cm flat-bottom covered weighing dish in the following manner: Cut into short lengths and add ca 2 g asbestos fibers of the type used in preparing Gooch crucibles and an 8 cm stirring rod with flattened end. Dry dish, asbestos, and rod in air oven 1 hr at 100° and tare. Weigh into dish, to the nearest mg, 9–10 g of prepd sample. Add 20 ml of distd H_2O and mix sample thoroly with asbestos. Support end of stirring rod on edge of dish and evap. just to dryness on steam bath, stirring once while still moist. Drop rod into dish and heat 4 hrs in air oven at 100°. Cover dish, cool in desiccator, and weigh promptly.

Referring to Table 1, it can be seen that the results on the two samples of canned fish were much more uniform than those on the three samples of fresh fish. This may be due in part to the ease of preparing a finely divided uniform mixture with a Waring blendor. The fresh fish could not be ground satisfactorily in a blendor, and the next best method available was a meat grinder with holes 2 mm in diameter. The resulting mixture was lumpy; this made it difficult to obtain a representative sample and to reincorporate the moisture that separates on thawing. In the case of fresh halibut and fresh salmon, the average results by the forced draft oven were significantly higher than by the other two methods (not all collaborators agreed on this point, however). The Associate Referee believes that if the fresh fish could be as finely ground as the canned samples, much closer agreement among collaborators would be obtained.

The results by the forced draft oven were extremely gratifying, particularly since this is a relatively new piece of equipment in many laboratories and very little was known about the performance of six different ovens of

TABLE 1.—*Total solids in 5 collaborative samples of fish—comparison of 3 different oven methods*[a]

COLLABORATOR	CANNED PINK SALMON			FRESH HALIBUT			FRESH CHINOOK SALMON			FRESH ROCKFISH			CANNED CHINOOK SALMON		
	AIR OVEN, 4 HRS	FORCED DRAFT OVEN, 1 HR	VACUUM OVEN, 4 HRS	AIR OVEN, 4 HRS	FORCED DRAFT OVEN, 1 HR	VACUUM OVEN, 4 HRS	AIR OVEN, 4 HRS	FORCED DRAFT OVEN, 1 HR	VACUUM OVEN, 4 HRS	AIR OVEN, 4 HRS	FORCED DRAFT OVEN, 1 HR	VACUUM OVEN, 4 HRS	AIR OVEN, 4 HRS	FORCED DRAFT OVEN, 1 HR	VACUUM OVEN, 4 HRS
R. N. Allen, Gorton-Pew Fisheries, Gloucester, Mass.	28.20														
G. Ivor Jones, Nat. Canners Assn., Seattle	28.62 28.65			20.93 20.72			26.29 26.37			23.96 23.91			32.50 32.69		
D. Menschenfreund, L. A. District, Food & Drug Admin.	28.53 28.49	28.57 28.59	28.49 28.47	19.64 19.48	20.35 20.23	19.51 19.31	25.32 25.26	26.53 26.31	25.25 25.38	22.04 21.85	22.56 22.62	21.87 21.82	32.72 32.90	32.81 32.74	32.70 32.67
D. W. Johnson, Denver District, Food & Drug Admin.[b]	28.79 28.76	28.62 28.57	28.56 28.60	20.46 20.59	20.73 20.80	20.43 20.55	26.99 26.63	27.50 27.33	27.13	23.21 23.59	23.27 23.37	23.37 23.56	32.62 32.69	32.67 32.72	32.85 32.83
M. C. Harrigan, Boston District, Food & Drug Admin.	28.46 28.45	28.63 28.64	28.44 28.42												
H. J. Meuron, S. F. District, Food & Drug Admin.	28.73 28.74	28.90 28.89	28.74 28.82	19.94 19.94	20.18 20.16	19.95 19.84	26.30 26.32	26.28 26.31	26.06 26.18	22.50 22.47	22.45 22.59	22.49 22.60	32.64 32.68	32.67 32.64	32.64 32.65
S. H. Perlmutter, Minneapolis District, Food & Drug Admin.	28.89 28.98	28.89 28.88	28.89 28.86	19.90 19.54	19.94 19.81	19.96 19.80	24.96 25.79	26.32 26.30	26.32 26.16	23.03 22.59	22.86 22.76	22.74 22.82	32.67 32.64	32.59 32.78	32.64 32.63
J. A. Ross, Seattle District, Food & Drug Admin.	28.57 28.58	28.54 28.51	28.52 28.52	19.84 19.98	20.15 20.15	19.82 19.96	26.16 26.23	26.23 26.20	26.07 25.92	22.51 22.49	22.48 22.50	22.33 22.38	32.70 32.66	32.70 32.69	32.66 32.66
H. M. Risley, Seattle District, Food & Drug Admin.	28.41 28.50	28.62 28.54	28.61 28.66	20.01 20.08	20.45 20.23	20.32 20.15	26.38 26.33	26.09 26.15	26.13 26.28	22.67 22.78	22.91 22.80	22.70 22.75	32.68 32.71	32.60 32.64	32.59 32.60
Total number of determinations	17	14	14	14	12	12	14	12	11	14	12	12	14	12	12
Average (%)	28.61	28.68	28.61	20.08	20.27	19.97	26.10	26.46	26.08	22.83	22.76	22.62	32.68	32.69	32.68

[a] All at 100°C.
[b] First sample only; T. C. Dunn Denver District, remaining 4 samples.

possibly two or more different makes. No instructions were given as to the amount of draft or venting to be used, since it was not known whether all ovens had the same adjustments. Where this type of oven is available, it will result in a substantial saving in time at no sacrifice in accuracy.

The results by the vacuum oven method were obtained for comparative purposes. No advantage is seen in this type of oven over a simple air oven which should be available in any laboratory.

None of the collaborators reported any serious difficulty with the method, and although some obtained somewhat higher results with the forced draft oven, this was not generally true. It is believed that if apparent complete dryness is obtained by the steam bath, together with adequate stirring as directed, results will be satisfactory regardless of the type of oven.

ETHER EXTRACT

Last year the Associate Referee suggested a rapid sorting method for the determination of ether extract in canned salmon, with the recommendation that it be subjected to collaborative study and comparison with the official method. The method was originally developed for the rapid determination of ether extract of the drained meat of canned salmon. The entire contents of several cans of salmon were taken for collaborative study because of the ease of preparing a finely divided uniform mixture with a Waring blendor. The additional water did not seem to interfere with the method, although each collaborator used a slightly different means of transferring the weighed sample to the centrifuge bottle.

Two different collaborative samples were prepared and sent out. The first was pink salmon with approximately 7 per cent fat, and the other was Chinook salmon with about 10 per cent fat. The pink salmon was preserved with Zephiran chloride solution as described above under TOTAL SOLIDS, and the Chinook salmon was preserved by freezing. The subdivisions examined for ether extract were the same as those examined for total solids as reported above. Each collaborator was asked to determine ether extract in duplicate by the following method and also by the official method (*Official Methods of Analysis*, 7th Ed., 1950, **18.9** and **18.10**).

RAPID METHOD—ETHER EXTRACT IN CANNED FISH

Accurately weigh 5 g of ground and mixed sample into a 250 ml centrifuge bottle. Add, in the following order, 10 ml (ca 10 g) of Ottawa sand (predominantly 20 mesh), 10 ml (ca 10 g) of anhyd. Na_2SO_4, 25 ml of ethyl ether, and 25 ml of petr. ether. Stopper, fastening stopper in place with wide rubber band, and shake vigorously by hand 1 min. to break up and disperse fish solids. Shake 30 min. in a mechanical shaker vigorously enough to keep solids in suspension. Centrifuge to form tight cake under ether (ca 10 min. at 1200 r.p.m.), and carefully pipet 20 ml of clear ether layer into tared 50 ml beaker and evap. to dryness on steam bath (direct a gentle stream of air into beaker to prevent bumping). Heat 15–20 min. in air oven at 100°; then cool in air and weigh. Calc. % fat according to the following formula, which corrects for the vol. of fat contained in the aliquot:

$$\% \text{ fat} = \frac{W(50)(100)}{\left(20 - \frac{W}{0.92}\right)(S)}$$

where W = wt of fat in aliquot; 50 = ml of solvent used; 20 = ml of aliquot; 0.92 = sp. gr. of salmon oil; and S = wt of sample.

All collaborators except one obtained significantly higher results by the rapid method, as shown in Table 2. The reason for this is not clear, although one analyst suggested that possibly the alcohol present in C.P. ether would account for part of the difference. The hazard involved in

TABLE 2.—*Comparison of ether extract by rapid method with official method*[a]

COLLABORATOR	CANNED PINK SALMON		CANNED CHINOOK SALMON	
	OFFICIAL METHOD	RAPID METHOD	OFFICIAL METHOD	RAPID METHOD
R. N. Allen, Gorton-Pew Fisheries, Gloucester, Mass.	6.56	6.71		
G. Ivor Jones, Nat. Canners Assn., Seattle, Wash.	6.79 6.78	7.41 7.38	10.08 10.06	10.33 10.17
D. Menschenfreund, Los Angeles District, FDA	6.81 6.84	6.88 6.81	10.08 10.04	10.00 10.00 10.14
D. W. Johnson, Denver District, FDA	6.93 6.80	6.98 6.94		
T. C. Dunn, Denver District, FDA			10.08 10.01	10.72 10.24
M. C. Harrigan, Boston District, FDA	7.04 7.08	7.85 7.64		
H. J. Meuron, San Francisco District, FDA	6.91 6.97	7.84 7.77	10.14 10.08	9.95 10.27
S. H. Perlmutter, Minneapolis District, FDA	7.11 6.93	7.25 7.45	10.14 10.07	10.38 10.31
J. A. Ross, Seattle District, FDA	6.84 6.80	7.32 7.30	10.16 10.27	10.41 10.20
H. M. Risley, Seattle District, FDA	6.79 6.78	7.34 7.28	9.98 10.04	10.31 10.12
Total No. Determinations Average	17 6.87	17 7.30	14 10.09	15 10.29

[a] Ether extract—per cent.

storing and handling anhydrous ether does not warrant recommending its use in a rapid method, particularly when the results parallel those of the official method so closely. The second sample (Chinook salmon) showed much better agreement among collaborators and between methods than the first. This may be due in part to a greater familiarity resulting in better technique, or possibly to the method used in preserving the samples. No specific instructions were issued on weighing out the portions for analysis, and the collaborators devised their own methods. (The consistency of the sample determines this point.) When the drained flesh of canned salmon (for which the method was designed) is examined, the material is dry enough to be weighed in a tared dish and scraped into the centrifuge bottle. When the free liquid is included, the material is "soupy." One collaborator used a collapsible tube as a weighing bottle; another washed the material out of the weighing dish into the bottle with a small amount of distilled water. No particular difficulty with the method was reported, and some collaborators commented favorably, particularly with regard to the saving of time in comparison with the official method.

RECOMMENDATIONS

It is recommended*—

(1) That the method for total solids in fish and other marine products be adopted, first action.

(2) That the method provide for a mechanical convection oven at 100°C. for one hour, to be used as an alternate to an air oven if desired.

(3) That additional collaborative work be done on other species of fresh, frozen, and canned marine products (except oysters).

(4) That collaborative work be continued on the suggested rapid method for ether extract in canned fish, and that the method be extended to fish other than canned salmon.

REPORT ON SOLIDS IN RAW OYSTERS

By JOHN P. TRAYNOR (Food and Drug Administration, Department of Health, Education, and Welfare, Baltimore 2, Md.), *Associate Referee*

In accordance with the recommendations of the Referee at the October, 1952 meeting of the A.O.A.C., a study has been made of methods to reduce the time required for the determination of solids in raw oysters.

Preliminary work consisted of comparing the results obtained by the present A.O.A.C. method and by drying in the forced draft oven. Drying in the air oven at 130° for varying periods of time was also studied.

* For report of Subcommittee C and action of the Association, see *This Journal*, 37, 70, 96 (1954).

TABLE 1.—*Per cent solids at different locations of dishes in forced draft oven*

SAMPLE NO.	FORCED DRAFT OVEN								AVERAGE INDIVIDUAL VARIATIONS BETWEEN DUPLICATES		AVERAGE VARIATIONS FROM AVERAGE OF ALL DETNS	
	NO STEAM BATH				STEAM BATH							
	POSITION								NO STEAM BATH	STEAM BATH	NO STEAM BATH	STEAM BATH
	1	2	3	4	5	6	7	8				
Two-Thirds Draft for 2 Hours												
1	11.79	11.78	—	—	—	—	—	—	0.01	—	0.01	
2	11.59	11.55	—	—	—	—	—	—	0.04	—	0.02	—
3	9.29	9.38	9.35	9.31	9.43	9.40	9.37	9.37	0.07	0.02	0.03	0.02
4	14.84	14.83	14.74	14.70	14.70	14.64	14.67	14.74	0.03	0.07	0.05	0.03
5	15.44	15.40	14.95	15.07	15.24	15.25	15.24	15.08	0.08	0.08	0.21	0.06
6	15.09	15.18	14.89	14.87	15.12	15.06	15.11	14.99	0.06	0.09	0.10	0.05
								Av.	0.05	0.04	0.07	0.04
Full Draft for 1.5 Hours												
7	12.90[a]	12.92[a]	12.90	12.87	13.07	12.91	12.96	13.03	0.03	0.12	0.01	0.06
8	12.38	12.42	12.31	12.45	12.58	12.34	12.29	12.25	0.04	0.14	0.05	0.11
9	12.53	12.54	12.60	12.56	12.95	12.57	12.54	12.50	0.03	0.21	0.02	0.16
10	12.81	12.81	12.89	12.82	13.00	12.83	12.81	12.79	0.04	0.10	0.03	0.07
11	11.00	11.08	11.29	11.21	11.21	11.22	11.18	11.14	0.08	0.03	0.10	0.03
12	10.84	10.68	10.81	10.81	10.95	10.76	10.85	10.80	0.08	0.12	0.05	0.06
								Av.	0.05	0.12	0.04	0.08

[a] Two hours' drying (all values in column).

A. FORCED DRAFT OVEN DRYING

The following factors were considered in investigating the forced draft oven:

(1) Position of dishes in the oven.
(2) Preliminary drying of oysters on the steam bath.
(3) Drying without preliminary treatment on the steam bath.
(4) Amount of draft utilized.
(5) Adequate time for drying.

A series of determinations was made in which the dishes in the forced draft oven were located at different positions (Fig. 1). Evaluation of the results shown in Table 1 indicated that position of the dishes in the oven had little effect on the results.

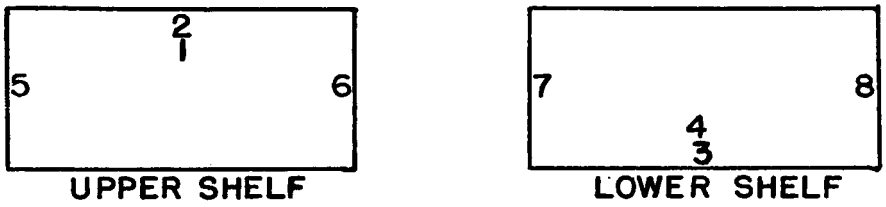


FIG. 1.—Position diagram. *No steam bath:* Positions 1 & 2, and 3 & 4 are duplicates. *With steam bath:* Positions 5 & 6, and 7 & 8 are duplicates.

Early work also indicated (Table 2) that it was unnecessary to dry the oysters on the steam bath prior to heating in the oven.

It was believed that the time of drying and possibly the amount of draft were the only factors that required detailed study. Although it might be desirable to specify the amount of heated air (100°) in cubic feet per minute passing over the samples, most forced draft ovens are not calibrated, and different models vary in their draft. When the Hot Pack Oven No. 1306 was used, it was found that drying for two hours at two-thirds draft or for one and one-half hours at full draft produced solids in close agreement with solids by the official A.O.A.C. method. These data are shown in Tables 3 and 4.

Since this forced draft drying eliminated pre-drying on the steam bath and the total drying time was thus one to two hours shorter than the official method, the Referee agreed that it should be studied collaboratively.

INSTRUCTIONS TO COLLABORATORS

Drain 1 gallon of oysters in the manner indicated in *Official Methods of Analysis*, 7th Ed., **18.1 (f), 18.2,** and **18.3,** and combine the oysters and liquid without regard to the % of drained liquid. Take a representative sample (approximately 1 qt.) and grind 2 to 3 min. in the Waring Blendor; this is sample A.

TABLE 2.—*Solids with and without predrying on steam bath*

SAMPLE NO.	SOLIDS BY FORCED DRAFT OVEN				VARIATIONS BETWEEN AVERAGES OF PREHEATED AND NON-PREHEATED SAMPLES
	NO STEAM BATH		STEAM BATH		
	NO. OF SUBS	AV. SOLIDS	NO. OF SUBS	AV. SOLIDS	
			⅔ Draft for 2 Hours		
		per cent		*per cent*	
1	2	11.79	—	—	—
2	2	11.57	—	—	—
3	4	9.33	4	9.39	0.06
4	4	14.78	4	14.69	0.09
5	4	15.22	4	15.12	0.10
6	4	15.01	4	15.07	0.06
					Av. 0.08
			Full Draft for 1.5 Hours		
		per cent		*per cent*	
1	2	12.87	4	12.99	0.12
2	2	12.38	4	12.37	0.01
3	2	12.58	4	12.64	0.06
4	2	12.86	4	12.86	0.00
5	2	11.25	4	11.19	0.06
6	2	10.81	4	10.84	0.03
					Av. 0.05

TABLE 3.—*Solids by A.O.A.C. and by two hours' drying at two-thirds draft (100°C.)*

SAMPLE NO.	NO. SUBS BY FORCED DRAFT	TOTAL SOLIDS		VARIATION FROM A.O.A.C. METHOD
		A.O.A.C. METHOD, (AV.) ALL SUBS	FORCED DRAFT OVEN (⅔ DRAFT), (AV.) ALL SUBS	
		per cent	*per cent*	
1	2	11.88	11.79	−.09
2	2	11.44	11.57	+.13
3	4	9.35	9.33	−.02
4	4	14.80	14.78	−.02
5	4	15.09	15.22	+.13
6	4	15.14	15.01	−.13
			(Av. of 20 Subs)	±.00

TABLE 4.—*Solids by A.O.A.C. and by one and one-half hours' drying at full draft (100°C.)*

SAMPLE NO.	NO. SUBS BY FORCED DRAFT	TOTAL SOLIDS		VARIATION FROM OFFICIAL METHOD
		A.O.A.C. METHOD (AV. OF 2)	FORCED DRAFT OVEN, FULL DRAFT, 1.5 HRS	
		per cent	*per cent*	
1	2	13.06	12.87	−.19
2	2	12.50	12.38	−.12
3	2	12.87	12.58	−.29
4	2	13.00	12.86	−.14
5	2	11.12	11.25	+.13
6	2	10.82	10.81	−.01
			(Av. of 12 Subs)	−.10

(*a*) Determine solids in duplicate on sample A by **18.4** with these exceptions: Dry on the steam bath exactly 15 min.[1] (this is sufficient to evaporate just to dryness) and then dry in regular oven exactly 3 hours at 98–100°, cool, and weigh.

(*b*) Next weigh 2 portions (ca 10 g each) from Sample A for moisture determination in the forced draft oven. Do *not* dry these samples on the steam bath; insert them immediately after weighing into forced draft oven and dry at 98–100° for 1.5 hrs, using full draft. Cool and weigh.

(*c*) Weigh out 2 more samples (approximately 10 g each) from sample A for moisture determination in the forced draft oven. Do *not* dry these samples on the steam bath; insert them into the forced draft oven immediately after weighing and dry at 98–100° for 2 hrs, using 2/3 draft. Cool and weigh.

If time permits, run samples B, C, and D or more, if possible, following the above procedures.

Note.—Be sure regular oven is at 100° before putting samples in. See that the adjustable air intake and exhaust are opened wide enough to operate the forced draft oven at 2/3 and at capacity flow. Mark the adjustable damper control in units of thirds so that the 2/3 draft will always be at the same point for each determination.

The results obtained by 7 collaborators, using 5 different forced draft ovens, are shown in Table 5.

In order to illustrate the difference between duplicate determinations, the data have been summarized in Table 6.

Tables 5 and 6 indicate that oysters may be dried by the forced draft oven without previous drying of the oysters on the steam bath. Less handling of the dishes is thus required, and a saving in time of 20 to 40 minutes is effected.

Whether to dry at full draft for 1.5 hours or at two-thirds draft for two hours seems to have been answered adequately by the 7 collaborators.

[1] Prevent contact of metal of the oyster dish and metal of the steam bath to avoid electrolysis and consequent changes in weight.

TABLE 5.—*Total solids by official method and by forced draft oven*

COLLABORATOR	SAMPLE		A.O.A.C. METHOD, AV. OF 2 DETNS	FORCED DRAFT OVEN				
				TYPE FORCED DRAFT OVEN	FULL DRAFT 1.5 HRS		¾ DRAFT 2 HRS	
	NO.	DRAINED LIQUID			SOLIDS, AV. OF 2 DETNS	VARIATION FROM OFFICIAL METHOD	SOLIDS, AV. OF 2 DETNS	VARIATION FROM OFFICIAL METHOD
		per cent	*per cent*		*per cent*	*per cent*	*per cent*	*per cent*
1	INV 99–808K	5.5	11.48	Freas 601	11.46	−.02	11.42	−.06
2	26–242L	2.9	14.52		14.61	+.09	14.58	+.06
2A	67–031L	6.4	14.41		14.34	−.07	14.31	−.10
2B	67–032L	3.1	15.25	FC 701M	15.15	−.10	15.09	−.16
	67–033L	2.3	16.15		16.13	−.02	16.04	−.11
3A	56–725L	3.9	10.31	Freas 621	10.35	+.04	10.25	−.06
3B	56–725L	3.9	10.29		10.34	+.05	10.27	−.02
3A	56–726L	4.3	12.65		12.63	−.02	12.55	−.10
3B	56–726L	4.3	12.60		12.64	+.04	12.55	−.05
4	16–704L	0.3	7.72	Freas 621	7.77	+.05	7.72	±.00
5	82–476K	2.7	10.19	Hot Pack	10.33	+.14	10.26	+.07
	82–477K	2.4	11.69	1306	11.76	+.07	11.66	−.03
					Av. (12 samples)	+.02		−.05

TABLE 6.—*Differences in duplicate determinations by official method and forced draft methods*

COLLABORATOR	SAMPLE NO.	A.O.A.C. METHOD	FORCED DRAFT OVEN	
			FULL DRAFT, 1.5 HRS	⅔ DRAFT, 2 HRS
1	INV 99–808 K	0	0.05	0
2	26–242 L	0	0.04	0.02
2A (A.O.A.C.)	67–031 L	0.03	0	0.03
2B (Forced Draft)	67–032 L	0.05	0.06	0.04
	67–033 L	0	0.03	0.00
3A	56–725 L	0.07	0.02	0.00
3B	56–725 L	0.04	0.01	0.01
3A	56–726 L	0.04	0.01	0.00
3B	56–726 L	0.01	0.03	0.01
4	16–704 L	0.01	0.03	0.03
5	82–476 K	0.03	0.05	0.01
	82–472 K	0.05	0.07	0.01
Av. (12 Samples)		0.03	0.03	0.01

All of them were able to adjust their 5 different ovens to either two-thirds or full draft and to obtain satisfactory results.

Table 5 shows a variation of +.02 per cent from the A.O.A.C. method on an average of 12 samples by drying at full draft for 1.5 hours at 100°, and a variation of −.05 per cent from the A.O.A.C. method on an average of 12 samples by drying at two-thirds draft for two hours.

Seven collaborators, in determining solids in oysters, used the forced draft oven for samples not previously dried on the steam bath. Results both at full draft and at two-thirds draft are in excellent agreement with those obtained with the A.O.A.C. method. A saving of approximately two hours per determination is effected by the forced draft method.

Reports of the collaborators indicated approval of the method. None of the seven reported any difficulty with the procedure.

B. AIR OVEN DRYING AT 130°C

One of the methods which was suggested to the Associate Referee involved use of a higher temperature. A study was accordingly made of the feasibility of determining solids in oysters by drying at 130° in the regular convection oven for intervals of 1 hour, 1.5 hours, and 2 hours.

Twenty-six samples were analyzed in duplicate by this method. It was found to be unsatisfactory for the following reasons:

(1) Variations in results obtained by this method and by the official method were too great.

(2) There was no saving in time because preliminary drying on the steam bath was required.

(3) The oyster material dried at 130° showed darkening and browning.

Because of these factors, it was decided to discontinue any further work on the 130° method.

C. VACUUM OVEN METHOD

Because of the volume of work involved in this experiment, the Associate Referee was unable to obtain any results for the vacuum oven.

RECOMMENDATIONS

It is recommended*—

(1) That the forced draft oven method utilizing full draft for 1.5 hours at 100° be adopted as an alternative procedure. The proposed procedure shall read:

"Make duplicate detns. Weigh quickly 10 g of meats, liquid, or mixed meat and liquid, in flat-bottom metal dish ca 9 cm in diam. Spread sample evenly over bottom of dish, insert directly in preheated forced draft oven set at full draft, and dry 1.5 hrs at 100°C. Cool in desiccator and weigh promptly."

(2) That further study be carried on with the view of decreasing the time necessary for the determination of solids in oysters and that such studies be in line with the recommendations made by Subcommittee C, *This Journal*, **36**, 56 (1953).

ACKNOWLEDGMENT

Sincere thanks are extended to the following collaborators, all of the Food and Drug Administration, without whose generous efforts this work would not have been possible:

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C. D. Schiffman, Atlanta District
F. E. Yarnall, Kansas City District

REPORT ON DISINFECTANTS

By L. S. STUART (U. S. Department of Agriculture, Insecticide Division, Livestock Branch, Washington 25, D. C.), *Referee*

During the year, a number of collaborative studies involving specific products were conducted with individual commercial laboratories on the

* For report of Subcommittee C and action of the Association, see *This Journal*, 37, 70–71 (1954).

use-dilution method for testing disinfectants, accepted as first action in 1952. Good agreement has been obtained in all of these studies.

Some objections have been raised to the method, chief of which appears to originate with manufacturers and formulators who employ phenol coefficient scale standardizations on concentrates in all of their operations. There is considerable evidence to indicate that the phenol coefficient scale is widely used in this connection, even when it is not employed in arriving at the safe use-dilutions recommended in the labeling for disinfecting. It has been claimed that use in labeling of true coefficient numbers, even though they cannot be confirmed in the use-dilution method, has a fundamental value to the manufacturer and cannot be considered misleading to the consumer except in those cases where the required directions for use are out of line with practical disinfecting values as indicated by the confirmation method. This argument has not been resolved, but it may well be that the method may be limited in application to checking the reliability of the phenol coefficient as an index to practical disinfecting values and in the final determination of the acceptability of the maximum dilution recommended in the required directions given for disinfecting where specific directions for precleaning are not also given.

RECOMMENDATIONS

A substantial amount of collaborative work was done on the fungicidal test and the recommendations* made by the Associate Referee should be adopted. It is recommended also that the studies on the resistance of the specified test culture, planned for the coming year, be expanded to investigate the possibility of accepting *Microsporum audouini* as an alternate test organism. This fungus appears to be the infectious agent of greatest concern to public health officials responsible for enforcement of local health ordinances pertaining to barber shops and beauty parlors, and might be more acceptable than *T. interdigitale* in evaluating claims made for products sold for use in such establishments.

Progress has been made toward the standardization of culture media used in disinfectant testing, and work during the coming year should provide information in this respect for some specific changes in the accepted methods.

Another Associate Referee should be appointed to investigate, during the coming year, the desirability of accepting the "sporicidal test" reported by Ortenzio, Stuart, and Friedl (1953) as a first action method.[1]

* For report of Subcommittee A and action of the Association, see *This Journal*, 37, 62 (1954).

[1] Ortenzio, L. F., Stuart, L. S., and Friedl, J. L., *This Journal*, 36, 480 (1953).

REPORT ON FUNGICIDE TESTING

By L. F. ORTENZIO (U. S. Department of Agriculture, Insecticide Division, Livestock Branch, Washington 25, D. C.), *Associate Referee*

Bacteriologist have frequently complained that they could not produce spores of *Trichophyton interdigitale* within the resistance range specified in the present first action A.O.A.C. fungicidal test.[1] Details of the present method have been studied by the Associate Referee and four collaborators to determine the desirability of various changes suggested to overcome this objection. There have also been reports of difficulties in preparing and standardizing the spore suspension prescribed, and because of this, various techniques to improve this procedure have also been made a part of these investigations.

In the present method, primary spore suspensions are prepared by removing the mycelial mat from the surface of five 10 to 15 day agar plate cultures by means of a sterile spatula or heavy flattened wire and suspending this in 25 ml of physiological saline, shaking with glass beads to free the conidia from the mycelium, and filtering through sterile absorbent cotton to remove the hyphal elements. This primary suspension is then employed for making up the standardized test suspension which contains 5 million conidia per ml.

This procedure was compared directly with a modified procedure in which the 5 mycelial mats were harvested from agar plates as specified, but were transferred to a heat-sterilized glass tissue grinder (Arthur H. Thomas Company, Size B) and macerated with 25 ml of sterile saline. The 25 ml of macerated mycelia in saline were then filtered through cotton.

In preliminary trials, primary spore suspensions prepared according to the present first action method were found to contain from 70 to 115 million spores per ml, while those prepared by using the tissue grinder contained from 135 to 150 million spores per ml.

Some investigators have reported that the resistance of the fungus spores in the method seemed to be higher when the fungus was grown on agar without adjustment of the *p*H to 5.6–5.8, the present specification. This claim was investigated carefully, and no significant differences could be found either in the production of spores or in their resistance to phenol when the culture was propagated on agar adjusted to *p*H 5.6–5.8 and on unadjusted agar with a *p*H varying from 6.1–6.3. The results of this study are presented in Table 1.

The results given in Table 1 also show the increased yield or recovery of conidia from the mycelial mats when they are harvested and macerated

[1] *Official Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Box 540, Benjamin Franklin Station, Washington 4, D. C., pp. 91–92.

TABLE 1.—*Spore count and resistance of conidia harvested from adjusted and unadjusted neopeptone agar media*

CULTURE AGAR	NO. OF TESTS	SPORE COUNT/ML OF PRIMARY SUSPENSION AS HARVESTED BY:		RESISTANCE TO PHENOL IN TEST SUSPENSION, 5 MILLION SPORES/ML	
		PRESENT METHOD	TISSUE GRINDER METHOD	PRESENT METHOD	TISSUE GRINDER METHOD
		million	*million*	*Max. 10 min. killing dln. at 20°C.*	
Adjusted *p*H 5.6–5.8	1	70	135	1:65	1:65
	2	96	150	1:65	1:65
	3	80	145	1:65	1:60
	4	115	155	1:65	1:65
	5	105	130	1:65	1:65
	Av.	93.2 ± 14.6	143 ± 9.6	1:65	1:64
Unadjusted *p*H 6.1–6.3	1	80	150	1:65	1.65
	2	90	155	1:65	1:65
	3	105	130	1:65	1:65
	4	85	125	1:65	1:65
	5	90	140	1:65	1:65
	Av.	90.0 ± 6.0	140.0 ± 10.0	1:65	1:65

with a tissue grinder. The average deviation figures indicate fairly clearly that this method of harvesting will provide for a more uniform recovery of spores. It is interesting to note that, in this particular study, no culture was found which had a 10 minute resistance at 20°C. to phenol at a dilution of 1:60. The required 10 minute killing dilutions in the method must fall within the range of 1:45 to 1:55.

In collaborative studies, 5 laboratories determined the resistance of the test culture conidia to phenol, once each month over a five-month period. The conidia were propagated and harvested by the present method. The results indicated quite clearly that the resistance range presently specified is out of line with the normally encountered resistance of the culture. The results of these studies are summarized in Table 2.

Table 2 demonstrates that in the work of 4 out of the 5 collaborators, the dilution which killed in 10 minutes but not in 5 minutes ranged from 1:65 to 1:55. The results of Collaborator 4 were somewhat out of line. They showed the organism to be considerably less resistant to phenol. It should be noted that only one collaborator obtained a consistent resistance within the range specified over the five-month period. Collaborator 2 obtained this resistance in only one test. Collaborators 3, 4, and 5 did not obtain the required resistance in any of the tests.

Some testing laboratories have developed the practice of storing the primary macerated suspension of harvested spores in the refrigerator at

TABLE 2.—*Results of collaborative studies on the 10 minute resistance at 20°C. of Trichophyton interdigitale to phenol*

DETERMINATION	COLLABORATORS BY NUMBERS				
	1	2	3	4	5
	Maximum dilutions of phenol killing in 10 min. at 20°C.				
1	1:55	1:55	1:65	1:75	1:65
2	1:55	1:65	1:65	1:70	1:65
3	1:55	1:65	1:65	1:75	1:65
4	1:55	1:60	1:60	1:70	1:65
5	1:55	1:60	1:60	1:70	1:65

temperatures of 2–10°C. and employing this stock suspension for preparing aliquots of test spores when they are needed. There is no provision for this practice in the present method. It should be acknowledged, however, that it constitutes a convenience of great practical value to most large testing laboratories. Therefore, it was investigated quite carefully.

Eight stock suspensions of spores were prepared by harvesting mycelial mats with the use of the tissue grinder method. The suspensions were stored for periods up to seven weeks in the refrigerator at 2–10°C. Resistance to phenol was determined on standardized spore suspensions made from the stock suspensions immediately after harvesting and after one, three, five, and seven weeks' storage. The results are given in Table 3.

TABLE 3.—*Phenol resistance of standardized spore suspensions made from refrigerated primary spore suspensions*

STOCK SPORE SUSPENSION NO.	STORAGE TIME IN WEEKS				
	0	1	3	5	7
	Maximum 10 minute killing dilution at 20°C.				
1	1:65	1:60	1:55	1:55	1:55
2	1:65	1:60	1:55	1:55	1:55
3	1:65	1:65	1:60	1:60	1:60
4	1:65	1:65	1:60	1:55	1:55
5	1:65	1:60	1:55	1:55	1:55
6	1:65	1:65	1:60	1:55	1:55
7	1:65	1:60	1:55	1:55	—
8	1:60	1:55	1:55	—	—

The data shown in Table 3 are especially interesting in view of the resistance range specified in the present method. It can be seen that none of the freshly prepared spore suspensions possessed the required resistance to phenol. However, the spores in all but one of these suspensions developed a resistance within the prescribed range upon storage in the

refrigerator from periods ranging from one to five weeks. The cause for this is not known.

The observation that there is a definite increase in the resistance of spores stored in saline suspension in the refrigerator for two weeks was confirmed by two collaborators. One collaborator reported no change in resistance after storage for ten days at 2–5°C.

It would appear from the data given in the three tables that the test culture has a resistance to phenol considerably different from the present requirements of the method. Apparently the specified culture has decreased in resistance to phenol during the years since the initial work was conducted in developing the method. Fortunately, by aging the spores in the refrigerator, suspensions within the range specified can still be obtained with reasonable consistency. It may eventually be necessary to lower the phenol resistance requirements, but such an action would not seem wise until more information is available concerning the effect of such a change on the relation of the endpoints in the method to practical fungicidal values.

Calculations based on counts made on primary suspensions of spore crops harvested as specified in the present method indicate clearly that the maceration of one mycelial mat in 20–25 ml of physiological saline provides a suspension containing too small a number of spores for convenient and accurate standardization of the test suspension of 5 million conidia/ml. It is apparent from the data on hand that 5 mycelial mats macerated in each 20–25 ml of physiological saline provide primary stock spore suspensions of about the proper density for the easy preparation and accurate standardization of the specified test suspension.

RECOMMENDATION

It is recommended* that the present method be amended, first action, as follows:

(1) That the test culture be maintained and propagated on neopeptone agar at *p*H 6.1–6.3 and that a heat-sterilized glass tissue grinder be permitted for use in preparing stock suspensions of conidiospores.

(2) That the use of aged, harvested conidia stored in saline suspensions at 2–10°C. for periods up to four weeks be permitted in preparing standardized test suspensions of spores.

(3) That neopeptone broth at *p*H 6.1–6.3 be used for subculturing conidia exposed to the fungicides.

(4) That the method be changed to specifiy the maceration of 5 (petri dish) mycelial mats in 20–25 ml of physiological saline in lieu of the present stipulation of "each" in the preparation of primary suspensions of conidia.

* For report of Subcommittee A and action of the Association, see *This Journal*, 37, 62, 82 (1954).

(5) That further studies on the phenol resistance of the test culture be conducted, including an evaluation of the effect of phenol resistance on the relation of endpoints in the method to the effective concentrations in actual use of commonly accepted commercial fungicides, to determine if the specified resistance level of the spores to phenol should be lowered.

ACKNOWLEDGMENT

The Associate Referee gratefully acknowledges the cooperation of the following collaborators:

Dr. C. M. Brewer and Dr. S. Molinas, Food and Drug Administration, Washington, D. C.
Dr. Haldane Gee, Foster D. Snell, Inc., New York, N. Y.
Mrs. Eleanore S. Wright, Lehn and Fink Corporation, Bloomfield, N. J.
Dr. George R. Goetchius, Rohm and Haas Company, Philadelphia, Pa.

REPORT ON MEDIA INGREDIENTS FOR DISINFECTANT TESTING

STANDARDIZATION OF BACTERIOLOGICAL CULTURE MEDIA

By MICHAEL J. PELCZAR, JR. (Department of Bacteriology, University of Maryland, College Park, Md.), *Associate Referee*

Studies have been continued since the last report with a view toward improving the medium employed for phenol coefficient determinations. It is desired that the constituents of this medium will ultimately be of materials with established specifications. Only then can a "standard" medium be provided for the A.O.A.C. procedure for determination of phenol coefficients.

Since previous experiments indicated that the meat extract in the currently employed medium might be dispensable, media with peptone(s) as their basic constituent were investigated. Peptones, prepared from various types of proteinaceous raw materials, were supplemented individually and in combinations with inorganic salts. In some instances glucose and *l*-cystine were added.

The experimental media were evaluated by determining the phenol resistance of the two test organisms (*Micrococcus pyogenes* var. *aureus* and *Salmonella typhosa*) when they were maintained in the manner prescribed by the A.O.A.C. The F.D.A. broth currently employed for phenol coefficient determinations was used simultaneously as a control.

Of the numerous media investigated, the following appeared to be most promising: peptone (pancreatic digest of casein, U.S.P. XIII), 2.0%;

NaCl, 0.5%; KH_2PO_4, 0.06%; K_2HPO_4, 0.14%; glucose, 0.05%; and *l*-cystine, 0.01%.

Sufficient collaborative data have not yet been compiled to recommend* this formula for adoption. However, more extensive test studies are contemplated for the next year.

REPORT ON ECONOMIC POISONS

By THOMAS H. HARRIS (Pesticide Regulation Section, Plant Pest Control Branch, Agricultural Research Service, U. S. Department of Agriculture, Washington 25, D. C.), *Referee*

During the past year, J. J. T. Graham resigned as Referee on economic poisons. Mr. Graham, an Associate Referee from 1917 to 1919, inclusive, served continuously for 33 years as Referee. The present Referee desires in this first report to express appreciation on behalf of the Economic Poisons Section for the many years of valuable service which Mr. Graham rendered. It is reassuring, however, that he expects to maintain an active interest in the Association and has kindly offered to make available to the present Referee the benefits of his advice and council.

At the 1951 meeting, the official mercury reduction methods for Pyrethrin I were modified by substitution of hydrochloric acid for sulfuric acid in the neutralization following the saponification. This modification was, however, subsequently rescinded. The Referee has reviewed the correspondence and data on which these actions were based, and is of the opinion that the Association was rather hasty in both the adoption and rescission of this modification. The Referee and Associate Referee, therefore, feel that the proposed modification merits further evaluation.

There were several changes in Associate Referee assignments following the last meeting; however, an up-to-date list of Associate Referees was published in the February, 1953 issue of the *Journal* of the Association. The various Associate Referees have been quite active in their assignments, as their reports indicate. Several collaborative programs have already been outlined for the coming year and the Referee appreciates the interest and effort put forth by the Associate Referees.

The Referee on Dairy Products has suggested that the following editorial amendment be added to method **5.142–5.143** (Chloramine T): After "Cl" in the last line add "To convert active Cl to available Cl, multiply active Cl by 2." This was suggested following correspondence with the American Public Health Association because of the desire to have the chemical methods of the two groups identical. The Referee concurs in this suggestion. An explanation of the term "available chlorine," which is

* For report of Subcommittee A and action of the Association, see *This Journal*, 37, 62 (1954).

sometimes confusing, appears in the United States Dispensatory, 24th Edition, page 1079.

In anticipation of the Eighth Edition of *Official Methods of Analysis*, routine changes in the Pesticides section are embodied in the recommendations of the Referee.

RECOMMENDATIONS

It is recommended*—

(1) That the study of analytical methods for the determination of pyrethrins be continued and that the recently proposed hydrochloric acid modification of the official mercury reduction method be further evaluated.

(2) That the revisions in method **5.112** (Pyrethrin II) (*This Journal*, **35**, 64 (1952)), adopted first action, be made official.

(3) That the first action method **5.113–5.114** (Pyrethrum Extracts in Mineral Oil) be made official so that it will have the same status as the corresponding method for Pyrethrum powder.

(4) That methods for the determination of rotenone, dieldrin, aldrin, piperonyl butoxide, phenolic disinfectants, dithiocarbamates, and systemic insecticides be further studied.

(5) That the proposed modification of method **5.149** (*gamma*-BHC) be studied collaboratively.

(6) That the modified Eble method for warfarin be given further collaborative study.

(7) That collaborative studies of methods for the determination of physical properties of insecticidal powders be initiated.

(8) That methods proposed by the Associate Referee for the determination of volatility hazards of hormone-type herbicides be studied collaboratively.

(9) That methods **5.21, 5.22, 5.23,** and **5.24** (fluorine), **5.124** (sulfur), and **5.8, 5.9,** and **5.37** (arsenic), now first action, be made official.

(10) That the method for the determination of total chlorine in esters of 2,4-D and 2,4,5-T in liquid herbicides by the Parr Bomb–boric acid procedure (*This Journal*, **36**, 367, 379 (1953)), adopted as first action, be made official.

(11) That the O'Keefe and Averell method for technical parathion, parathion dusts, and emulsifiable concentrates, as modified by the Associate Referee, be subjected to further collaborative study.

(12) That methods for higher boiling phenols be studied further with special attention to those of petroleum origin.

(13) That method **5.158–5.160** (tetraethyl pyrophosphate) be continued as first action, pending consultation with users of the method.

* For report of Subcommittee A and action of the Association, see *This Journal*, **37**, 61, 62 (1954).

(14) That method **5.127** (*alpha*-naphthyl thiourea) be deleted from *Official Methods of Analysis* because of the substitution and use of more effective rodenticides.

(15) That the name of this chapter be changed from "Economic Poisons" to "Pesticides."

REPORT ON BENZENE HEXACHLORIDE

By IRWIN HORNSTEIN (U. S. Department of Agriculture, Agricultural Research Service, Bureau of Entomology and Plant Quarantine, Beltsville, Md.), *Associate Referee*

In last year's report (1) mention was made of a committee appointed at a joint meeting of technical representatives of industry and government to study and, if possible, to improve the chromatographic procedure for gamma-isomer determinations in benzene hexachloride as described in *Official Methods of Analysis*, 7th Ed.

The committee set as its goal an accuracy of ± 3 per cent of the gamma isomer present. A modified procedure based on a method suggested by Rosin and Radan (2) appears to meet this degree of accuracy and is being submitted for collaborative study in comparison with the present A.O.A.C. chromatographic procedure.

This modified chromatographic procedure is divided into three steps:

(A) A partition chromatographic separation on silicic acid, using nitromethane, normal hexane, and a dye indicator. This separation yields a relatively pure main gamma cut and an impure gamma fraction, the latter consisting of the eluant immediately preceding and following the main gamma band.

(B) A cryoscopic correction, which is applied to the pure gamma cut. This cut contains seldom less than 90 per cent and usually at least 95 per cent of the gamma isomer placed on the column.

(C) A polarographic assay of the impure gamma fraction. This fraction seldom contains more than 10 per cent of the gamma isomer.

Although the method as finally developed appears to give satisfactory results, each of the steps outlined above presents its own difficulties.

In step (A) the silicic acid used as the columnar material varies widely from batch to batch. This variability, whether due to differences in particle size or water content, affects the rate of flow through the column and thus the separation of the isomers. The variability of the silicic acid has been minimized by specifying that, when a new batch of silicic acid is obtained, a known benzene hexachloride sample should be analyzed with the new silicic acid as the columnar material. The flow rate and packing

characteristics should be similar to those of a known satisfactory silicic acid. Moreover, the main gamma cut, when chromatographed on this silicic acid, should contain at least 90 per cent of the total gamma isomer and have a minimum melting point of 109°C. Only those batches of silicic acid meeting these requirements are used.

In step (B), because of eutectic formation, there may be a relatively wide melting-point range. Best results are obtained when the melting point is taken as the temperature at which the last crystal disappears and the depression in the melting point as the difference between 112°C. and this temperature. Since the correction is concerned only with impurities of roughly 5 per cent, an error even as high as 10 per cent in determining the impurities will result in no more than a ± 0.5 per cent error in the gamma isomer present.

In the polarographic step (C), Dragt's method (3) for determining the gamma isomer is followed. A material of unusually high α-heptachlorocyclohexane content will give a high gamma correction, since it will be eluted in the tail run and, when polarographed, will reduce at a half-wave potential similar to that of the gamma isomer. However, under present manufacturing conditions for benzene hexachloride, adequate temperature control is maintained, and thus products containing large excesses of overchlorinated materials are not usually encountered.

In Table 1 are given results obtained by the committee, using this modified chromatographic procedure on a commercial sample of technical benzene hexachloride assumed to contain 13.10 per cent of the gamma isomer, as determined by numerous infrared analyses.

If the gamma-isomer content of 13.10 per cent for this sample is accepted as correct, values of 13.10 ± 0.39 per cent have the desired accuracy of ± 3 per cent. Six of the seven reporting laboratories obtained results

TABLE 1.—*Analysis of technical benzene hexachloride by the modified chromatographic procedure*

LABORATORY	NO. OF RUNS	GAMMA ISOMER			
		AVERAGE	RANGE	AVERAGE DEVIATION	DEVIATION OF AVERAGE FROM INFRARED VALUE
		per cent	*per cent*	*per cent*	*per cent*
1	6	12.87	12.65–13.03	0.14	−0.23
2	3	13.10	13.00–13.20	.07	0
3	7	13.46	13.08–13.80	.22	+ .36
4	5	13.06	12.84–13.18	.10	− .04
5	6	13.27	13.14–13.39	.07	+ .17
6	6	12.80	12.50–13.00	.18	− .30
7[a]	2	11.83	11.75–11.93	.09	−1.21
	2	11.74	11.73–11.74	.01	−1.36

[a] Analyses were made by two analysts.

within this range. The results of the seventh laboratory were considerably out of line. No satisfactory explanation for this deviation was discernible, but it is possible that a poor batch of silicic acid caused improper separation of the gamma fraction.

The results of further collaborative studies will indicate whether the present A.O.A.C. chromatographic procedure should be modified in line with the suggestions outlined.

The committee, mentioned in last year's report, which was studying the radioactive-isotope dilution method for gamma-isomer determinations in benzene hexachloride has not yet completed its study.

No recommendation* is made at the present time for an analytical method for lindane.

REFERENCES

(1) HORNSTEIN, I., *This Journal*, **36**, 367 (1953).
(2) ROSIN, J., and RADAN, G. B., *Anal. Chem.*, **25**, 817 (1953).
(3) DRAGT, G., *ibid.*, **20**, 737 (1947).

REPORT ON PARATHION

By PAUL A. GIANG (U.S. Department of Agriculture, Agricultural Research Service, Bureau of Entomology and Plant Quarantine, Beltsville, Md.), *Associate Referee*

Last year it was recommended that the O'Keefe and Averell titration method for parathion be modified to include the use of the potentiometric endpoint technique described by the Associate Referee and be further studied collaboratively, and that the investigations of the analysis of parathion emulsifiable concentrate be continued (1).

In following these recommendations, the Associate Referee has made a number of determinations on various technical and emulsifiable parathion samples by the O'Keefe and Averell titration method (2). This method has been found quite satisfactory for the analysis of technical parathion, and, with some modifications, should also be applicable for the analysis of parathion emulsifiable concentrates.

Accordingly, in June, 1953, one sample of 25 per cent emulsifiable parathion and one of technical parathion were sent to each of eight laboratories for collaborative study. Seven of these laboratories have thus far reported their results, and their data are given in Table 1.

In general, all the collaborators were satisfied with the O'Keefe and Averell titration method for the analysis of either technical or emulsifiable parathion. However, the following improvements have been suggested:

* For report of Subcommittee A and action of the Association, see *This Journal*, **37**, 62 (1954).

TABLE 1.—*Results of the 1953 collaborative study of parathion samples*

ANALYST	TECHNICAL PARATHION		EMULSIFIABLE PARATHION	
	PARATHION	*p*-NITROPHENOL	PARATHION	*p*-NITROPHENOL
	per cent	*per cent*	*per cent*	*per cent*
1	94.80	—	25.87	—
	94.52	—	25.67	—
	94.62	—	25.81	—
2	—	0.46	—	0.91
	—	0.47	—	0.90
	96.38	—	26.96	—
	96.10	—	26.87	—
	93.23	—	26.45	—
	94.56	0.46	26.68	—
	95.24	0.49	27.19	0.85
	95.45	0.46	26.91	0.89
	94.90	0.45	—	—
3	95.5	0.09	26.2	0.37
	95.6	0.09	26.5	0.37
	95.8	0.09	26.3	0.37
	95.7	0.09	24.2	—
	95.5	0.09	24.4	—
	95.8	0.09	—	—
4	94.44	—	26.25	—
	95.32	—	25.76	—
	95.11	—	26.11	—
5	94.1	0.12	26.1	0.39
	94.6	0.12	26.0	0.38
6	95.06	0.08	25.60	—
	94.75	0.08	25.67	—
	95.56	0.08	26.26	0.18
	95.72	0.08	26.21	0.21
	95.42	0.12	26.34	0.19
7	94.5	0.14	26.70	0.40
	94.7	0.13	26.54	0.40
	95.1	0.14	26.58	0.39
	96.3	0.13	26.67	0.38
	95.3	0.14	26.67	0.39
	94.9			
	95.7			
	95.2			
Average:	95.16 ± 0.59		26.19 ± 0.98	

(A) In the reduction of parathion, 2 grams of zinc should be used. The time of reduction before the addition of 10 ml of concentrated hydrochloric acid should be from forty-five minutes to one hour, and heating should then be continued for ten minutes longer.

(B) The reduced mixture should be filtered before being titrated, and the 100 ml of water usually added for dilution may be used in transferring the mixture and rinsing the beaker.

(C) Three suggestions were made for the determination of the *p*-nitrophenol content in an emulsifiable parathion sample: (*a*) Determine the *p*-nitrophenol directly on a separate portion of the sample in 100 ml of ethanol to which 10 ml of 1 *N* alcoholic potassium hydroxide has been added; (*b*) use 20 ml of 1 per cent sodium carbonate and 2 grams of anhydrous sodium sulfate for each extraction and then wait fifteen to thirty minutes for the ether and aqueous layers to separate and become clear; (*c*) extract the ether solution of the parathion sample with 10 ml of 1 per cent sodium carbonate and 2 grams of anhydrous sodium sulfate four times, or until the aqueous layer becomes colorless. (Procedures (*a*) and (*b*) tend to give too high results.)

All the suggestions have since been tried by the Associate Referee, and he believes that suggestions (A) and (B) should be incorporated into the O'Keefe and Averell titration method for the determination of technical parathion, parathion dust, or emulsifiable parathion. Because many different techniques have been reported for the extraction of *p*-nitrophenol from the emulsifiable parathion sample, and because the results vary so widely, the Associate Referee believes that a detailed procedure such as the one described in the following paragraph should be included in the next collaborative study.

DETERMINATION OF FREE P-NITROPHENOL

Weigh accurately from weighing pipet 2.5–3.5 g of the emulsifiable parathion sample into a 400 ml tall-form beaker. Heat on steam bath for 30 min., passing gentle stream of air over surface of sample to hasten evapn of the solvent. Cool to room temp., and with aid of 150 ml ether, transfer sample to 250 ml separatory funnel. Ext. ether soln with 10 ml cold 1% Na_2CO_3 soln plus 2 g anhyd. Na_2SO_4 4 times, or until aq. layer becomes colorless. Collect aq. layer in 200 ml volumetric flask, and proceed with detn of *p*-nitrophenol by procedure given for technical parathion (2). The addn of anhyd. Na_2SO_4 will prevent emulsion formation in the separatory funnel.

COLLABORATORS

The Associate Referee thanks the following collaborators for their cooperation in this year's study:

Boyd L. Samuel, Division of Chemistry, Virginia Department of Agriculture and Immigration, Richmond, Va.

H. A. Thomson, U. S. Rubber Co., Naugatuck Chemicals Division, Elmira, Ontario, Canada
P. R. Averell, American Cyanamid Co., Stamford, Conn.
Charles V. Marshall, Ontario Department of Agriculture, Ottawa, Ontario, Canada
R. L. Caswell, Production and Marketing Administration, U. S. Department of Agriculture, Beltsville, Md.
Nahit H. Teoman, Bureau of Entomology and Plant Quarantine, U. S. Department of Agriculture, Beltsville, Md.
Lloyd G. Keirstead, Connecticut Agricultural Experiment Station, New Haven, Conn.

RECOMMENDATIONS

Because of the wide disagreement in the results reported this year, it is recommended*—

(1) That further collaborative studies be made of the O'Keefe and Averell titration method, modified as suggested in this report, with the use of a potentiometric titration technique for the determination of technical parathion, parathion dust, and parathion emulsifiable concentrate.

(2) That further collaborative studies be made of the modified procedure for the extraction of *p*-nitrophenol content of parathion emulsifiable concentrate.

REFERENCES

(1) GIANG, P. A., *This Journal*, **36**, 384 (1953).
(2) EDWARDS, F. I., *ibid.*, **35**, 381 (1952).

REPORT ON PYRETHRINS

By DAVID KELSEY (Pesticide Regulation Section, Plant Pest Control Branch, Agricultural Research Service, Department of Agriculture, Washington 25, D. C.), *Associate Referee*

It was recommended at the 1952 meeting of the Association (1) that a further investigation should be made of the official mercury reduction method for determination of pyrethrins. In 1951, a modification of this method was recommended and adopted, first action, by the Association (2). This modification, which resulted in substantially higher values for Pyrethrin I (average increase: 14.6 per cent), was subsequently rescinded at the 1952 meeting (3); at the same time a recommendation was made for further study of the problem.

* For report of Subcommittee A and action of the Association, see *This Journal*, **37**, 62 (1954).

Accordingly, a joint collaborative program was established for this purpose during 1953 with the Insecticide Chemical Analyses Committee of the Chemical Specialties Manufacturers Association. The program, however, is to be directed primarily toward the development of an entirely new method of analysis for the pyrethrins, rather than toward any further modification of the existing mercury reduction method, unless there is evidence that such a modification could improve the precision and reliability of the method. In such a case, the usual collaborative procedure would be observed. A joint collaborative program has also been established to determine the relationship between the analytical results of the collaborative study on methods and the results of biological tests. The pyrethrin content of the dilutions for these biological tests will be determined on the basis of the results of the analytical methods under consideration.

It has been decided to study commercial 2000 mg concentrates from Kenya and Congo flowers. The analytical methods to be considered first are the spectrophotometric method originally proposed by Beckley (4) and modified by Shukis, *et al.* (5), and a modification of the ethylenediamine method currently used in the determination of allethrin (6). Since both of these methods determine total pyrethrins (both I and II) rather than the separate determination of Pyrethrin I and Pyrethrin II possible with the mercury reduction method, each sample is to be analyzed by the official method as well as by the new methods under consideration. Samples have been sent to seventeen American and foreign collaborators for analysis and to ten laboratories for biological testing.

The modification of the official mercury reduction method referred to at the beginning of this report called for the use of hydrochloric acid instead of sulfuric acid in the neutralization of the saponifying alkali. It was felt that the higher value for Pyrethrin I found when hydrochloric acid was used was due to active constituents which were retained by the barium sulfate precipitate when the official method was used. On the basis of work done with pure chrysanthemum acid during 1952, following considerable opposition to the modification from representatives of the principal manufacturers of pyrethrum insecticides, the action of the Association in adopting this modification, first action, was rescinded (3). Mitchell (7) has now shown that an acidic material which can be recovered from the barium sulfate residues, which are normally discarded in the analysis of pyrethrum products by the mercury reduction method, can be shown to account almost exactly for the difference between results obtained with hydrochloric acid, as proposed by the modification, and those obtained using sulfuric acid. The insecticidal activity of the ester from which this acid was obtained, however, has not been demonstrated or disproved. It is not possible, therefore, to say that this acid was derived only from insecticidally-inactive esters.

It is recommended* that the investigation of analytical methods for the determination of pyrethrins, including the hydrochloric acid modification of the official mercury reduction method, be continued.

REFERENCES

(1) KELSEY, D., *This Journal*, **36**, 369 (1953).
(2) *This Journal*, **35**, 44 (1952).
(3) *Ibid.*, **36**, 49 (1953).
(4) BECKLEY, V. A., *Pyrethrum Post*, **1**, No. 3, 5 (1949), and **2**, No. 1, 23 (1950).
(5) SHUKIS, A. J., CRISTI, D., and WACHS, H., *Soap Sanit. Chemicals*, **27**, No. 11, 124 (1951).
(6) JOHNSON, J. B., *Anal. Chem.*, **25**, 1207 (1953).
(7) MITCHELL, WM., *J. Sci. Food Agr.*, **4**, 246 (1953).

REPORT ON ROTENONE

By ROMEO PAYFER (Laboratory Services, Plant Products Division, Department of Agriculture, Ottawa, Ontario, Canada), *Associate Referee*

In view of last-minute findings, the Associate Referee has attempted to establish a mathematical formula for rotenone comparable to the Morton-Stubbs formula for vitamin A (1, 2).

In the analysis of a known compound which is mixed with an impurity of unknown absorption, it is possible to eliminate approximately the effect of the impurity by taking the absorbance at three wavelengths, $\lambda_1 < \lambda_0 < \lambda_2$, in such a way as to obtain C_0:

$$A_{\lambda 0} = a_0 C_0 + A'_{\lambda 0}$$
$$A_{\lambda 1} = a_1 C_0 + A'_{\lambda 1}$$
$$A_{\lambda 2} = a_2 C_0 + A'_{\lambda 2}$$

The values λ_1 and λ_2 must be as widely separated as possible in order to obtain reproducibility, yet close enough so that the impurity is represented by a straight line. The treatment follows that of Morton and Stubbs (1) and is not detailed here. It yields correction factors which are applied to the observed absorbances at the three selected wavelengths. When the factors are established and the curves are traced, a rotenone analysis can be made in approximately thirty minutes.

In the preliminary work, rotenone was purified until a product was obtained with a mixed melting point of 161–162°C. The rotenone standard curve, which has a peak at 294 millimicrons at a concentration of 5 micro-

* For report of Subcommittee A and action of the Association, see *This Journal*, **37**, 62 (1954).

grams per ml, was next established, using a Beckman Model DU spectrophotometer. Rotenone was then extracted from derris root of unknown source, and the absorption curves, at a concentration of 2 micrograms per ml, were then measured. Both curves are shown in Figure 1. The two adjacent wavelengths were selected as 284 and 304 millimicrons and the required factors for analysis were then established.

The new extraction procedure is as follows:

PROCEDURE

Accurately weigh a sample, contg not more than 0.05 g of rotenone; place in a 125 ml standard taper Erlenmeyer flask, and add 100 ml of acetone. Place the closed flask on a magnetic stirrer and stir for 10 min. Filter the soln thru a Whatman No. 31 paper or equivalent, or transfer a portion of the homogenous sample to a Maizel-Green reaction vessel[1] and centrifuge until the soln is clear.

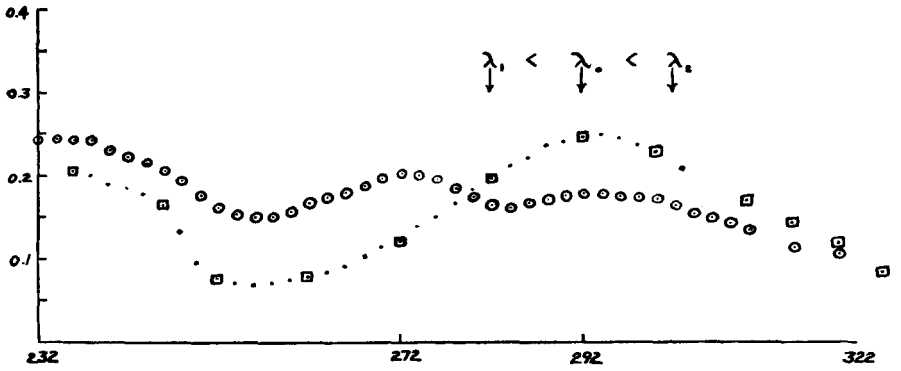

FIG. 1.—Comparison of curves for rotenone. ⊙ represents derris root extracts, 2 mmg/ml. ⊡ represents pure rotenone, 5 mmg/ml.

Take an aliquot of 1 ml for detn and place in a 100 ml volumetric flask. Warm the flask (not exceeding 60°C.) on an H_2O bath and evap. the solvent with a stream of air. Add 50 ml of 95% alcohol, warm slightly to dissolve the rotenone and resins, cool to room temperature, and make to vol.

Read absorbance at 284, 294, and 304 mμ, using 95% alcohol as a blank, and calc. % rotenone.

NOTES

(1) The instrument should be zeroed (with sensitivity control turned left 3½ turns, after being fully turned to the right or clockwise) by means of final slit adjustment, which is then ca 0.8 mm.

(2) Factors (as examples only): $7.02\ A_{294} - 3.5\ A_{284} - 3.5\ A_{304}$ = corrected absorbance for pure rotenone.

(3) Corrected absorbance may be read from the standard rotenone curve.

[1] Wilkins-Anderson Co., Chicago, Ill., No. 7105 MG, is suitable.

DISCUSSION

Use of the Waring blendor was suggested as a way to shorten the time of extraction from four–eight hours to five minutes. However, results of last year's collaborative study showed that the solvent, chloroform, tended to dissolve the bushing of the blendor, and a magnetic stirrer was used instead. The extractant was analyzed for rotenone by the official method and by the proposed ultraviolet procedure (Table 1). Eighteen commercial samples were treated in the same way; the trend of results for all samples was the same.

TABLE 1.—*Determination of rotenone by the ultraviolet procedure*[a]

ROTENONE GUARANTEED PRESENT	ROTENONE FOUND		
	A.O.A.C. METHOD	ULTRAVIOLET METHOD	
		EXTN BY MAGNETIC STIRRER	EXTN BY SHAKING
1.0	0.73	0.96	0.92
		0.88	0.82
	0.63	0.94	0.98

[a] Composition of commercial sample: Derris root, 14.3%; talc, 85.7%.

In addition, samples were prepared by adding rotenone to talc and also to derris root, in order to study the extraction time with the magnetic stirrer. It was concluded that ten minutes was sufficient time to extract rotenone. Different solvents (chloroform, carbon tetrachloride, and acetone) were also studied, and acetone was selected as the solvent to be used. A shorter extraction period is advantageous because less resin is thereby extracted.

The procedure was later checked by repeating some of the experiments in their entirety. The standard curve was again established by using the same purified rotenone employed originally. However, it was found that the peak had shifted by seven millimicrons to a new peak at 287 mμ. Since one physical constant of rotenone was found to have changed within a few months, a melting point determination was made, and it was discovered that the melting point had shifted from 161–162°C. to 153–158°C. The alcoholic rotenone solutions used in the original determination were retested; however, the maximum had remained constant at 294 mμ.

In view of these findings, it may thus be concluded that rotenone, like many organic compounds, is stable in solution but undergoes change with time when allowed to remain in crystalline form. The ultraviolet absorb-

ance of rotenone is very promising as a means for the qualitative determination of the compound, but further study of the procedure must be made. The purification of rotenone must be standardized in such a way that the same quality product can be obtained for the establishment of empirical factors in each laboratory. In the light of these studies, the question arises as to the stability of commercial products containing rotenone.

ACKNOWLEDGMENT

The determination of rotenone by three wavelengths, as well as use of a magnetic stirrer, was suggested by H. A. MacLeod, who also made the tests necessary to establish the empirical factors.

RECOMMENDATIONS

It is recommended*—

(1) That this new procedure be studied collaboratively by as many laboratories as possible, since the method depends upon the establishment of empirical factors.

(2) That a standard for rotenone, whether crystalline or in solution, be clearly and precisely defined.

(3) That the method of preparation of this standard be given in detail.

REFERENCES

(1) MORTON, R. A., and STUBBS, A. L., *Analyst*, **71**, 348 (1946).
(2) GRIDGEMAN, N. T., *ibid.*, **76**, 449 (1951).

REPORT ON DIELDRIN

HYDROGEN BROMIDE METHOD

By J. B. MCDEVITT, Jr. (Louisiana Agricultural Experiment Station, Baton Rouge 3, La.), *Associate Referee*

The Associate Referee made a preliminary study of the hydrogen bromide method (1) as applied to recrystallized dieldrin and dieldrin in commercial insecticides.

The results were erratic. Low results were obtained on recrystallized dieldrin, and the results on dieldrin in commercial insecticides were high.

It is recommended* that further study be made of methods for dieldrin.

* For report of Subcommittee A and action of the Association, see *This Journal*, 37, 62 (1954).

REFERENCE

(1) Shell Method Series 549, Shell Chemical Corporation, Julius Hyman and Co. Division, Denver 1, Colo.

REPORT ON RODENTICIDES

By J. B. LaClair (California State Department of Agriculture, Bureau of Chemistry, Sacramento 14, Calif.), *Associate Referee*

The 1952 report on rodenticides[1] included the recommendation that a collaborative study be undertaken on the determination of warfarin in warfarin concentrates. This collaborative effort was initiated early in 1953, and three samples containing known percentages by weight of pure warfarin in cornstarch were sent to collaborators to be analyzed in triplicate by the following procedure.

METHOD—0.5% WARFARIN CONCENTRATES

Place 0.600 g of sample in a 100 ml centrifuge tube. Add 50 ml of ethyl ether from a pipet, stopper tightly, and shake on a mechanical shaker for 30 min. Place tube in centrifuge and spin a few min. or until ether soln is clear. Pipet 2.0 ml aliquot of the ether soln into centrifuge tube, or glass-stoppered $6 \times \frac{3}{4}$ in. test tube, contg 10.0 ml of 1% Na pyrophosphate ($Na_4P_2O_7 \cdot 10H_2O$) soln. Stopper tube and shake vigorously for 2 min. Place tube in centrifuge and spin until lower aq. layer is quite clear. Draw off upper ether layer, using a fine-tipped glass tube attached to an aspirator. Add ca 2 ml ethyl ether to tube, stopper, shake vigorously, and centrifuge to separate phases. Completely draw off ether layer and any emulsion at interface. If aq. soln is not colorless, or if there is any turbidity, repeat ether extn.

Make final extn, using petr. ether (previously purified by shaking with 1% Na pyrophosphate soln and dried by passing through anhyd. Na_2SO_4). Completely draw off petr. ether layer and draw air over surface of aq. soln for few sec. to evap. last trace of solvent.

Fill a 1 cm silica cuvette with the aq. soln and det. absorbance at 308 mμ on a Beckman Model DU spectrophotometer, using for the check setting a 1% soln of Na pyrophosphate which has been extd with ethyl ether and purified petr. ether.

Absorbance at 308 m$\mu \times 0.90 =$ % warfarin.

DISCUSSION

In Table 1, the percentage recovery for each collaborator is calculated from the percentage of warfarin known to be present in the samples, and is the average of the recoveries for the three samples.

Deviation from the average percentage of warfarin found for the triplicate analyses is low, and the over-all recoveries are within the 5 per cent accuracy usually expected for spectrophotometric methods, but the reasons for low recoveries in some cases are difficult to explain.

[1] *This Journal*, 36, 373 (1953).

TABLE 1.—*Collaborative analysis of samples containing known percentages of pure warfarin in cornstarch*

	PER CENT WARFARIN FOUND BY COLLABORATORS[a]							AV. FOR ALL COLLABORATORS
	1	2	3	4	5	6	7	
Sample A (0.400% Warfarin)	0.375	0.408	0.359	0.392	0.397	0.346	0.396	
	0.376	0.403	0.367	0.378	0.399	0.343	0.394	
	0.373	0.402	0.375		0.398	0.347	0.398	
Av.:	0.375	0.404	0.367	0.385	0.398	0.345	0.396	0.381
Recovery, %	93.75	101.00	91.75	96.25	99.50	86.25	99.00	95.25
Sample B (0.600% Warfarin)	0.562	0.606	0.545	0.578	0.597	0.518	0.599	
	0.585	0.612	0.561	0.580	0.596	0.528	0.603	
	0.575	0.606	0.556		0.598	0.512	0.601	
Av.:	0.574	0.608	0.554	0.579	0.597	0.519	0.601	0.576
Recovery, %	95.67	101.33	92.33	96.50	99.50	86.50	99.83	96.00
Sample C (0.500% Warfarin)	0.482	0.516	0.459	0.494	0.490	0.442	0.503	
	0.482	0.510	0.464	0.492	0.491	0.441	0.497	
	0.484	0.515	0.468		0.495	0.437	0.506	
Av.:	0.483	0.514	0.464	0.493	0.492	0.440	0.502	0.484
Recovery, %	96.60	102.80	92.80	98.60	98.40	88.00	99.60	96.80
Collaborators' Av. Recovery:	95.34	101.71	92.29	97.12	99.13	86.92	99.48	

[a] Not listed in the same order as the list of collaborators.

Collaborators whose average recoveries were low obtained poor recoveries on all three samples; this tends to rule out the factor of nonuniformity of samples. Low recovery could be due to errors in spectrophotometer wavelength calibration. Table 2 shows the magnitude of error which could be caused by a faulty wavelength calibration.

The procedure as presented should be applicable to all commercial warfarin concentrates. Some of these concentrates contain materials other than cornstarch and warfarin, and the above purification steps are often necessary for a successful analysis.

TABLE 2.—*Warfarin recovery with erroneous spectrophotometer wavelength calibration*

WAVELENGTH, mμ[a]	ABSORBANCE	WARFARIN FOUND	RECOVERY
		per cent	*per cent*
303	0.606	0.545	97.15
304	0.613	0.552	98.40
305	0.618	0.556	99.11
306	0.620	0.558	99.47
307	0.6215	0.559	99.64
308	0.623	0.561	100.00
309	0.6215	0.559	99.64
310	0.618	0.556	99.11
311	0.611	0.550	98.04
312	0.6005	0.540	96.26
313	0.5865	0.528	94.12
314	0.572	0.515	91.80
315	0.552	0.497	88.59

[a] Assuming that the spectrophotometer is set on a wavelength of 308 mμ, but through calibration error the spectrophotometer is actually giving readings at the wavelengths listed.

The use of 100 ml centrifuge tubes in the procedure was suggested in order to allow ample head space above the liquid to permit better contact between concentrate and solvent during shaking. After the shaking period, the centrifuge tube is placed directly in a centrifuge. Extraction in any other type of vessel usually involves transferring some of the volatile ether solution to a centrifuge tube with subsequent loss of some ether. Most commercial concentrates require some centrifugation to clarify the ether solution.

There are certain advantages in using glass-stoppered $6\times\frac{3}{4}$ inch test tubes for extracting the warfarin from a 2 ml aliquot of the ether solution with pyrophosphate solution. All purification extractions can be made in these tubes and they can be placed in a centrifuge without having to be transferred to a centrifuge tube. Any transference of such a small volume of solution could cause an error.

It has been found that if all petroleum ether is not removed after the

final purification extraction, the thin film of petroleum ether will be transferred to the quartz cuvette as a suspension of minute droplets which cause scattering of the light beam.

The solution of warfarin in sodium pyrophosphate solution is quite stable and there is no need for speed in reading the solutions in the spectrophotometer. Any change which might occur would be the result of evaporation losses.

The 1952 report[1] on rodenticides discusses the chromatographic purification of ether extracts of prepared warfarin baits, in which Attapulgus clay and silicic acid are used as adsorbents. During the past year the Wisconsin Alumni Research Foundation reported to the author that some samples of Attapulgus clay, grade 200/up, had given extremely poor recoveries of warfarin, requiring up to 50 ml of ether for complete recovery. Their work with two samples of Atta-clay, also a product of the Attapulgus Clay Co., showed that this clay gave excellent results, and that all warfarin was recovered in the first 10 to 20 ml cuts. A sample of Attasol did not permit any warfarin to come through the column. The author had a similar experience with a five-pound can of Attapulgus clay, grade 200/up. The clay in the upper half of the can gave complete recovery of warfarin in the first 25 ml of ether. The clay in the lower portion of the can required 60 ml of ether for passage through the column and then recovery of warfarin was only 95 per cent complete.

All laboratories employing chromatographic purification of ether extracts should first test the clay with a standard warfarin solution in ether prior to use to determine if the warfarin can be completely recovered.

F. B. Coon of the Wisconsin Alumni Research Foundation has submitted a modification of the Eble procedure[2] which employs chromatographic purification to reduce blank values. The method is undergoing tests to determine its suitability for use in the analysis of pelleted baits which are difficult to extract completely with ether.

The procedure in brief is as follows:

MODIFIED EBLE PROCEDURE

DETERMINATION OF WARFARIN IN FINISHED BAITS

Shake 2 g of sample with 50 ml of 1% Na pyrophosphate soln for 1 hr. Centrifuge for 5 min. Shake 25 ml aliquot for 10 min. with 5 ml of 2.5 *N* HCl plus 50 ml of Skellysolve B-ethyl ether mixt. (80+20). Place 20 ml of ether phase in small beaker and evap. to dryness on H_2O bath, using gentle stream of air. Take up residue with three 1 ml portions of ethyl ether, and place each portion on a 2 g Attapulgus clay column. Collect 20–25 ml of ether from column and shake with 10 ml of 1% Na pyrophosphate soln. Remove ether and wash pyrophosphate soln with ether and then with Skellysolve B. Read at 308 mμ in a Beckman Model DU spectrophotometer.

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[1] The Eble method, though originally developed by Mr. Jack Eble in the Laboratories of Dr. K. P. Link, University of Wisconsin at Madison, was modified by E. F. Richter and F. B. Coon of the Wisconsin Alumni Research Foundation. This modified procedure is the one appearing in *This Journal*, **36**, 373 (1952).

SUMMARY

A procedure for the determination of warfarin in concentrates, containing approximately 0.5 per cent warfarin, was subjected to collaborative study on three samples containing known percentages of pure warfarin in cornstarch. The results obtained on an over-all average basis were within the 5 per cent limit of error expected of spectrophotometric procedures of this type, but on an individual collaborator basis the percentage recovery varied from 86.9 to 101.7 per cent.

The effect of error in wavelength calibration of a spectrophotometer has been demonstrated to be a possible source of low recoveries in warfarin determination.

The lack of uniform adsorption quality of Attapulgus clay makes it necessary to check each batch of clay chromatographically with a standard warfarin solution to determine recovery of warfarin before attempting its use in an actual analysis.

The Eble method for determining warfarin in prepared baits has been modified by the Wisconsin Alumni Research Foundation to include a chromatographic purification step.

COLLABORATORS

F. B. Coon, Wisconsin Alumni Research Foundation, Madison, Wis.
W. R. Flach, Eastern States Farmers' Exchange, Buffalo, N. Y.
Harry J. Fisher, Connecticut Agricultural Experiment Station, New Haven, Conn.
J. B. LaClair, California State Department of Agriculture, Bureau of Chemistry, Sacramento, Calif.
D. J. Mitchell, State Chemical Laboratory, University of South Dakota, Vermillion, S. D.
Boyd L. Samuel, Commonwealth of Virginia, Department of Agriculture and Immigration, Division of Chemistry, Richmond, Va.
John F. Weeks, Jr., Food and Drug Administration, Department of Health, Education and Welfare, New Orleans, La.

RECOMMENDATIONS

It is recommended*—

(1) That collaborative study of warfarin in warfarin concentrate be undertaken again next year with the hope that the percentage recovery reported in this year's work can be improved.

(2) That work be continued on checking and improving methods for the analysis of low percentage warfarin baits.

* For report of Subcommittee A and action of the Association, see *This Journal*, **37**, 62 (1954).

REPORT ON METHODS FOR DETERMINING PHENOLS IN PHENOLIC DISINFECTANTS

By FRANK A. SPURR (Pathological Division, Bureau of Animal Industry, U. S. Department of Agriculture, Washington 25, D. C.), *Associate Referee*

There are several methods, based on similar techniques, by which the phenols of coal-tar origin can be determined successfully. These methods appear to give consistently low results when they are applied to phenolic disinfectants containing the complex phenolic bodies derived from petroleum.

The present study of methods for the determination of phenols in phenolic disinfectants has thus been confined to the type of phenolic disinfectants which contain these petroleum-derived phenols. A method for the determination of this type of phenols is now under study and appears to produce satisfactorily reproducible results.

It is recommended* that further study of the methods for the higher boiling phenols, especially those of petroleum origin, be continued for another year.

REPORT ON PHYSICAL PROPERTIES OF INSECTICIDE POWDERS

By ERNEST L. GOODEN (U. S. Department of Agriculture, Agricultural Research Service, Entomology Research Branch, Beltsville, Md.), *Associate Referee*

Progress has been made in the development of methods for physical evaluation of insecticide powders in the past year for two general types of material: (1) granulated insecticides, and (2) water-dispersible powders. In each type the active ingredients upon which most attention was focused were DDT and dieldrin. The physical principles involved, however, are largely general; the change from one active ingredient to another among the new chlorinated synthetics calls for only minor adjustments in physical requirements.

GRANULATED INSECTICIDES

This type of product is a coarse powder, relatively non-dusty, designed for surface treatment either of dry soil or of marshland by broadcasting from an airplane or by other convenient means. Its granulometric char-

* For report of Subcommittee A and action of the Association, see *This Journal*, 37 ,62 (1954).

acteristics are such that it will fall readily through any ordinary blanket of vegetation and arrive promptly at the ground surface. The material is commonly prepared by impregnating a granular clay with a solution of the toxic agent. The preliminary requirements and test methods that have been worked out with respect to physical properties are given below; the paragraphs have been numbered in conformity with the Federal Specification system.

REQUIREMENTS

3.2.1 *General.*—The product shall consist essentially of dry granular aggregates of microscopic particles, free of objectionable dustiness, but capable of being broken down into subaggregates and ultimate particles with reasonable ease, to facilitate further distribution of the toxicant through natural weathering processes subsequent to application of the product upon the soil.

3.2.2 *Grain size distribution.*—The grain-size distribution of the product, determined by dry sieve test as specified in **4.3.2.1**, shall come within the following limits:

MICRONS	U. S. NO.	PER CENT PASSING
1000	18	90 minimum
250	60	25 maximum
149	100	10 maximum

3.2.3 *Grain breakdown.*—Not less than 60% of the granulated insecticide shall pass a 250 μ (U. S. No. 60) sieve by the (water spray) wet sieve test, described in **4.3.2.2.**

3.2.4 *Bulk density.*—The bulk density of the granulated insecticide as determined by the Scott volumeter **(4.3.3)** shall be not less than 0.70 g/cc.

TEST PROCEDURES

4.3.2 *Sieve tests.*—Sieves shall conform to the requirements of Federal Specification RR-S-366.

4.3.2.1 *Dry sieve test.*—Screen a 20 g sample through a nest of 8 in. U. S. Standard sieves of the designated mesh sizes, using a single-eccentric type mechanical shaker which imparts to the sieve a rotary motion and tapping action of uniform speed of ca 300 gyrations and ca 150 taps per min. Continue the screening for 15 min. Weigh the residues and calculate the percentage passing thru each sieve.

4.3.2.2 *Wet sieve test.*—Place a 1 g sample on the sieve and wash for 10 min. with an oscillating spray of water from a conventional bath-type spray hose connected to a water source with pressure adjusted so as to maintain a head of about 2 feet of water. (The control may be a manual adjustment of the faucet valve, in conjunction with a simple overflow standpipe, the top of which is ca 2 feet above the level of the sprinkler head when the sprayer is in use.)

Transfer the residue from the sieve to a filter, dry and weigh the residue, and calculate by difference the percentage of material passing through the sieve.

4.3.3 *Bulk density.*—Determine the bulk density of the granulated insecticide with the Scott volumeter, taking the average of three runs, or more if needed to establish a reliable average value. In case the wire screen supplied for use in the funnel is too fine in mesh for the nature of the powder, a coarser screen may be substituted.

These requirements and methods are subject to continual review in the light of developing experience in this relatively new physical type of formulation. One point that needs particular attention is the possible adaptation to the use of organic carriers, such as tobacco waste.

WATER-DISPERSIBLE POWDERS

Water-dispersible powders are manufactured in this country for two types of market, American and international. The wettable DDT powder for American consumption, if sold to the United States Government for either civilian or military use, is covered by the Federal Specification O-I-568;[1] that for use by other countries is generally expected to comply with the World Health Organization specification WHO/SIFORM/1.[2] (Respective specifications for dieldrin are similar to those for DDT.) The principal difference in requirements is that the WHO powders are much more finely divided than the Federal-specification type, or are at least more finely dispersible in the test suspension and therefore much slower in settling. This slower-settling powder is more difficult to manufacture, and difficulty has been experienced in obtaining material that complies originally with the specification and retains its physical quality in transit and in storage. This difficulty and the divergence between the two standards of quality have combined to cause confusion in the wettable-powder market. The following points for the improvement of test methods have been under special consideration in the last year as a means of easing procurement problems, and considerable progress has been made.

(1) Redevelopment of methods of evaluating dispersibility and/or suspensibility in order to put the two types of specifications on a common testing procedure.

(2) Improvement of both types of specifications, especially those of the WHO, with respect to testing for physical stability under hot storage conditions.

(3) Improvement of reproducibility.

(4) Modification of the suspensibility test to eliminate chemical analysis from the essentially physical test.

(5) Investigation of the effects of water temperature on the behavior of powder in suspension.

The work briefly reported herein has been almost entirely governmental, including cooperation of the Bureau of Entomology and Plant Quarantine with the World Health Organization. The specification requirements and test methods have been in such a highly unsettled state that an orderly program of domestic collaboration has so far been impos-

[1] May be obtained from the Superintendent of Documents, U. S. Government Printing Office, Washington 25, D. C.

[2] Report No. 54 of the Expert Committee on Insecticides, approved for December, 1951. May be obtained from the World Health Organization, Regional Office, 1501 New Hampshire Ave., N.W., Washington, D. C.

sible. One industrial laboratory has volunteered for collaboration; it is hoped that others will offer their cooperation and that an appropriate program may soon be set up.

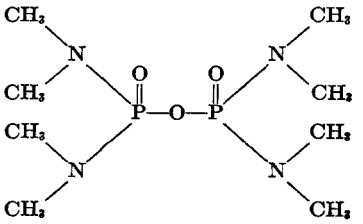
It is recommended* that collaborative studies of the physical properties of insecticide powders be initiated.

REPORT ON SYSTEMIC INSECTICIDES

By PAUL A. GIANG (U. S. Department of Agriculture, Agricultural Research Service, Entomology Research Branch, Beltsville, Md.),
Associate Referee

Interest in the study of systemic insecticides (insecticides that are taken up by a growing plant in quantities great enough to destroy insects that feed on it) may be said to date back to 1947, when the organic phosphorus and fluorine compounds synthesized by Schrader in Germany during World War II were reported by Martin and Shaw (1). The systemic properties of some of Schrader's compounds have since been confirmed by workers in England and in this country. In general, all these systemic compounds are effective aphidicides and miticides. At the present time, however, only three of Schrader's compounds and one compound synthesized by Hartley (2) in England have proved promising enough for commercial production. These four compounds will be described briefly.

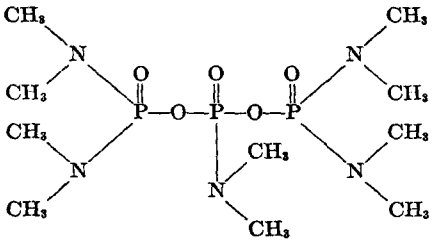
(1) *Schradan* (octamethyl pyrophosphoramide or OMPA):



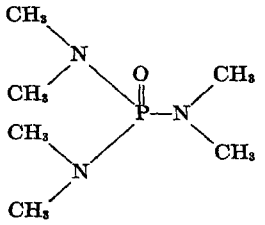
Schradan has been intensively studied by W. E. Ripper and his co-workers in England (3), who call it a selective insecticide because it kills insects that feed on the plants but not those that feed on the plant pests. Schradan is being produced on a commercial scale in England by Pest Control, Ltd., and in experimental amounts in this country by the Dow Chemical Company and the Monsanto Chemical Company. The experimental data (4) indicate that all the preparations of technical schradan also contain a con-

* For report of Subcommittee A and action of the Association, see *This Journal*, 37, 62 (1954).

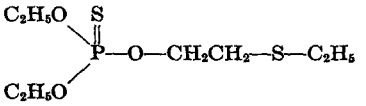
siderable amount of decamethyl triphosphoramide, which is reported to be insecticidally active:



They may also contain a lesser amount of hexamethyl phosphoramide, which is not insecticidally active:

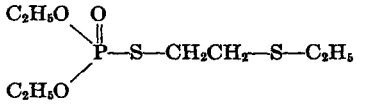


(2) *Demeton* (O-[2-(ethylmercapto)ethyl]-O,O-diethyl thiophosphate):



Demeton is a systemic insecticide that has been extensively studied in this country and abroad. "Demeton" is the new common name for the active constituent of Systox. The Pittsburgh Coke and Chemical Company manufactures this compound in experimental amounts. In addition to being a systemic insecticide, it is also a contact insecticide and a fumigant, acting somewhat like parathion (5). However, the systemic action is its most important economic function.

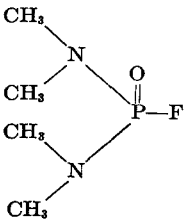
Recent experiments (6) indicate that technical demeton contains an isomer in which oxygen is interchanged with sulfur as follows:



This so-called oxygen isomer is much more soluble in water and is about 10 times as toxic as demeton itself (6, 7), both to insects and to warm-blooded animals.

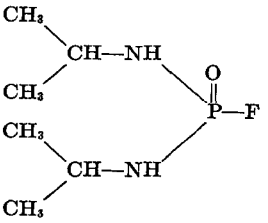
The third and fourth systemic insecticides, *dimefox* and *mipafox*, are produced commercially by Pest Control, Ltd. Like demeton, both compounds can also be used as contact insecticides or as fumigants.

(3) *Dimefox* (*bis*-(dimethylamino)fluorophosphine oxide or Pestox 14):



Dimefox is more toxic than schradan to warm-blooded animals and insects, but its insecticidal action in the plant does not last as long. An appreciable part of this compound absorbed by the plant roots is later given off as vapor by the leaves (8). Dimefox is best known in a formulation called Hanane, which is used to kill mealybugs infesting cocoa plants on the Gold Coast in Africa (9). This formulation has given phenomenal control of swollen shoot disease carried by mealybugs to cocoa trees (10).

(4) *Mipafox* (*bis*-(isopropylamino)fluorophosphine oxide, Isopestox or Pestox 15):



Mipafox, which is not one of Schrader's compounds, was first synthesized by Hartley in England in 1950 (2). It is claimed to be "26 times less toxic than parathion, nine times less toxic than nicotine, and only twice as toxic as DDT" (11). In the summer, mipafox completely disappears from an actively growing plant within sixteen days after its application, in contrast to schradan and demeton, which last from five to six weeks (12, 13). Recently two cases of human paralysis (similar to ginger paralysis caused by triortho-cresyl-phosphate) were reported (14, 15), and for this reason, mipafox has been withdrawn from the market (15).

The four compounds are inhibitors of cholinesterase, an enzyme generally present in nerve tissues. Their insecticidal activity can probably be largely accounted for on the basis of the enzymatic inhibition (16, 17). All the work on the toxicity to warm-blooded animals of these compounds

(18) indicates that extreme care must be exercised in handling them, a fact which should be kept in mind when samples are analyzed.

Methods of analysis of any of these systemic insecticides, whether in the form of technical materials, formulations, or extractives from plant materials, have not been previously studied by this Association. Some of the methods currently in use or under development will therefore be reviewed in this report. Three methods are known for schradan and three for demeton,[1] but no specific method has been reported for either dimefox or mipafox, although these two chemicals can be analyzed by one or more of the methods described for schradan (Hartley, personal communication) with some modifications.

ANALYTICAL METHODS FOR SCHRADAN

Selective extraction and hydrolysis method (Hartley, *et al.* (4)).—This method of analysis is based on the different speeds of alkaline hydrolysis and the partition coefficients in $CHCl_3$ and CCl_4 of the three main components of technical schradan. Of these three components, decamethyl triphosphoramide hydrolyzes readily in a *N* NaOH solution at 100°C.; octamethyl pyrophosphoramide and hexamethyl phosphoramide hydrolyze only after a much longer time. Hexamethyl phosphoramide is extracted with CCl_4; the other two are extracted readily with $CHCl_3$. The dimethylamine obtained from hydrolysis can then be distilled and estimated by titrating with standard HCl.

Hydrolysis method (David, *et al.* (20)).—This method was devised for the determination of schradan in plant residues. It is based upon the fact that schradan can be easily hydrolyzed to dimethylamine and orthophosphate in an acid medium, and the orthophosphate ion resulting from the hydrolysis can then be determined by the molybdenum colorimetric method of Allen (21).

Dimethylamine colorimetric method (Hall, *et al.* (22)).—This method was developed principally for the estimation of schradan in plant materials. The procedure utilizes acid hydrolysis, which splits schradan into dimethylamine and phosphoric acid; the dimethylamine is then determined by the formation of a colored complex with copper reagent in an alkaline CS_2—$CHCl_3$ medium.

ANALYTICAL METHODS FOR DEMETON

Differential hydrolysis method (Pittsburgh Coke and Chemical Company (23)).—This method is based upon the principle that the oxygen isomer in technical demeton that has been dissolved in an acetone-indicator solution can be easily hydrolyzed in 50.00 ml of 0.1 *N* NaOH solution at room temperature, whereas demeton itself can be hydrolyzed in the same manner only when heat is applied. The amount of each of the isomers present in a sample can be obtained by back-titrating with standard 0.1 *N* HCl the NaOH which remains after hydrolysis.

Nitroprusside method (Ritchie (24)).—This method was developed for the determination of technical demeton or demeton formulations. It is a modification of the McCarthy and Sullivan (25) colorimetric procedure for methionine. It is possible that a modification of the method might be used for the determination of demeton in plant residues. This method consists mainly in the development of a red color by adding an aqueous sodium nitroprusside reagent to a MeOH and NaOH solution

[1] Since the presentation of this report at the 67th annual meeting of this Association, a partition chromatographic method for the determination of demeton has been published (19).

of demeton and then acidifying the solution with phosphoric acid; after the solution has stood in an ice bath for 5 min., the intensity of the color can be read in a spectrophotometer.

Enzymatic method (Giang and Hall (26)).—This is a general method for determining all the organic phosphorus insecticides that have a strong inhibitive action on cholinesterase; it is not a specific method for demeton. However, it has recently been used, in various modified forms, for the estimation of demeton in plant materials. An aliquot of the extractive of the plant material is allowed to react with a standard cholinesterase buffered solution at *p*H 8 for 30 min. at 25°C. Partial inhibition of the enzyme is effected by the extractive. As a substrate for the enzyme to act upon, a standard acetylcholine chloride solution is added. The enzyme and substrate are stirred together at 25°C. for 60 min. The acidity resulting from the hydrolysis of the substrate is then measured with a *p*H meter and converted to per cent inhibition, which is related to micrograms of the insecticide present.

In general, the comments of investigators indicate dissatisfaction with all the methods described. The objections are mostly centered on either the time consumed by certain methods or the lack of accuracy of the results.

RECOMMENDATIONS

It is recommended*—

(1) That all the methods described in this report be investigated with a view to overcoming the reported objections, and that any of the methods that show promise be studied collaboratively.

(2) That the survey of new methods for the analysis of all systemic insecticides be continued.

REFERENCES

(1) MARTIN, H., and SHAW, H., Final Report of the British Intelligence Objectives Sub-Committee (London), **1095**, 1947, 93 pp.
(2) ANON., *Chemistry and Industry*, **7**, 123 (1951).
(3) RIPPER, W. E., *Mfg. Chemist*, **21**, 65 (1950).
(4) HARTLEY, G. S., HEATH, D. F., HULME, J. M., POUND, D. W., and WHITTAKER, M., *J. Sci. Food Agr.*, **2**, 303 (1951).
(5) GOODMAN, A., *World Crops*, **4**, 350 (1952).
(6) HARTLEY, G. S., *ibid.*, **4**, 397 (1952).
(7) RIPPER, W. E., *Systemic Insecticides.* Address to the IIIrd International Congress, Crop Protection (Paris), Pest Control, Ltd., Cambridge, England, 1952, 56 pp.
(8) DAVID, W. A. L., *Ann. Appl. Biol.*, **39**, 203 (1952).
(9) WEST, J., *Cocoa, Chocolate and Confectionery Alliance Report*, 1951, p. 86.
(10) ROSS, S. D., and BROATCH, J. D., *ibid.*, p. 164.
(11) ANON., *Gardeners' Chron.*, **79** (3346), 57 (1951).
(12) IVY, E. E., *Agr. Chemicals*, **8**, 47 (1953).
(13) KELLY, R. A., *Entomol. Soc. Ontario, Ann. Rept.*, **81**, 86 (1951).
(14) BIDSTRUP, P. L., BONNELL, J. A., and BECKETT, A. G., *Brit. Med. J.*, **1**, 1068 (1953).
(15) DAVID, D. R., *Proc. Roy. Soc. Med.*, **45**, 570 (1952).

* For report of Subcommittee A and action of the Association, see *This Journal*, 37 ,62 (1954).

(16) FRAWLEY, J. O., HAGAN, E. G., and FITZHUGH, O. G., *J. Pharmacol. Exptl. Therap.* **105**, 156 (1952).
(17) DUBOIS, K. P., *Bull. Am. Soc. Hosp. Pharm.*, **9**, 169 (1952).
(18) ———, and COON, J. M., *Arch. Ind. Hyg. and Occupational Med.*, **6**, 9 (1952).
(19) GARDNER, K., and HEATH, D. F., *Anal. Chem.*, **25**, 1849 (1953).
(20) DAVID, A., HARTLEY, G. S., HEATH, D. F., and POUND, D. W., *J. Sci. Food Agr.*, **2**, 310 (1951).
(21) ALLEN, R. J. L., *Biochem. J.*, **34**, 858 (1940).
(22) HALL, S. A., STOHLMAN, J. W., IIIrd, and SCHECHTER, M. S., *Anal. Chem.*, **23**, 1866 (1951).
(23) Pittsburgh Coke and Chemical Company, ***TM*-60**, April 20, 1953.
(24) RITCHIE, GEORGE D., "A Colorimetric Method of Analysis of 0,0-Diethyl-0-(*beta*-Ethylmercapto)Ethyl Thiophosphate (Systox)." Presented as a Thesis. Cornell University, Ithaca, N. Y., February, 1953.
(25) MCCARTHY, T. E., and SULLIVAN, M. X., *J. Biol. Chem.*, **141**, 871 (1941).
(26) GIANG, P. A., and HALL, S. A., *Anal. Chem.*, **23**, 1830 (1951).

REPORT ON HERBICIDES

EXTENT OF VOLATILITY OF ESTERS OF 2,4-D AND 2,4,5-T ACID

By E. A. WALKER (Pesticide Regulation Section, Plant Pest Control Branch, Agricultural Research Service, U. S. Department of Agriculture, Washington 25, D. C.), *Associate Referee*

The Insecticide Division of the Livestock Branch, U. S. Department of Agriculture, has developed a method[1] for evaluating the volatility of proprietary formulations of 2,4-D and/or 2,4,5-T herbicide chemicals for potential vapor hazards in an open system. In the method, a measured stream of forced air is passed over the herbicide liquid and directed toward a test plant (Rutgers tomato) contained in a paper bag. After being exposed for periods of two, four, and sixteen hours, the plants are removed and readings are taken on epinastic response and other effects. The plants remain in the open and are examined again at intervals of twenty-four, forty-eight, and seventy-two hours for evidence of formative effects.

By this method, investigators examined more than 80 samples of proprietary ester formulations in establishing a basis for classification of volatile and low volatile ester formulations of 2,4-D and/or 2,4,5-T. It was found that the butyl, isopropyl, amyl or pentyl, and alkyl (from mixed alcohols containing 3 to 5 carbon atoms) esters are volatile and that esters which are low-volatile at ordinary temperatures (70–75°F.) include butoxyethanol, tetrahydrofurfuryl, isoocytl, and propylene glycol butyl

[1] *Agricultural Chemicals*, August, 1953.

ether esters. This list in no way constitutes all of the formulations that are available for testing.

The Associate Referee recommends* that collaborative study be planned and conducted during the coming year with a view to acceptance and adoption of this method.

No reports were given on aldrin; allethrin; chlordane and toxaphene; DDT and related compounds; dithiocarbamates; piperonyl butoxide; and quaternary ammonium compounds.

REPORT ON MOISTURE IN COFFEE

By GEORGE SCHWARTZMAN (Food and Drug Administration, Department of Health, Education, and Welfare, New York 14, N. Y.), *Associate Referee*

ROASTED COFFEE

The specificity and rapidity of the Karl Fischer reagent for moisture determinations in foodstuffs and other products has given it wide acceptance in many laboratories. The theoretical aspects of this method and its application to many materials has been reviewed by Mitchell (1).

Johnson (2), and Schroeder and Nair (3) used dry methanol as the extraction solvent for moisture in dehydrated foods. This technique required heating or refluxing with the solvent for varying lengths of time in order to obtain complete extraction of the moisture. McComb and McCready (4) substituted formamide for methanol, and found that its use greatly reduced the length of time necessary for the sample to be in contact with the solvent.

This report is a preliminary study of the use of the Karl Fischer reagent for the determination of moisture in roasted ground coffee, and a comparison of this method with the classical air-oven method of drying at 105°C. for five hours. Two solvents, methanol and formamide, were used as extractants for moisture. Visual titrations could not be used since the solvents would darken on contact with the coffee and mask the endpoint. The dead-stop endpoint with direct titration appeared to be the most convenient electrometric technique and was used throughout this study. A commercially roasted No. 2 Santos coffee was ground to 40 mesh in a Labconco Mill and used for the experiments.

APPARATUS

A cathode-ray titrimeter[1] consisting of a rectifier, amplifier, and "magic-eye" tube assembled in a cabinet was built, using the circuit diagram of Kieselbach (5). All

* For report of Subcommittee A and action of the Association, see *This Journal*, 37, 62 (1954).

[1] The Associate Referee is greatly indebted to David Firestone of this laboratory for planning and constructing the cathode ray titrimeter and the platinum electrodes.

parts were purchased from a radio supply house and are standard radio components. The platinum electrodes were constructed as follows: A pair of 20 gauge platinum wires were soldered to 18 gauge copper wires and sealed through soft glass tubes. The platinum wires were ground off almost flush with the glass to prevent breakage close to the seal. The electrodes were connected to the instrument by means of a shielded cable. The "eye" was set so that it was initially closed and the endpoint was taken as the point where the addition of a drop of reagent caused it to remain open for thirty seconds. The most satisfactory method of agitation was a magnetic stirrer with a "flea," inside the titration flask. The "flea" consisted of several pieces of wire clip sealed in glass tubing, and was kept with the titration flask throughout all operations. Two all-glass automatic burets, protected by drying tubes containing Drierite, were used; one dispensed the extraction solvent and the other the Karl Fischer reagent. The titration flasks were 250 ml glass-stoppered Erlenmeyers; they were rinsed with cleaning solution, thoroughly washed with water and dried overnight in a 105° oven, stoppered, and allowed to cool before use. For titrating, the flask was fitted with a four-hole rubber stopper to accommodate the buret tip, the two platinum electrodes, and a vent tube filled with desiccant.

REAGENTS

(a) *Karl Fischer reagent* (6).—Add 125 g of resublimed iodine to a soln contg 670 ml of dried methanol and 170 ml of dried pyridine. Pass SO_2 into 100 ml of dried pyridine in a glass cylinder, protected from atmospheric moisture and cooled in an ice bath, until the vol. reaches 200 ml. Slowly add this soln, with shaking, to the cooled iodine-methanol-pyridine mixt. Shake well to dissolve the iodine and transfer to an automatic buret. Methanol (7) for use in the reagent was dried by treating 1 l with 5 g of Mg turnings and 0.1 g of $HgCl_2$, and distg as soon as the Mg turnings had dissolved. The pyridine (8) was dried with solid NaOH and distd. Precautions were taken to protect the distn systems from atmospheric moisture.

(b) *Methanol.*—Reagent grade.

(c) *Formamide.*—Technical grade was found to be sufficiently dry to be used as a solvent.

PROCEDURE

The Karl Fischer reagent gradually deteriorates and should be standardized before each series of detns. Place 25 ml of methanol or 20 ml of formamide in the titration flask and det. the reagent blank. To the same flask, add a drop of H_2O from a weighing buret, det. its weight by difference to the nearest 0.1 mg, and again titrate. Calculate the strength of the Karl Fischer reagent in terms of mg of H_2O per ml of reagent. Three blanks and three standardizations were detd for each series of experiments.

For the following detns 1 g samples of coffee were used and the moisture content was calcd as follows:

$$\frac{(\text{ml K.F.}_{\text{sample}} - \text{ml K.F.}_{\text{blank}})(\text{ml } H_2O/\text{ml K.F.}) \times 100}{\text{Sample wt in mg}} = \% \text{ moisture}$$

In the first series of experiments the moisture was extracted with methanol at room temperature. Table 1 shows the results obtained by varying the length of time the methanol was in contact with the coffee. The moisture content of this coffee was 3.55 per cent when determined by drying at 105° for five hours.

TABLE 1—*Effect of time of standing in methanol at room temperature*

HOURS	PER CENT MOISTURE
1	2.45, 2.35
2	2.61, 2.57
3	2.52, 2.58
4	2.75, 2.79
24	3.51, 3.46
30	3.49, 3.63

A similar series of experiments on additional samples of the same coffee was performed in which the coffee and methanol were heated in a paraffin oil bath at $60 \pm 5°$ in lightly-stoppered titration flasks. The flasks were heated for varying lengths of time, cooled to room temperature, and titrated as above. These results are shown in Table 2.

TABLE 2.—*Effect of time of standing in methanol at $60 \pm 5°C$.*

MINUTES	PER CENT MOISTURE
5	2.43, 2.47
10	2.77, 2.85
20	3.02 —
30	3.27, 3.22
45	3.45, 3.48
60	3.65, 3.62

One determination was made using formamide at elevated temperatures. The titration flask with sample and solvent was placed in a paraffin bath that had been heated to $150 \pm 10°$. After forty seconds, it was removed from the bath, cooled, and titrated. Moisture on three other samples was determined by stirring sample and formamide on the magnetic stirrer for varying times at room temperature. These results are reported in Table 3.

TABLE 3.—*Use of formamide as solvent*

TIME	PER CENT MOISTURE
40 seconds at $150 \pm 10°$	3.36, 3.33
5 minutes stirring[a]	2.07
10 minutes stirring[a]	2.21
20 minutes stirring[a]	2.66

[a] At room temperature. Magnetic stirrer used and flasks tightly stoppered.

GREEN COFFEE

A small amount of exploratory work has been done on the determination of moisture in the green bean. The principal difficulty is in the grinding;

the beans may be so wet that they cannot be fractured by any type of mill. This is overcome by using a method similar to the one used by the Swiss Society of Analytical Chemists (9).

This modified method is as follows: Weigh accurately a 25 g sample of the unground green bean into an aluminum dish ca 70 mm×30 mm with close-fitting cover. Heat the sample in an air-oven at 105° for 4.5 hrs. Replace the cover, cool dish in a desiccator, and weigh. Calc. % loss in weight. Then grind the partially dried coffee in a Wiley mill through the 20 mesh screen, and weigh a 5 g sample into the same type dish as above. Heat sample for an addnl 5 hrs, cool, and weigh. Again calc. % loss in wt. Add the two figures and report the total as % moisture in the green bean.

This method was compared with the toluene distillation method, using 30 g of unground green beans and a five hour reflux.

The results are shown in Table 4.

TABLE 4.—*Comparison of air-oven and toluene distillation methods on green coffee*[a]

AIR-OVEN METHOD (PER CENT MOISTURE)		TOLUENE DISTILLATION METHOD (PER CENT MOISTURE)
25 g for 4.5 hours	6.16, 6.09	
Ground in Wiley Mill through 20 mesh sieve and heated for 5 hours (5 g sample)	2.38, 2.47	7.69, 7.75
Per cent moisture (total)	8.54, 8.56	

[a] No. 2 Yemen Coffee used.

SUMMARY

Results by the Karl Fischer method, using methanol as a solvent at room temperature for thirty hours, were comparable to those from the five-hour air-oven method. The time required for a single determination makes the use of methanol at room temperature of very little value in routine work. The use of methanol at 60 ± 5° reduces the time to one hour. On the basis of the limited observations, formamide as a solvent shows the greatest promise for routine use.

With coffee, as with most natural products, the results obtained by the Karl Fischer method cannot be correlated with the true moisture, because of the lack of an absolute method.

Plans are being made to investigate the use of methanol at temperatures higher than 60°C. Work will also continue with the use of formamide for longer periods of time at 150°C. Several other solvents are under consideration.

The modified Swiss procedure of partially drying the green bean and then grinding it, also shows promise as a method for the determination of moisture in green coffee.

It is recommended* that this study be continued.

* For report of Subcommittee C and action of the Association, see *This Journal*, **37**, 69 (1954).

REFERENCES

(1) MITCHELL, J., JR., *Anal Chem.*, **23**, 1069 (1951).
(2) JOHNSON, C. M., *Ind. Eng. Chem. Anal. Ed.*, **17**, 312 (1945).
(3) SCHROEDER. C. W., and NAIR, J. H., *Anal. Chem.*, **20**, 452 (1948).
(4) MCCOMB, E. A., and MCCREADY, R. M., *This Journal*, **35**, 437 (1952).
(5) KIESELBACH, R., *Anal. Chem.*, **21**, 1578 (1949).
(6) *United States Pharmacopeia*, Fourteenth Revision, Mack Publishing Co., Easton, Pa., 1950, p. 796.
(7) GILMAN, H., and BLATT, A. H., *Organic Synthesis*, Coll. Vol. I, 2nd Ed., John Wiley and Sons, Inc., New York, 1941, p. 220.
(8) *Ibid.*, p. 100.
(9) *Schweizerisches Lebensmittelbuch*, 2nd Ed., 1909, p. 158.

No report was given on chlorogenic acid in coffee.

REPORT ON ALCOHOLIC BEVERAGES

By RICHARD L. RYAN (Alcohol and Tobacco Tax Division Laboratory, Internal Revenue Service, Washington 25, D. C.), *Referee*

The following recommendations* have been made by the Associate Referees and the Referee:

MALT BEVERAGES, BREWING MATERIALS, AND ALLIED PRODUCTS

It is recommended—

(1) That methods for the determination of sugars in beer be studied.

(2) That the study of the wet-ash orthophenanthroline method for iron in beer be continued.

(3) That the first action direct, non-ash procedure for iron in beer adopted two years ago, modified to provide for α, α'-dipyridyl as an optional reagent, be further studied by the Associate Referee.

(4) That the direct, non-ash method of Stone[1] for copper in beer be studied collaboratively.

(5) That collaborative studies of methods for the determination of moisture, total nitrogen, and P_2O_5 in yeast be continued.

(6) That work on methods for the measurement of turbidity in beer be resumed.

(7) That the method for alpha-amylase in malt, as presented in the report of the Associate Referee, be adopted, first action.

* For report of Subcommittee D and action of the Association, see *This Journal*, **37**, 75, 76 (1954).
[1] STONE, I., *Ind. Eng. Chem., Anal. Ed.*, **14**, 479 (1942).

WINES

It is recommended—

(1) That collaborative studies of the applications of paper chromatography in wine analysis be discontinued.

(2) That the spectrophotometric method for the determination of phosphates in wines and spirits, *This Journal*, **35**, 257 (1952), be adopted as official.

(3) That the spectrophotometric method for the determination of tannins in wines and spirits, *This Journal*, **35**, 255 (1952), be adopted, first action.

(4) That studies on the determination of color in wines be discontinued.

(5) That the study of methods for the determination of tartrates in wines be continued.

(6) That the method for "total alkalinity of the ash" be studied collaboratively.

DISTILLED LIQUORS

It is recommended—

(1) That the official method, **9.29**, for methanol by the immersion refractometer be reviewed in the light of the findings of Beyer and Reeves, *This Journal*, **28**, 800 (1945).

(2) That the study of the methods for methanol in distilled liquors and drugs be continued with a view to correlating the procedures.

(3) That the investigation of the higher alcohols in distilled spirits by chromatography be discontinued.

(4) That a study be made to adapt the "Dye Color Method for Beer", *Official Methods of Analysis*, 7th Ed., **10.3**, for use as a color reference solution for distilled liquors.

(5) That a study of the naturally occurring sugars in aged liquors be undertaken.

CORDIALS AND LIQUEURS

It is recommended—

(1) That the method for citric acid in fruits and fruit products, *This Journal*, **34**, 75 (1951), be adopted, first action.

(2) That the method for tartaric acid in fruits and fruit products, *This Journal*, **36**, 79 (1953), be adopted, first action.

(3) That the method for malic acid in fruits and fruit products, *This Journal*, **36**, 80 (1953), be further studied.

REPORT ON CORDIALS AND LIQUEURS

DETERMINATION OF CHARACTERISTIC ACIDS

By JOHN B. WILSON (Division of Food, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.), *Associate Referee*

Following the suggestion of Subcommittee D to study the application to cordials of the newly modified methods for fruit acids in fruit products, the Associate Referee prepared two synthetic cordials and submitted them to two collaborators, *viz.*, C. G. Hatmaker, Food and Drug Administration, and Alex P. Mathers, Internal Revenue Service. The composition of the samples was as follows:

	Peach	*Raspberry*
Sugar	400 g	300 g
Citric Acid	5.8 g	3.0 g
Malic Acid	3.2 g	0 g
Tartaric Acid	0 g	4.0 g
Alcohol	300 ml	275 ml
Flavoring		*Imitation Raspberry*
Benzaldehyde	0.5 ml	*Flavor* 0.2 g
Peach Aldehyde	0.6 ml	
Oil Cognac	0.4 ml	
Water	q.s.	q.s.
	1000 ml	1000 ml

The acidity and solids content of the samples were such that 20 ml of the peach and 30 ml of the raspberry fulfilled the conditions outlined in section **9.56,** "Preparation of Sample", and no evaporation was required. Collaborators were instructed to use these quantities of sample for the several determinations and to continue as in **9.56,** beginning: "to ca 30 ml, add 6 ml of 1 *N* NaOH . . . ". Transfer 200 ml of filtrate to a centrifuge bottle and proceed as in *This Journal,* **36,** 79 (1953), under DETERMINATION, beginning: "To soln in centrifuge bottle . . . " to "filter thru folded paper". They were directed to proceed next as follows:

For citric acid, proceed as in *This Journal,* **34,** 75 (1951), beginning: "Evaporate 200 ml . . . ".

For tartaric acid, proceed as in *This Journal,* **36,** 79 (1953), under DETERMINATION, line 12: "Transfer 100 ml of clear filtrate . . . ".

For malic acid, proceed as in *This Journal,* **36,** 80 (1953), under LAEVO-MALIC ACID, beginning "Conc. filtrate from tartaric acid . . . ".

The results obtained by the collaborators are given in Table 1. Recoveries of citric and tartaric acid as such warrant adoption of the methods used, first action. The recoveries of malic acid indicate the necessity of

TABLE 1.—*Determination of fruit acids in cordials*

	IMITATION PEACH			IMITATION RASPBERRY		
	ACID, G PER 100 ML			ACID, G PER 100 ML		
	CITRIC	MALIC	TARTARIC	CITRIC	MALIC	TARTARIC
Added	0.530	0.320	0.000	0.274	0.000	0.400
Found (H)	0.482	0.306	0.000	0.252	0.000	0.386
	0.464	0.306	0.000	0.252	0.000	0.378
(M)	0.475	0.225	0.000	0.248	0.000	0.394
	0.473	0.207	0.000	0.247	0.000	0.383
	0.478	0.199	0.024	0.252		
	0.475		0.024	0.254		
(W)	0.506	0.306	0.000	0.254	0.000	0.400
	0.500					
Av.	0.482	0.258	0.007	0.251	0.000	0.388
Per Cent Recovery	91	84		92		97

further study, since one collaborator found only about two-thirds of the malic acid present in the sample of imitation peach cordial.

RECOMMENDATIONS

It is recommended*—

(1) That the method for the determination of citric acid in cordials and liqueurs, as given in this report, be adopted, first action.

(2) That the method for the determination of tartaric acid in cordials and liqueurs, as given in this report, be adopted, first action.

(3) That the method for the determination of *l*-malic acid in cordials liqueurs, as given in this report, be given further collaborative study.

REPORT ON ALPHA–AMYLASE IN MALT

By DWIGHT B. WEST (J. E. Siebel Sons' Co., Inc., Chicago 30, Ill.),
Associate Referee

Since about 1937, the American Society of Brewing Chemists has cooperated with the Association of Official Agricultural Chemists in the development of methods of analysis of mutual interest to both groups.

* For report of Subcommittee D and action of the Association, see *This Journal*, 37, 76 (1954).

The method presented below for the determination of alpha-amylase in malt was developed and collaboratively tested by the Alpha-Amylase Subcommittee of the A.S.B.C. in the period from 1944 to 1952.

DEVELOPMENT OF METHOD

There are three general methods available for specifically determining alpha-amylase: (*a*) The measurement of dextrinizing time in the presence of an excess of beta-amylase, as by the method of Sandstedt, Kneen, and Blish (1); (*b*) a liquefaction procedure such as that of Redfern and Landis (2); and (*c*) determination by the diastatic power method after differential inactivation of beta-amylase as described by Graesser and Dax (3). In 1949, the A.S.B.C. Subcommittee, after careful consideration of these different methods, concluded that the dextrinizing method was the simplest and most accurate, and was the method in most general use.

The procedure of Sandstedt, Kneen, and Blish specified 30°C. as the reaction temperature, but this method was modified by Olson, Evans, and Dickson (4) for use at 20°C. To conform with the official method (*Official Methods of Analysis*, 7th Ed., 1950, **10.60–10.63**) the reaction temperature was kept at 20°C. The alpha-amylase unit is defined as the quantity of alpha-amylase which will dextrinize soluble starch in the presence of excess beta-amylase to the specified endpoint at the rate of one gram per hour at 20°C.

The method as presented below, except for the portions concerned with the extraction of the malt for analysis and the dilution of this extract for further use, were collaboratively tested and accepted by the 1949 Alpha-Amylase Subcommittee. This report is published in full in the 1949 Proceedings of the American Society of Brewing Chemists (7).

As Olson, Lowry, and Dickson (5) and Witt and Ohle (6) had demonstrated considerable variation in alpha-amylase values obtained by using distilled water and various dilute salt solutions in the extraction of the malt and the dilution of the extract, the work in 1949, in part, and in 1950, 1951, and 1952 was concerned primarily with these problems. Detailed reports are found in the Proceedings of the American Society of Brewing Chemists for those years (7–10).

A condensed version of the report of the A.S.B.C. Alpha-Amylase Subcommittee for 1952 follows:

Six barley malt samples, selected to cover a wide range of alpha-amylase values and including both brewers and distillers type malts, and a sample of malt syrup were given to each collaborator. All collaborators also received the same sample of Merck's Lintner soluble starch (Lot No. 11249) and Wallerstein's Beta-Amylase (Lot No. 454951), but were instructed to use their own comparators and alpha-amylase color standards. The individual malt samples were prepared from a large lot of each malt

by repeated division in a Boerner divider. Each sample was sealed in a sanitary tin can and stored in the refrigerator until analyzed.

Each collaborator was instructed to determine alpha-amylase on the six malts and the syrup by the method described below. Also, each collaborator was instructed to vary the method for the six malts by using 500 ml of 0.4 per cent sodium bicarbonate in place of the 500 ml of 0.5 per cen+ sodium chloride in the preparation of the malt infusion and by diluting the 20 ml of malt infusion to 100 ml with distilled water in place of the 0.5 per cent sodium chloride solution. Each infusion for each malt was made in duplicate with separately weighed samples of malt, rather than by running duplicate analyses on a single infusion.

ALPHA-AMYLASE IN MALT

REAGENTS

(a) *Special starch.*—Use Merck's soluble Lintner starch, special for diastatic power determination.

(b) *Beta-amylase.*—The special beta-amylase powder free from alpha-amylase made by the Wallerstein Laboratories, 180 Madison Avenue, New York, N. Y. should be used. This prepn has been standardized to 2000°L. and should comply with following specifications: At addn level used, there must not be a variation greater than 5% in dextrinization of standard malt infusion when 1 and 3-day old substrates are compared. Further, a substrate prepd by adding twice the level of beta-amylase indicated must not deviate by more than 5% from that prepd with the recommended level after 24 hrs standing. Store powder in tightly closed bottle in the refrigerator. To avoid moisture condensation on the cold enzyme prepn, allow bottle to warm to room temp. before opening.

(c) *Stock iodine soln.*—Dissolve 5.50 g I crystals (A.C.S.) and 11.0 g KI in H_2O and dil. to 250 ml. Store in dark bottle and make fresh soln monthly.

(d) *Dil. iodine soln.*—Dissolve 20.0 g KI in H_2O, add 2.00 ml of stock I soln and dil. to 500 ml. A series of 13×100 mm test tubes contg 5 ml of the dil. I soln must be made up beforehand and attemperated at 20° in readiness for testing. An all glass automatic pipet such as the Machlett type is recommended for rapidly dispensing this soln.

(e) *Buffer soln.*—Dissolve 120 ml glacial acetic acid and 164 g anhyd. Na acetate in H_2O and dil. to 1 l.

(f) *Sodium chloride soln.*—0.5%. Dissolve 5 g reagent NaCl in one l H_2O. This soln need not be made up in a volumetric flask.

(g) *Buffered limit-dextrin (alpha-amylodextrin) substrate.*—Prep. a suspension of 10.00 g (dry weight) of Merck's soluble starch in cold H_2O and pour slowly into boiling H_2O. Boil with stirring for 1–2 min., cool, and add 25 ml of buffer soln and 250 mg of beta-amylase dissolved in a small amount of H_2O. Make up to 500 ml, sat. with toluene, and store at or close to 20°C. for not less than 18 hrs nor more than 72 hrs before use.

APPARATUS

(a) *Constant temperature bath.*—Set at 20±0.05°C.

(b) *Reference color standard.*—Use the special Alpha-Amylase Color Disc (catalog 620-S5) made by Hellige Inc., 3718 Northern Blvd., Long Island City, New York.

(c) *Comparator.*—Either the standard Hellige comparator (catalog 607) or the pocket comparator (catalog 605) with prism attachment (catalog 605-A) may be

used. The comparator shall be illuminated with a 100 watt frosted lamp mounted in such a manner that direct rays from the lamp do not shine in the operator's eyes. The lamp shall be placed 6 inches from the rear opal glass of the comparator.

(d) *Comparison tubes.*—Use precision bore square tubes with 13 mm viewing depth. A tube filled with distd H_2O should be placed behind the color disc.

The alpha-amylase color disc is correct only when used with the specified 13 mm viewing depth. Precision bore square tubes are specified to obviate need for individual calibration of test tubes and to insure use of standard viewing depth. The 13 mm precision square tubes are supplied as standard equipment with the Hellige Comparator and are also used with the Coleman Universal Spectrophotometer. They may be secured from either Hellige, Inc., distributors of the Coleman instrument, or Fisher and Porter Co., Hatboro, Pa.

DETERMINATION

Preparation of malt infusion.—Ext. 25 g (± 0.05 g) of finely ground malt exactly as in **10.62**, paragraph 1, but use 500 ml of 0.5% NaCl soln instead of distd H_2O. Dil. 20 ml of malt infusion to 100 ml with 0.5% NaCl soln.

Dextrinization.—The buffered limit-dextrin substrate and dild malt infusion should be attemperated and dild to final vol. at 20°C.

Transfer 20.0 ml of substrate soln at 20°C. to a 50 ml Erlenmeyer flask, add 5 ml of 0.5% NaCl soln and again adjust temp. to 20°C. Add 5 ml dild malt infusion at 20°C., blowing it in and counting time from the instant the first of the dild malt infusion reaches the starch substrate in flask. After 10 min. reaction time, add 1 ml of hydrolyzing mixt. to 5 ml of dil. I soln at 20°C., shake, pour into the 13 mm square tube and compare with the alpha-amylase color disc in the comparator. At appropriate intervals remove addnl 1 ml aliquots of the hydrolyzing mixt., add to dil. I soln, mix, and compare with color disc until the alpha-amylase color is reached.

During the initial stages of the reaction it is not necessary that the 1 ml sample be measured precisely before addn to the dil. I soln. As the end-point is approached make the addn accurately with a 1 ml pipet. (A fast flowing pipet such as a 1 ml bacteriological pipet is recommended for withdrawing the 1 ml aliquot.) Blow contents of the pipet into the I soln. Near the end-point, take readings every 0.5 min. on the min. or half min. In case two readings 0.5 min. apart show that one is darker than the alpha-amylase color disc and the other is lighter, then the endpoint is recorded at the nearest quarter min. Shake out the 13 mm square tube used for color comparison between successive readings.

For accuracy and convenience it is desirable that dextrinization times fall between 10 and 30 min. With malts of low alpha-amylase activity it may be necessary to use 10 ml of the dild infusion. In this case, do not add 5 ml of the NaCl soln. The final vol. of the reaction mixt. should always be 30 ml.

CALCULATION OF ALPHA-AMYLASE ACTIVITY

From the time interval necessary for dextrinization and the wt of malt represented by the infusion aliquot taken, calc. alpha-amylase units. An alpha-amylase unit is defined as the quantity of alpha-amylase which will dextrinize soluble starch in the presence of an excess of beta-amylase at the rate of one g per hr at 20°C.

$$20^\circ\ D.U.\ (\text{as is basis}) = \frac{24}{W \times T}$$

$$20^\circ\ D.U.\ (\text{dry basis}) = \frac{D.U.\ (\text{as is}) \times 100}{100 - M}$$

where: W = wt in g of malt in aliquot taken; T = dextrinizing time in min.; M = % moisture in sample; and 24 is the wt of starch employed (0.4 g) multiplied by 1 hr (60 min.).

Example:

$W = 0.05$ g; $T = 20$ min.; Temp. = 20°C.

$$20° \; D.U. \text{ (as is)} = \frac{24}{0.05 \times 20} = 24.0$$

NOTES

(1) Slight differences in color discrimination among different operators may be minimized by the use of the prism attachment, by maintenance of a 6–10 inch reading distance between the eye and the comparator, and by the experience gained with continued practice.

(2) Care should be taken to prevent the tubes containing the reaction mixture plus iodine from changing temperature while colors are being compared. If color comparisons are made immediately after the addition of the reaction mixture to the iodine, there will be essentially no temperature change and no change in color.

DISCUSSION OF RESULTS

The collaborators' results were checked for obvious errors and rounded off to the same decimal. All values were calculated on an "as is" basis to avoid the introduction of any additional error from the determination of moisture.

Table 1 lists the mean results for the alpha-amylase determinations for each collaborator for each malt and for the malt syrup.

Other than as a check of the accuracy of the primary method itself, the work reported in this table was designed to answer the question of whether sodium chloride or sodium bicarbonate was more desirable as an aid to extraction. This question is answered by the data for the standard deviation among replicates. The statistical data are collected in Table 2 for greater ease of comparison.

The variance of the sodium chloride infusion is much more uniform or homogeneous than the variance of the sodium bicarbonate infusion. However, if the results are pooled or combined, then exactly the same standard deviation is found among replicates for both methods. On the basis of uniformity, however, sodium chloride infusion is to be preferred. The coefficient of variation for the malt syrup is lower than the variation for the malts.

Table 2 also shows the pooled standard deviation among operators as 1.68 20° dextrinizing units for the sodium chloride extraction and 1.88 for the sodium bicarbonate extraction. From these values and the figures for the coefficient of variation among operators, it is seen that the largest proportion of the total variability in the alpha-amylase determination is caused by differences among operators and only a small part by the actual method itself.

TABLE 1.—*Collaborative data for alpha-amylase determination*

COLLABORATOR	ALPHA-AMYLASE, 20° D.U. MALT												ALPHA-AMYLASE, 20° D.U. SIRUP
	A		B		C		D		E		F		
	NaCl	$NaHCO_3$	NaCl	$NaHCO_3$	NaCl	$NaHCO_3$	NaCl	$NaHCO_3$	NaCl	$NaHCO_3$	NaCl	$NaHCO_3$	
1	22.7	25.1	26.2	28.9	26.0	26.5	38.8	38.8	25.8	27.1	19.1	20.4	10.6
2	23.2	22.9	25.8	25.1	23.0	23.7	35.9	35.9	25.0	25.8	21.0	20.3	9.8
3	20.5	24.3	23.9	25.8	22.9	23.3	35.3	35.9	24.0	24.6	19.0	18.9	9.9
4	23.9	25.3	31.0	33.7	21.7	24.0	36.9	38.4	24.0	27.0	18.2	19.9	9.9
5	24.0	24.2	26.5	28.0	24.6	24.9	37.6	37.6	24.9	26.3	19.5	21.7	10.0
6	25.1	26.1	28.5	29.6	26.5	29.3	38.8	40.9	25.1	26.3	21.6	22.6	10.6
7	24.8	26.1	26.7	28.5	25.1	26.5	38.4	38.4	27.4	28.5	19.4	20.2	10.9
8	24.8	23.7	29.1	25.7	27.4	23.4	35.0	35.0	26.7	27.8	19.2	20.1	10.7
9	26.5	28.0	26.5	28.0	27.2	28.2	40.9	43.2	28.7	29.1	21.8	23.6	11.7
10	26.0	27.7	26.5	27.8	26.9	27.2	36.9	39.3	27.9	28.5	22.6	22.9	11.0
11	22.9	23.7	26.1	27.6	23.4	24.0	36.6	37.6	25.8	26.7	18.5	20.7	9.9
Average:	24.02	25.19	26.96	28.05	24.95	25.54	37.36	38.26	25.98	27.05	19.99	21.01	10.43
SDbO[a]	1.66	1.59	1.88	2.30	1.94	2.10	1.74	2.28	1.41	1.31	1.39	1.44	0.6
CVbO[b]	6.91	6.31	6.97	8.20	7.78	8.22	4.66	5.96	5.43	4.84	6.95	6.85	5.8
SDbR[c]	0.45	0.65	0.32	0.63	0.59	0.22	0.44	0.82	0.57	0.28	0.74	0.27	0.11
CVbR[d]	1.9	2.58	1.19	2.24	2.37	0.87	1.19	2.14	2.18	1.04	3.70	1.28	1.1

[a] Standard deviation among operatiors.
[b] Coefficient of variation among operators.
[c] Standard deviation among replicates.
[d] Coefficient of variation among replicates.

TABLE 2.—*Comparison of variability of sodium chloride and sodium bicarbonate extraction methods*

MALT	SDbR[a]		CVbR		SDbO		CVbO	
	NaCl	$NaHCO_3$	NaCl	$NaHCO_3$	NaCl	$NaHCO_3$	NaCl	$NaHCO_3$
A	0.45	0.65	1.86	2.58	1.66	1.59	6.9	6.3
B	0.32	0.63	1.19	2.24	1.88	2.30	7.0	8.2
C	0.59	0.22	2.37	0.87	1.94	2.10	7.8	8.2
D	0.44	0.82	1.19	2.14	1.74	2.28	4.7	6.0
E	0.57	0.28	2.18	1.04	1.41	1.31	5.4	4.8
F	0.74	0.27	3.70	1.28	1.39	1.44	7.0	6.9
Pooled	0.53	0.53			1.68	1.88		
Sirup	0.11		1.1		0.60		5.8	

[a] For explanation of headings, see footnotes to Table 1.

Table 3 gives a comparison of the average replicate and operator coefficients of variability for the alpha-amylase method obtained by the subcommittee during the years indicated. The variability among replicates has not changed much during these years. It should be noted that the validity of calculating average values for the coefficient of variability is questionable, but it is the only means of inter-comparison among years.

TABLE 3.—*Mean variabilities of alpha-amylase values*

YEAR	CVbR	CVbO
1946	2.9	11.4
1949	2.3	6.5
1950	2.6	9.2
1951	2.6	6.9
1952	2.1	6.6

TABLE 4.—*Summary of alpha-amylase values for six malts submitted by A.S.B.C. Malt Analysis Check Service*

MALT SAMPLE DESIGNATION	ALPHA-AMYLASE (20°C. DEXTRINIZING UNITS, DRY BASIS)			
	MEAN VALUE	MINIMUM VALUE	MAXIMUM VALUE	STANDARD DEVIATION
E–52	28.02	18.5	34.3	3.67
F–52	27.33	17.8	34.0	3.91
G–52	26.13	18.5	29.4	2.30
H–52	27.94	20.8	31.9	3.11
A–53	30.17	26.3	37.0	2.48
B–53	29.60	23.8	35.4	2.19

The variability among operators has gradually improved during these years.

The American Society of Brewing Chemists maintains a Malt Analysis Check Service which furnishes participating laboratories with two malt samples every three months. The data of the various items of analysis are collected, compiled, and published by a committee. Since the adoption by the A.S.B.C. of the alpha-amylase method in May, 1952, six samples of malt have been analyzed for alpha-amylase by approximately 30 malting and brewing laboratories. Table 4 is a summary of alpha-amylase results for these 6 malt samples.

The standard deviation for these six samples is greater than the value for the 1952 sub-committee results, but it is noted that the figure is decreasing as the analysts become more familiar with the method.

COMMENTS AND RECOMMENDATION

The alpha-amylase method as described above was accepted by the American Society of Brewing Chemists at its annual meeting in May, 1952 after several years of detailed collaborative study. The method is now accepted and in use by the majority of malting and brewing laboratories in this country. The method gives very good intralaboratory checks and good interlaboratory results.

It is recommended* that the 20°C. dextrinizing unit alpha-amylase in malt method as described above be adopted, first action.

COLLABORATORS

(A.S.B.C. Alpha-Amylase Subcommittee)

Sutton Redfern, Fleischmann Laboratories, New York, N. Y., *Chairman*
R. F. Bawden, Rahr Malting Co., Manitowoc, Wis.
E. B. Bensing, Hiram Walker & Sons, Inc., Peoria, Ill.
H. A. Conner, National Distillers Products Corp., Cincinnati, Ohio
L. E. Ehrnst, Froedtert Grain & Malting Co., Inc., Milwaukee, Wis.
L. A. Hunt, Joseph Schlitz Brewing Co., Milwaukee, Wis.
Stephen Jozsa, Fleischmann Malting Co., Chicago, Ill.
Eric Kneen, Kurth Malting Co., Milwaukee, Wis.
R. L. Ohle, Northwestern Malt & Grain Co., Chicago, Ill.
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Katherine Whitehouse, Joseph E. Seagram & Sons, Inc., Louisville, Ky.

REFERENCES

(1) SANDSTEDT, R. M., KNEEN, E., and BLISH, M. J., *Cereal Chem.*, **16**, 712 (1939).
(2) REDFERN, S., and LANDIS, Q., *ibid.*, **23**, 1 (1946).
(3) GRAESSER, F. R., and DAX, P. J., *Wallerstein Lab. Commun.*, **9**, 43 (1946).
(4) OLSON, W. J., EVANS, R., and DICKSON, A. D., *Cereal Chem.*, **21**, 533 (1944).

* For report of Subcommittee D and action of the Association, see *This Journal*, **37**, 75 (1954).

(5) ———, LOWRY, M. T., and DICKSON, A. D., *Amer. Soc. Brewing Chemists Proc.*, **1948**, p. 13
(6) WITT, P. R., and OHLE, R. L., *ibid.*, **1947**, p. 45.
(7) *Amer. Soc. Brewing Chemists Proc.*, **1949**, p. 165.
(8) *Ibid.* **1950**, p. 175.
(9) *Ibid.*, **1951**, p. 153.
(10) *Ibid.*, **1952**, p. 145.

REPORT ON SPECTROPHOTOMETRIC DETERMINATION OF PHOSPHATES IN WINES AND SPIRITS

By MAYNARD J. PRO (Alcohol and Tobacco Tax Division, Internal Revenue Service, Washington 25, D. C.), *Associate Referee*

The method published in *This Journal*, **35**, 257 (1952) was subjected to collaborative study.

Wine samples consisting of blackberry, cherry, apple, and a dry and a sweet vermouth were forwarded to five collaborators. In addition to analyzing the samples spectrophotometrically, two collaborators were asked to determine the phosphate contents volumetrically by the method given in *Official Methods of Analysis*, 7th Ed., **2.11**, **2.12**, and **2.13 (a)**. A Coleman 10-S spectrophotometer was used by one chemist and Beckman DU spectrophotometers by the others. The results obtained by these chemists were received over a period of approximately two years and are presented in Table 1.

TABLE 1.—*Determination of phosphates in wines and spirits by the spectrophotometric and volumetric methods*

COLLABORATORS	SAMPLES (MG P_2O_5/100 ML)									
	DRY VERMOUTH 1		SWEET VERMOUTH 2		BLACKBERRY 3		APPLE 4		CHERRY 5	
	SPECTRO-PHOTO-METRIC	A.O.A.C. VOLU-METRIC	SPECTRO-PHOTO-METRIC	A.O.A.C. VOLU-METRIC	SPECTRO-PHOTO-METRIC	A.O.A.C. VOLU-METRIC	SPECTRO-PHOTO-METRIC	A.O.A.C. VOLU-METRIC	SPECTRO-PHOTO-METRIC	A.O.A.C. VOLU-METRIC
A	29.3	35.2	26.0	31.6	16.4	19.7	12.0	15.6	18.0	22.5
B[a]	31.5		27.7		17.0		12.4		19.8	
C	29.5		26.9		16.4		12.6		19.1	
D	29.7	30.0	26.4	26.7	16.2	17.4	12.5	12.9	18.6	18.3
E	29.1		26.7		16.6		12.7		18.6	
σ[b]	0.98		0.66		0.32		0.32		0.67	

Average coefficient of standard deviation:[c] 0.64

[a] Coleman 10-S spectrophotometer.

σ = Coefficient of Standard Deviation = $\sqrt{\frac{\Sigma X^2 - \frac{(\Sigma X)^2}{n}}{n-1}}$ [c] Average $\sigma = \sqrt{\frac{\Sigma\sigma^2}{n}}$.

TABLE 2.—*Comparison of spectrophotometric and volumetric analyses of wine containing added potassium dihydrogen phosphate*

SAMPLES		RESULTS (MG P_2O_5/100 ML WINE)	
WINE	MG P_2O_5 ADDED[a]	SPECTROPHOTOMETRIC	VOLUMETRIC A.O.A.C.
Blackberry	0.00	10.50	10.72
	5.73	15.65	15.72
	11.45	21.45	21.28
	28.63	38.85	38.22
Red Grape Wine	0.00	54.80	55.11
	11.45	65.40	64.91
Sweet Vermouth	0.00	26.90	27.58
	28.63	54.15	54.07
Dry Vermouth	0.00	26.90	28.39
	28.63	54.30	54.46

[a] 1.00 ml of this solution contained 11.45 mg of P_2O_5.

Correlation of these data shows that the coefficient of standard deviation for each sample and the average coefficient of standard deviation were less than 1 mg of phosphate per 100 ml of wine. The volumetric analysis by one collaborator indicated that the methods were not in agreement. The volumetric data submitted by another collaborator agreed with his spectrophotometric results and with those obtained by the other chemists. Since one set of volumetric data did not agree with all the other results, a more lengthy evaluation of each method was undertaken.

Four new wine samples were prepared in this laboratory, consisting of red grape and blackberry wines, and a dry and a sweet vermouth. Varving quantities of standard potassium dihydrogen phosphate solution, accurately measured, were added to these wines, and the solutions were given to two collaborators. One chemist analyzed the samples spectrophotometrically and the other analyzed them volumetrically. The results of these increment experiments are presented in Table 2.

The data indicate that both methods can be used to determine phosphates with an accuracy better than ± 1 mg of phosphate per 100 ml of wine. Due to speed and ease of manipulation, the spectrophotometric method is preferred.

In view of the above considerations, it is recommended* that the spectrophotometric method for the determination of phosphates in wines and spirits be adopted as official.

* For report of Subcommittee D and action of the Association, see *This Journal*, 37, 75 (1954).

REPORT ON SPECTROPHOTOMETRIC DETERMINATION OF TANNINS IN WINES AND SPIRITS

By MAYNARD J. PRO (Alcohol and Tobacco Tax Division, Internal Revenue Service, Washington 25, D. C.), *Associate Referee*

The method published in *This Journal*, **35**, 255 (1952); **37**, 89 (1954) was subjected to collaborative study.

Samples were prepared in the Alcohol and Tobacco Tax Division Laboratory and forwarded to five collaborators. The samples consisted of sweet and dry vermouth; blackberry, apple, and cherry wines; a whisky; and a brandy. Four collaborators used Beckman Model DU spectrophotometers and one a Coleman 10-S spectrophotometer. The results presented in Table 1 were received from these chemists over a two-year period.

Five samples with tannic acid contents of less than 50 mg per 100 ml had coefficients of standard deviation of less than ± 5 mg. A blackberry and a cherry wine containing more than 100 mg of tannic acid per 100 ml had larger coefficients of standard deviation. Re-analysis of samples 3 and 5 for tannins twelve months after preparation produced tannin values approximately 20 mg higher than those obtained when the wines were first opened. A large precipitate was also noted, indicating that wines subjected to aging, light, exposure to air, and some enzymatic action undergo changes in their tannin-like constituents. This is more noticeable in the highly colored wines. Reproducible results can be obtained for wines regardless of age or treatment, but to be consistent from one analyst to another, they must be analyzed at the same time.

TABLE 1.—*Determination of tannins in wines and spirits by a spectrophotometric method*

COLLABORATORS	SAMPLES (MG TANNIC ACID/100)						
	DRY VERMOUTH	SWEET VERMOUTH	BLACKBERRY	APPLE	CHERRY	BRANDY	WHISKY
	1	2	3	4	5	6	7
1	26.5	56.5	140.7	44.5	118.5	23.5	29.5
2	27.2	50.0	142.5	37.0	131.0	25.0	31.0
3[a]	26.8	47.8	116.0	35.8	91.9	26.8	33.4
4	26.5	49.7	139.0	38.5	87.2	24.9	30.4
5	26.7	51.5	147.3	38.2	91.4	23.9	29.4
σ[b]	0.32	3.30	12.18	3.30	19.50	4.0	1.70

[a] Coleman 10-S spectrophotometer.

σ = Coefficient of Standard Deviation = $\sqrt{\dfrac{\Sigma X^2 - \dfrac{(\Sigma X)^2}{n}}{n-1}}$

TABLE 2.—*Determination of tannins in samples within two weeks of their preparation*

COLLABORATORS	SAMPLES				
	DRY VERMOUTH	WHISKY	BRANDY	ELDERBERRY	BLACKBERRY
	1	2	3	4	5
1[a]	27.0	36.8	26.7	235.0	189.0
2	29.5	41.3	29.4	276.0	224.0
3	30.2	41.7	28.5	271.0	225.0
4	30.2	41.5	28.5	270.0	216.0
σ	0.39	0.45	0.60	3.20	4.90

[a] Coleman 10-S spectrophotometer.

Fresh samples were prepared in this laboratory and immediately sent to four collaborators. The samples consisted of dry vermouth, whisky, and brandy, and elderberry and blackberry wines. The data presented in Table 2 represent the analyses of these samples performed within two weeks of their preparation.

Data submitted by collaborator 1 were eliminated because he indicated that the spectrophotometer was not operating properly. The coefficient of standard deviation for the five samples was less than ± 5 mg. of tannic acid per 100 ml.

SUMMARY AND RECOMMENDATION

Tannin-like materials in wines and spirits calculated as tannic acid can be determined spectrophotometrically with an accuracy of better than ± 5 mg per 100 ml. This method is much more rapid and has greater accuracy over a wider range than the comparison method in *Official Methods of Analysis*, 7th Ed., **9.37**.

In view of the above considerations, it is recommended* that the spectrophotometric method for the determination of tannins in wines and spirits be adopted, first action.

REPORT ON WINES

TOTAL ALKALINITY OF THE ASH

By PETER VALAER (Alcohol and Tobacco Tax Division Laboratory, Internal Revenue Service, Washington 25, D. C.), *Associate Referee*

At the 1952 meeting of the Association, a new determination was suggested for fruit juices, wines, and similar products to replace the method for "Alkalinity of water-insoluble ash."

* For report of Subcommittee D and action of the Association, see *This Journal*, 37, 75 (1954).

PROCEDURE

Dissolve the ash of a wine in 10 ml of 0.1 *N* H_2SO_4, with stirring. Add ca 50 ml of boiling H_2O, allow to cool somewhat, add 4 drops of methyl orange indicator, and titrate with 0.1 *N* NaOH. Calc. results as ml of 0.1 *N* H_2SO_4 required to neutralize the total alkalinity of ash from 100 ml of wine.

Two official methods for determining the alkalinity of ash are listed in *Official Methods of Analysis:* Alkalinity of water-soluble ash, and alkalinity of water-insoluble ash. The first method is quite satisfactory; the second, although satisfactory for some fruit juices and wines, is not as useful for others. For instance, with apple juices and wines, the water-soluble ash is only slightly less than the total alkalinity, and the method thus has no practical application. With berry wines such as blackberry, however, there is invariably a substantial difference between the alkalinity of the water-soluble ash and the total alkalinity, usually a 3:4 ratio, which may often have a significant interpretation.

Since the last meeting, some preliminary collaborative work was done with the "Total alkalinity of the ash" method described. Results obtained by nine collaborators are given in Table 1.

TABLE 1.—*Analysis of blackberry wine by "Total alkalinity of the ash" method*

COLLABORATOR NO.	ML 0.1 *N* NaOH PER 100 ML WINE
1	38.4
2	38.0
3	35.0
4	36.0
5	36.7
6	36.0
7	38.0
8	38.0
9	36.1
Average:	36.92
Range: 35–38.4	
Maximum Deviation: 3.4	

The general opinion of the collaborators toward the method was very favorable. Among the nine collaborators, the spread in results was 3.4 ml; better results might have been obtained with an indicator with a sharper endpoint. A number of indicators were tried, and methyl orange was selected for use in the determination. One of the collaborators suggested the use of methyl purple, which has a *p*H range of 4.8–5.4 and produces a gray tint preceding either endpoint, thus warning the operator of its approach. When tests were made on ash from the same sample, practically the same results were obtained with each indicator, although the endpoint of

methyl purple seemed more distinct and conclusive. The Associate Referee believes that a smaller range of values would have resulted from the use of methyl purple.

It is recommended* that collaborative work be continued to decide if the determination "Total alkalinity of ash" is acceptable, and if so, whether methyl purple or methyl orange is a more suitable indicator.

No reports were given on distilled spirits; higher alcohols in distilled spirits by chromatography; inorganic elements in beer; malt beverages, sirups, extracts, and brewing materials; methanol; and non-volatile acids in wines by chromatography.

REPORT ON FLAVORS AND NON-ALCOHOLIC BEVERAGES

By JOHN B. WILSON (Division of Food, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.), *Referee*

Only the Associate Referee on Vanilla Extracts and Imitations submitted a report this year. The Referee concurs in his recommendation to drop the gravimetric method for vanilla and coumarin.

When the Wichmann lead number was adopted as an official method (*This Journal*, **8**, 690 (1925)), it was suggested that the Winton method be retained until data had been collected for the newer and more efficacious method. Such data has been obtained, and since the deletion of the gravimetric method for vanillin and coumarin makes it unnecessary to obtain a defecated alcohol-free solution of vanilla extracts for the determination of these flavoring substances, the deletion of the Winton lead number method is also recommended at this time.

In addition, the procedures for Color Value and Residual Color have fallen into disuse, and these procedures are also being dropped. It is not intended to drop the method for color insoluble in amyl alcohol, since this method frequently discloses addition of artificial color to these products.

RECOMMENDATIONS

It is recommended†—

(1) That the official method for vanillin and coumarin (gravimetric), **19.4** and **19.5**, be deleted, first action.

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* For report of Subcommittee D and action of the Association, see *This Journal*, **37**, 75 (1954).
† For report of Subcommittee D and action of the Association, see *This Journal*, **37**, 78 (1954).

(2) That the official Winton method for Lead Number, **19.8,** be deleted, first action.

(3) That the procedure for Color Value, **19.17,** be deleted, first action.

(4) That the procedure for Residual Color after precipitation with lead acetate, **19.18,** be deleted, first action.

REPORT ON VANILLA EXTRACTS AND IMITATIONS

By LUTHER G. ENSMINGER (Food and Drug Administration, Department of Health, Education, and Welfare, Cincinnati 2, Ohio), *Associate Referee*

The present official gravimetric method (**19.4** and **19.5**) for determination of vanillin and coumarin in vanilla flavorings has given low recoveries on authentic solutions of these components. The Associate Referee therefore subjected the method to collaborative study this year.

Two 120 ml samples, one an authentic vanilla extract (sample 53-1), and the second an imitation vanilla flavoring (sample 53-2), were analyzed in duplicate as unknowns by eleven collaborators for vanillin and coumarin, using method **19.4** and **19.5.** The vanilla extract was obtained from a Cincinnati manufacturer, and was made from a 50–50 blend of No. 3 Bourbon beans and Mexican cuts. The retaining solvent contained 35 per cent ethyl alcohol but no glycol. The imitation vanilla flavor was prepared by the Associate Referee to contain the following ingredients:

	per cent
Vanillin	0.400
Coumarin	0.130
Ethyl Alcohol	3.5
Sucrose	12.5
Caramel	0.4

COLLABORATIVE RESULTS

Vanillin.—Collaborative results for the vanillin in the two samples are shown in Table 1. It should be noted that some collaborators purified the vanillin residue as directed in the method while others did not. Certain collaborators reported both types of recoveries. Hence, the unpurified and purified recoveries are listed and analyzed separately.

An average of about 33 per cent loss in recovery was sustained by purification of the extract residue by ether as compared to approximately 24 per cent for the imitation residue. Substances other than vanillin were extracted from both flavorings but in greater quantity from the vanilla extract.

TABLE 1.—*Recoveries of vanillin from flavorings by method* 19.5

COLLABORATOR	VANILLIN (G/100 ML)[a]			
	SAMPLE 53-1 (EXTRACT)		SAMPLE 53-2 (IMITATION)	
	UNPURIFIED	PURIFIED	UNPURIFIED	PURIFIED
1	0.259	0.141	0.496	0.298
	0.230	0.140	0.472	0.302
2	0.15	—	0.36	—
	0.16	—	0.37	—
3	—	0.128	0.410	—
	—	0.138	0.384	—
4	—	0.064	—	0.208
	—	0.062	—	0.203
5	0.148	0.126	0.324	0.296
	0.180	0.124	0.350	0.296
6	—	0.139	0.372	—
	—	0.136	0.384	—
7	0.160	—	0.352	—
	0.161	—	0.360	—
8	0.166	0.093	0.360	—
	0.166	—	0.370	0.359
9	0.166	0.124	0.494	0.350
	0.165	0.138	0.372	0.350
10	0.134	0.124	0.347	—
	0.140	0.130	0.352	—
11	0.206	0.091	0.425	0.295
	0.224	0.099	0.413	0.297
Average Recovery	0.176	0.117	0.388	0.296
Average Per Cent Recovery	—	—	97.0	74.0
Range	0.134–0.259	0.062–0.141	0.324–0.496	0.203–0.359
Standard Deviation	.035	.026	.049	.051

[a] Sample 53-1 contained 0.171 g vanillin/100 ml and sample 53-2 contained 0.397 g vanillin/100 ml, by the official photometric method.

The variability of results is rather high in all cases, as evidenced by the wide ranges and high standard deviations. Also, the average recovery of vanillin from the authentic imitation is 97 per cent, unpurified and 74 per

cent, purified. Three recoveries of unpurified vanillin are 20 to 24 per cent above the actual concentration of vanillin, which indicates that much excess material may at times be extracted with vanillin even from imitations.

Criticisms were voiced by three collaborators against this method with the claim that vanillin is lost to some degree, first, when alcohol is driven

TABLE 2.—*Recoveries of coumarin from flavorings by method* ***19.5***

COLLABORATOR	COUMARIN (G/100 ML)[a]	
	SAMPLE 53-1	SAMPLE 53-2
1	0.008	0.098
	0.008	0.102
2	None	0.10
	None	0.11
3	0.014	0.119
	0.012	0.108
4	0.010	0.092
	0.008	0.092
5	0.012	0.108
	0.010	0.106
6	None	0.139
	None	0.136
7	0.005	0.098
	0.005	0.099
8	0.026	0.107
	0.021	0.110
9	0.006	0.110
	0.008	0.109
10	0.016	0.099
	0.015	0.099
11	0.001	0.076
	0.002	0.076
Average Recovery	0.009	0.104
Average Per Cent Recovery	—	80.0
Range	0.000–0.026	0.076–0.139
Standard Deviation	0.007	0.015

[a] Sample 53-1 contained 0.032 g coumarin/100 ml and sample 53-2 contained 0.123 g coumarin/100 ml, by the official photometric method.

from the flavoring with heat, and second, when ether purification of the vanillin residue is performed. Several collaborators indicated they had difficulty in obtaining a pure vanillin residue from the extract.

Coumarin.—Table 2 contains the recoveries of coumarin by the collaborators. Several collaborators expressed some concern over the purity of the residue from the extract that was weighed as coumarin. An average of 2 mg of residue did not provide adequate amounts for melting point determinations. Coumarin residues from the imitation flavoring were fairly pure, as indicated by melting points.

Collaborator 6 obtained coumarin recoveries of 105 and 107 per cent from the imitation, while all others obtained considerably less than 100 per cent. The over-all average was 80 per cent. Variability of results was also great as indicated by the wide range (0.076–0.139 g/100 ml). Thus method **19.4** and **19.5** did not give consistent and satisfactory recoveries of coumarin from the imitation flavoring.

ACKNOWLEDGMENT

The Associate Referee appreciates the kind cooperation of the following analysts of the Food and Drug Administration:

Juanita E. Breit, Minneapolis District
John H. Bornmann, Chicago District
E. C. Deal, New Orleans District
Charles H. Eisenberg, St. Louis District
William J. McCarthy and Thomas F. Osberger, Cincinnati District
Paul Mills, San Francisco District
Fred C. Minsher, Philadelphia District
J. E. Roe, Denver District
John P. Traynor, Baltimore District

RECOMMENDATIONS

It is recommended*—

(1) That method **19.4** and **19.5** for vanillin and coumarin be deleted, first action.

(2) That ultraviolet methods for vanillin and coumarin be further studied.

No reports were given on beta-ionone, emulsion flavors, organic solvents in flavors, peel oils in citrus juices, and propylene glycol.

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* For report of Subcommittee D and action of the Association, see *This Journal*, **37**, 78 (1954).

REPORT ON VEGETABLE DRUGS AND THEIR DERIVATIVES

By PAUL S. JORGENSEN (Food and Drug Administration, Department of Health, Education, and Welfare, San Francisco 2, Calif.), *Referee*

RECOMMENDATIONS*

Aminophylline and phenobarbital.—The Associate Referee conducted studies on the following three methods for the determination of aminophylline and phenobarbital in admixture: (1) The chromatographic separation of the two ingredients; (2) separation by solvent extraction with spectrophotometric determination; and (3) spectrophotometric two-color analysis without prior separation. It was concluded that, of the three methods studied, the solvent extraction with spectrophotometric measurement gave the best results. Average recovery by seven collaborators was 102.9 per cent for phenobarbital and 99.3 per cent for aminophylline. The Associate Referee recommends that the method be adopted, first action, and that the subject be closed. The Referee concurs in this recommendation.

Quinine and strychnine.—A report was not received from the Associate Referee this year. Last year's report described a method entirely satisfactory for these two drugs when they were freshly mixed. The present problem is to demonstrate that the method will apply equally well when the drugs have been mixed for two or three years. It may be proved that what has heretofore been considered a deficiency in the method is in fact due to deterioration of the alkaloids. It is therefore considered that this problem will require a year or two more for solution, and it is recommended that the topic be continued.

Aminopyrine, ephedrine, and phenobarbital.—In a private communication, the present Associate Referee has requested that, due to a change in position and the urgency of other interests, he be relieved of the Associate Refereeship. With sincere thanks for his past interest in this work, the Referee recommends that the topic be reassigned.

REPORT ON AMINOPHYLLINE AND PHENOBARBITAL

By RUPERT HYATT (Food and Drug Administration, Department of Health, Education, and Welfare, Cincinnati 2, Ohio), *Associate Referee*

The work done on this problem was directed toward finding a satisfactory method for assay. It was suggested that chromatographic tech-

* For report of Subcommittee B and action of the Association, see *This Journal*, 37, 65 (1954).

niques might be useful. Studies were made on three methods, *viz.*, chromatographic separation, separation by extraction, and spectrophotometric two-color analysis without separating the aminophylline and phenobarbital.

Good results for aminophylline were obtained by all three of the methods. The theophylline was determined and calculated to aminophylline. The first method gave low results for phenobarbital, and the third method produced high results. The third method is quick and easy since it consists merely of reading a dilute solution at 240.5 and 274.5 millimicrons. However, it could not be assumed that no interferences would be present. The second method was therefore found to give the best results.

Vials were prepared containing weighed amounts of ingredients, *viz.*, 16.0 mg of phenobarbital and 100.0 mg of aminophylline plus a small amount of starch to simulate tablet granulation. The aminophylline assayed 80.0 per cent anhydrous theophylline by direct reading. This figure multiplied by 1.33 gave 106.4 mg of aminophylline per vial. Results from five collaborators and from the Associate Referee are shown in Table 1. The details of the procedure, sent to the collaborators, are as follows:

TABLE 1.—*Assay of aminophylline and phenobarbital by extraction method*

COLLABORATOR	AMINOPHYLLINE, MG PER VIAL		PHENOBARBITAL, MG PER VIAL	
1	107.3	107.3	17.33	17.45
2	99.5	99.3	16.3	15.2
3	107.5	108.4	17.0	17.2
4	101.3	99.9, 106.4	15.9	16.2, 15.6
5	109.4	108.2	17.1	16.9
6	108.1	106.3	15.9	16.2
7	106.5	109.0	15.4	17.5
Average	105.63		16.48	
Lowest	99.3		15.2	
Highest	109.4		17.5	
Recovery, % (average)	99.3		103.0	

METHOD

Weigh portion of powd. sample equivalent to ca 15 mg of phenobarbital and transfer to separator containing 25 ml of HCl (1+1). Add 60 ml of ether, shake, and let stand to clear. Pass the acid soln successively through 2 other separators, each of which contains 50 ml of ether, shake, and let stand as before. Transfer ether-washed acid soln to a 500 ml volumetric flask. Wash the 3 ether solns successively with two 10 ml portions of HCl (1+1) and add to contents of volumetric flask. Reserve total contents of flask for detn of theophylline.

Combine ether solns and evap. to dryness. Dissolve residue in 5 ml of *N* NaOH and H_2O. Transfer to a 200 ml volumetric flask, test for alkalinity of soln, make to mark, and mix. Filter if necessary and transfer a 10 ml aliquot to a 100 ml volumetric flask, make to mark, and mix. Det. the absorbances at 240.5 and 274.5 mμ.

The reading at 274.5 mμ is made in order to correct for the small amount of theophylline extracted with the phenobarbital. Read the alkaline soln of phenobarbital the same day it is prepared.

Det. the absorptivities of known phenobarbital and theophylline solns, each contg 1.0 mg/100 ml, using the same alkali concn as the sample soln. Read against a water blank at 240.5 and 274.5 mμ. Calc. the concn of phenobarbital (C_P) in g/100 ml by the equation.

$$C_P = \frac{a_4 \times A_{240.5} - a_2 \times A_{274.5}}{a_1 a_4 - a_2 a_3}$$

and concn of theophylline (C_T) in g/100 ml by the equation:

$$C_T = \frac{a_1 \times A_{274.5} - a_3 \times A_{240.5}}{a_1 a_4 - a_2 a_3}$$

where a_1 = absorptivity for phenobarbital at 240.5 mμ; a_3 = absorptivity for phenobarbital at 274.5 mμ; a_2 = absorptivity for theophylline at 240.5 mμ; a_4 = absorptivity for theophylline at 274.5 mμ; C_T = the amount of theophylline extracted with phenobarbital by the ether; and A = absorbance.

Convert concns into mg of phenobarbital and theophylline in the original sample taken for analysis. Next det. the theophylline in the acid-aqueous ext. Dil. acid ext. in 500 ml flask to mark with H_2O and mix.

Transfer a 5 ml aliquot or an aliquot contg 0.5–1.0 mg of theophylline to a 100 ml volumetric flask, make to mark with H_2O, and mix. Read at 271 mμ (the point of maximum absorption found for theophylline in acid soln). Using an H_2O blank, det. the absorptivity for theophylline in a known soln at the same *p*H as the sample soln. Calc. theophylline in g/100 ml by the equation:

$$C_{Ta} = \frac{A_{271}}{a}$$

where C_{Ta} is the concn in g/100 ml of the theophylline in acid soln and a is absorptivity of the standard theophylline at 271 mμ in acid soln.

Convert concn to mg in original sample, and add theophylline detd with phenobarbital above for total amount in sample.

Convert theophylline to aminophylline by multiplying the total theophylline by 1.33. Calculation may also be made on the basis of the U.S.P. range for anhyd. theophylline in aminophylline.

Report final results in mg per vial (or tablet).

Use the contents of one vial for analysis.

Instead of obtaining pure theophylline and phenobarbital and determining spectrophotometric data, the following a values may be used: $a_1 = 432$; $a_3 = 13$; $a_2 = 220$; $a_4 = 665$; and $a_{1\,cm}^{1\%}$ for theophylline (acid *p*H) = 574.

COLLABORATORS

The cooperation of the following collaborators, all of Food and Drug Administration, is gratefully acknowledged: H. E. Gakenheimer, D. W. Johnson, M. A. McEniry, F. A. Rotondaro, D. M. Taylor, and A. C. Thomson.

RECOMMENDATION

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

* For report of Subcommittee B and action of the Association, see *This Journal*, 37, 65 (1954).

No report was given on quinine and strychnine.

REPORT ON SYNTHETIC DRUGS

By F. C. SINTON (Food and Drug Administration, Department of Health, Education, and Welfare, New York 14, N. Y.), *Referee*

RECOMMENDATIONS*

Acetophenetidin and Caffeine.—The Associate Referee has submitted a report in which a method employing chromatographic separation of acetophenetidin and caffeine was studied collaboratively. The results were quite encouraging but did disclose that erratic findings may be obtained at abnormally high summer temperatures. The Associate Referee has recommended that the topic be continued in order to eliminate this potential error. The Referee concurs.

Propadrine Hydrochloride.—The Associate Referee has developed a method for the determination of propadrine in aqueous solution and capsules, and has recommended collaborative study. He has recommended that study of methods for the determination in elixirs and nasal jellies be continued, and further, that the name of the subject be changed to Phenylpropanolamine Hydrochloride. The Referee concurs in these recommendations.

Amphetamines.—The Associate Referee has continued collaborative study on the determination of total amphetamine and the determination of stereochemical composition of amphetamine. He has recommended that the methods be adopted as first action and that methods be investigated for the determination of total amphetamine based on the ultraviolet absorption spectrum of that substance. The Referee concurs.

Aspirin and Phenobarbital.—The Associate Referee reported that erratic results were obtained by a method sent to collaborators. He is studying several possible methods for the separation of these compounds as well as for other barbiturates. The Referee recommends that the study be continued.

Sulfonamide Derivatives.—The Associate Referee has submitted a report including a collaborative study for the determination of sulfadiazine in the presence of other sulfonamides and for the determination of sulfadiazine and sulfamerazine in mixtures. The results were in good agreement with one exception. The discrepancy in this instance was found to be due to loss in volume at the stage where the directions require "Stopper and heat 1 hour at 100°C." Repeated determinations by the same collaborator

* For report of Subcommittee B and action of the Association, see *This Journal*, 37, 65, 66 (1954).

showed good agreement with known composition, following compensation of loss during heating by addition of an equal volume of water. The final results were in good agreement with theory, and the Associate Referee recommends adoption of the methods as first action. The Referee concurs but suggests the addition to the method of some statement that will caution the operator regarding possible volume loss during the hour-long heating. The Associate Referee has also recommended that the procedure for titration of sulfonamides in organic solvents be studied collaboratively. The Referee concurs.

Steroid Estrogens.—The Associate Referee has submitted results of analyses which show that the method for ketosteroids (gravimetric) has yielded consistently higher results than the colorimetric procedure, due to naturally occurring contaminants related to the steroid estrogens and extracted with them from pregnant mare's urine. He has recommended that the status of the method for the determination of ketosteroids (gravimetric) be changed from first action to an optional or semi-quantitative procedure and has proposed some changes in the wording of the method. The Referee concurs and recommends that the subject be continued with a study of methods for conjugated estrogens.

Methylene Blue.—The Associate Referee assigned to this topic submitted his resignation. Since the present day therapeutic use of methylene blue appears to be quite limited, it is recommended that this subject be discontinued in order to devote attention to more important problems.

Tuinal.—The Associate Referee was unable to perform the additional work recommended by Subcommittee B but hopes to do so next year. It is recommended that the subject be continued.

Diphenhydramine and Tripelennamine Hydrochlorides.—No report was received. It is recommended that the topic be reassigned and continued.

Spectrophotometric Methods.—It is recommended that the topic be continued.

REPORT ON ACETOPHENETIDIN AND CAFFEINE

CHROMATOGRAPHIC SEPARATION

By GORDON SMITH (Food and Drug Administration, Department of Health, Education, and Welfare, New York 14, N. Y.), *Associate Referee*

A method for acetophenetidin and caffeine, employing chromatographic separation followed by spectrophotometric determination of each substance, has been subjected to collaborative study. This method is based on the work of Higuchi and Patel,[1] but represents a substantial alteration of

[1] HIGUCHI, T., and PATEL, K., *J. Am. Pharm. Assoc., Sci. Ed.*, **41**, 171 (1952).

their method. These investigators used silicic acid as adsorbent, water as the stationary phase, an isopropyl ether–chloroform mixture (3+1) as eluent for phenacetin,[2] and chloroform as eluent for caffeine. In the method discussed here, Celite is the adsorbent and an ethyl ether–chloroform mixture is the eluent for phenacetin. A partition tube 25 mm in diameter is used in place of a 20 mm tube.

These alterations had the purpose of utilizing apparatus and materials already used in other A.O.A.C. methods. Other advantages expected were as follows:

(1) Ethyl ether could be evaporated simply at low temperatures if it were necessary to substitute another solvent for reading in the spectrophotometer. (Actually this was not found necessary.)

(2) Ethyl ether does not have appreciable absorbance at the wavelength used, whereas commercial isopropyl ether does.

(3) Celite permits simple, effective control of the rate of flow.

(4) Changes in tube and adsorbent together result in a much shorter column.

Details of the method are as follows:

ACETYLSALICYLIC ACID, ACETOPHENETIDIN, AND CAFFEINE

APPARATUS

(a) *Spectrophotometer.*—Capable of indicating spectral bands of the order of 2 mμ or less in the region of 230–300 mμ.

(b) *Chromatographic tube.*—Fuse a 6 cm length of 6–8 mm glass tubing to the end of a piece of 25 mm tubing about 25 cm in length. (A 25×250 mm test tube may be used.) Constrict stem slightly 1.5–2 cm below the joint. A shorter partition tube, down to ca 20 cm, may be used.

(c) *Plunger.*—Flatten one end of an 8–9 mm glass rod, about 40 cm long, to a circular head of diam. 1–2 mm less than internal diam. of the chromatographic tube.

REAGENTS

(a) *Ether–$CHCl_3$ mixture (9+1).*—Mix 1000 ml of ether and 110 ml of $CHCl_3$. (This is usually ample for 2 samples.) One batch of this mixt. should be used thruout, and all $CHCl_3$ used in the method should preferably be from one bottle.

(b) *Celite No. 545.*—Johns-Manville Corp.

(c) *Acetophenetidin standard soln.*—0.500 mg/100 ml. Dissolve 100.0 mg of pure acetophenetidin in the ether–$CHCl_3$ mixt. and dil. to exactly 100 ml. Dil. a 5 ml aliquot to exactly 100 ml. Dil. a 10 ml aliquot of resulting soln to exactly 100 ml. Use ether–$CHCl_3$ mixt. for dilns.

(d) *Caffeine standard soln.*—1.000 mg/100 ml. Dissolve 100.0 mg of pure caffeine in $CHCl_3$ and make to exactly 100 ml. Dil. a 10 ml aliquot with $CHCl_3$ to exactly 100 ml. Dil. a 10 ml aliquot of this soln with $CHCl_3$ to exactly 100 ml.

Absorbance of both standard solns is to be read at about the same time as that of the sample solns. They should be made up on the same day.

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[2] The shorter name is used here, although the longer name is used in the detailed method to conform to official usage.

SEPARATIONS

Weigh accurately an amount of powd. sample to contain about 20 mg of caffeine and treat as in **32.85(a)**, halving quantities of cooled $NaHCO_3$ and of $CHCl_3$ used, but using 2 ml of $NaHCO_3$ for wash as directed. Combine $CHCl_3$ exts in 150 ml beaker. Retain $NaHCO_3$ solns for aspirin detn if desired. Evap. $CHCl_3$ soln contg acetophenetidin and caffeine on steam bath, without boiling, to a vol. of 8–10 ml. Cool, and transfer to a 100 ml volumetric flask, using only ether from a wash bottle to complete transfer. Dil. with ether to bottom of neck and mix; then dil. to mark and mix.

Pack fine glass wool rather tightly into stem of chromatographic tube to a height of 1–1.5 cm above constriction. Above this, pack loosely another 2–2.5 cm of glass wool. Support tube vertically, preferably in hood, and attach to outlet stem a rubber tube with pinch clamp, to regulate flow during packing. In a 400 ml beaker mix 20 g of Celite and 20 ml of H_2O until moisture appears to be evenly distributed. Add 140 ml of ether–$CHCl_3$ mixt. and stir until suspended solid appears homogeneous. Pack tube by transferring Celite to it in portions with a spatula, maintaining at all times enough free liquid in tube to suspend solid loosely, adding not more than 5 cm of loosely suspended solid at a time, and packing this firmly with plunger in increments of not more than 1 cm of packed material. Avoid enclosures of air or free liquid. Keep Celite covered with liquid at all times except during actual transit from beaker to tube. A little addnl ether–$CHCl_3$ mixt. may be used if needed. Rate of flow thru finished column should be between 8 and 16 drops per 10 sec. when liquid level is 3–5 cm above column.

With packing complete, and with some liquid remaining above solid column, remove rubber tube and wash stem with $CHCl_3$ and then with ether. As soon as last of supernatant liquid sinks into solid column, place a 100 ml volumetric flask under tube in receiving position and at once pipet a 5 ml aliquot of the acetophenetidin–caffeine soln on to top of column. As soon as this sinks into solid, add 10 ml of the ether–$CHCl_3$ mixt., washing down inside of tube. As soon as this sinks, add the first of three 20 ml portions of same mixt., adding the other two successively at time when height of liquid has dropped to about 1 cm. (This 60 ml may be added in more and smaller portions, if preferred, keeping height of liquid within same limits.) Just before last of liquid sinks, wash stem with ether–$CHCl_3$ mixt., and 15–20 sec. after it sinks remove the 100 ml volumetric flask, substitute another, and at once begin to add $CHCl_3$ to tube. Add a total of 60 ml in same manner specified for the 60 ml of mixt. When last of $CHCl_3$ eluent disappears, wash stem with $CHCl_3$ and remove flask. First flask contains the acetophenetidin; the second contains the caffeine.

In case of delay between packing and use of column, maintain some ether–$CHCl_3$ mixt. at top. Outlet may be kept closed.

DETERMINATIONS

Acetylsalicyclic acid.—Proceed as in **32.85.**

Acetophenetidin.—Dil. soln in first flask with ether–$CHCl_3$ mixt. to mark and mix. Dil. aliquot contg ca 0.5 mg of acetophenetidin with same mixt. to exactly 100 ml and mix. Measure absorbance of resulting soln against the ether–$CHCl_3$ mixt. as blank, at 249 mμ. Fill silica cell to about 5 mm above window and make reading between 4 and 6 min. from time soln is placed in cell. Measure absorbance of acetophenetidin standard soln in same manner, within same time limits. Calc. amount of acetophenetidin in sample.

$$\text{Per cent acetophenetidin} = \frac{\text{absorbance sample}}{\text{absorbance std}} \times \frac{10}{\text{wt sample}}$$

Caffeine.—Dil. soln in second flask with $CHCl_3$ to bottom of neck, and mix. Insert a thermometer and cool soln to room temp. Remove thermometer, washing adhering soln into flask with $CHCl_3$. Dil. to mark with $CHCl_3$ and mix. Measure absorbance against $CHCl_3$ blank at 276 mμ. Make reading between 2 and 6 min. from time soln is placed in cell. Measure absorbance of caffeine standard soln in same manner within same time limits. Calc. amount of caffeine in sample.

$$\text{Per cent caffeine} = \frac{\text{absorbance sample}}{\text{absorbance std}} \times \frac{2}{\text{wt sample}}.$$

DISCUSSION

Efficacy of the ether-water system in separating phenacetin from caffeine may be expected from a consideration of the respective solubilities of the two substances in the solvents involved. These data, taken from the U.S.P. and calculated in grams per 100 ml, are shown in Table 1.

TABLE 1.—*Comparative solubilities of phenacetin and caffeine*

	SOLUBILITIES IN G PER 100 ML			RATIOS	
	H_2O (*W*)	ETHER (*E*)	$CHCl_3$ (*C*)	*E/W*	*C/W*
Phenacetin	0.077	0.769	6.67	10.0	8.66
Caffeine	2.00	0.167	16.7	0.089	8.35

If any tendency of the adsorbent to hold back either solute is disregarded, the rate at which each solute moves down the column, in ether elution, should be proportional in some manner to E/W, and the rate at which they draw apart might be expected to have some relation to the difference between the two values of E/W. The same suppositions apply to elution with chloroform and the ratios C/W. Here the closeness of the two ratios would indicate no separation in any column of practical height.

These suppositions are in general accord with what takes place in practice. From a Celite-water column, ether elutes phenacetin well ahead of caffeine; chloroform elutes both without separation. In elution with mixtures of the two solvents, the blank zone or "safety factor" between the phenacetin and the caffeine increases with increase in the percentage of ether. Four portion-wise elutions (with 10 ml portions) under varied quantitative conditions supplied the data shown in Table 2. In each case the weight of water was the same as that of Celite. The phenacetin and caffeine were placed on the column in 5 ml of an ether-chloroform mixture.

Ether effects the separation, but some less volatile solvent, such as chloroform, must be mixed with it in some amount to make the method workable at high summer temperatures.

TABLE 2.—*Rates of elution of phenacetin and caffeine*

Wt of adsorbent	30 g	20 g	20 g	15 g
Ht of column	21 cm	14 cm	14 cm	10.5 cm
Wt of phenacetin	25 mg	5 mg	5 mg	32.5 mg
Wt of caffeine	5 mg	1 mg	1 mg	2.5 mg
Ratio of ether to $CHCl_3$	3–1	9–1	pure ether	pure ether
Vol. to remove all phenacetin	70 ml	50 ml	50 ml	40 ml
Threshold vol. for caffeine	100 ml	>100 ml	>160 ml	>110 ml

COLLABORATIVE STUDY

A review of products in Gutman's *Modern Drug Encyclopedia* showed the phenacetin-caffeine ratio therein varying from 2:1 to 13:1, but in "A-P-C" mixtures without additional ingredients only a 5:1 ratio was found. It has been found that ether elution will separate a 13:1 mixture as readily as a 5:1 mixture, and undoubtedly elution with the 9:1 solvent mixture will do likewise. (This has not been checked.) However, it was not deemed necessary to ask collaborators to run other than a 5:1 mixture. An "A-P-C" mixture was made up with the composition shown in Table 3. Shown also is the melting range of each ingredient and the moisture as determined by U.S.P. methods.

TABLE 3.—*Melting range and moisture content of APC mixture*

	COMPOSITION	MELTING RANGE, °C.	MOISTURE
	per cent		
Aspirin	43.75	133.5–135	none
Phenacetin	31.25	135 –137	none
Caffeine	6.25	236 –238	0.04%
Starch	18.75	—	—

Collaborators were not asked to determine aspirin, since it was added merely to simulate completely the usual conditions of analysis. Results of the collaborative study are shown in Table 4.

The first two determinations (a) by collaborator 1 were made at very high room temperatures, 35°–37° (95°–99°F), and there were some difficulties which were attributed to the behavior of the ether-chloroform mixture at these high temperatures. They are somewhat higher than allowed for in designing the method. This collaborator repeated the determinations, but not at the same high temperatures. The later results (b), obtained at about 27°–29° (low 80's F), are closer to theory. Development of a pale yellow color in the solutions was reported in both instances.

Collaborator 3 chromatographed 2 aliquots from each duplicate sample,

TABLE 4.—*Analysis of phenacetin and caffeine by modified Higuchi-Patel method*

COLLABORATOR	PHENACETIN	CAFFEINE
	per cent	*per cent*
1 (a)	32.8	6.97
	32.1	6.86
(b)	31.4	6.22
	30.6	6.27
2	32.0	6.5
3	30.96	6.34
	31.22	6.43
	31.19	6.18
	31.46	6.20
4	31.52	6.16
	31.49	6.20
5	31.58	6.25
	31.25	6.29
6	31.12	6.03
	31.48	6.11
		6.18
		6.30
7	31.15	6.18
	31.29	6.12
All 7 omitting 1 (a)		
Max.	32.0	6.5
Min.	30.6	6.03
Av.	31.31	6.23
Last 5		
Max.	31.58	6.43
Min.	30.96	6.03
Av.	31.31	6.21
Theoretical %	31.25	6.25

reporting 4 results. Samples were not read on the same day as standards, but this did not affect the results measurably. Collaborator 6 repeated the chromatographing and the caffeine determination because of an accident to the first caffeine standard. For the first two caffeine results, standard and sample were read on different days; for the last two results, on the same day.

COMMENTS

Comments by the first collaborator had to do with the temperature difficulty and the intrusion of some color.

Collaborator 2 commented at some length, stating that satisfactory recoveries could be obtained after several preliminary runs were made to learn the peculiarities of the method, that there was occasional clouding of the caffeine eluate by moisture, requiring filtration, and that careful control of the ether-chloroform ratio is necessary because of different absorbances of phenacetin in these two solvents. He also described a method used by him, employing a small column of dry silicic acid and Celite, with chloroform and alcohol as eluents, and eluting by suction.

Collaborators 3 and 4 said that they were satisfied with the method and encountered no difficulties.

Most of the results are quite satisfactory, especially when it is considered that they represent the outcome of two separations, one extraction, and one chromatographic procedure. However, the difficulties encountered by one collaborator at very high summer temperatures cannot be ignored. It is thought that the method should be revised sufficiently to make it workable at any temperature it may encounter. This might be accomplished merely by increasing the amount of chloroform in the eluent for phenacetin, but the use of isopropyl ether as an eluent will also be studied.

It is recommended* that the subject be continued.

ACKNOWLEDGMENT

The Associate Referee wishes to thank the following collaborators, all of the Food and Drug Administration, for the excellent cooperation received from them and from their Districts: Rupert Hyatt, Cincinnati District; Mary A. McEniry, St. Louis District; Felice A. Rotondaro, Philadelphia District; J. Phyllis Skyrme, Boston District; Robert D. Stanley, Chicago District; and Sidney Williams, Boston District.

REPORT ON PHENYLPROPANOLAMINE HYDROCHLORIDE

By Arthur W. Steers (Food and Drug Administration, Department of Health, Education, and Welfare, Los Angeles 15, Calif.), *Associate Referee*

Phenylpropanolamine hydrochloride (Propadrine hydrochloride) has been under consideration for several years but no report has previously

* For report of Subcommittee B and action of the Association, see *This Journal*, 37, 65 (1954).

been made by the Associate Referee. Preliminary work has included the determination of some of the physical properties of the compound and a survey of methods which may be applicable to the analysis of this compound in simple aqueous solutions, capsules, elixirs, and nasal jellies. These preparations containing phenylpropanolamine hydrochloride, with the exception of the nasal jelly, are listed in *New and Nonofficial Remedies*, 1952. Phenylpropanolamine hydrochloride is also found on the market in combinations with vinbarbital, codeine phosphate, methadone hydrochloride, and methapyrilene hydrochloride. Its determination in the latter combinations is not under consideration at the present time.

Extraction with an immiscible solvent seemed to offer the best approach for the isolation and determination of phenylpropanolamine hydrochloride. This required some knowledge of the solubility of the salt and base in water and in organic solvents. *New and Nonofficial Remedies*, 1952, gives the solubility of phenylpropanolamine hydrochloride as freely soluble in alcohol and water; and insoluble in benzene, chloroform, and ether. It was determined that one gram of the base is soluble in approximately 43 ml of water, 9 ml of chloroform, 45 ml of ether, 200 ml of benzene, and 3000 ml of petroleum ether. It is evident from these data that the base would not be readily extracted from aqueous solutions with organic solvents. It was found, however, that by nearly saturating the aqueous solution with sodium chloride, the base can be readily extracted with either chloroform or ether.

A method of analysis for aqueous solutions and capsules has been developed and tested by the Associate Referee with satisfactory results. The method has not yet been submitted to collaborative study.

METHOD

If the product is an aqueous soln, transfer an accurately measured sample contg ca 50 mg of phenylpropanolamine·HCl to separator and dil. to ca 20 ml. Make solution alk. to litmus with 10% NaOH soln; then add 0.5 ml excess. Nearly sat. with NaCl and ext. by shaking with four 20 ml portions of $CHCl_3$. Combine $CHCl_3$ exts in second separator and wash by shaking with 10 ml of satd NaCl soln. Filter $CHCl_3$ thru pledget of cotton into glass-stoppered flask. Shake NaCl soln with addnl 15 ml of $CHCl_3$ and draw off thru same cotton into main $CHCl_3$ soln. To the flask add 20.0 ml of 0.02 *N* H_2SO_4 and shake. Add a few glass beads and evap. the $CHCl_3$ on a steam bath in current of air. Cool and titrate excess acid with 0.02 *N* NaOH, using 1 drop of methyl red indicator. 1 ml of 0.02 *N* H_2SO_4 = 0.003753 g of phenylpropanolamine·HCl.

If the product is in capsule form, accurately weigh quantity of capsule contents contg ca 0.5 g of phenylpropanolamine·HCl. Transfer to 100 ml volumetric flask, dil. to ca 75 ml with H_2O, add 1 ml of 10% HCl, and allow to stand 30 min., shaking occasionally. Make to vol. with H_2O and mix thoroly. Filter thru a suitable dry filter into a dry flask, rejecting the first portion. Transfer to separator an accurately measured aliquot of filtrate equivalent to ca 50 mg of the drug and dil. to ca 20 ml. Proceed as directed above for analysis of aq. solns, beginning "Make soln alk. to litmus with 10% NaOH. . . ."

DISCUSSION

No collaborative work with the proposed methods was completed, but preliminary determinations by the Associate Referee on aqueous solutions and capsules gave recoveries between 99 and 100 per cent.

Attempts to extract diluted elixir or nasal jelly with chloroform or ether, after it was made alkaline, resulted in emulsions which it was necessary to centrifuge. In an effort to avoid emulsions, the solutions were given a preliminary hydrolysis by heating with dilute hydrochloric acid for fifteen minutes on the steam bath. This did not completely overcome the difficulty, and further work is required to perfect a method.

RECOMMENDATIONS

It is recommended*—

(1) That the proposed methods for the determination of phenylpropanolamine hydrochloride in aqueous solutions and capsules be submitted for collaborative study.

(2) That the study of methods for the determination of phenylpropanolamine hydrochloride in elixirs and nasal jellies be continued.

(3) That in keeping with the desirability of using generic rather than brand names the title of the subject be changed from "Propadrine hydrochloride" to "phenylpropanolamine hydrochloride."

REPORT ON AMPHETAMINES

By LLEWELLYN H. WELSH (Division of Pharmaceutical Chemistry, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D.C.), *Associate Referee*

The 1952 collaborative study (1) of the determination of amphetamines showed that amphetamine in a dilute aqueous solution, such as that resulting from a titration, may be quantitatively converted to its acetyl derivative and isolated as such by chloroform extraction. It also showed that the acetyl derivative thus obtained may be examined polariscopically to obtain an accurate measurement of the stereochemical composition of the amphetamine originally present. However, since it was considered necessary to continue the investigation for several reasons, which included exploration of the possibility of applying thermal analysis to acetylamphetamine in order to obtain a measure of stereochemical composition, no recommendation was made regarding adoption of these methods.

The 1952 study also included comparision of two methods for the determination of total amphetamine in tablets. These two methods were

* For report of Subcommittee B and action of the Association, see *This Journal*, 37, 65, 66 (1954).

essentially the U.S.P. titrimetric method (2), but one was a modification designed to substantially shorten the time required for analysis. Collaborative results obtained by both methods were virtually identical but were about 3 per cent lower than the theoretical. It was recommended that the study be continued in order to improve recoveries of total amphetamine. The following analytical procedure for amphetamine in tablets consists of methods of analysis studied collaboratively in 1952 and 1953.

TOTAL AMPHETAMINE

TITRIMETRIC METHOD

Accurately weigh not fewer than 20 tablets and det. av. wt per tablet. Grind tablets in mortar until the resulting powder will pass an 80-mesh sieve. Accurately weigh sufficient powder to provide 125 mg of amphetamine sulfate (or equivalent of other amphetamine salt), transfer to 100 ml beaker, add 15 ml of H_2O, and stir the mixt. for 15 min. Using rod or policeman transfer as much of the suspension as possible to a fritted glass funnel (medium porosity 40 mm disk is convenient) and filter with suction into a suitable vessel. Break suction and, with portions of H_2O totaling 15 ml, rinse as much adherent material as possible from beaker into funnel. Triturate mixt. in funnel to a uniform paste, and re-apply suction. Make transfer and filtration quant. by repeating the washing 4 addnl times with 10 ml portions of H_2O. With aid of small portions of H_2O, quantitatively transfer filtrate to 100 ml volumetric flask, make to vol., and mix. Transfer a 40 ml aliquot to separatory funnel, add 1 ml of 10% NaOH, and ext. with six 25 ml portions of ether. Wash combined ether extracts with two 5 ml portions of H_2O, and ext. the combined washings with two 10 ml portions of ether. Combine ethereal washings with the main ethereal ext., filter thru a pledget of cotton into a 250 ml separatory funnel, thoroly rinse with ether the separatory funnel which contained the unfiltered ethereal extracts, and pass these rinsings thru the filter so they combine with the filtered ethereal extracts. Ext. filtrate with exactly 20 ml of 0.02 *N* H_2SO_4, and drain the acid extract into 200 ml Erlenmeyer flask. Wash ether with 10, 5, and 5 ml portions of H_2O, combine washings with acid ext. and heat the whole on steam bath until dissolved ether is expelled. Cool, and titrate the soln with 0.02 *N* NaOH in presence of methyl red indicator. Calc. % amphetamine sulfate in sample and amount per tablet: 1 ml of 0.02 *N* H_2SO_4 = 3.658 mg of amphetamine sulfate.

CONFIRMATORY GRAVIMETRIC DETERMINATION

In a 250 ml separatory funnel combine the titrated soln with a 50 ml aliquot of the 60 ml of unused aq. ext. remaining in the volumetric flask, acidify by dropwise addition of 0.1 *N* H_2SO_4, and ext. with three 10 ml portions of CCl_4. Discard CCl_4 extracts. To the aq. soln, which has been sepd from as much CCl_4 as practicable, add 4.10 g of $NaHCO_3$ and swirl funnel until salt has mostly dissolved. Rapidly introduce into funnel 1.0 ml of acetic anhydride, A.C.S. reagent grade, by blowing in reagent from a 1 ml pipet. Immediately stopper funnel securely and shake vigorously until evolution of CO_2 has nearly ceased (release pressure in funnel frequently during shaking by opening stopcock). Add another 1.0 ml portion of anhydride, and continue to shake funnel until evolution of CO_2 has ceased (5–10 min. after addn of second portion of anhydride). Allow mixt. to stand 5 min. and completely ext. the acetylamphetamine by shakeouts with 50 ml portions of $CHCl_3$ (4 should be sufficient; test for complete ext. with a fifth shakeout). Filter extracts thru pledget of cotton, rinse filter with $CHCl_3$, conc. filtrate to a small vol. on steam-bath in current of air, quantitatively transfer concentrate to tared 50 ml beaker by rinsing

with small portions of $CHCl_3$, and continue the concentration until solvent is removed. Heat residue of acetylamphetamine in an oven (not forced-draft type) at 80° for 1 hr, cool in desiccator, and weigh. Calc. % amphetamine salt in sample and amount per tablet: Acetylamphetamine × 1.0395 = amphetamine sulfate.

Induce crystallization in the residue, if it has not crystallized spontaneously, by trituration, adding a small seed crystal of racemic acetylamphetamine if necessary. Powder the crystalline derivative finely and mix well. The pure acetyl derivative of *d*-amphetamine melts at 124.5–125°C.; that of the racemic substance melts at 93-93.5°C.

STEREOCHEMICAL COMPOSITION OF TOTAL AMPHETAMINE

POLARIMETRIC METHOD

Accurately weigh 90 mg of the derivative, transfer quantitatively to 5 ml volumetric flask, and make to vol. with U.S.P. $CHCl_3$. Det. optical rotation of soln in a semi-micro 2 dm tube (bore ca 4.5 mm, vol. 3–4 ml) at the same temp. at which the soln was made to vol. Acetyl-*d*-amphetamine is levorotatory in $CHCl_3$.

In measuring rotation with polariscope, take 10 readings on soln and calc. the av. to 0.001°. In the same way, det. the av. reading with same tube filled with U.S.P. $CHCl_3$, and use the av. zero-point reading thus obtained to correct the av. reading given by the soln.

If saccharimeter is used instead of polariscope, estimate all readings to 0.05 division, calc. the av. to 0.01 division, correct for zero-point, and multiply value so obtained by 0.3468 to obtain rotation, α, in angular degrees.

Calc. the specific rotation, $[\alpha]$, to 0.1° by the equation

$$[\alpha] = \frac{100\ \alpha}{c \times l}$$

in which c is concn of acetylamphetamine in g/100 ml and l is length of tube in decimeters.

Determine the % *d*-amphetamine by means of the equation $\%\ d = 50 + (50[\alpha]/44)$ in which $[\alpha]$ is the specific rotation of the acetyl derivative from the sample, and 44 is the specific rotation of pure acetyl-*d*-amphetamine, in which the sign of rotation has been ignored.

CONFIRMATORY THERMAL ANALYSIS

In a melting point tube with an internal diam. of 2–3 mm at the bottom and a length of ca 70 mm, place sufficient finely-powd. acetyl-*dl*-amphetamine (ca 8 mg) to form column 5 to 6 mm high after tube and contents have been tapped firmly several times on a hard surface.

Select a thermometer the range of which includes temperatures between 90 and 130°C. and whose graduations will permit readings to 0.5° with the aid of a low-power hand lens. An Anschütz-type thermometer is convenient but not necessary. The thermometer need not be calibrated, but if not, it is important that the same thermometer be used in determining the standard m.p. curve and the m.p. of the derivative from the sample.

Fix melting point tube securely to the thermometer by two small rubber bands, one of which should be placed near the top of m.p. tube and the other as far down as possible without allowing liquid[1] in bath to touch the band. Suitable bands may be cut from rubber tubing of the proper size. Adjust m.p. tube so that the middle of column of specimen coincides approximately with middle of thermometer bulb.

[1] DC 200 Silicone fluid, viscosity grade 20 centistokes at 25°C., Dow Corning Corporation, Midland, Michigan, is convenient for a bath liquid.

Support assembly in a mechanically-stirred m.p. bath. Raise temp. of bath rapidly until it is about 5° below anticipated m.p. (temp. at which specimen becomes entirely a clear liquid); then regulate heating carefully so that the rise in temp. does not exceed 0.5° per min. After specimen begins to melt, stir continuously with a chromel wire (0.4 mm diam.; flatten the lower end for a distance of ca 3 mm and bend flattened portion at a right angle ca 1 mm from tip so as to form a hoe-like stirrer) while inspecting it carefully with ca 10× hand lens. Note temp. at which the last crystalline material disappears and record it as the m.p. Detection of the disappearance of solid phase is facilitated by observing the specimen in a beam of light transmitted through it from the rear of the apparatus. Remove tube and thermometer from bath, induce the melt to solidify by stirring (seeding if necessary), and repeat detn. Replicate detns will not differ by more than 1° if carefully carried out.

Following the same procedure, det. m.p. of pure acetyl-*d*-amphetamine.

Prep. a series of standard mixtures of acetyl-*d*- and acetyl-*l*-amphetamine with the following compositions expressed in mg: 80 *d*+20 *dl*, 60 *d*+40 *dl*, 40 *d*+60 *dl*, 20 *d*+80 *dl*. These mixtures contain, resp., 90%, 80%, 70%, and 60% of *d*-isomer. In each case accurately weigh out each component into small (18×55 mm) test tube and hold tube in bath heated to 130–135°C. until contents have completely melted. Stir molten contents with small stainless steel spatula until well mixed; then withdraw tube from bath and continue to stir until melt has completely solidified. Transfer solidified material as completely as possible to small mortar, powder finely, and mix thoroly. Det. m. p. of each mixt. in the manner described above. In each mixt. the beginning of fusion (softening, appearance of liquid phase) will be noted at ca the same temp. (ca 93°), but the temp. at which the system becomes entirely liquid (m.p.) will depend on the composition of the mixt. Unlike the m.p. detns of pure *dl*- and *d*-derivatives, it is not important to stir the mixtures continuously after the first evidence of fusion. After considerable liquid phase has formed, the specimen should be stirred occasionally as the solid phase diminishes in size. Stirring should be continuous during ca the last 2 min. of the detn, i.e., during inspection in anticipation of the disappearance of the last portion of crystalline matter. Any solid matter adhering to walls of tube above melt should be pushed down into melt by the wire stirrer.

On coordinate paper, plot the av. m.p. (ordinate) of each specimen against the composition (abscissa) expressed as % acetyl-*d*-amphetamine, and draw a smooth curve which is in best conformity with the 6 plotted points.

Det. m.p. of the derivative obtained from the tablets, and, by referring to the standard curve, estimate the % *d*-isomer present.

DISCUSSION

The collaborative sample of simulated tablet mixture used in the 1952 study was an 80-mesh powder containing 2.00 per cent of a mixture of amphetamine sulfates of the composition 75 per cent *d*, 25 per cent *l*. The remaining 98 per cent of the sample consisted of lactose, 49 per cent; potato starch, 20 per cent; tricalcium phosphate, 20 per cent; talc, 10 per cent; and stearic acid, 1 per cent. The collaborative sample used in the 1953 study differed only in that the amphetamine sulfate had the composition 85 per cent *d*, 15 per cent *l*.

The *confirmatory gravimetric* determination of total amphetamine was carried out collaboratively in 1952 by a procedure which varied from that

described above only in that the acetylation was carried out on the solution resulting from the pooling of duplicate titrations instead of on the composite of a single titrated solution and an aliquot of the unused aqueous extract which remains in the 100 ml volumetric flask. The latter procedure permits duplicate gravimetric determinations to be obtained from duplicate weighings of powdered tablet mixture, whereas the former yields only one confirmatory gravimetric determination from a set of duplicate weighings. In the 1953 study, the derivative was prepared as directed above for use in the determination of stereochemical composition, but instructions to collaborators did not direct that isolation of the derivative be carried out on a quantitative basis.

The results of the 1952 study involving four collaborators showed that 97.4—100.3 per cent (average, 99.4 per cent) of the amphetamine found present titrimetrically could be recovered gravimetrically as the acetyl derivative. It has been the experience of the Associate Referee that the minor modification of the 1952 method, discussed above, has no measurable effect on recoveries.

The *titrimetric method* for total amphetamine, and the determination of stereochemical composition by the *polarimetric method* and by *confirmatory thermal analysis* were studied collaboratively this year. The results of collaborators are listed in Table 1.

The *titrimetric method* for total amphetamine is fundamentally a modification of "Method B" employed in last year's study. Investigation by the Associate Referee showed that the low recoveries reported in the 1952 study were due to incomplete extraction of amphetamine sulfate from the

TABLE 1.—*Collaborative results, 1953*

COLLABORATOR	TOTAL AMPHETAMINE SULFATE, %		*d*-AMPHETAMINE SULFATE, %			
	FOUND, TITRIMETRIC	RECOVERY	POLARIMETRICALLY		BY THERMAL ANALYSIS	
			FOUND	RECOVERY	FOUND	RECOVERY
H. Fischbach[a]	1.97	98.5	86.9	102.2	84	99
	1.98	99.0	86.1	101.3	85	100
P. Sanders[a]	1.99	99.5	85.3	100.4	84	99
	1.98	99.0	85.2	100.2	85	100
J. C. Molitor[a]	1.98	99.0	85.9	101.1	84	99
Associate Referee	1.97	98.5	84.7	99.6	85	100
	1.97	98.5	85.0	100.0	86	101
Average	1.977	98.9	85.57	100.7	84.7	99.6

[a] Food and Drug Administration, Washington, D. C.

collaborative sample. The modification achieves more efficient extraction by specifying that the powdered sample be treated with approximately twice as much water. Recoveries of collaborators were within the range 98.5—99.5 per cent, and averaged 98.9 per cent.

The *polarimetric method* for the determination of stereochemical composition is the same as that studied last year. Recoveries of *d*-amphetamine sulfate by collaborators ranged between 99.6 and 102.2 per cent, and averaged 100.7 per cent. In the 1952 study, recoveries of four collaborators were in the range 95—100 per cent and averaged 97.9 per cent.

The determination of stereochemical composition by *thermal analysis* is based on the method of H. Rheinboldt and co-workers (3) for establishing phase diagrams of binary systems. Recoveries of the *d*-isomer by collaborators were within the range 99—100 per cent, and averaged 99.6 per cent.

The procedure for thermal analysis, as described, does not *per se* define stereochemical composition, since the same melting point characteristics are exhibited by a mixture in which acetyl-*d*-amphetamine predominates by a given amount and a mixture in which acetyl-*l*-amphetamine predominates by the same amount. Distinction between the two compositions is possible only by correlating supplementary mixed melting point data or by determining the sign of rotation of the mixture. For this reason, the procedure is a *confirmatory* one, to be employed in conjunction with (and not as a substitute for) the polariscopic method.

RECOMMENDATIONS

It is recommended* that first action status be accorded the following methods:

(1) The titrimetric determination of total amphetamine;

(2) The confirmatory gravimetric determination of total amphetamine as the acetyl derivative;

(3) The determination of stereochemical composition of amphetamine by polarimetric examination of the acetyl derivative;

(4) The confirmatory determination of stereochemical composition by thermal analysis of the acetyl derivative.

In the 1952 report, it was recommended that investigation be undertaken (*a*) to eliminate difficulties associated with the A.O.A.C. method for the determination of total amphetamine as its benzoyl derivative, and (*b*) to develop a titrimetric method for total amphetamine in which the drug is separated from tablet mixtures by distillation.

Reappraisal of the circumstances leads the Associate Referee to recommend that (*a*) be discontinued and that, instead of (*b*), methods be

* For report of Subcommittee B and action of the Association, see *This Journal*, **37**, 66 (1954).

investigated for the determination of total amphetamine which are based on the ultraviolet absorption spectrum of that substance.

REFERENCES

(1) WELSH, L. H., *This Journal*, **36**, 714 (1953).
(2) The United States Pharmacopeia, Fourteenth Revision, Mack Publishing Co., Easton, Pa., 1950, p. 47.
(3) RHEINBOLDT, H., *et al.*, *J. prakt. Chem.*, **111**, 242 (1925); **112**, 187, 199, 348 (1926).

REPORT ON SEPARATION OF ASPIRIN AND PHENOBARBITAL

By T. E. BYERS (Food and Drug Administration, Department of Health, Education, and Welfare, Cincinnati 2, Ohio),
Associate Referee

This study was undertaken to develop a quantitative separation of phenobarbital from aspirin in commercial products (capsules and tablets).

The ratio of aspirin to phenobarbital in products on the market ranges from 6–20 parts aspirin to 1 part phenobarbital. This preponderance of aspirin as well as the similarity of their physical properties caused difficulty in separation with ordinary extraction procedures.

A partition or absorption chromatographic separation procedure seemed feasible, so several combinations of adsorbent and immobile solvent were tried.

A Celite column containing 2 *M* K_2HPO_4 as the immobile solvent, as described by Banes in his method for the determination of phenobarbital in the presence of salicylates, did not completely retain the aspirin. Varying the concentrations of K_2HPO_4 in the immobile solvent did not remedy the situation.

A silicic acid column was used as follows: 5 ml of chloroform containing 40 mg of aspirin was placed in the column and portions of 10 ml of chloroform eluate were collected. Each portion was analyzed for aspirin spectrophotometrically. A small amount of aspirin (1–1.5 mg) passed through the column, practically all in the first 25 ml eluted. Five ml of chloroform containing 2 mg of phenobarbital was passed through a similar column, and 10 ml portions of eluate were collected as before. Each portion was evaporated to dryness, the phenobarbital was dissolved in 1 ml of 0.1 ml *N* sodium hydroxide and made to 100 ml with water, and the resulting solution was analyzed spectrophotometrically.

It was found that the phenobarbital was eluted in from 30 to 70 ml of chloroform.

From these data it seemed feasible to discard the first 25 ml of eluate, which contained the aspirin, and collect the next 75 ml which contained the phenobarbital. Several authentic mixtures of phenobarbital and aspirin were analyzed by this method with good reproduction of results. Two samples were sent out for collaboration: one contained 2.0 g of aspirin and 100 mg of phenobarbital; the other was a commercial capsule containing 5 grains of aspirin and 3/4 grain of phenobarbital.

With a few exceptions, collaborative results were very erratic, and the phenobarbital recovered was generally lower than the actual amount present. The only explanation appeared to be that some phenobarbital was eluted in the first 25 ml and thus lost.

To overcome such a difficulty, it would seem practical to retain all the eluate and correct for the aspirin present by a spectrophotometric two-component analysis. However, there are two shortcomings in such a method:

(1) The slope of the absorbance curve for aspirin at the point of maximum absorbance for phenobarbital (240.5 mμ) is very steep, and offers a chance for some error in correction.

(2) Some aspirin is hydrolyzed to salicylic acid, which has a much higher specific absorbance than aspirin, so that any correction for aspirin on such a basis would be in error. While this difficulty might be overcome by hydrolyzing all aspirin to salicylic acid, such treatment may destroy some of the phenobarbital and change its absorbance.

More work will be necessary on this problem. Several possible methods for the separation of these compounds as well as other barbiturates are now being studied.

It is recommended* that the study of methods for the separation of phenobarbital and aspirin be continued.

REPORT ON ANALYSIS OF DRUGS BY INFRARED SPECTROPHOTOMETRY

NITROGLYCERINE, MEPERIDINE, ATROPINE, CODEINE, AND AMIDONE

By JONAS CAROL (Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D.C.), *Associate Referee*

The application of infrared spectrophotometry to the analysis of pharmaceutical preparations has been limited except in the case of steroid

* For report of Subcommittee B and action of the Association, see *This Journal*, 37, 66 (1954).

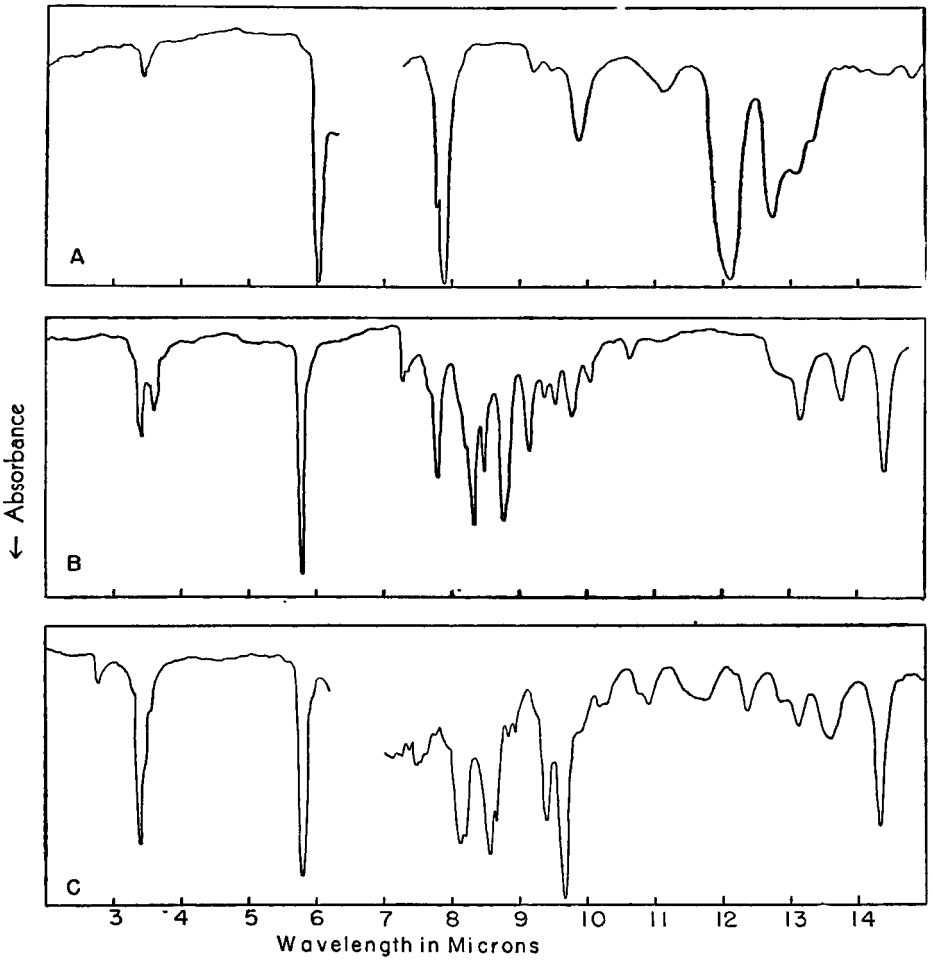

FIG. 1.—Infrared absorption spectra of (A) Nitroglycerin, (B) Meperidine; and (C) Atropine, in carbon disulfide solution.

hormones (1–4). This neglect is no doubt due largely to the expensive equipment required and to the technical difficulties frequently encountered in the preparation of samples. Infrared spectrophotometry, however, offers a basis for highly specific analyses since no two compounds, except enantiomorphs, have identical spectra.

Quantitative analyses can be made easily for substances soluble (to 0.5 per cent or more) in suitable infrared transparent solvents.[1] Absorbances

[1] Carbon disulfide and carbon tetrachloride are the only two solvents that are appreciably transparent (in thickness up to 1.0 mm) from 2–15 μ. A number of solvents are transparent in limited regions of the spectrum. Chloroform may be used from 5–6.5μ.

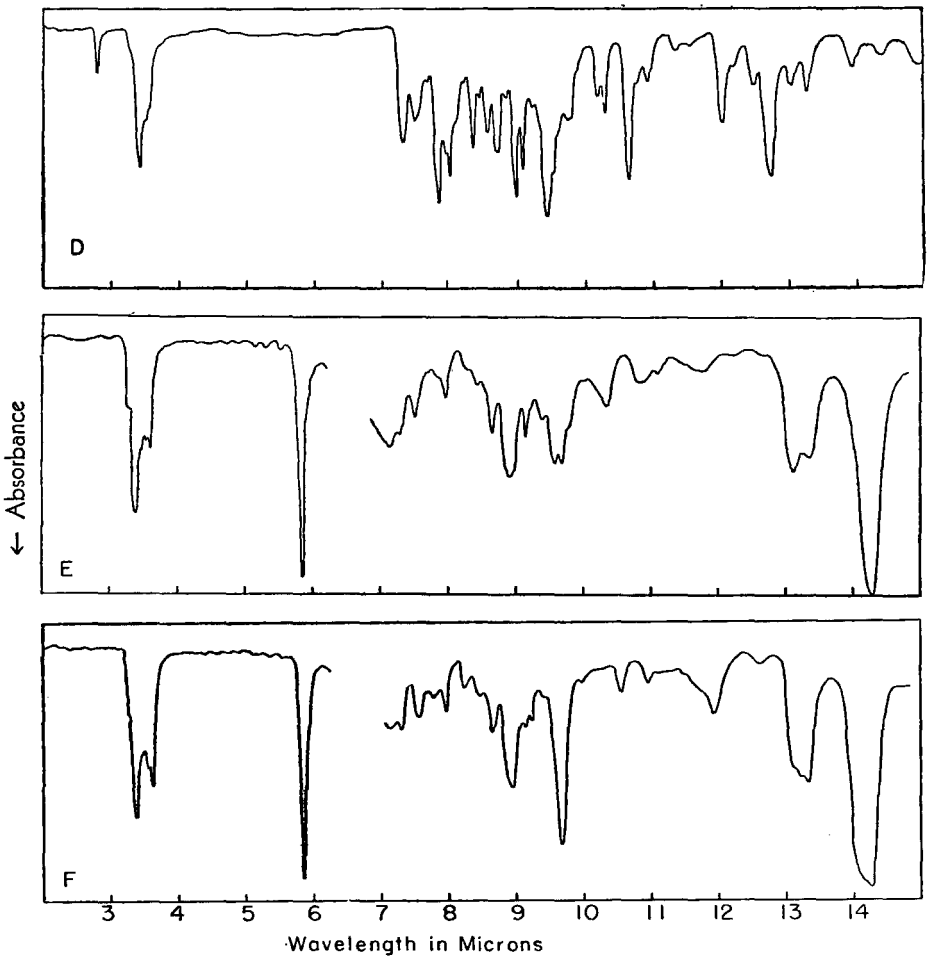

FIG. 2.—Infrared absorption spectra of (D) Codeine; (E) Amidone; and (F) Isoamidone, in carbon disulfide solution.

of the standard and sample solutions at the selected wavelengths are measured, using liquid-tight cells (matched or of known difference in thickness). However, many drugs are almost insoluble in carbon disulfide or carbon tetrachloride. These substances can be determined by one of the following methods of sample preparation: Formation of a soluble derivative (5), preparation of a suspension in carbon disulfide by the use of aluminum stearate (6), mineral oil mulls with internal standards (7), or the newly developed pressed potassium bromide disk technique (8, 9).

The present work deals only with drugs easily soluble in carbon disulfide. The absorption spectrum of each compound was recorded from 2–15μ with a Perkin-Elmer Model 21 spectrophotometer. These spectra are shown in Figs. 1 and 2. The concentration of the sample solutions, cell thickness, and suggested wavelengths for quantitative measurements are shown in Table 1.

TABLE 1.—*Infrared data for six pharmaceutical compounds*

COMPOUND	CONCN IN MG PER ML CS_2	CELL THICKNESS	SUGGESTED WAVELENGTHS, μ
		mm	
Nitroglycerin[a]	3.0	1.0	6.05, 7.90
Meperidine[b]	5.0	1.0	7.58, 8.35, 8.76, 14.38
Atropine U.S.P.	10.0	0.5	8.12, 8.55, 9.66, 14.32
Codeine[c]	5.0	1.0	7.82, 8.95, 10.61, 12.70
Amidone[d]	10.0	1.0	9.56, 14.26
Isoamidone[e]	10.0	1.0	9.66, 14.25

[a] Extracted from commercial nitroglycerin tablets, 0.6 mg.
[b] Extracted from commercial meperidine hydrochloride tablets, 50 mg.
[c] Anhydrous codeine crystallized from ether.
[d] Extracted from *dl*-amidone hydrochloride (Mallinckrodt).
[e] Extracted from *l*-isoamidone hydrochloride (Winthrop-Stearns).

A general procedure is proposed for the analysis of these (and other carbon disulfide-soluble) substances where they occur as the only active ingredient in a pharmaceutical preparation. A method for atropine sulfate in tablets is presented to illustrate the general procedure. A series of solutions containing 1–5 mg. of atropine per ml of carbon disulfide was prepared. The absorbance of each, relative to a solvent blank, was determined with 1.0 mm cells at each of the wavelengths suggested for atropine in Table 1. These results (Table 2) show good adherence to Beer's law at each wavelength. In the proposed method, absorbance measurements are made at 8.66μ.

TABLE 2.—*Data showing adherence of atropine solutions to Beer's law at four measuring wavelengths*

	ABSORBANCE AT:			
MG/ML	8.12μ	8.55μ	8.66μ	14.32μ
1	0.085	0.091	0.170	0.061
2	0.174	0.180	0.351	0.120
3	0.278	0.291	0.540	0.202
4	0.355	0.374	0.700	0.268
5	0.434	0.458	0.820	0.325

METHOD

APPARATUS

(a) *Recording spectrophotometer.*—Suitable for measuring absorbance from 2–15 μ.

(b) *Absorption cells.*—Two 1.0 mm liquid-tight absorption cells fitted with NaCl windows. (The cells need not be matched, but their difference in absorbance should be measured prior to analysis.)

REAGENTS

(a) *Atropine.*—U.S.P. grade.

(b) *Ammonium hydroxide.*—10% solution (v/v).

(c) *Carbon disulfide.*—A.S.C. reagent grade.

DETERMINATION

Weigh a counted number of tablets and reduce to a fine powder without appreciable loss. Weigh accurately a portion of the powder equivalent to 10–12 mg of atropine and transfer to a 125 ml separator. Add 5 ml of H_2O, mix thoroly, and make distinctly alk. with dil. NH_4OH soln. Ext. with 4 successive 25 ml portions of CS_2, filtering each thru a dry filter into a 150 ml beaker. Carefully evap. the combined ext. to ca 3 ml and transfer to a 5 ml volumetric flask with the aid of small amounts of CS_2. Dil. to vol. and mix thoroly. Carefully weigh 25 mg of atropine, transfer to a 10 ml volumetric flask, dissolve in CS_2, dil. to vol., and mix thoroly. Det. the absorbance of the sample soln and standard soln relative to solvent blanks at 9.66 μ. Compare the recorded spectra of each soln from 2–15 μ in order to ascertain the identity of the sample.

$$\text{Atropine sulfate in sample} = \frac{A \text{ sample}}{A \text{ standard}} \times 2.5 \times 1.2$$

Analysis was made on four commercial samples of atropine sulfate tablets by this procedure. These results are compared to those obtained by the U.S.P. XIV assay in Table 3.

TABLE 3.—*Comparison of analysis of atropine sulfate tablets by infrared spectrophotometry and by U.S.P. assay*

SAMPLE	ATROPINE SULFATE MG/TABLET		
	DECLARED	U.S.P. XIV ASSAY	I.R. ANALYSIS
1	0.40	0.41	0.41
2	0.65	0.62	0.60
3	0.33	0.34	0.35
4	?	0.34	0.34

These results indicate that the proposed method has an accuracy equal to the U.S.P. XIV assay and in addition offers qualitative proof of identity by comparison of the spectra of standard and sample.

It is recommended* that the infrared procedure for the analysis of

* For report of Subcommittee B and action of the Association, see *This Journal*, 37, 66 (1954).

pharmaceutical preparations be tested, using preparations of nitroglycerin, meperidine, codeine, and amidone, and that samples of these and of atropine be submitted to collaborative study.

REFERENCES

(1) PLEAT, G. B., HARLEY, J. H., and WEBERLEY, S. E., *J. Am. Pharm. Assoc. Sci. Ed.*, **40**, 107 (1951).
(2) WASHBURN, W. H., and KRUEGER, E. O., *ibid.*, **40**, 291 (1951).
(3) FURCHGOTT, R. F., *et al.*, *J. Biol. Chem.*, **163**, 375 (1946).
(4) JONES, R. N., *et al.*, *J. Am. Chem. Soc.*, **70**, 2024 (1948).
(5) CAROL, J., MOLITOR, J. C., and HAENNI, E. O., *J. Am. Pharm. Assoc., Sci. Ed.*, **37**, 173 (1948).
(6) DOLINSKY, M., *This Journal*, **34**, 749 (1951).
(7) BARNES, R. B., *et al.*, *Anal. Chem.*, **19**, 620 (1947).
(8) SCHIEDT, V., *Z. Naturforsch.*, **5**, 270 (1952).
(9) SISTER MIRIAM MICHAEL (Stimson), *J. Am. Chem. Soc.*, **74**, 1805 (1952).

REPORT ON SULFONAMIDE DERIVATIVES

By HARRY W. CONROY (Food and Drug Administration, Department of Health, Education, and Welfare, Kansas City 6, Mo.), *Associate Referee*

Committee B (1) recommended that the study of methods for sulfanilamide derivatives be continued with investigation of recently advocated titrimetric procedures in organic solvents. A study of the method of Fritz and Keen (2) for the determination of sulfa drugs and sulfonamides by titration in organic solvents was made by the Associate Referee. In addition, as suggested by the Referee, collaborative studies were made of the thiobarbituric acid method of Kohn (3) as modified by Shepherd (4) for the determination of sulfadiazine, and of a spectrophotometric method (5) for the determination of sulfamerazine in the presence of sulfadiazine.

TITRATION OF SULFONAMIDES IN ORGANIC SOLVENTS

The titration of sulfa drugs in organic solvents has been reported to furnish an accurate, general method for their determination. The procedure involves solution of the sulfonamide in a suitable solvent (dimethylformamide or butylamine) and titration with sodium methoxide and the use of thymol blue indicator with dimethylformamide or azo violet indicator with butylamine.

Six sulfonamides were assayed by the above procedure and the effect of common tablet excipients was determined. The sulfonamides used in the

determinations were U.S.P. or N.F. quality with the exception of sulfamethazine which complied with N.N.R. tests for identity and purity. Dimethylformamide and butylamine were obtained commercially and required neutralization immediately before use. The results obtained are given in Table 1.

TABLE 1.—*Assay of sulfonamides by titration in organic solvents*

COMPOUND	WEIGHT SAMPLE	N/10 BASE	PURITY	SOLVENT	INDICATOR
	g	*ml*	*per cent*		
Sulfanilamide	0.1547	8.94	99.5	$BuNH_2$	Azo violet
Sulfathiazole	0.2043	7.95	99.4	DMF	Thymol blue
Sulfamerazine	0.2693	10.18	99.9	DMF	Thymol blue
Sulfadiazine	0.2029	8.08	99.7	DMF	Thymol blue
Sulfapyridine	0.2111	8.47	100.0	DMF	Thymol blue
Sulfamethazine	0.2092	7.45	99.1	DMF	Thymol blue

The titration of sulfonamides in the presence of excipients found in tablet preparations showed that stearic acid was the only commonly encountered interfering substance. Some experiments were made which indicated that stearic acid could be eliminated, with subsequent titration of the sulfonamide. Starch, lactose, stearates, sodium bicarbonate and calcium carbonate did not affect the titration result. The procedure is not adapted to the titration of the salts of the sulfonamides.

While the titration of sulfonamides in organic solvents permits a more accurate determination than the nitrite titration, the latter method is more specific and convenient to use. Precautions must be taken to exclude carbon dioxide while titrating the sulfa drugs in dimethylformamide or butylamine since these solvents absorb carbon dioxide. The benzene-methanol solution of sodium methylate requires frequent standardization. The cost of dimethylformamide and butylamine necessitates the use of smaller sample weights and titration volumes. In view of the desirability of a collaborative study of the following spectrophotometric methods and since insufficient time was available, no collaborative study was made of the titrimetric procedure in organic solvents.

SPECTROPHOTOMETRIC DETERMINATION OF SULFADIAZINE AND SULFAMERAZINE

A collaborative study was made of the determination of sulfadiazine and sulfamerazine mixed together with excipients (Sample A) and the determination of sulfadiazine mixed with seven other sulfonamides and excipients (Sample B). The determination of sulfadiazine on samples A and B was based on its reaction with 2-thiobarbituric acid to form a red color with subsequent absorbance measurement at 532 millimicrons. The

reaction is reported to be specific for sulfadiazine in mixtures of sulfonamides, and known interferences do not appear to be likely constitutents of sulfa drug preparations. Sulfamerazine was determined in sample A by its absorption of ultraviolet light at 305 millimicrons, correcting the absorbance of the sample for that of the known sulfadiazine. The following method was sent to collaborators:

SULFADIAZINE AND SULFAMERAZINE

REAGENTS

(a) *Citrate buffer soln.*—37 g Na citrate dihydrate and 32 ml concd HCl dild to 250 ml with H_2O.

(b) *2-Thiobarbituric acid soln.*—Recrystallize twice from hot H_2O. Dissolve 5 g of this recrystallized acid in 5 ml 4 *N* NaOH dild with 500 ml H_2O. Add 250 ml citrate buffer soln and adjust *p*H to 2.0 to obtain final reagent. Reagent is stable when stored in glass-stoppered bottle in refrigerator.

DETERMINATION

Prep. sample soln contg ca 0.1 g mixed sulfonamides by intermittent shaking with 50 ml *N* HCl during a 10 min. period. Filter, if necessary, to obtain a clear soln and dil. filtrate and washings to 100 ml. To 5 ml of this soln add 7.5 ml *N* HCl and dil. to 100 ml. (The soln obtained should contain ca 5 mg mixed sulfonamides per 100 ml 0.1 *N* HCl.) To 1.0 ml of this soln in a glass-stoppered test tube add 10.0 ml of buffered 2-thiobarbituric acid soln **(b)**. Stopper and heat 1 hr at 100°C. Weigh tubes before and after heating and compensate for any loss of moisture during heating by addn of H_2O. Treat similarly a 1.0 ml standard contg 25 mmg sulfadiazine in 0.1 *N* HCl and a 1.0 ml sample of acid for a blank. Det. absorbances at 532 mμ relative to the blank and calc. the sulfadiazine content of sample.

Det. the absorbances at 305 mμ of the prepd soln and of sulfadiazine and sulfamerazine standards each contg 5.0 mg pure sulfonamide per 100 ml 0.1 *N* HCl, relative to the blank acid. Subtract from sample value the absorbance due to the sulfadiazine content and calc. quantity of sulfamerazine in sample. The ultraviolet spectrum of the sample should be examined to assure absence of background interference.

CALCULATIONS

Sulfadiazine:

$$\text{Mmg sulfadiazine in 1 ml aliquot} = \frac{\text{Absorbance sample}}{\text{Absorbance standard}} \times 25$$

or:

$$\text{Mg sulfadiazine in 5 mg sample per 100 ml 0.1 } N \text{ HCl} = \frac{100 \times \text{mmg detd}}{1000}$$

Sulfamerazine: Det. absorbance due to sulfadiazine, A_2. $A_2 = A_1C_2/C_1$ where A_1 = absorbance of 5 mg sulfadiazine standard; $C_1 = 5$ (mg sulfadiazine standard /100 ml 0.1 *N* HCl); C_2 = mg sulfadiazine calcd in 5 mg sample/100 ml of 0.1 *N* HCl. Deduct the val. calcd for A_2 from the absorbance obtained for the 5 mg sample in 0.1 *N* HCl. Then:

Mg sulfamerazine in the 5 mg sample/100 ml

$$= 5.0 \times \frac{\text{corrected sample absorbance}}{\text{absorbance of 5 mg sulfamerazine standard}}$$

DETERMINATION OF SULFADIAZINE IN THE PRESENCE OF OTHER SULFONAMIDES

Det. sulfadiazine as directed above from a prepd soln contg ca 25 mmg. of sulfadiazine per ml of 0.1 *N* HCl.

COLLABORATIVE STUDY

Collaborators were supplied with purified 2-thiobarbituric acid, twice recrystallized as directed in the method, and with sulfadiazine and sulfamerazine of U.S.P. quality for preparation of standard dilutions. Sample A was composed of 35 per cent sulfamerazine, 35 per cent sulfadiazine, 15 per cent lactose, 14.5 per cent starch, and 0.5 per cent stearic acid. Sample B contained 50 per cent sulfadiazine, 5 per cent each of sulfanilamide, sulfathiazole, sulfapyridine, sulfamerazine, sulfaguanidine, sulfamethazine, and sulfathalidine, and 7.50 per cent lactose, 7.25 per cent starch, and 0.25 per cent stearic acid. Collaborators were requested to determine sulfadiazine and sulfamerazine on sample A and to determine sulfadiazine on sample B. In order to ascertain the proper amount of sample needed to provide 0.1 gram of mixed sulfonamides in the case of sample A, the collaborator was requested to make a nitrite titration, using the average factor for sulfadiazine and sulfamerazine. The amount of sample B specified was 0.1 gram of the sample as received. The following results obtained by collaborators are shown in Table 2:

TABLE 2.—*Collaborative results*

COLLABORATOR	SAMPLE A				SAMPLE B	
	SULFAMERAZINE		SULFADIAZINE		SULFADIAZINE	
	PRESENT	FOUND	PRESENT	FOUND	PRESENT	FOUND
	per cent		*per cent*		*per cent*	
1	35	34.6	35	34.3	50	49.4
2		34.8		34.7		50.0
3		34.7		34.4		49.6
4		34.4		33.4		48.6
5		34.8		34.6		48.3

COMMENTS OF COLLABORATORS

Collaborator 2: This analyst reported that the 2-thiobarbituric acid reagent was difficultly soluble and tended to precipitate out on storage.

Collaborator 3: The same comment was made as above, regarding the 2-thiobarbituric acid reagent. This analyst and collaborator 4 found no background interference in sample A.

Collaborator 5: The same comment was made in regard to 2-thiobarbituric acid as given by collaborators 2 and 3. He stated that the reagent still appeared to be usable. This analyst had had previous experience with the method and believed that complete solution of the sulfonamides in normal HCl can not be attained in a short time. He suggested that a smaller volume of a higher concentra-

tion of HCl be used and then diluted to 0.1 *N* HCl. He found an appreciable and variable loss of moisture from the glass-stoppered tubes during one hour of heating. To obviate this difficulty the tubes were weighed before and after heating and the loss of moisture was compensated for by the addition of water.

DISCUSSION

The directions for preparation of sample solution specify the use of a 50 ml portion of normal HCl per 0.1 gram of mixed sulfonamides, which is shaken intermittently for ten minutes. Preliminary tests on 0.1 gram portions of sulfadiazine and sulfamerazine mixed in equal amounts had shown that 50 ml of normal HCl dissolved the sulfonamides in less than two minutes when shaken intermittently.

Several collaborators found that the 2-thiobarbituric acid was difficultly soluble and formed a precipitate on storage in the refrigerator. It was found that the result obtained by the use of such a solution, which was filtered before use, duplicated the values obtained with the fresh reagent.

All collaborators obtained results which were reasonably close to the composition of the samples submitted to them. One collaborator, whose first results were unsatisfactory, repeated the analysis and discovered that the glass-stoppered test tubes, used in the colorimetric determination of sulfadiazine, lost appreciable and variable amounts of moisture when heated for one hour at 100°C. When the tubes were weighed before and after heating and the loss in moisture was compensated for by the addition of water, satisfactory results were obtained.

The presence of seven other sulfonamides did not interfere in the colorimetric determination of sulfadiazine.

RECOMMENDATIONS

It is recommended*—

(1) That the method for determination of sulfadiazine in the presence of other sulfonamides be adopted, first action.

(2) That the method for the determination of sulfadiazine and sulfamerazine in mixture be adopted, first action.

(3) That the procedure for titration of sulfonamides in organic solvents be studied collaboratively.

ACKNOWLEDGMENT

The Associate Referee wishes to thank the following chemists of the Food and Drug Administration for participating in the collaborative work: Theodore S. Smith, Kansas City; Arthur C. Thomson, Cincinnati; John R. Schnably, Chicago; and Arthur Kramer, New York.

* For report of Subcommittee B and action of the Association, see *This Journal*, 37, 66 (1954).

REFERENCES

(1) *This Journal*, **36**, 53 (1953).
(2) FRITZ, J. S., and KEEN, R. T., *Anal. Chem.*, **24**, 308 (1952).
(3) KOHN, H. I., *Proc. Soc. Exptl. Biol. Med.*, **63**, 146 (1946).
(4) SHEPHERD, R. G., *Anal. Chem.*, **20**, 1150 (1948).
(5) BANES, D., unpublished report.

REPORT ON STEROID ESTROGENS

By PAUL M. SANDERS (Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D.C.), *Associate Referee*

Since the last report on this subject (1), the two first action methods for ketosteroids have been utilized in many analyses made on commercial products in this laboratory. It has been noted in this work that the method for the determination of ketosteroids (gravimetric) has yielded consistently higher results than those obtained by the colorimetric procedure. This variation has also been corroborated by the infrared-spectrophotometric assay for ketosteroids of Carol, *et al.* (2). The close agreement between the gravimetric and colorimetric procedures for ketosteroids obtained in the collaborative study conducted by Haenni (1) resulted from the use of solutions containing mixtures of pure estrogens. Good agreement is also obtained in the analysis of commercial products made from *highly refined* estrogens. The variation between the two procedures increases when less refined products are assayed. Naturally occurring contaminants related to the steroid estrogens and extracted with them from pregnant mares' urine are probably the major source of error in the gravimetric procedure.

The assay results of 62 samples obtained with both procedures are presented in Table 1. The average per cent of ketosteroids obtained by the colorimetric procedure relative to the gravimetric procedure is 83 per cent. The range of variation is large (59—100 per cent), and indicates the inaccuracy and unpredictability of the gravimetric procedure as an assay for pharmaceutical products.

Because of these data, the Associate Referee feels justified in recommending the changing of the status of the method for the determination of ketosteroids (gravimetric) from that of first action to merely an optional semi-quantitative estimation.

RECOMMENDATIONS

It is recommended* that the method for the determination of ketosteroids (gravimetric) as given in *This Journal*, **34**, 81 (1951) be revised as follows:

† For report of Subcommittee B and action of the Association, see *This Journal*, **37**, 66, 116 (1954).

TABLE 1.—*Analysis of ketosteroids by gravimetric and by colorimetric procedures*

PRODUCT	TOTAL KETOSTEROIDS		PER CENT OF GRAVIMETRIC
	GRAVIMETRIC	SPECTROPHOTOMETRIC[a]	
Estrogens in oil	0.96[b]	0.89	93
Estrogens, capsules	0.93	0.72	77
	1.10	1.02	93
Conj. estrogens, tablets	0.41	0.34	83
	1.22	1.08	88
	1.16	0.89	77
	1.13	0.94	83
	1.07	0.90	84
	1.15	0.97	84
	1.12	0.86	77
	1.24	0.86	69
	1.26	0.94	75
Conj. estrogens, powder	29.5	17.6	59
	21.8	16.3	75
	18.7	16.9	90
	17.5	13.8	79
	17.4	13.5	78
Conj. estrogens, granules	7.5	6.3	84
Estrogens, aqueous–suspension	1.30	0.96	74
	0.98	0.88	90
	1.10	0.88	80
	1.35	1.01	75
	0.51	0.43	84
	0.78	0.58	74
	0.93	0.93	100
	1.97	1.85	94
Estrogens, in oil	0.14	0.09	64
Estrogens, powder	21.9	20.5	94
Estrogens, aqueous suspension	0.78	0.67	86
	1.02	0.89	87
	0.81	0.65	80
	0.74	0.57	77
	0.58	0.51	88
	0.76	0.70	92
	0.83	0.76	92
	0.83	0.80	96
	1.30	1.12	86

TABLE 1.—(*Continued*)

PRODUCT	TOTAL KETOSTEROIDS		PER CENT OF GRAVIMETRIC
	GRAVIMETRIC	SPECTROPHOTOMETRIC[a]	
Conj. estrogens, tablets	1.36	1.20	88
	0.91	0.73	80
	1.31	1.09	83
	1.41	1.21	86
	1.36	1.22	90
	0.80	0.75	94
	1.40	1.18	84
	1.35	1.23	91
	0.33	0.32	97
	0.77	0.69	90
	1.42	1.20	85
	0.95	0.77	81
	0.41	0.34	83
	1.30	1.23	95
	0.48	0.36	75
	0.80	0.77	96
	0.92	0.89	96
	0.53	0.45	85
	0.76	0.57	75
	0.43	0.31	72
	0.45	0.31	69
	0.78	0.75	96
Conj. estrogens, powder	17.7	16.0	90
	19.7	17.5	89
	16.7	14.1	84
		Average:	87

[a] Total of estrone, equilin, and equilenin, determined by the colorimetric or infrared procedure.
[b] Free estrogens: expressed in mg/cc or capsule. Conjugated estrogens: expressed in mg/tablet or gram powder.

(1) That the two determinations for ketosteroids (gravimetric and colorimetric) be combined.

(2) That the word "(gravimetric)" in the last two lines of page 81 be changed to "(Colorimetric)".

(3) That the two sentences at the end of the first paragraph on page 83, beginning with "Dry in a vacuum. . ." to end of paragraph, be deleted, and that the sentence "Weigh and prepare an alcoholic solution of the ketosteroids from the residue to contain 90–120 micrograms of ketosteroids per ml." be inserted in their place.

(4) That the sentence in the middle of page 83, beginning with "(7)", be deleted.

(5) That the following footnote be placed at bottom of page 83:

"Optional—Dry in a vacuum dessicator for semiquantitative estimate of ketosteroid weight."

(6) That the title SAMPLE SOLUTION and the accompanying sentence on page 84 be deleted.

REFERENCES

(1) SCHURMAN, I., *This Journal*, **34**, 581 (1951).
(2) CAROL, J., MOLITOR, J. C., and HAENNI, E. O., *J. Am. Pharm. Assoc.*, **37**, 173 (1948).

No reports were given on amobarbital sodium and secobarbital sodium; diphenhydramine and tripelennamine hydrochlorides; methylene blue; and synthetic estrogens.

REPORT ON MISCELLANEOUS DRUGS

By I. SCHURMAN (Food and Drug Administration, Department of Health, Education, and Welfare, Chicago 7, Ill.), *Referee*

RECOMMENDATIONS*

The Associate Referee on Preservatives and Bacteriostatic Agents in Ampul Solutions has resigned from the Food and Drug Administration, and thus no report was given on this subject. The Referee recommends that the subject be reassigned.

No reports were received on the following topics: mercury compounds, organic iodides and separation of halogens, and alkali metals. The Referee recommends that these topics be continued.

The Referee concurs with the recommendation for the adoption of the tables given in the Associate Referee's report for the identification of various drugs by their microscopic crystallographic properties.

REPORT ON MICROSCOPIC TESTS FOR ALKALOIDS AND SNYTHETICS

By WILLIAM V. EISENBERG (Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D.C.), *Associate Referee*

At the October, 1952 meeting the Associate Referee presented a table for the identification of crystalline sulfonamides and barbiturates by

* For reportof Subcommittee B and action of the Association, see *This Journal*, **37**, 66, 67 (1954).

TABLE 1.—*Optical-crystallographic properties of some crystalline drugs*[a]

COMPOUND	α	β	γ	OPTIC SIGN	EXTINCTION	ELONGATION	2 V	REMARKS
Antihistamines								
Antergan Hydrochloride	1.587	1.635	1.734	+				Short prisms. Inclined figs.
Anthallan Hydrochloride	1.505	1.585	1.617		p	−		Small rods & irregular fragments. No figs.
Bromothen Hydrochloride	1.617	1.654	1.734		i	+		Very small rods. Inclined figs. occas'l
Chlorcyclizine Monohydrochloride (Di-Paralene HCl)	1.590	1.610	1.665	+				Thin platy fragments. Op. Ax. figs. common
Chlorcyclizine Dihydrochloride (Perazil Dihydrochloride)	1.610	1.660	1.665	−	p	+	very small	Short rods & thin 6-sided plates. Op. Ax. figs. common
Chlorprophenpyridamine Maleate (Chlor-Trimeton Maleate)	1.533	n_i1.668	sl<1.734					Box-like prisms & irregular fragments. Figs. infrequent
Chlorothen Citrate (Tagathen)	1.583	1.603	1.645					Minute plates & shreds. Op. Ax. figs. rare
Chlorothen Hydrochloride	1.553	1.625	>1.734					Massive fragments, some rectangular. Op. Ax. figs. occas'l
Dimenhydrinate (Dramamine) (Unsatisfactory for optical-crystallographic study.)								Platy mat'l & rods
Diphenhydramine Hydrochloride (Benadryl HCl)	1.602	1.625	1.630	−	p	−		6-sided plates
p-Fluorobenzyl (D.P.E.) Hydrochloride	1.585	1.600	1.668	+	p	−	large	Rods & square plates. Op. Ax. figs. occas'l
Linadryl Hydrochloride	1.577	1.631	1.672	−	p,i	+		Elongated 6-sided & irregular fragments. Figs. rare
Methaphenilene Hydrochloride (Diatrine HCl)	1.604	1.675	1.733	−				Elongated 6-sided rods with obtuse ends. Op. Ax. figs. frequent
Methapyrilene Hydrochloride (Histadyl, Thenylene HCl)	1.588	1.654	>1.695 but <1.734	−				Thick hexagonal plates. Inclined Op. Ax. figs. frequent
Phenergan Hydrobromide	1.667	1.675	>1.733	+			small	Massive prisms, elongated or short & stubby
Phenergan Hydrochloride	1.617	1.691	1.733	−	p	+		Rods & irregular fragments
Pyrilamine Maleate (Unsatisfactory for optical-crystallographic study.)								Rods & irregular fragments
Pyrrolazote Hydrochloride	1.690		1.737					Stout prismatic forms. No figs.

[a] Abbreviations: Bx. Ac. =Acute bisectrix; Bx.Ob. =Obtuse bisectrix; fig. =figure; i =inclined; n_i =intermediate index; Op. Ax. =Optic Axis; p =parallel; s =symmetrical.

TABLE 1—(*continued*)

COMPOUND	α	β	γ	OPTIC SIGN	EXTINCTION	ELONGATION	2 V	REMARKS
Thenfanil Hydrochloride	1.590		1.680				large	Square plates & stubby prisms. Inclined figs. common
Thonzylamine Hydrochloride (Neohetramine HCl)	1.612	1.679	1.691	−	p	+		Rods & platy material
Tripelennamine Hydrochloride (Pyribenzamine HCl)	1.580	1.655	1.705	−				Rectangular plates & prisms from water. Op. Ax. figs. common
Barbiturates								
Allylbarbituric acid (5-allyl-5-isobutylbarbituric acid)	1.508	n_i1.521	1.577	+	p	+	moderate	Rods and plates
Alphenal (5-allyl-5-phenylbarbituric acid)	1.551	1.578	1.645	+	p	−	67°	Op. Ax. fig. common
Alurate (5-allyl-5-isopropylbarbituric acid)	1.522	—	1.602					Both n's common
Amytal (5-isoamyl-5-ethylbarbituric acid)	1.467	—	1.539		p	+		Both n's common
Amytal Sodium	(n) 1.505	—	—	Isotropic				
Barbital (diethylbarbituric acid)	1.445	1.548	1.580					All n's common
Barbital Sodium	1.512		1.615			+		Both n's common
Butallylonal [5-(2-bromallyl)-5-sec-butylbarbituric acid]	1.524	1.577	1.603	−	p	+	moderate	Rosettes of tiny rods and blades. Bx. Ac. figs. occas'l
Butethal (5-butyl-5-ethylbarbituric acid)	1.454	1.518	1.556	−	i		large	Rods & needles. Op. Ax and Bx. Ac. figs. common
Cyclobarbital (Phanodorn)(5-ethyl-5-cyclohexenylbarbituric acid)	1.515	1.546	1.621	+		±	69°	Bx. Ac. & Bx. Ob. figs. common
Cyclopal (5-cyclopentenyl-5-allylbarbituric acid)	1.520	1.575	1.626	−		−	85°	Bx. Ac. fig. common
Delvinal (5-ethyl-5-(1-methyl-1-butenyl) barbituric acid)	1.506	1.544	1.672	+	p	−	61°	Bx. Ac. fig. common
Dial (5,5-diallylbarbituric acid)	1.516	1.572	1.625	−	s		large	Op. Ax. fig. common
Hexethal (Ortal) (5-ethyl-5-*n*-hexylbarbituric acid)	1.473	1.519	1.549	−		−	76°	Bx. Ac. fig. common
Hexobarbital (Evipal) (5-cyclohexenyl-1,5-dimethylbarbituric acid)	1.546	1.608	1.634	−	p	+	64°	Bx. Ac. & Op. Ax. figs. common
Mephobarbital (Mebaral) (5-ethyl-1-methyl-5-phenylbarbituric acid)	1.594	1.610	1.651	+	p	−	65°	Bx. Ac. fig. common
Pentobarbital [5-ethyl-5-(1-methylbutyl) barbituric acid]	1.465	—	1.565	−	i		very large	———
Pentobarbital Sodium	1.477	—	1.523					———
Phenobarbital (5-ethyl-5-phenylbarbituric acid)	1.557	1.620	1.667		p	−		β very common

TABLE 1—(*continued*)

COMPOUND	α	β	γ	OPTIC SIGN	EXTINCTION	ELONGATION	2 V	REMARKS
Phenobarbital Sodium (unstable)								
Secobarbital (Seconal) [5-allyl-5-(1-methylbutyl barbituric acid]	1.487	1.557	1.563	−	p	+	31°	Bx. Ac. fig. common
Secobarbital Sodium	1.490	n_i1.500	1.525					n_i & γ common
Sigmodal (5-*s*-amyl-5-β-bromallylbarbituric acid)	1.519	1.583	1.634	−		+	80°	Bx. Ac. fig. common
Sulfonamides								
Sulfacetamide	1.559	1.564	1.727	+	s		21°	
Sulfadiazine	1.596	1.675	1.830	+	p, i	±	76°	
Sulfadiazine[c]	1.615	1.663	>1.734		p	±		Rods
Sulfaguanidine	1.606	1.663	1.734					Op. Ax. fig.
Sulfaguanidine monohydrate	1.586	1.649	1.731	+	p, i	±	86°	
Sulfallantoin (sulfanilamide+allantoin—addition product)	1.513	1.590	>1.690 <1.733					Op. Ax. fig.
Sulfamerazine	1.568	1.657	1.687	−	p	±	58°	Bx. Ac. fig. all n's common
Sulfamerazine[c]	1.587		1.675					
Sulfamethazine	1.584	1.623	1.778					Rods
Sulfamidazole (sulfanilamide+sulfathiazole–double crystal[b])	1.661	1.678	>1.733	+	p	−	small	Bx. Ac. fig.
Sulfanilamide phase B (anhyd.)	1.555	1.672	1.85	+	p	−		Stable form com'l prepns
Sulfanilamide HCl	1.540	1.655	1.690		p	−		Rods
Sulfapyridine, Phase I	1.670	1.736	1.813	+	p, i	±	88°	Tabular to equant stable form com'l prepns
Sulfapyridine[c]	1.680	1.733	>1.733					Op. Ax. fig.
Sulfapyridine Sodium Monohydrate	1.590		1.700		p	−		
Sulfasuxidine	1.578	1.676	1.710	−	i		58°	Rods
Sulfathiazole, Phase I	1.674	1.685	>1.733	+			small	α & β common
Sulfathiazole, Phase II	1.598	1.741	1.780	−	p, i	±	52°	Lath shaped
Sulfathiazole[c]	1.695	n_i1.733	>1.733					
Sulfathiazole Sodium Sesquihydrate	1.596		1.621					
Sympathomimetic Amines								
d-Amphetamine Hydrochloride (Dexedrine HCl)	1.560	1.592	1.622	+	p, i		very large	Large plates & rods. Op. Ax. figs. occas'l
dl-Amphetamine Phosphate, dibasic	1.549	1.589	1.655	+			large	Small platy crystals. Bx. Ac. figs. common

[b] Equimolecular proportions.

[c] The second set of optical properties in each case represents intermediate data which are quite commonly found in some commercial samples. They probably represent a hydrous form or merely a different common orientation of the crystal.

TABLE 1—(*continued*)

COMPOUND	α	β	γ	OPTIC SIGN	EXTINCTION	ELONGATION	2 V	REMARKS
d-Amphetamine Sulfate (Dexedrine SO_4)	1.505	1.545	1.608	+		−	small	6–8-sided plates
dl-Amphetamine Sulfate (Benzedrine SO_4)	1.520	1.535	1.580	+		−	very sm.	
Benzazoline Hydrochloride (Priscoline HCl)	1.586	1.604	1.703	+	i			6-sided plates. Inclined Op. Ax. figs. common
d-Desoxyephedrine Hydrochloride (Methamphetamine HCl)	1.530	1.537	1.615	+				Irregular fragments. Op. Ax. figs. frequent
dl-Desoxyephedrine Hydrochloride	1.535		1.620		p	−		Small 6-sided platy or rod-like crystals. No figs.
dl-Ephedrine Hydrochloride (Racephedrine HCl)	1.570	1.608	1.630	−				Irregular fragments. Occasional Op. Ax. figs.
l-Ephedrine Hydrochloride	1.530	1.603	1.638	−	p	−	70°	Elongated prisms & rods
l-Ephedrine Sulfate	1.540	1.565	1.587	+	p		large	6-sided plates & rods
dl-Epinephrine	1.551	1.599	1.736	+	p	−	moderate	Thin, blade-like, 6-sided crystals in rosettes. Bx. Ac. figs. common
l-Epinephrine	1.551	1.599	1.736	+	p	−	moderate	Rosettes of thin, blade-like, 6-sided crystals. Bx. Ac. figs. common
Hydroxyamphetamine Hydrobromide (Paredrine HBr)	1.560	1.680	1.734	−				Irregular fragments. Inclined Op. Ax. fig. frequent
Naphazoline Nitrate	1.560	1.619	>1.740	+	s, i			6-sided plates & irregular fragments. Bx. Ac. figs. common
Phenylpropylmethylamine Hydrochloride (Vonedrine HCl)	1.577		1.603		p	−		Small rod-like fragments. No figs.
Supriphen Hydrochloride	1.507	1.604	1.668	−	p	±		Rectangular rods. Bx. Ob. figs. common
dl-Synephrine base (Desoxyepinephrine)	1.546	1.604	1.725	+	s, i		large	Platy crystals, often diamond-shaped. Op. Ax. figs. common
Synephrine Hydrochloride	1.549	1.605	1.664	+	p, i		large	Large plates. Bx. Ac. figs. frequent
dl-Synephrine (+) Tartrate (Neutral Salt)	1.516	n_i1.620	1.689	+	i	+	large	Rods & plates. Partial Op. Ax. figs. common
Tuaminoheptane Sulfate (Tuamine Sulfate)	1.458_ω		1.468_ϵ	+	p			Irregular-shaped plates & fibrous flakes. Figs. frequent
Veritol Sulfate (Isodrine Sulfate)	1.516	1.552	1.645	+	s	+		Rhombohedral or 6-sided plates

TABLE 2.—*Determinative table for drugs, arranged according to ascending value of the lowest index*[a]

α	β	γ	COMPOUND
			Antihistamines
1.505	1.585	1.617	Anthallan Hydrochloride
1.533	n_i1.668	sl<1.735	Chlorphenpyridamine Maleate (Chlor-Trimeton Maleate)
1.553	1.625	>1.734	Chlorothen Hydrochloride
1.577	1.631	1.672	Linadryl Hydrochloride
1.580	1.655	1.705	Tripelennamine Hydrochloride (Pyribenzamine HCl)
1.583	1.603	1.645	Chlorothen Citrate (Tagathen)
1.585	1.600	1.668	p-Fluorobenzyl (D.P.E.) Hydrochloride
1.587	1.635	1.734	Antergan Hydrochloride
1.588	1.654	>1.695 but <1.734	Methapyrilene Hydrochloride (Histadyl, Thenylene HCl)
1.590	1.610	1.665	Chlorocyclizine Monohydrochloride (Di-Paralene HCl)
1.590		1.680	Thenfanil Hydrochloride
1.602	1.625	1.630	Diphenhydramine Hydrochloride (Benadryl HCl)
1.604	1.675	1.733	Metaphenilene Hydrochloride (Diatrine HCl)
1.610	1.660	1.665	Chlorcyclizine Dihydrochloride (Perazil di-HCl)
1.612	1.679	1.691	Thonzylamine Hydrochloride (Neohetramine HCl)
1.617	1.654	1.734	Bromothen Hydrochloride
1.617	1.691	1.733	Phenergan Hydrochloride
1.667	1.675	>1.733	Phenergan Hydrobromide
1.690		1.737	Pyrrolazote Hydrochloride
			Barbiturates
1.445	1.548	1.580	Barbital (Diethylbarbituric acid)
1.454	1.518	1.556	Butethal (Neonal)
1.465		1.565	Pentobarbital (Nembutal)
1.467		1.539	Amobarbital (Amytal)
1.473	1.519	1.549	Hexethal (Ortal)
1.476	1.573	1.610	Probarbital (Ipral)
1.477		1.523	Pentobarbital Sodium (Nembutal Sodium)
1.487	1.557	1.563	Secobarbital (Seconal)
1.490	n_i1.500	1.525	Secobarbital Sodium (Seconal Sodium)
(n) 1.505	Isotropic		Amobarbital Sodium (Amytal Sodium)
1.506	1.544	1.672	Vinbarbital (Delvinal)
1.508	n_i1.521	1.577	Allylbarbituric Acid (Sandoptal)
1.512		1.615	Barbital Sodium
1.515	1.546	1.621	Cyclobarbital (Phanodorn)
1.516	1.572	1.625	Diallylbarbituric Acid (Dial)

TABLE 2—(*continued*)

α	β	γ	COMPOUND
1.519	1.583	1.634	Sigmodal
1.520	1.575	1.626	Cyclopal
1.552		1.602	Aprobarbital (Alurate)
1.524	1.577	1.603	Butallylonal (Pernoston)
1.546	1.608	1.634	Hexobarbital (Evipal)
1.551	1.578	1.645	Alphenal
1.557	1.620	1.667	Phenobarbital (Luminal)
1.594	1.610	1.651	Mephobarbital (Mebaral)
		Sulfonamides	
1.513	1.590	>1.690 but <1.733	Sulfallantoin
1.540	1.655	1.690	Sulfanilamide HCl
1.555	1.672	1.85	Sulfanilamide, Phase B (anhydrous)
1.559	1.564	1.727	Sulfacetamide
1.568	1.657	1.687	Sulfamerazine
1.578	1.676	1.710	Succinylsulfathiazole (Sulfasuxidine)
1.584	1.623	>1.778	Sulfamethazine
1.586	1.649	1.731	Sulfaguanidine Monohydrate
1.587		1.675	Sulfamerazine
1.590		1.700	Sulfapyridine Sodium Monohydrate
1.596		1.621	Sulfathiazole Sodium Sesquihydrate
1.596	1.675	1.830	Sulfadiazine
1.598	1.741	1.780	Sulfathiazole, Phase II
1.606	1.663	1.734	Sulfaguanidine
1.615	1.663	>1.734	Sulfadiazine
1.661	1.678	>1.733	Sulfamidazole
1.670	1.736	1.813	Sulfapyridine, Phase I
1.674	1.685	>1.733	Sulfathiazole, Phase I
		Sympathomimetic Amines	
1.458_{ω}		1.468_{ϵ}	Tuaminoheptane Sulfate (Tuamine Sulfate)
1.505	1.545	1.608	d-Amphetamine Sulfate (Dexedrine SO_4)
1.507	1.604	1.668	Supriphen Hydrochloride
1.516	1.552	1.645	Veritol Sulfate (Isodrine Sulfate)
1.516	n_i1.620	1.689	dl-Synephrine (+) Tartrate (Neutral Salt)
1.520	1.535	1.580	dl-Amphetamine Sulfate (Benzedrine SO_4)
1.530	1.537	1.615	d-Desoxyephedrine Hydrochloride (Methamphetamine HCl)
1.530	1.603	1.638	l-Ephedrine Hydrochloride
1.535		1.620	dl-Desoxyephedrine Hydrochloride
1.540	1.565	1.587	l-Ephedrine Sulfate
1.546	1.604	1.725	dl-Synephrine base (Desoxyepinephrine)
1.549	1.589	1.655	dl-Amphetamine Phosphate, dibasic
1.549	1.605	1.664	Synephrine Hydrochloride

TABLE 2—(*continued*)

α	β	γ	COMPOUND
1.551	1.599	1.736	dl-Epinephrine
1.551	1.599	1.736	l-Epinephrine
1.560	1.592	1.622	d-Amphetamine Hydrochloride (Dexedrine HCl)
1.560	1.619	>1.740	Naphazoline Nitrate
1.560	1.680	1.734	Hydroxyamphetamine Hydrobromide (Paredrine HBr)
1.570	1.608	1.630	dl-Ephedrine Hydrochloride (Racephedrine HCl)
1.577		1.603	Phenylpropylmethylamine Hydrochloride (Vonedrine HCl)
1.586	1.604	1.703	Benzazoline Hydrochloride (Priscoline HCl)

[a] See Table 1, p. 110, for symbols.

means of their microscopic-crystallographic properties. During the past year, with the collaboration of Dr. Albert H. Tillson, the Associate Referee has compiled a table for the identification of antihistaminic crystalline drugs and crystalline sympathomimetic amines. Some sulfonamides and barbiturates in addition to those presented last year have been included. The data in the table have been compiled from the literature and unpublished data in the files of the Microanalytical Laboratory of the U. S. Food and Drug Administration.

The new identification tables of drugs and the additions to the tables presented last year are listed above. For ready use in the identification of unknown substances, the tables have also been organized in ascending order of the alpha index.

For next year's work, the Associate Referee recommends* the study and preparation of determinative tables of estrogenic and androgenic crystalline drugs.

No reports were given on glycols and related products; mercury compounds; organic iodides and separation of halogens; and preservatives and bacteriostatic agents in ampul solutions.

* For report of Subcommittee B and action of the Association, see *This Journal*, 37, 66 (1954).

REPORT ON PLANTS

By E. J. MILLER (Michigan Agricultural Experiment Station, East Lansing, Mich.), *Referee*

Several of the Associate Referees have submitted reports of their activities during the past year.

Eunice J. Heinen, Associate Referee on Sodium in Plants, has submitted data on sodium in a variety of tissues, obtained by the A.O.A.C. gravimetric method and by a flame photometric procedure.

Carroll L. Hoffpauir, Associate Referee on Starch in Plants, reported results of a collaborative study of the revised procedure on which he reported last year.

Kenneth T. Williams, Associate Referee on Sugar in Plants, together with Earl F. Potter, reported results of a second collaborative study in which the ion-exchange resin and official lead acetate methods of clarification of plant extracts for sugar analysis were compared.

No reports were received for boron, carotene, cobalt, copper, potassium, and zinc. It is hoped that these subjects can be reported on next year.

ACKNOWLEDGMENT

The General Referee wishes to extend his thanks to the Associate Referees for their cooperation during the past year.

RECOMMENDATIONS

It is recommended*—

(1) That the Associate Referees continue their investigations on the following subjects: boron, carotene, copper and cobalt, sampling, sodium, starch, sugar, and zinc.

(2) That the study of methods for sodium in plant materials be continued with special attention to solvents for its extraction, and to flame photometric procedures.

(3) That methods for sodium in plants be studied collaboratively.

(4) That the anthrone procedure for starch in plant materials be studied further.

(5) That the ion-exchange resin procedure for clarification of plant extracts containing sugars as described by the Associate Referee, *This Journal*, **36**, 401 (1953), be adopted, first action, for addition to the method for sugars, **6.48** (see *Changes in Methods*, **37**, 82 (1954).

* For report of Subcommittee A and action of the Association, see *This Journal*, **37**, 63 (1954).

REPORT ON SODIUM IN PLANTS*

By EUNICE J. HEINEN (Michigan Agricultural Experiment Station, East Lansing, Mich.), *Associate Referee*

Last year the Associate Referee submitted a report (1) in which was discussed a preliminary study of methods for determining sodium in plant materials. That report included values for sodium in several plant tissues obtained by use of the A.O.A.C. magnesium uranyl acetate method, **6.21,** (2) and the flame photometer. It was recommended that this comparative study be continued, with the inclusion of the analysis of a variety of plant materials; hence, the work during the past year has dealt primarily with a continuation of this investigation. A study was also made of sodium values obtained for these materials by use of the flame photometer, both with and without the prior removal of calcium from the solution.

All samples were given the same preliminary treatment for each of the methods. This included treating separately weighed portions with 1:10

TABLE 1.—*Comparison of sodium values obtained by the magnesium uranyl acetate and flame photometric methods*

PLANT TISSUE ANALYZED	PER CENT SODIUM FOUND[a]		
	A.O.A.C. MAGNESIUM URANYL ACETATE METHOD	FLAME PHOTOMETER METHOD	
		CALCIUM REMOVED	CALCIUM NOT REMOVED
Alfalfa hay	.029	.025	.030
Barley grain	.006	.008	—
Brome hay	.020	.017	.019
Celery plants	.98	1.08	1.06
Corn grain	.64	.64	.65
Ladino clover hay	.44	.46	.47
Oat grain	.012	.010	.018
Onion bulbs	.030	.029	.031
Pea grain	.045	.042	.065
Pea plants no. 1	.051	.054	.054
Pea plants no. 2	.021	.038	.032
Pea straw	.38	.44	.41
Potato tubers	.012	.011	.017
Red clover hay	.021	.017	.025
Reed canary grass	.015	.016	.015
Soybean grain	.009	.009	.014
Sugar beet pulp	.11	.13	.12
Timothy hay	.002	.005	.005
Wheat grain	.007	.007	.009

[a] Expressed on the air-dry basis.

* Published with the approval of the Director of the Michigan Agricultural Experiment Station as Journal Article No. 1549.

TABLE 2.—*Effect of certain anions on the flame photometric evaluation of sodium*

P.P.M. Na	FLAME PHOTOMETER READINGS ON INTERNAL STANDARD DIAL				
	soln NaCl in distd H_2O	soln NaCl in 0.01 *N* H_2SO_4	soln NaCl in 0.036 *N* H_3PO_4	soln NaCl in 0.35 *N* HCl	NaCl in soln 0.02 *N* in $(NH_4)_2C_2O_4$ and 0.35 *N* in NH_4Cl
0	0.4	0.4	0.4	0.4	2.3
2	12.4	—	—	12.4	12.4
4	25.2	—	—	25.2	24.8
5	29.5	29.5	29.5	—	—
7	41.7	—	—	41.0	41.0
10	54.7	54.7	54.7	54.7	55.5
12	70.2	—	—	70.1	70.2
16	80.6	82.0	79.6	81.8	82.0

sulfuric acid, drying on a steambath followed by several hours in an oven at 100–105°C., ashing in an electric muffle furnace at 500°C., and digesting the ash with hydrochloric acid. A Perkin-Elmer flame photometer, Model 52A, was used for making the photometric measurements, with acetylene gas as the fuel. From one set of solutions the calcium was removed as the oxalate as directed in *Official Methods of Analysis*, **6.12** (2). In both flame photometric procedures, 25 p.p.m. of lithium chloride was used as the internal standard. The top standard used throughout was 20 p.p.m. of sodium. The results obtained in this study are given in Table 1.

It will be noted that, in general, there is fairly good agreement in values obtained by the gravimetric and the two flame photometric methods. However, in a few instances, higher sodium values were obtained by the flame photometer when calcium was not previously removed. The interference of calcium in the flame photometric evaluation of sodium was mentioned by Toth, *et al.* (3). It was further investigated by Seay, *et al.* (4), who have found that the presence of calcium gives high values for sodium and that this calcium interference is greatest in the analysis of plant tissues containing very small quantities of sodium and large quantities of calcium.

During the study it was also thought advisable to determine if certain anions present in the reagents, as well as the phosphate ion in a quantity exceeding that found in normal plant ash, would have any effect on the sodium values obtained by use of the flame photometer. The results from this limited investigation are given in Table 2.

The results show that the anions in the quantities used did not affect the sodium values obtained in the flame photometer procedure.

RECOMMENDATIONS

It is recommended†—

† For report of Subcommittee A and action of the Association, see *This Journal*, **37**, 63 (1954).

(1) That the study be continued with special attention to flame photometric procedures.

(2) That a collaborative study be conducted of methods for determining sodium in plant materials.

(3) That the extraction of sodium from plant tissues by use of extracting solutions be given some attention.

REFERENCES

(1) HEINEN, E. J., *This Journal*, **36**, 392 (1953).
(2) *Official Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Box 540, Benjamin Franklin Station, Washington 4, D. C., 1950.
(3) TOTH, S. J., PRINCE, A. L., WALLACE, A., and MIKKELSEN, D. S., *Soil Sci.*, **66**, 459 (1948).
(4) SEAY, W. A., ATTOE, O. J., and TRUOG, E., *ibid.*, **71**, 83 (1951).

REPORT ON STARCH IN PLANTS

COLLABORATIVE STUDY OF ANTHRONE PROCEDURE

By CARROLL L. HOFFPAUIR (Southern Utilization Research Branch,* New Orleans 19, La.), *Associate Referee*

Previous reports have described the application of the anthrone–sulfuric acid reagent to the determination of starch in plant materials (1, 2). This method has been studied collaboratively in accordance with last year's recommendation.

Samples of alfalfa leaf meal, buckwheat leaves, and peanut meal, which had been used in previous investigations (3), were again selected for study because they offer a wide range of possible interferences. All three samples were air-dried plant tissue and consequently afforded a rigid test of the extraction procedure. They all contained considerable protein and nonstarch carbohydrates, both of which interfere unless removed by the purification steps of the method. In addition, the alfalfa meal was quite low in starch, while the peanut meal contained a moderate amount, and the buckwheat leaves had a fairly high starch content for this type of material.

The samples were ball-milled to pass a 100 mesh sieve and allowed to come to moisture equilibrium before distribution. The collaborators were requested to report for each sample two independently determined sets of duplicate values for starch by the modified procedure (2), a copy of which was supplied. Moisture values, obtained by drying 5 g samples for two hours at 101°C. in a forced draft oven, were also requested so the data

* One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Service, U. S. Department of Agriculture.

could be calculated to a dry basis. Five of the six collaborators returned complete reports; the sixth, however, encountered difficulty due to deterioration of anthrone and obtained only one set of duplicates. Since these values were quite erratic, they are not included in this report.

TABLE 1.—*Starch content of alfalfa, buckwheat leaves, and peanut meal (moisture-free basis)*

ANALYST	ALFALFA	BUCKWHEAT LEAVES	PEANUT MEAL
	per cent	*per cent*	*per cent*
1	1.36	9.1	8.0
	0.95	9.7	8.6
	1.00	10.1	7.3
	1.36	11.2	6.4
Av:	1.17	10.0	7.6
2	0.92	9.8	7.7
	1.13	9.6	7.7
	1.02	10.0	6.9
	1.08	—	7.6
	1.40	9.4	7.5
	1.13	8.5	7.8
Av:	1.11	9.5	7.5
3	0.43	9.7	7.5
	0.20	9.4	8.7
	0.10	10.1	8.0
	0.15	10.1	7.9
	0.58	10.9	8.1
	1.91	10.2	8.1
Av:	0.56	10.1	8.0
4	1.00	10.9	6.4
	0.73	10.2	8.0
	0.78	11.9	6.6
	0.97	12.3	6.1
Av:	0.87	11.3	6.8
5	0.69	12.4	7.0
	0.36	12.2	7.2
	0.31	10.6	6.4
	0.32	10.9	6.3
Av:	0.46	11.5	6.7
Total Av:	0.83	10.5	7.3

The values obtained by the collaborators (Table 1) show unsatisfactory precision. Although the method is rather complex, the first set of duplicates agree as well among individual determinations and among analysts as the succeeding sets. It appears, therefore, that the poor precision cannot be accounted for by the unfamiliarity of the analysts with the procedure.

The average values for all of the materials are appreciably higher than the corresponding values obtained in previous studies (2, 3) in which the same samples were analyzed by several other procedures. This suggests that interfering constituents were not completely eliminated in the purification of the starch prior to final colorimetric evaluation.

The comments of the collaborators indicated some difficulty in obtaining a satisfactory quality of anthrone, and the possibility of using carbothrone, a stabilized anthrone, was suggested. However, most of the collaborators seemed to consider that the method offered some promise.

It is recommended* that the anthrone procedure be submitted to further critical study.

ACKNOWLEDGMENT

The Associate Referee wishes to thank each of the following six collaborators for the interest and cooperation which made this report possible.

E. J. Benne and Cornelia Key, Michigan State College, East Lansing, Mich.
A. L. Potter, Jr., Western Utilization Research Branch, Albany, Calif.
R. B. Carson and I. Hoffman, Department of Agriculture, Ottawa, Ontario, Canada.
C. R. Joiner and Mary A. McEniry, Food and Drug Administration, St. Louis, Mo.
C. L. Ogg, Eastern Utilization Research Branch, Philadelphia, Pa.
Elizabeth R. McCall, Southern Utilization Research Branch, New Orleans, La.

REFERENCES

(1) HOFFPAUIR, C. L., *This Journal*, **35**, 398 (1952).
(2) ———, *ibid.*, **36**, 400 (1953).
(3) ———, *ibid.*, **34**, 698 (1951).

REPORT ON SUGARS IN PLANTS

By KENNETH T. WILLIAMS, *Associate Referee*, and EARL F. POTTER
(Western Utilization Research Branch,† Albany 6, Calif.)

The present report deals with the second collaborative study in which the ion-exchange resin and official lead acetate methods of clarification of

* For report of Subcommittee A and action of the Association, see *This Journal*, **37**, 63 (1954).
† Agricultural Research Service, U. S. Department of Agriculture.

TABLE 1.—*Results expressed as mg of dextrose in a 5 ml aliquot of clarified solution taken for the determination of sugar*[a]

COLLABORATOR	CLARIFIED WITH LEAD	CLARIFIED WITH ION-EXCHANGE RESINS
	Asparagus	
A	1st[b] 1.07, 1.09 2nd 1.05, 1.07–1.07 Av.	1st[b] 1.02, 1.02 2nd 1.03, 1.01–1.02 Av.
B	1st 1.08, 1.09 2nd 1.08, 1.08–1.08 Av.	1st 1.07, 1.05 2nd 1.06, 1.05–1.06 Av.
C	1st 1.04 2nd 1.03 1.04 Av.	1st 0.97 2nd 1.01 0.99 Av.
E. F. Potter	1st 0.99, 0.99 2nd 0.98, 0.98–0.99 Av.	1st 0.95, 0.96 2nd 0.96, 0.95–0.96 Av.
D	1st 0.92, 0.92 2nd 0.92, 0.91–0.92 Av.	1st 0.97, 0.94 2nd 0.93, 0.94–0.95 Av.
	Dehydrated Cabbage	
A	1st 1.31, 1.31 2nd 1.31, 1.34–1.32 Av.	1st 1.20, 1.18 2nd 1.20, 1.20–1.20 Av.
B	1st 1.26, 1.26 2nd 1.27, 1.26–1.26 Av.	1st 1.18, 1.19 2nd 1.18, 1.18–1.18 Av.
C	1st 1.24 2nd 1.24 1.24 Av.	1st 1.19 2nd 1.21 1.20 Av.
E. F. Potter	1st 1.22, 1.22 2nd 1.22, 1.22–1.22 Av.	1st 1.14, 1.15 2nd 1.16, 1.16–1.15 Av.
D	1st 1.20, 1.20 2nd 1.20, 1.19–1.20 Av.	1st 1.16, 1.13 2nd 1.20, 1.14–1.16 Av.
	Mixed Lawn Grass	
A	1st 1.14, 1.14 2nd 1.17, 1.13–1.15 Av.	1st 0.96, 1.00 2nd 1.01, 1.02–1.00 Av.
B	1st 1.10, 1.11 2nd 1.12, 1.12–1.11 Av.	1st 1.07, 1.07 2nd 1.08, 1.10–1.08 Av.
C[c]	1st 1.17 2nd 1.18 1.18 Av.	1st 1.26 2nd 1.27 1.27 Av.
E. F. Potter	1st 1.20, 1.18 2nd 1.20, 1.18–1.19 Av.	1st 1.01, 1.01 2nd 1.00, 1.00–1.01 Av.
D	1st 1.01, 1.05 2nd 1.03 Av.	1st 1.10, 1.02 2nd 1.08, 1.06–1.07 Av.

[a] All values were taken from standard curves prepared by the collaborators.
[b] Aliquot of A.
[c] These solutions were centrifuged instead of the regular filtration through Celite.

TABLE 1—(*continued*)

COLLABORATOR	CLARIFIED WITH LEAD		CLARIFIED WITH ION-EXCHANGE RESINS	
	Strawberries			
A	1st	1.08, 1.07	1st	1.03, 1.03
	2nd	1.07, 1.08–1.08 Av.	2nd	0.98, 1.00–1.01 Av.
B	1st	1.13, 1.13	1st	1.13, 1.12
	2nd	1.12, 1.12–1.13 Av.	2nd	1.12, 1.12–1.12 Av.
C	1st	1.11	1st	1.10
	2nd	1.11 1.11 Av.	2nd	1.12 1.11 Av.
E. F. Potter	1st	1.04, 1.04,	1st	1.09, 1.09
	2nd	1.04, 1.03–1.04 Av.	2nd	1.09, 1.09–1.09 Av.
D	1st	1.00, 1.01	1st	1.01, 1.01
	2nd	1.00, 0.99–1.00 Av.	2nd	1.01, 1.01–1.01 Av.

plant extracts for sugar analysis were compared. The results are in agreement with last year's collaborative study and previous progress reports (1).

MATERIALS FURNISHED COLLABORATORS

(a) *Plant extracts.*—Four plant extracts prepared with hot 80% alcohol.
(b) *Celite analytical filter aid.*[1]—(Johns-Manville).
(c) *Cation exchange resin.*—Amberlite IR-120 (Fisher Scientific Co.).
(d) *Anion resin.*—Duolite A-4 (Chemical Process Co., Redwood City, Calif.).
(e) *Dextrose.*—U. S. Bureau of Standards.

INSTRUCTIONS TO COLLABORATORS

The instructions were essentially the same as those furnished last year (1).

DISCUSSION

Four plant materials, very different from each other and from those used last year, were chosen for the collaborative study of the ion-exchange method of clarification. The same design of experiment as last year was used to give a direct comparison of the official neutral lead acetate and the proposed ion-exchange methods.

The results of the second collaborative study show again that the proposed resin method removes the non-sugar reducing materials as well as the official neutral lead acetate method, and in fact is superior to the lead acetate method for some materials (see Table 1).

The authors believe that the resin clarification is accomplished with less effort on the part of the analyst. This is especially true when a large num-

[1] Mention of commerical products does not imply that they are endorsed or recommended by the Department of Agriculture over others not mentioned.

ber of samples are to be clarified at one time. Some collaborators volunteered the opinion that the ion-exchange procedure was easier than the lead acetate procedure; none reported it more difficult.

The filtration of the grass solution through Celite was reported by some collaborators to be difficult. This is in line with our experience. The difficulty resulted from the evaporation of the large volume necessary to provide a sufficient quantity of sample for replicate resin and lead clarification procedures.

COLLABORATORS

The following is a list of collaborators (the order has no bearing on the letter designation used above):

C. H. Van Etten, Northern Utilization Research Branch, Peoria, Ill.
E. J. Benne and Eunice Heinen, Michigan State College, East Lansing, Mich.
Elizabeth R. McCall, Southern Utilization Research Branch, New Orleans, La.
C. L. Ogg and Mrs. Gaspar, Eastern Utilization Research Branch, Philadelphia, Pa.

RECOMMENDATION

It is recommended* that the ion-exchange resin method of clarification be adopted, first action, for addition to the method for sugars, **6.48.**

REFERENCE

(1) WILLIAMS, K. T., and POTTER, E. F., *This Journal*, **36**, 401 (1953); **35**, 402 (1952); **34**, 700 (1951); and **33**, 816 (1950).

No reports were given on boron, carotene, copper and cobalt, sampling, or zinc.

REPORT ON METHODS INVOLVING EMISSION SPECTROSCOPY

By W. T. MATHIS (Connecticut Agricultural Experiment Station, New Haven 4, Conn.), *Referee*

In the last report on this subject,[1] schemes for presenting spectrographic procedure were suggested. The description of the general principles of the successive steps of instrumentation and photometry was considered. The alternative is to describe specific techniques in detail. Since both of these modes of presentation possess merit, they have been used collectively in the suggested procedures submitted herewith. The preference for one over

* For report of Subcommittee A and action of the Association, see *This Journal*, 37, 63 (1954).
[1] *This Journal*, 36, 411 (1953).

the other depends upon the equipment available to the potential user.

The two methods described were selected from among several available procedures, for the following reasons: (*a*) Their scopes cover determination of major elements as well as the common minor elements in plant materials; (*b*) both methods have been used for several years and results have been found satisfactory in comparison with routine chemical operations; and (*c*) they illustrate the wide differences that can exist among procedures capable of producing satisfactory results.

Unfortunately, it has not been possible to follow the usual A.O.A.C. collaborative procedure for evaluation of these methods because of the inability of potential collaborators to meet the specific equipment requirements. It is believed, however, that comparative performance of the two methods is indicated with reasonable validity by data published in *This Journal*, **35**, 406 (1952). Methods 1[2] and 2[3] were used by laboratories 12 and 1, respectively, in that study. It is recommended* that methods 1 and 2, DC arc excitation and AC spark excitation, respectively, be adopted, first action. They are described in detail in *Changes in Methods*, **37**, 83 (1954).

Certain general considerations might supplement the detailed directions of Methods 1 and 2:

Instrumental Technique.—If neither of these methods can be followed in detail because of equipment limitations, or if the particular analytical problem involves determination of elements not within their scopes, the following procedure is recommended: Determine experimentally to the limits of the facilities available the potential of various electrode preparations and excitation conditions with relation to element detectability and general concentration requirements. If a set of conditions shows promise, make a preliminary check for reproducibility of line indices.

For determination of the very minute amounts of some of the elements present in plant material, preliminary chemical separation and concentration may be necessary. A satisfactory procedure, using 8-hydroxyquinoline, is described by Mitchell.[4] The trace element concentrate obtained may be combined with a suitable matrix and the subsequent treatment adapted to the basic conditions of the regular instrumental technique.

Selection of analysis lines on the basis of the intensity desired and the freedom from spectral interference by other elements is facilitated by preparing a spectrum of each component element at the average concentration level at which it occurs in the material to be analyzed. Align the spec-

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[2] Submitted by Alston W. Specht, now in charge of the spectrochemical work, Horticultural Crops Research Branch, Agricultural Research Service, Beltsville, Md. The technique is based in part upon the important pioneer work done at Beltsville by B.C. Brunstetter and A.T. Myers.

[3] Developed by W. T. Mathis, Connecticut Agricultural Experiment Station, New Haven, Conn.

[4] MITCHELL, R. L., *The Spectrographic Analysis of Soils, Plants and Related Materials*, Commonwealth Bureau of Soil Science, Harpenden, England, 1948.

* For report of Subcommittee B and action of the Association, see *This Journal*, **37**, 65 (1954).

tra collectively for comparison, preferably by exposure on the same film or plate.

Precision.—Standardize all conditions of the technique and determine the reproducibility of results by running a suggested twenty successive exposures on a sample of representative composition. For each element, calculate the standard deviation of a single exposure and divide by the square root of the number of individual exposures that will be averaged in practice to constitute a determination. From this estimate of the standard deviation of a single determination, calculate the coefficient of variation for each element.

The following upper limits are tentatively suggested for errors of precision in spectrographic determinations of plant materials. These limits are satisfactory in relation to other routine methods, or to practical requirements:

Coefficients of variation for K, Ca, and P, 5.0; Mg, Mn, Fe, Al, Na, and Cu, 10.0; B, 15.0.

Methods 1 and 2 are potentially capable of meeting these requirements.

Accuracy.—A precise technique is essential, but it is by no means the only factor involved in ultimate accuracy. The reliability and appropriateness of the standards and the judgment used in the reference procedure are of utmost importance. Failure in any of these respects can result in a serious calibration error with an otherwise satisfactory method.

Synthetic standards should be carefully prepared from analytical reagent grade chemicals, free from moisture contamination. Collective blanks should be run for minor and trace elements. Values assigned to natural standards should preferably be confirmed by more than one laboratory.

The necessity for matrix similarity between standard and sample, or for a closely controlled correction system for matrix differences, has been stressed in the methods and is again emphasized. Correction scales should be checked frequently against standards which closely match the particular types of plant materials being analyzed.

It should be kept in mind that the errors of precision of the technique apply to reference exposures as well as to the samples. For this reason, fiducial adjustments should be based upon as many reference exposures as it is convenient to include in each run of samples.

No report was given on tobacco.

No report was given on radioactivity.

TUESDAY—AFTERNOON SESSION

REPORT ON EXTRANEOUS MATERIALS IN FOOD AND DRUG PRODUCTS

By KENTON L. HARRIS (U. S. Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D.C.), *Referee*

The Referee concurs with the recommendations* of the Associate Referee on Extraneous Materials in Drugs, Spices, and Miscellaneous Products that the method for extraneous materials in ground capsicums (*This Journal*, **36**, 90 (1953) as amended in *This Journal*, **36**, 301 (1953)) be adopted, first action. The Referee also concurs with the recommendation that work be continued on the development of methods for the separation of filth elements from spices.

The Referee concurs with the Associate Referee on Extraneous Materials in Baked Products, Prepared Cereals, and Alimentary Pastes, who has suggested a new method to replace the present **35.28** (**b**), that method **35.28** (**a**) be reworded as described in the Associate Referee's report, and who recommends that this revised wording be adopted, first action. The Referee agrees that this change should be made and recommends that the new method **35.28** (**b**) as described in the Associate Referee's report be studied collaboratively during the coming year. The Associate Referee recommends that the title of section **35.32** and **35.33** be amended to read "Whole and Degerminated Corn Meal, Corn Grits, Rye Meal, Wheat Meal, Whole Wheat Flour, Farina, and Semolina." The Referee concurs in this recommendation. The changes suggested for section **35.33** require a rewording of this method as given by the Associate Referee, and the Referee recommends that this revised section **35.33** be adopted, first action. The Referee concurs in the Associate Referee's recommendation that sections **35.34** and **35.35** be deleted and that **35.36** be amended as follows:

"**35.36**(**a**) *Cream corn meal* (*corn flour*).—Proceed as directed in **35.29**(**a**)."

The Referee concurs in the recommendation that section **35.29** (**b**) be deleted.

The report of the Referee on Dairy and Egg Products consists of a progress report on the identification of dung and plant fragments in dairy products. It is recommended that this work be continued.

The Referee does not concur that the staining technique used with ruthenium red be studied collaboratively.

It is recommended that the work on Dairy and Egg Products be divided so that there will be an Associate Referee on Extraneous Matter in Eggs

* For report of Subcommittee D and action of the Association, see *This Journal*, **37**, 77, 78 (1954).

and another Associate Referee on Extraneous Matter in Dairy Products.

The Referee concurs in the recommendations made by the Associate Referee on Extraneous Materials in Nut Products.

The Referee concurs in the recommendations made in the report on Sediment Tests in Milk and Cream.

The Referee agrees with the recommendation of the Associate Referee on Identification of Insect Contaminants in Food and Drug Products that the work on methods for insect fragment identification be continued and that the collaborative studies be expanded.

The Referee agrees with the recommendation of the Associate Referee on Extraneous Materials in Vegetable Products that the title of the method on the identification of rot fragments (*This Journal*, **35**, 96 (1952)) be changed from "Rot Fragments in Tomato Products—First Action" to "Rot Fragments in Comminuted Tomato Products—First Action."

It is recommended that work on the recovery of fly eggs and maggots from tomato products be continued as suitable material becomes available.

This year, more than ever, the work in the field of extraneous materials in food and drug products has advanced at an accelerated pace. The Referee is especially grateful for the excellent work accomplished by the Associate Referees and their collaborators.

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

By WILLIAM V. EISENBERG (Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.), *Associate Referee*

It is recommended that method **35.87**[1] for extraneous materials in ground capsicums, modified by the addition of one step which requires boiling the pancreatin-digested material as reported during the October, 1952 meetings and published in *This Journal*, **36**, 301 (1953), be adopted, first action.

The method, as modified, has been in use by Food and Drug Administration analysts for the past two years and has been found to give clean separations and satisfactory recoveries.

Changes in method **35.82** have been suggested both as to procedure and sample size for various spices listed. It is recommended* that special methods more suitable for separating out filth elements so as to permit satisfactory microscopic examination, and which will permit the use of larger samples of these spices in some instances, be studied collaboratively during the coming year.

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[1] *This Journal*, **36**, 90 (1953).

* For report of Subcommittee D and action of the Association, see *This Journal*, **37**, 77 (1954).

REPORT ON EXTRANEOUS MATERIALS IN DAIRY PRODUCTS

By DOROTHY B. SCOTT (Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.), *Associate Referee*

An empirical method of staining plant and dung fragments in dairy products with ruthenium red as a stain for pectic substances has been reported as part of a symposium on Extraneous Materials in Foods and Drugs.† Degradation of pectic substances in plant tissue is caused by the enzymatic action of microorganisms in the intestinal tract of ruminant animals.

RECOMMENDATIONS

It is recommended*—

(1) That the technique of staining plant and dung fragments with ruthenium red be submitted for collaborative study.

(2) That investigation be continued to find an adequate staining technique for the determination of dung fragments in dairy products.

REPORT ON EXTRANEOUS MATERIALS IN NUT PRODUCTS

By MARYVEE G. YAKOWITZ (Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.), *Associate Referee*

A method for determining filth in finely-granulated nutmeats which introduces the use of a new reagent,[1] *viz.*, the tetrasodium salt of ethylene diamine tetraacetic acid, has been developed and subjected to collaborative study.

METHOD

Weigh 100 g of sample into 400 ml beaker. Cover nutmeats with petr. ether cover beaker, and boil gently 30 min. Add sufficient ether to maintain the original vol. Decant ether thru 7 cm filter paper on Büchner funnel. Add ca 200 ml of $CHCl_3$ to beaker and allow to settle 10–15 min. Remove floating nutmeats with spoon to 15 cm filter paper in Büchner funnel. Decant $CHCl_3$ thru 15 cm filter paper, being careful not to disturb any heavy residue in bottom of beaker. Repeat extn with small quantities of $CHCl_3$–CCl_4 (1+1) mixt. until heavy residue becomes relatively free of nutmeat particles. Transfer residue, if any, from beaker to ashless filter paper and examine for heavy filth. Ignite and weigh to det. sand and dirt.

* For report of Subcommittee D and action of the Association, see *This Journal*, 37, 77 (1954).
† SCOTT, D. B., *This Journal*, 37, 177 (1954).
[1] Bersworth Chemical Company, Framingham, Mass; Alrose Chemical Co., Providence, R. I.; and other supply houses.

Transfer nutmeats to smooth sheet of paper and retain 15 cm filter paper to be rinsed later as directed. Dry overnight at room temp. or in an oven at ca 80° for 1 hr. Place dry nutmeats in a 2 l Wildman trap flask, assisting the transfer and rinsing the 15 cm filter paper with 300 ml of 60% alcohol. Stir slightly and allow mixt. to soak 10 min. Add 250 ml of Tween 80[2]–60% alcohol soln (40 ml of Tween 80+210 ml of 60% alcohol) and mix. Add 75 ml of gasoline. Quickly add 5 g of powdered tetrasodium salt of ethylenediamine tetraacetic acid to 250 ml of 60% alcohol and, before completely dissolving, add to trap flask. Immediately stir for 2 min. in the usual manner to mix in gasoline. Fill flask with 60% alcohol. (Add reagents, mix gasoline, and fill flask with 60% alcohol without time interruption. Operate only one flask at a time during these steps.) Stir occasionally during only the first 15 min. after the flask is filled. Allow flask to stand strictly undisturbed 1 addnl hr. Trap off and filter, using only 60% alcohol as a rinse. Repeat the above extn, using 40 ml of gasoline and 1.5 hours' standing. Examine filters microscopically.

COLLABORATIVE STUDY

Four 100 g defatted samples of finely-granulated black walnuts, each containing 26 rodent hair fragments (1–2 mm) and 20 added insect fragments, were examined as unknowns by five collaborators using both the

TABLE 1.—*Recovery of filth from granulated black walnuts (26 rodent hair fragments and 20 insect fragments added to each sample)*

COLLABORATOR	BY A.O.A.C. METHOD 35.22(c)				BY PROPOSED METHOD			
	RODENT HAIR FRAGMENTS	WHOLE INSECTS OR EQUIVALENT	INSECT FRAGMENTS ADDED BY ASSOC. REF.[a]	INSECT FRAGMENTS "ORIGINALLY" PRESENT	RODENT HAIR FRAGMENTS	WHOLE INSECTS OR EQUIVALENT	INSECT FRAGMENTS ADDED BY ASSOC. REF.[a]	INSECT FRAGMENTS "ORIGINALLY" PRESENT
1	18	2	15	4	23	7	20	17
	19	5	15	15	19	3	19	9
2	12	6	16	9	19	6	15	10
	9	5	15	2	7	3	11	8
3	9	0	7	4	15	8	13	10
	5	0	8	7	10	3	12	4
4	12	3	20	13	22	10	19	20
	10	4	19	17	15	3	15	4
5	17	3	10	6	4	4	9	5
	4	3	18	4	16	0	10	2
Average recovery	11.5	3.1	14.3	8.1	15.0	4.7	14.3	8.9
Average % recovery	44.2	—	71.5	—	57.7	—	71.5	—

[a] Added insect fragments consisted of adult Rust Red beetle antennae and legs with femur and tibia intact, all readily distinguishable from insect fragments originally present in the nutmeats.

[2] Atlas Powder Company, Wilmington, Del.

present method, 35.22 (c), and the proposed method. In addition to the added filth, certain originally-occurring filth elements were present in the samples. The results obtained are shown in Table 1.

All collaborators felt that the proposed method possessed certain definite advantages over the official method. The chief points mentioned were that the new method is simpler, permitting filtering each extraction on one filter paper; that the filter papers obtained are much cleaner and thus easier to examine microscopically; and that better recovery of filth fragments is obtained.

The Associate Referee concludes that the proposed method for determining heavy and light filth in granulated nutmeats possesses the following advantages over the official method: (*a*) It is considerably faster and easier to perform; (*b*) the insect fragment recovery is at least equal to that obtained by the present method; (*c*) the rodent hair recovery by the proposed method is greater.

ACKNOWLEDGMENT

The Associate Referee appreciates the kind cooperation of the following collaborators of the Food and Drug Administration: Luther G. Ensminger, Cincinnati; Robert E. O'Neill, Atlanta; Garland L. Reed, Baltimore; J. E. Roe, Denver; and W. W. Wallace, Seattle.

RECOMMENDATIONS

It is recommended*—

(1) That method **35.22(c)**, an alternative procedure for heavy and light filth in shelled nuts, be dropped and that the method for heavy and light filth in granulated nutmeats be adopted, first action.

(2) That the method for "Light Filth in Spanish Peanuts," *This Journal*, **35**, 94 (1952), be designated "Light Filth in Peanuts with Adhering Testa."

(3) That the first procedure in section **35.22** (**b**) for light filth in shelled nuts beginning "Weigh 100 g into suitable beaker" and ending "Trap off, filter, and examine microscopically" be deleted. This method essentially duplicates the method which follows it and is less satisfactory from the standpoint of filth recovery.

* For report of Subcommittee D and action of the Association, see *This Journal*, **37**, 78 (1954).

REPORT ON EXTRANEOUS MATERIALS IN BAKED PRODUCTS, PREPARED CEREALS, AND ALIMENTARY PASTES

By J. FRANK NICHOLSON (Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.), *Associate Referee*

From time to time, there have been misinterpretations of the wording of method **35.28** (**a**), *Official Methods of Analysis*, 7th Ed. A re-wording of this method to clarify the meaning of the procedure was published in *This Journal*, **37**, 120 (1954).

A new method which serves the same purposes as **35.28** (**b**) has been developed and is as follows:

METHOD

35.28(b). *Direct trapping to show insect fragments and rodent hairs.*—Weigh 100 g sample. Break alimentary pastes and soft bakery material into pieces, separate crust, and crumble into fine pieces. In addition, if glazed coating is present, peel off coating in thin layers from crust and shred. Place sample in a 1500 ml beaker, add approximately 500 ml of 40° water, and let stand ca 1.5 hrs at ca 40° in an H_2O bath with occasional vigorous stirring. Adjust to *p*H 8 with Na_3PO_4 soln. Add 100 ml of pancreatin soln, and readjust to *p*H 8 after 15 min. Let stand 1–2 hrs at ca 37° (not over 40°), occasionally readjusting to *p*H 8 and stirring. Add 6–8 drops of formaldehyde solution and digest overnight at 30–37°. Cool, and proceed as follows:

(1) *Low fat sample.*—Transfer the digested material to a 2 l Wildman trap flask and adjust final vol. to about 900 ml with H_2O. Trap off twice in the usual manner, using 25 and 15 ml portions of gasoline, respectively. Combine trappings in beaker, transfer contents of beaker to trap flask, and fill with H_2O. Stir, and after 30 min. trap off into a beaker. Add sufficient concentrated HCl to trappings in beaker to make 2% acid. Boil to digest starchy material. Filter and examine microscopically.

(2) *High fat sample (except pie crust).*—Transfer the digested material to a 2 l Wildman trap flask, using a minimum amount of hot H_2O to rinse sides of the 1500 ml beaker free from fat. Adjust the final vol. to about 900 ml with H_2O. Trap off twice with 15 ml portions of gasoline in the usual manner. Chocolate products, even though sepd from icing and filling, may still require a wash to remove excess cocoa. If so, return each ext. separately to a trap flask, fill with H_2O, stir, and after 30 min. trap off. Make each final trapping 2% acid with HCl and boil to digest starchy material. Filter each ext. separately with the aid of boiling H_2O and hot alcohol from wash bottles. Examine microscopically.

(3) *Pie crust.*—Transfer the digested material to a 2 l Wildman trap flask and adjust the vol. as directed under **35.28(b)** (2) above. Add 5–10 ml gasoline, mix, and fill flask with H_2O. Trap off excess fat repeatedly, as it separates, into a 600 ml beaker until interface level rises to position in neck of flask for trapping. Let stand 20 min. and trap off into same beaker. Add 20 ml of gasoline to trap flask and make second extn. Make trappings 2% acid with HCl and boil to digest starchy material. Partially cool, and from a wash bottle cautiously add petr. ether around inner rim of beaker to liquefy fat. Filter with the aid of boiling H_2O, hot alcohol, and petr. ether. Examine microscopically.

A revision of section **35.33** was published in *This Journal*, **37**, 120 (1954).

These changes are all based on collaborative work done on these methods by individuals engaged in this type of work, or on changes which were found to be necessary during the corn and wheat surveys published in *This Journal*, **35**, 115 (1952) and **36**, 1037 (1953).

RECOMMENDATIONS

It is recommended*—

(1) That the method described in this report be substituted for **35.28** (**b**).

(2) That the title of **35.32** and **35.33** be amended to read "Whole and Degerminated Corn Meal, Corn Grits, Rye Meal, Wheat Meal, Whole Wheat Flour, Farina, and Semolina."

(3) That sections **35.34** and **35.35** be deleted and that **35.36** be amended as follows: "**35.36** (**a**) *Cream corn meal (corn flour).*—Proceed as in **35.39** (**a**)."

(4) That section **35.39** (**b**) be deleted.

REPORT ON EXTRANEOUS MATERIALS IN VEGETABLE PRODUCTS

By L. G. ENSMINGER and FRANK R. SMITH, *Associate Referee* (Food and Drug Administration, Department of Health, Education, and Welfare, Cincinnati 2, Ohio, and Washington 25, D. C.)

There is only one minor change in the section on extraneous materials in vegetable products this year. Although section **35.66** is entitled "Rot in Canned Tomatoes", a method entitled "Rot Fragments in Tomato Products—First Action" was added at the 1951 meeting. At first glance, this might seeem to be a duplication, and it is therefore recommended that the following change be made:

The title of the new method[1] should be changed from "Rot Fragments in Tomato Products—First Action" to "Rot Fragments in Comminuted Tomato Products—First Action."

Method **35.67** (**a**), with certain variations in manipulations, was employed to determine relative efficiency in separating vinegar fly eggs from tomato catsup by two- and six-liter separators. These modified manipulations are discussed below in regard to their application to each separate experiment.

* For report of Subcommittee D and action of the Association, see *This Journal*, **37**, 78 (1954).
[1] *This Journal*, **35**, 96 (1952).

Three codes of one brand of catsup were collected from a distributor and were composited into one 3-gallon batch. In each experiment, 100 gram subs from the continually mixed batch were alternately placed in two-liter and six-liter separators. Six globe-shaped separators of each capacity were used. Gasoline was then added at the rate of 30 ml to the two-liter and 50 ml to the six-liter separators. At each hourly draw-off 75 ml of liquid was drained from the two-liter separators and 100 ml was taken from the six-liter separators. The drainings were next filtered through 10XX black-dyed bolting cloth for microscopic examination at 20×. Only vinegar fly eggs were counted, since other fly eggs and maggots were present in insignificant numbers.

EXPERIMENT 1

After a 100 g sub was poured into each separator, the weighing beaker was rinsed with water and rinsings were placed in the separator. Gasoline was then added, and, without mixing the sample with gasoline, water was forcibly added from the faucet. The contents of the separator were then swirled every 15 minutes for 2 hours, and draw-offs were taken at the end of each hour. Recoveries are given in Table 1. The time for examination of cloths microscopically is also included for comparison.

Excessive tomato material settled and was drawn off from both 2 and 6 l flasks; considerably more material was taken from the 2 l size. As a result, much time was required for microscopic examination, especially since the eggs were quite translucent (not white) and were difficult to see.

Very little advantage was obtained in the way of increased recovery, but much microscopic examination time was saved by using the six-liter separators.

EXPERIMENT 2

The only deviation from the procedure used in Experiment 1 was the thorough mixing of the gasoline, catsup, and beaker water rinsings before adding water forcibly from the faucet. Table 2 gives the recoveries.

TABLE 1.—*Vinegar fly egg recoveries, Experiment 1*

DRAW-OFF	VINEGAR FLY EGGS								EXAMINATION TIME, HRS.
	1	2	3	4	5	6	TOTAL	AV.	
(a) Two-Liter Separator									
First Hour	34	17	17	31	37	26	162	27.0	2.7
Second Hour	1	2	0	0	1	0	4	0.7	1.8
Total:	35	19	17	31	38	26	166	27.7	4.5
(b) Six-Liter Separators									
First Hour	35	25	27	32	28	36	180	30.0	1.9
Second Hour	0	0	0	1	0	0	1	0.3	0.7
Total:	35	25	27	33	28	36	181	30.3	2.6

TABLE 2.—*Vinegar fly egg recoveries, Experiment 2*

DRAW-OFF	VINEGAR FLY EGGS								EXAMINATION TIME, HRS.
	1	2	3	4	5	6	TOTAL	AV.	
(a) Two-Liter Separator									
First Hour	31	12	27	27	28	23	148	24.6	1.8
Second Hour	1	11	5	0	2	3	22	3.7	1.2
Total:	32	23	32	27	30	26	170	28.3	3.0
(b) Six-Liter Separators									
First Hour	29	50	43	29	31	40	222	37.0	1.0
Second Hour	0	2	0	0	9	0	11	1.8	0.7
Total:	29	52	43	29	40	40	233	38.8	1.7

Mixing gasoline, rinsings, and catsup well before adding water from faucet reduced settling of pulp, decreased examination time, and increased recoveries, which are definite advantages over Experiment 1. Experiment 2 also increased the advantage of the 6 l separator over the 2 l in the average recovery of eggs (10.5 eggs more with the 6 l).

EXPERIMENT 3

In adding the catsup to the separators, the remaining catsup in the weighing beaker was scraped out into these separators with a flat-end glass rod. Catsup which could not be scraped out was negligible. Gasoline was added and mixed thoroughly before any water was used. To mix gasoline and sample most efficiently, each separator was tipped almost horizontally and swirled, coating the sides with the mixture. In this manner, tomato particles were better exposed to gasoline.

Otherwise, the procedure was the same as in above experiments (i.e. adding

TABLE 3.—*Vinegar fly egg recoveries, Experiment 3*

DRAW-OFF	VINEGAR FLY EGGS								EXAMINATION TIME, HRS.
	1	2	3	4	5	6	TOTAL	AV.	
(a) Two-Liter Separators									
First Hour	39	26	24	20	45	14	168	28.0	0.5
Second Hour	2	6	6	10	1	10	35	5.8	0.4
Total:	41	32	30	30	46	24	203	33.8	0.9
(b) Six-Liter Separators									
First Hour	33	37	40	24	39	30	203	33.8	0.3
Second Hour	10	15	10	35	12	7	89	14.8	0.3
Total:	43	52	50	59	51	37	292	48.7	0.6

faucet water, swirling, drawing off, etc.). Table 3 contains the results.

Experiment 3 shows that a thorough mixing of the gasoline with comminuted sample before addition of any water is decidedly important. Tomato material rose to the top of the mixtures in the separators, and eggs came down more efficiently than in the other experiments. Still, the six-liter separators hold the advantage over the two-liter in both egg recovery and time consumed for examination.

Observations indicate that in a flask containing a great deal of suspended tomato matter throughout the water phase, there are some fly eggs that will never settle and be normally recovered. In other words, the buoyancy of eggs varies. When this material can be made to rise more completely, less impedance to egg settling results, and the more buoyant eggs can come down more readily, resulting in greater recoveries. Also, with little tomato material settling, filter cloths are much cleaner with resulting savings in analytical time and eye strain. Six-liter flasks, with greater dilutions, enhance these advantages.

Experiment 3 shows that it is important to mix the catsup and gasoline thoroughly before addition of any water—a critical point in method **35.67**(a).

EXPERIMENT 4

A new phase of the procedure studied was the drawing off of settlings at the end of each 15 minute period just prior to the normal swirling operation. 50 ml and 75 ml portions were taken from the 2 l and 6 l separators, respectively, at each draw-off. Other than this operation, all conditions of analysis were identical with those in Experiment 3. The recoveries are listed in Table 4 by individual successive 15 minute draw-offs for the first hour only. The second hour's 4 draw-offs were combined in the case of 6 l separators, and for the 2 l separators the fifth draw-off was combined with the sixth, and the seventh with the eighth. No time was recorded during analysis as previously.

TABLE 4.—*Vinegar fly egg recoveries, Experiment 4*

15 MINUTE DRAW-OFF	VINEGAR FLY EGGS							
	1	2	3	4	5	6	TOTAL	AV.
(a) Two-Liter Separators								
1	15	14	17	12	12	11	81	13.5
2	5	10	12	7	16	16	66	11.0
3	9	6	3	7	11	11	47	7.8
4	4	4	4	8	4	3	27	4.5
5–6	5	2	7	5	4	8	31	5.2
7–8	0	2	1	1	2	0	6	1.0
Total:	38	38	44	40	49	49	258	43.0
(b) Six-Liter Separators								
1	29	23	30	34	47	40	203	33.8
2	20	7	8	13	6	6	60	10.0
3	3	6	3	3	5	4	24	4.0
4	1	3	1	3	1	3	12	2.0
5–8	1	4	0	1	1	3	10	1.7
Total:	54	43	42	54	60	56	309	51.5

The 15-minute draw-off operation increased even more the recovery of eggs by both 2 and 6 l separators; the greatest improvement was shown for the 2 l separators. However, the 6 l size still retained superiority in total recovery. In the case of the 6 l separators, 96.7% of the eggs recovered were drawn off during the first hour, compared to 85.6% for the 2 l separators, proving that the larger size gives quicker recoveries. Also, four draw-offs (1 hour period from each 6 l separator were combined and put through one cloth without difficulty, while two draw-offs combined from the 2 l separators gave considerable residue.

SUMMARY AND RECOMMENDATION

Four experiments were run on one batch of tomato catsup by method **35.67 (a)** to determine the importance of various manipulations and the relative efficiencies of two- and six-liter separators in the recovery of vinegar fly eggs from tomato catsup.

It was found for both two- and six-liter separators that (*a*) thoroughly mixing gasoline and catsup before any water was added improved egg recoveries and reduced filter residues, and (*b*) drawing off settlings every fifteen minutes hastened as well as increased recoveries.

Also, the six-liter separators showed the following advantages when compared to the two-liter:

(*a*) Higher egg recoveries were obtained in all experiments.
(*b*) Eggs were recovered more quickly.
(*c*) Filter residues were reduced with consequent savings in examination time and eye strain.

It is recommended* that work on the recovery of fly eggs and maggots from tomato products be continued as suitable material becomes available.

(Concerning section **35.67(b)**, John Bornmann of Chicago District, U. S. Food and Drug Administration, has conducted some investigations on the use of the Waring blendor instead of a No. 6 or No. 8 sieve for pulping the canned tomatoes. He found that one second in the blendor was satisfactory for pulping the tomatoes but neither the fly egg nor maggot recovery appeared to be as good as when the sieve was used. His work was not extensive enough to be conclusive and it is recommended* that the subject be further investigated when suitable material is available.)

REPORT ON SEDIMENT TESTS IN MILK AND CREAM

By CURTIS R. JOINER (Food and Drug Administration, Department of Health, Education, and Welfare, St. Louis 1, Mo.),
Associate Referee

Last year a method for the preparation of fine standard sediment pads was adopted, first action (1). A brief description of the essential require-

* For report of Subcommittee D and action of the Association, see *This Journal*, 37, 77 (1954).

ments, or performance standards, of a suitable filtering apparatus was recommended for adoption (2). However, the General Referee felt that the description was inadequate because of its brevity and lack of specificity (3). Several collaborators had expressed this opinion also, and Subcommittee D concurred in the General Referee's recommendation (4). After some consideration and discussion of the matter with the General Referee,

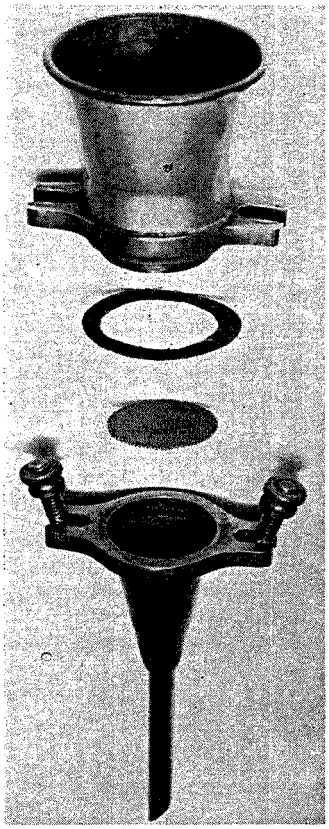

FIG. 1.—Sediment filtering apparatus, unassembled.

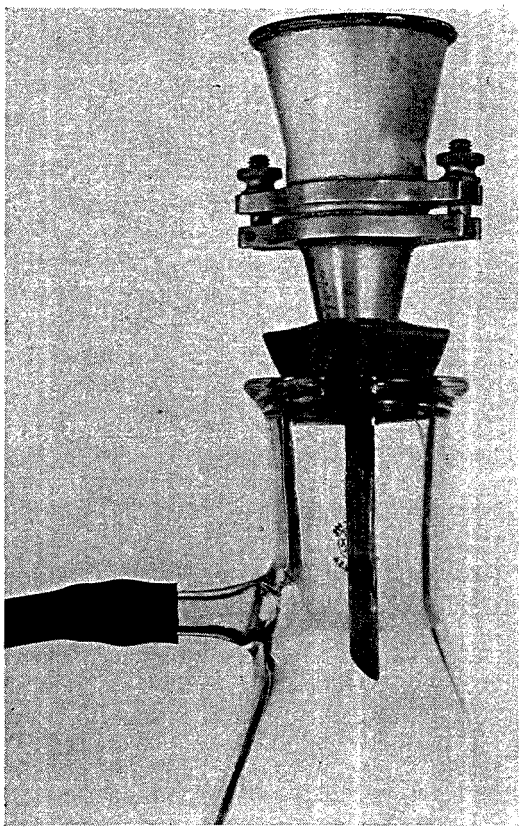

FIG. 2.—Sediment filtering apparatus, assembled.

the Associate Referee decided that the description should be supplemented with photographs of a suitable apparatus, since good photographs are usually more informative than a detailed description.

Two photographs, one of the assembled apparatus, and one of the same device unassembled to show its component parts, are submitted as Figures 1 and 2. The minor editorial changes that will be necessitated by the adoption of the photographs and the description are also submitted.

RECOMMENDATIONS

It is recommended*—

(1) That the following description and the accompanying photographs be adopted and be designated **35.9(c)**:

Sediment filtering apparatus.—App. must hold $1\frac{1}{4}''$ sediment disk and have effective filtering area $1\frac{1}{8}''$ in diam. This $1\frac{1}{8}''$ area must be unobstructed except for wire screen, or wire screen and perforated plate support for filter disk. App. should be supported in filter flask so vacuum can be used for rapid filtration or flask air outlet closed to stop filtration. App. should have ca 80° funnel with min. capacity of 80 ml and max. capacity of 450 ml. Test app. by filtering H_2O suspension of C thru standard disk. Disk should have clean, sharply defined border. When sediment suspension is filtered, sediment should be evenly distributed over disk with no pattern formation. Figures 1 and 2 are photographs of a suitable app.

(2) That the present section **35.9(c)** be changed to **35.9(d)** and the sentence beginning at the eighth line of the third paragraph: "Mix thoroly and pass mixt. thru standard sediment disk in filtering device having filtering area measuring $1\frac{1}{8}''$ in diam." be changed to: "Mix thoroly and pass mixt. thru standard sediment disk in filtering app. (**c**)."

(3) That the present section **35.9(d)** be changed to **35.9(e)**, and that the text be changed as follows:

In the next to the last line of the first paragraph and in the first and last lines of the second paragraph, "(**c**)" should be changed to "(**d**)."

In the second line of the second paragraph, "(**c**)" should be inserted after "apparatus."

In the seventh line of the second paragraph, "funnel" should be changed to "apparatus (**c**)."

(4) That the present section **35.9(e)** be changed to **35.9(f)**, and that it be modified as follows:

In the fourth line, "(**c**)" should be changed to "(**d**) or (**e**)".

The following sentence should be inserted just before the last sentence of this paragraph: "The standards that more nearly resemble the test disk being graded should be used in each case."

(5) That the complete method **35.9**, modified as recommended above, be adopted, first action.

REFERENCES

(1) *This Journal*, **36**, 87 (1953).
(2) JOINER, C. R. *ibid.*, **36**, 310 (1953).
(3) HARRIS, K. L., *ibid.*, **36**, 300 (1953).
(4) *This Journal*, **36**, 62 (1953).

* For report of Subcommittee D and action of the Association, see *This Journal*, **37**, 77 (1954).

REPORT ON IDENTIFICATION OF INSECT CONTAMINANTS IN FOOD AND DRUG PRODUCTS

By O. L. KURTZ (Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.), *Associate Referee*

Since the inclusion of a section on extraneous materials in the Sixth Edition of *Official Methods of Analysis*, A.O.A.C., improvements have been made in standardizing filth recovery procedures. At the 1952 meeting the Referee recommended that a section entitled "Methods for the Identification of Insect Contaminants in Food and Drug Products" be adopted and an Associate Referee appointed to this section.

It has become increasingly evident that the qualitative aspects of filth findings are as important as the quantitative findings. The ultimate objective of this project is the identification of insects responsible for the characteristic fragments recovered from food products. Identification of whole insects isolated from food and drugs is not too difficult and follows standard references. However, recognition and identification of insect fragments, especially when they are minute, imposes more of a problem. The fragments that break from the insect exoskeleton generally do not have the taxonomic features commonly used for identifying and differentiating insect orders, families, genera, and species. Since there is no specific literature for the identification of fragments recovered from food products and since the characters used will often differ from those applied by the taxonomist, the results of this investigation will rest squarely upon individual findings.

Work has been done on characterization of rodent and other fur hairs and some preliminary reports have been published on insect fragment identification. An approach to the subject published in *This Journal*[1] illustrates in part the work contemplated in this report and also supplies literature references which deal specifically with insect fragment identification.

Since the insect problem is an extensive one, dealing with the identification of both adult and immature stages, the work to be undertaken has been organized on a long-range basis. A work list citing the major insect contaminants and food products has been sent to several collaborators. Only a limited number of items and only the more common insects that may contaminate food products are included, although, in some instances, accidental and associated contaminants are given. To undertake at one time the study of all the insects that commonly infest all commodities would be impossible. Therefore, an outline of a few principal categories which are of the greatest regulatory and economic significance is provided.

[1] *This Journal*, 37, 167 (1954).

The first studies which will cover the storage- and factory-type insects have been organized under seven major categories and are indicated by the Roman numerals in the outline below. As this identification program unfolds, other food commodities and their insect contaminants will be considered for study.

These seven major categories have been broken down into working units, one or more units to be undertaken by one or two collaborators. The further subdivision of each working unit will depend entirely on the facts recovered from the particular study; i.e., the degree of breakdown will depend almost entirely on the diagnostic features which a fragment can provide.

WORKING UNITS OF MAJOR CATEGORIES

(I) *Adult storage beetles and weevils:* Infesting and associated with grain, grain products, and milled products.
- (A) Head capsule
- (B) Head appendages (mouthparts, antennae, etc.)
- (C) Thorax
- (D) Wings
- (E) Legs
- (F) Abdominal appendages

(II) *Adult moths:* Infesting stored grain, grain products, and milled products. Contamination by this stage will result generally from those moths that reach maturity within a grain seed.

(III) *Larvae of storage beetles, weevils, and moths:* (I and II above).
- (A) Head capsule
- (B) Head appendages (mouthparts, antennae, etc.)
- (C) Thorax and abdomen and their appendages

(IV) *Adult diptera:* (Flies) associated with processing of fruits and vegetables. Except for food commodities which will be considered at a later date, only the general characteristic features of some of the common families—Drosophilids, Muscids, Sarcophagids, Calliphorids, etc.—are of concern here.
- (A) Head
- (B) Thorax
- (C) Wings—the vestigial second pair (halteres) are also characteristic for fly recognition and occasionally fly identification.
- (D) Legs
- (E) Abdomen

(V) *Maggots of IV:* In some groups of maggots, the three body regions cannot be easily differentiated as they can in other groups. In the early phases of fragment identification, a general family classification is sufficient except in certain specific instances. Many foods are, however, contaminated by a specific maggot infestation, and a more detailed classification will be necessary for such products.

(VI) *Adult cockroaches (German, American, and Oriental):* This group of insects does not have a so-called larval stage. The immature form is the nymph which closely resembles the adult and differs most obviously by size and shortened wing covers.
- (A) Head capsule

(B) Head appendages (mouthparts, antennae, etc.)
(C) Thorax
(D) Legs
(E) Wings
(F) Abdomen with appendages

RECOMMENDATIONS

It is recommended*—
(1) That the methods for insect fragment identification be continued.
(2) That collaborative work be expanded.

No reports were given on extraneous materials in beverage materials, fruit products, and miscellaneous materials.

REPORT ON NUTRITIONAL ADJUNCTS

By O. L. KLINE (Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.), *Referee*

In past years, the work in this section has been concerned with the development of biological, microbiological, and chemical methods for the vitamins. With important developments in the field of nutrition relating to the supplementing effect of amino acids in both experimental and practical rations for animals, to the effect of antibiotics on the promotion of growth of swine and poultry under field conditions, and to the practice of improving consumer acceptability of poultry by the inclusion of plant pigments in poultry rations, it is clear that the A.O.A.C. can be of important service in the development of pertinent standard procedures. The proposal was made and approved for broadening the subject matter of this section, and the title of "Nutritional Adjuncts" has been adopted for the section. This is a convenient arrangement in view of the fact that many of those analysts generally familiar with the development of methods for vitamins are also well versed in the determination of amino acids, the determination of biological value of proteins, the estimation of growth effect of antibiotics, and the chemical or physical-chemical methods for estimating plant pigments.

During the past year a beginning was made on this broader program, and work was reported by the Associate Referee on Determination of Xanthophylls in Mixed Feeds. Studies are planned during the coming year for inquiring into the suitability of methods for determining antibiotics in feed mixtures, and for collaboratively evaluating methods for those amino acids that are now used commercially as feed supplements.

* For report of Subcommittee D and action of the Association, see *This Journal*, 37, 78 (1954).

There has been important activity in the study of vitamin A this year. To quote from the Associate Referee report by Dr. Morgareidge, "The methodology of vitamin A assay at low levels in foods and feeds has remained a matter of unfinished business on the agenda of everyone concerned with the problem." There have been important new findings in the technology of chromatography, and the Associate Referee on Vitamin A in Margarine has critically examined the feasibility of a two-column alumina system. The results of this collaborative study, supplemented by the findings described in the contributed papers[1] presented by Wilkie and Jones and by Rosner and Kan, provide a basis and suggest the direction for further work. The Referee concurs in the recommendation* that these studies be continued.

The report on Vitamin A in Mixed Feeds points up the problem that has arisen with the appearance of dry supplements of synthetic vitamin A stabilized with water-soluble coatings. This year's study warrants continued attention to a suitable saponification step and justifies retention of the first action status of the method. The Referee concurs in the recommendation to this effect.

The work on the determination of carotene continues to be important both as a guide to analysts who have difficulty with the method, and as a means of developing improvements. The Referee concurs in the recommendation that the work be continued.

Although the work on xanthophyll in mixed feeds was begun late in the year, the Associate Referee has made an admirable survey of methods and of laboratories interested in collaborating, and has presented a sound plan for further study. The Referee concurs in his recommendation.

The chemical method for the determination of thiamine by the thiochrome procedure is well established in *Official Methods of Analysis*. With routine examination of enriched flours it is found unnecessary to go through the enzyme and chromatographic steps of the procedure. Studies carried out by the Associate Referee this year have established the feasibility of a short method that is highly desirable in routine examinations which are a necessary part of control programs. The Associate Referee's statement is interpreted as a recommendation of first action status for the short method as applied to enriched flour, and the Referee concurs in this, as well as in the further recommendation that studies be extended to other enriched cereal products.

During the past year it was called to the attention of the Referee that certain types of drum-dried concentrates offered as a source of riboflavin caused difficulty when the A.O.A.C. method for riboflavin was applied to them. An Associate Referee was appointed to study this problem and to carry out collaborative work if necessary. His report points out that the

[1] *This Journal*, 37, 880 and 887 (1954).
* For report of Subcommittee A and action of the Association, see *This Journal*, 37, 64 (1954).

A.O.A.C. method as it appears in *Official Methods of Analysis* is quite adequate for this purpose.

Also, during the year, evidence appeared in the literature that the procedure described for the extraction of samples in the chemical method for determining niacin in foods was inadequate when applied to certain types of cereal products. A careful study of this problem, including a comparison of chemical and microbiological results, was carried out in the Associate Referee's laboratory, resulting in a recommendation for modification of the extraction procedure in the chemical method. The Referee concurs in this recommendation.

Information that has come to the Food and Drug Administration during the last few months has focused attention on the vitamin B_6 content of infant foods, and has emphasized the importance of accurate methods for the determination of this vitamin in food products. The study of the cause of variation between chemical and microbiological results planned by the Associate Referee is an important one.

Development of knowledge relating to the structure of enzyme systems containing pantothenic acid and the means of releasing bound pantothenic acid for proper microbiological or chemical determinations have been reviewed. The Associate Referee has reported on plans for a critical study of the microbiological method during the coming year, and the Referee concurs in his recommendation.

Important advances have been made on the assay of vitamin B_{12}. Because of the small amounts of this vitamin that are used in the treatment of human disease and in the supplementation of animal diets, and because of the extremely small amounts required in the microbiological assay, important new problems have had to be solved in this field. Those who have taken part in this difficult study deserve commendation for the progress that has been made. The Referee concurs in the recommendation offered by the Associate Referee that the method studied, applied to test materials containing 0.1 mmg or more of vitamin B_{12} per gram or cc, be adopted, first action. This method is now applicable to a wider range of test materials and includes those materials for which a method was adopted, first action, last year. The method reported this year should replace the previous one.

Finally, the Referee wishes to express his appreciation for the fine cooperation of all those who have taken part in the program of work of this section during the past year. Continued effort is important if we are to keep pace with the new developments in the field of nutrition.

REPORT ON VITAMIN A IN MIXED FEEDS

By MAXWELL L. COOLEY (General Mills, Inc., Minneapolis, Minn.),
Associate Referee

The status of the method for the determination of vitamin A in mixed feeds[1] was continued as first action. Subsequent reports (2–4) show that improvements in the procedure have resulted in more effective application of the method.

The assay of stabilized vitamin A products in feeds has been found satisfactory (3, 4), provided these products have been stabilized with materials which are soluble in hexane, the extracting solvent. However, because of the availability and use of dry vitamin A products in feeds which are stabilized by embedding the vitamin A in a matrix of either gelatin or pectin, a modification of the method is necessary, since these protective coatings (gelatin and pectin) are not soluble in hexane or other common solvents. A tentative procedure employing saponification was therefore developed for investigation.

In the present study the number of collaborators was limited to seven. The procedure under study required much more time for assay completion, with such a consequent large increase in laboratory work that many former participants could not satisfactorily fit the project into their program. Furthermore, the relatively small number of collaborators working on this study was considered desirable to facilitate exchange of ideas.

TABLE 1.—*Composition of experimental feed mixtures*

	SAMPLE					
	1	2	3	4	5	6
Ground corn	15	15	15	15	15	15
Wheat middlings	30	30	30	30	30	30
Soybean oil meal	38	25	25	36	36	36
Ground oats	5	5	5	5	5	5
Dehydrated alfalfa meal	2	15	15	4	4	4
Meat scraps	5	5	5	5	5	5
Fish meal (Vitamin A-free)	5	5	5	5	5	5
Vitamin A derived from:	F.L.O.[a]	F.L.O.	F.L.O.	Oil Soluble Dry A	Pectin Dry A	Gelatin Dry A
Vitamin A (calculated units of A per g):	5.0	9.0	9.0	9.0	6.5	6.5

[a] Fish liver oil.

[1] *This Journal*, 34, 97 (1951).

DESCRIPTION OF COLLABORATIVE SAMPLES

Six samples of feed were sent to each laboratory with a complete description of the procedure to be used for the vitamin A assay. The vitamin A in samples 1, 2, and 3 was derived from fish liver oil while sample 4 contained a stabilized, dry vitamin A product using an oil-soluble material as the protective coating. The vitamin A in sample 5 was derived from a product employing a pectin protective coating, and in sample 6 the source of vitamin A was a dry vitamin A material in which the matrix was gelatin.

The composition of the experimental feed mixtures is given in Table 1, which shows that samples 2 and 3 are identical. Samples 4, 5, and 6 vary only as to source and potency of the dry vitamin A materials used.

The following procedure was sent to each collaborator:

TENTATIVE PROCEDURE FOR VITAMIN A IN MIXED FEEDS

(with saponification)

APPARATUS

(a) *Photoelectric colorimeter.*—Evelyn colorimeter, equipped with 620 mμ glass filter, or Coleman spectrophotometer has been found satisfactory. An instrument with direct reading, deflecting type galvanometer is necessary.

(b) *Chromatographic tubes.*—Cylinder 23×200 mm, sealed to 5×80 mm tube. (Wilkens-Anderson Co., Chicago, Ill., or equivalent.)

(c) *Chromatographic assembly.*—Fisher Filtrator or any other suitable suction device for collecting eluate from adsorption column.

(d) *Extraction-saponification apparatus.*—Any suitable ground-glass joint refluxing apparatus (do not use rubber connections) arranged so that refluxing flask (125–150 ml capacity) may be immersed in a boiling water or steam bath.

(e) *Automatic pipet.*—10 ml. (Scientific Glass Apparatus Co., Bloomfield, N. J. Catalog No. J-2123 or equivalent.) This pipet should deliver rapidly through opening 3–4 mm in diam. (A suitable size graduated cylinder may also be used effectively for dispensing Carr-Price reagent.)

(f) *Ultraviolet lamp.*—Long wavelength such as Fisher Scientific Company Catalog No. 11-984-1.

REAGENTS

(a) *Hexane.*—B.p. 60–71° C. (Skellysolve B).

(b) *Ethanol.*—95%.

(c) *Acetone.*—Reagent grade.

(d) *Potassium hydroxide soln.*—50% aq. soln, w/v.

(e) *Acetone.*—10% in hexane (Skellysolve B).

(f) *Absorbent.*—Equal parts by wt of diatomaceous earth (Johns-Manville Hyflo Super-Cel) and magnesia (Micron brand #2641, Westvaco Chlorine Products Corporation, Newark, Calif.).

(g) *Anhyd. sodium sulfate.*—Reagent grade.

(h) *Chloroform.*—Reagent grade (purify by distn if necessary to prevent color interference).

(i) *Antimony trichloride reagent (Carr-Price).*—Prep. by dissolving 20 g of $SbCl_3$ in sufficient $CHCl_3$ to make 100 ml. Add 3 ml of acetic anhydride and filter if necessary.

PREPARATION OF ADSORPTION COLUMN

Connect chromatographic assembly to source of vacuum. Ordinarily an efficient water pump will be sufficient. Place *small* amount of cotton at bottom of chromatographic tube and pack tightly to depth of 7 cm with uniformly blended mixt.

of equal parts by wt of Hyflo Super-Cel and magnesia. To assure a homogeneous blend of these components, mix by rubbing out lumps by hand or run the mixt. thru a kitchen flour sifter and roll on paper at least 50 times. Add the mixt. in several portions, tamping well with a stopper or similar device. Keep suction on column during packing. Do not wet column before passage of soln thru column.

DETERMINATION

Extraction and saponification.—Weigh 10 g of feed into refluxing flask. Add 60 ml of ethanol and 15 ml of 50% KOH soln. Reflux on boiling H_2O or steam bath for 30 min. Cool and filter with suction into 500–1000 ml filter flask. Wash residue with 3 separate 25 ml portions of hexane. Transfer filtrate and washings to 500 ml separatory funnel. Add 60 ml of H_2O and shake at least 0.5 min. Allow layers to separate and drain the lower aq.-alcohol phase into 250 ml Erlenmeyer flask (labeled "A"). Collect the hexane layer in another 250 ml Erlenmeyer flask (labeled "B"). Return the aq.-alcohol layer from flask "A" to separatory funnel and re-ext. vitamin A and carotenoids by shaking with 30 ml of hexane. Again collect the lower layer in flask "A" and the upper layer in flask "B." Repeat extn with two addnl 30 ml portions of hexane. Collect all hexane extns in flask "B" and return them to separatory funnel, discarding the aq.-alcohol portion in flask "A." Wash hexane ext. in separatory funnel with 3 separate 100 ml portions of H_2O, pouring the H_2O thru the hexane layer and draining off washings each time. Drain off all washings and pass the hexane soln of vitamin A and carotenoids thru filter paper and funnel contg ca 5 g of anhyd. Na_2SO_4 into clean, dry 250 ml Erlenmeyer flask. Place boiling chip in the flask and diminish vol. to 25–30 ml, using reduced pressure and hot H_2O bath.

Chromatography.—Draw the soln thru adsorption column with suction. Elute with sufficient 10% acetone in hexane so that all of the carotene band passes thru column and is collected. This may require at least 50–75 ml of eluant. Substitute another eluate receiver beneath the adsorption column and elute colorless vitamin A band with more 10% acetone in hexane. This may require addnl 100–200 ml of eluant. The complete elution of vitamin A is ascertained by use of an ultraviolet light source. The vitamin A fluoresces and is quite readily observed in subdued light.[2] (Caution: Do not expose vitamin A to ultraviolet light except for intermittent examination.) Make first eluate contg the carotene up to a suitable vol. with hexane and mix. Do likewise with the second eluate contg the vitamin A.

Colorimetry for carotene.—Read the carotene present in the first eluate or soln at 440 mμ. Calc. as mmg of carotene per g of feed.

Colorimetry for vitamin A.—Prep. a colorimetric curve based on the U.S.P. Vitamin A Reference Standard which has been saponified and chromatographed according to above procedure. Evap. (with mild heat and reduced pressure) a suitable aliquot of the vitamin A soln and dissolve residue in sufficient $CHCl_3$ so that 1 ml after addn of $SbCl_3$ reagent will give transmittance readings which are within the range of 30–65%. Set colorimeter at 100% transmission, using blank comprised of 1 ml of $CHCl_3$ and 10 ml of Carr-Price reagent. Place assay tube in colorimeter and add rapidly 10 ml of Carr-Price reagent. Using 620 mμ wavelength, take maximum colorimetric reading (color begins to fade within 3–5 sec.). Apply readings to vitamin A curve described above and calc. units of vitamin A per g of feed.

Note.—A gelatin capsule of the U.S.P. Vitamin A Reference Standard (containing 2500 units of vitamin A) may be saponified, extracted, and chromatographed by the procedure as given. Carotene (crystalline or from dehydrated alfalfa meal)

[2] A comparatively weaker and narrower fluorescent band sometimes precedes the carotene. This does not appear to be vitamin A. The vitamin A band follows and is distinct from the carotene band.

may be added prior to saponification if subsequent preliminary examination of behavior of the carotene and vitamin A bands in the adsorption column is desired. The vitamin A lost by use of the proposed chromatography has been found in the Referee's laboratory to be 12–15%, compared with use of the saponification step only (omitting chromatography).

In the above saponification procedure the vitamin A ester is changed to the vitamin A alcohol. With the previous procedure (2), no saponification is used and the vitamin A is extracted and chromatographed in the ester form. In this case the chromatography is based on the fact that vitamin A ester is less strongly adsorbed than beta-carotene and just precedes the carotene down the adsorption column. However, vitamin A alcohol is more strongly adsorbed than carotene and consequently follows the carotene in the chromatogram. Thus, when saponification is used the chromatography is changed.

The above procedure should be applicable to the determination of vitamin A in feeds, no matter whether the vitamin A is derived from gelatinized or pectinized dry A products or from oil-soluble materials. Inasmuch as the saponification of the feed sample also extracts carotenoids, the method may be useful for the simultaneous determination of carotene and vitamin A in feeds.

Collaborator 2 commented that the proposed saponification and chromatography causes considerable loss of vitamin A, probably because the alcohol form is not as stable as the ester. Several antioxidants including pyrogallol, hydroquinone, and cottonseed oil were used in the Referee's laboratory during the saponification step with no noticeable improvement in stability.

Collaborator 1 states that several standards run through the procedure show only about 3 per cent loss when compared with values obtained without saponification and chromatography. Furthermore, this laboratory suggests that one shaking with saturated salt solution after the final water washing of the hexane extracts in the separatory funnel will clarify the solution and eliminate the filtration through anhydrous sodium sulfate.

Three participating laboratories indicated they had difficulty with the adsorbent used and with the separation of the carotene from the vitamin A.

DISCUSSION OF COLLABORATIVE DATA

Table 2 presents the average values of all vitamin A results received for the six feed samples compared with the calculated value for each sample. Results reported on samples 2 and 3 (duplicates) indicate a generally fair agreement within each laboratory on identical samples when the proposed procedure is used.

Samples 1 through 4 contained hexane-extractable vitamin A, and four of the seven collaborators determined the vitamin A in these samples by the previous procedure, which does not employ saponification. These re-

TABLE 2.—*Average values found for vitamin A in collaborative samples, using proposed procedure (with saponification)*

COLLABORATOR NO.	UNITS OF VITAMIN A PER GRAM					
	SAMPLE NO.					
	1	2	3	4	5	6
1	4.2	8.5	7.8	9.5	5.6	9.3
2	5.1	8.4	6.5	10.5	6.3	7.8
3	4.5	7.8	8.5	8.7	6.3	6.5
4	4.4	8.7	8.8	8.0	7.1	7.1
5	5.5	10.2	9.3	8.9	7.5	7.4
6	2.7	4.6	10.3	5.0	2.9	1.0
7	4.7	8.5	8.3	9.3	6.6	8.0
Calcd value	5.0	9.0	9.0	9.0	6.5	6.5
Mean	4.44	8.1	8.5	8.56	6.04	6.73

sults are given in Table 3. Although the data are limited, agreement within each laboratory also is fairly good in identical samples. Examination of reports on previous studies of the method for vitamin A in feeds (2, 3) indicates that the proposed procedure does not substantially reduce the erratic results obtained on samples where both methods are applicable. The mean values for each sample in Tables 2 and 3 generally agree fairly well with the theoretical potency in each case.

In Table 4 are presented the average values of all results, obtained by the proposed procedure, which were reported for carotene in the collaborative samples. Included are results obtained on these samples by two laboratories using the A.O.A.C. carotene method (1). Even though the latter results appear to be slightly higher than those obtained by the

TABLE 3.—*Average values found for vitamin A in collaborative samples, using A.O.A.C. procedure (no saponification)*

COLLABORATOR NO.	UNITS OF VITAMIN A PER GRAM					
	SAMPLE NO.					
	1	2	3	4	5[a]	6[a]
2	3.5	9.6	11.8	14.6	—	—
3	5.1	10.5	9.6	9.2	—	—
4	5.1	5.3	5.4	8.5	—	—
5	5.4	10.5	10.5	—	—	—
Calcd value	5.0	9.0	9.0	9.0	—	—
Mean	4.77	8.97	9.32	10.77	—	—

[a] The vitamin A in Samples 5 and 6 was not extractable by hexane. Therefore the present A.O.A.C. method is not applicable to the determination of vitamin A in these samples.

TABLE 4.—*Average values found for carotene in collaborative samples, using proposed procedure (with saponification)*

COLLABORATOR NO.	MICROGRAMS OF CAROTENE PER GRAM					
	SAMPLE NO.					
	1	2	3	4	5	6
1	1.7	13.0	12.1	3.1	3.4	3.1
2	2.7	19.8	24.8	5.8	4.9	4.9
3	2.3	12.9	12.9	3.3	3.2	3.3
4	1.9	12.2	12.1	3.6	3.5	3.4
5	1.8	12.9	12.6	3.0	3.0	3.0
6	2.3	14.0	13.9	3.5	3.3	3.4
7	2.3	14.1	13.6	3.6	3.5	3.6
Mean	2.14	14.13	14.57	3.55	3.54	3.53
A.O.A.C. Carotene Method[a]	2.2	15.1	15.0	3.5	3.7	3.6

[a] Two laboratories reported additional carotene results on the collaborative samples, using the official A.O.A.C. carotene procedure. These results were in close agreement between laboratories and the averages were used.

collaborative method, the agreement is close enough to be considered practical.

SUMMARY

The collaborative study of a procedure for determining vitamin A in mixed feeds was continued in 1953. A tentative revision of the procedure, requiring saponification to permit extraction of vitamin A from gelatinized and pectinized dry vitamin A products, was used in this study. Results of this investigation indicated similar, but no better, accuracy in applying the method than was found in previous studies on the shorter procedure (no saponification).

The saponification procedure under study is apparently effective in extracting vitamin A from all of the materials used as sources of vitamin A in the collaborative samples.

Carotene was determined in this investigation, and results indicate that application of the method may be satisfactory as a means of determining the total vitamin A activity in a feed sample.

The saponification procedure is much longer than the previous method. Unless a saponification procedure can be developed which demonstrates a greater accuracy and dependability than has been experienced with the present first action method, many laboratories indicate reluctance to discard the shorter method. In cases where saponification is required, this step should perhaps be included in the procedure as optional.

The Referee recommends* that the method (omitting saponification) be

* For report of Subcommittee A and action of the Association, see *This Journal*, 37, 64 (1954).

continued first action and that a saponification procedure be subjected to further collaborative study, including collaboration on samples of feed containing the stabilized vitamin A products mentioned in this report.

LIST OF COLLABORATORS

L. C. Callaghan, General Mills, Inc., Minneapolis, Minn.
H. A. Ellenberger, Lime Crest Research Laboratory, Newton, N. J.
C. H. Krieger, D. Cotora, and E. Richter, Wisconsin Alumni Research Foundation, Madison, Wis.
I. Olcott and Grace Blumer, Dawe's Laboratories, Inc., Chicago, Ill.
D. B. Parrish, Kansas State College, Department of Chemistry, Manhattan, Kan.
D. M. Stalter, Ohio State Department of Agriculture, Reynoldsburg, Ohio.
J. B. Wilkie, Food and Drug Administration, Washington, D.C.

REFERENCES

(1) *Official Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Box 540, Benjamin Franklin Station, Washington 4, D. C., 1950, p. 769.
(2) COOLEY, M. L., *This Journal*, **34**, 370 (1951).
(3) ———, *ibid.*, **35**, 706 (1952).
(4) ———, *ibid.*, **36**, 812 (1953).

REPORT ON THE DETERMINATION OF VITAMIN A IN MARGARINE

By KENNETH MORGAREIDGE (Food Research Laboratories, Inc., 48–14 Thirty-third Street, Long Island City, N. Y.), *Associate Referee*

Despite all that has been said and written on this subject in the past ten years, the methodology of vitamin A assay at low levels in foods and feeds has remained a matter of unfinished business on the agenda of everyone concerned with the problem. This has been especially true in the case of margarine. Biological assay, which presumably still constitutes the only official procedure applicable to this food, is rarely used, for obvious reasons. At the manufacturing control level, reliance my be placed on direct spectrophotometry where suitable blanks are available from the same ingredients used in the finished batch of margarine. Lacking such blanks, other physico-chemical methods have been employed with the realization that they entail certain difficulties which have not been satisfactorily overcome. Attesting to this is the fact that an official method for vitamin A in margarine has not yet been accepted by this Association.

In reviewing the history of the problem, the Associate Referee has been impressed with the rapid advances reported within the past few years in the use of chromatography for the separation and purification of many types of unstable compounds. Studies have been published on the chroma-

tography of vitamin A in particular (1–4), and a number of reports indicating progress in the application of the general technique to the margarine problem have appeared (5–9).

Ideally, this approach would be advantageous, provided the resulting fractions represent all of the vitamin A originally present in the aliquot taken for analysis, and that such fractions contain little or none of the usual extraneous materials which interfere with the application of direct spectrophotometric measurement. The elimination of the need for controversial formulas designed to correct for non-vitamin A absorption in the region of 325 mμ would be a definite advantage.

In view of this, it was decided to employ the time available during the past year in a limited survey of the most recent advances in this field. While a formal collaborative effort could not be considered in the time remaining after completion of these preliminary studies, Dr. Lawrence Rosner of the Laboratory of Vitamin Technology, and Dr. Eldon E. Rice, of Swift and Company, through the National Association of Margarine Manufacturers, and Dr. John B. Wilkie of the Food and Drug Administration were able to participate. The Associate Referee also wishes to acknowledge the valuable asistance of Mr. Joseph M. Icken of the staff of Food Research Laboratories, Inc. in carrying out the investigations reported here.

EXPERIMENTAL

The excellent results reported by the Dutch workers, Boldingh and Drost (8), made it appear desirable to repeat their procedure as applied to American margarines and to evaluate their method from the standpoint of applicability to A.O.A.C. needs. Unpublished information made available by Dr. R. J. Taylor, Unilever Ltd., Port Sunlight, England, further substantiated the feasibility of this procedure. The novelty of the chromatography employed lies in the use of alumina prepared in two different ways and set up in tandem columns, one above the other. The original apparatus (8) has been modified to permit easy and rapid operation, in particular by the addition of a side outlet tube near the bottom of the upper column which permits the separation of a forerun fraction without dismantling the unit.

Briefly, an unsaponifiable extract of margarine in dry petroleum ether is passed first through water-weakened alumina in the upper column under 1 to 2 pounds nitrogen pressure. The vitamin A band is followed visually on the column by means of its fluorescence under a long wavelength ultraviolet light source. The forerun, containing the coloring matter (dyes or carotene) is separated through the side-arm and its components may be determined separately. The vitamin A band is then transferred to the lower column of alkali-treated alumina where its passage is again followed by fluorescence. The portion of the final eluate con-

TABLE 1.—*Absorbance data, as fractions of a_{max}, for vitamin A alcohol in isopropanol*

WAVELENGTH	SOURCE OF VITAMIN A		
	CRYSTALLINE-SYNTHETIC[a]	CHROMATOGRAPHED[d]	
		U.S.P. REF.[b]	MARGARINE EXTRACT[c]
mμ		a_λ/a_{max}	
280	0.229	0.247	0.397
285	0.289	0.316	0.450
290	0.381	0.400	0.511
295	0.489	0.517	0.609
300	0.582	0.614	0.676
305	0.723	0.737	0.744
310	0.855	0.866	0.881
315	0.904	0.914	0.918
320	0.962	0.967	0.960
324	1.000	1.000	1.000
325	1.000	1.000	1.000
326	0.993	0.996	0.992
330	0.935	0.934	0.928
334	0.853	0.843	0.851
335	0.833	0.821	0.825
340	0.721	0.702	0.715
345	0.580	0.552	0.578
350	0.432	0.417	0.436
360	0.223	0.218	0.248

[a] Data given by Cama, Collins, and Morton, *Biochem. J.*, **50**, 48 (1951).
[b] Unsaponifiable extract of U.S.P. Reference Standard.
[c] Unsaponifiable extract of commercial colored margarine.
[d] Chromatography after Boldingh and Drost, *J. Am. Oil Chemists. Soc.*, **28**, 480 (1951).

taining the vitamin A is isolated in a separate receiver, the solvent is evaporated under nitrogen, and the residue is taken up in pure isopropanol to an appropriate volume for spectrophotometry. If the procedure has been properly carried out, the resulting vitamin A curve shows very little distortion and, at the present time, it is considered appropriate to multiply the observed a_{325} value by 1900 to obtain units of vitamin A.

RESULTS AND DISCUSSION

Table 1 shows a comparison of a typical ultraviolet absorption curve obtained from a commercial margarine sample with that of the U.S.P. Reference Standard, after both had been chromatographed as described. The curve for pure vitamin A alcohol in isopropanol given by Cama, Collins, and Morton (10) is also shown. It will be noted that very little non-vitamin A absorption is evident in the chromatographed margarine extract.

TABLE 2.—*Replicate assay of colored margarine samples by double column chromatography, units per lb.*

ASSAY	I[a]			II		
	POTENCY	R_{310}[b]	R_{324}[c]	POTENCY	R_{310}	R_{324}
1	11,800	0.865	0.889	17,700	0.864	0.879
2	12,000	0.869	0.891	17,400	0.867	0.882
3	11,800	0.870	0.890	17,400	0.873	0.876

[a] Coloring agent in this sample was carotene, the vitamin A equivalence of which has been omitted from this tabulation.
[b] Ratio of a_{310}/a_{325}.
[c] Ratio of a_{324}/a_{325}.

When increments of vitamin A acetate (as the U.S.P. Reference Standard solution) were added to margarine samples prior to saponification, the amount recovered as additional absorption at 325 mμ averaged 107 per cent in three trials.

The reproducibility of the procedure may be judged from the results obtained on two commercial samples of market margarine on which separate determinations were conducted on three successive days. The data are given in Table 2. Judged from this, the method is capable of quite satisfactory precision.

A number of observations made on the over-all behavior of the chromatographic columns in the Associate Referee's laboratory during the course of this work should be mentioned at this point. The efficiency of the two alumina preparations employed in tandem is very good. It was found that 27 per cent of the unsaponifiable solids, on a weight basis, are eluted in the final vitamin A fraction. Twenty-five per cent of the original solids are removed through the side-arm below the upper column. Thus, roughly half of the total unsaponifiable matter is retained, distributed between the two aluminas. Spectrophotometric evidence shows that considerable amounts of material with a peak absorption between 285 and 295 mμ are eluted with the vitamin A from the upper column (water-weakened alumina). Essentially all of this material is removed on the alkaline alumina in the lower column. It was further found that the length of the upper column of adsorbent could be varied considerably without much effect. The length of the alkaline alumina column, however, is more critical. Columns which are too long result in low recovery of the vitamin, while short columns permit the 290 mμ-absorbing material to pass through.

The statement is often made that the practice of chromatography is still more of an art than a science and there may be some justification for this point of view. Admittedly, a certain amount of skill must be acquired through practice on the part of the operator. Probably the most important factor in the successful use of alumina in separating vitamin A is the

TABLE 3.—*Collaborative study of single column method for vitamin A as applied to commercial colored margarine samples (all values reported without correction)*

SAMPLE	LABORATORY			
	1	2	3	4[c]
	Units/pound$\times 10^{-3}$			
A	14.5	15.3	15.3 (17.2)[a]	14.0
	14.8	15.3	15.7 (17.0)	14.3
B	13.4	14.8	12.0 (15.2)	14.8
	13.1	14.5	13.7 (15.2)	13.0
C[b]	10.4	10.7	8.1 (11.1)	8.6
	10.4	10.1	11.1 (11.1)	8.6
	11.4	10.1	8.7 (10.5)	—

[a] Values given in parentheses were obtained in the Associate Referee's laboratory by the Boldingh-Drost double column method.
[b] Coloring agent in this sample was carotene, the vitamin A equivalent of which has been omitted from this tabulation.
[c] The analyst of laboratory No. 4 employed a procedure which differed in the method of chromatography from that of laboratories 1, 2, and 3.

faithfulness with which the empirically derived procedures are followed, at least until considerable experience has been acquired. No difficulty has been encountered in this laboratory in preparing a series of successive batches of both types of adsorbent which have given uniformly good results. On the other hand, it has been found that there are a number of things to be avoided. This is a subject which will be elaborated on in subsequent reports.

It has been felt by some workers that the use of two different alumina adsorbents in tandem columns requiring a moderately complex job of glass-blowing makes for an unwieldy method for routine use. The Associate Referee recognizes and appreciates the basis for this impression although, in actual practice, the Boldingh and Drost technique can be handled with satisfactory rapidity. From the point of view of the routine control laboratory, a simpler technique would have obvious advantages, provided no serious difficulties were encountered. Efforts in this direction have been in progress, and a limited collaboration was undertaken to provide preliminary information on a single column method in which only the alkali-treated alumina is used. The results of this work are shown in Table 3, and it can be seen that encouraging progress has been made despite the fact that interlaboratory agreement still requires appreciable improvement. The details of the procedure used in this collaborative study are due to Dr. Lawrence Rosner whose contributed paper on the subject appears in *This Journal*, **37**, 887 (1954).

In conclusion, it may be said that a chromatographic method based on the use of specially prepared alumina offers distinct advantages over previously proposed procedures for the determination of vitamin A in mar-

garine. Much work remains to be done before a decision is reached as to the most suitable procedure to be recommended for recognition by this Association. A problem which still requires serious consideration is that of the validity of a correction for extraneous absorption as applied to chromatographically purified vitamin A fractions. There may be some reason to doubt the applicability to this type of extract of the current U.S.P. and A.O.A.C. fixation wavelengths in the Morton-Stubbs equation.

It is recommended* that studies be continued on the determination of vitamin A in margarine along the lines indicated in the foregoing report. A suitable procedure should be prepared and submitted to expanded collaborative trial.

REFERENCES

(1) BARUA, R. K., and MORTON, R. A., *Biochem. J.*, **45**, 308 (1949).
(2) GRIDGEMAN, N. T., GIBSON, G. P., and SAVAGE, J. P., *The Analyst*, **73**, 662 (1948).
(3) HJARDE, W., *Acta Chim. Scand.*, **4**, 628 (1950).
(4) EWING, D. T., SHARP, L. H., and BIRD, O. D., *Anal. Chem.*, **25**, 599 (1953).
(5) WILKIE J. B., and DEWITT, J. B., *This Journal*, **28**, 174 (1945).
(6) WILKIE, J. B., *ibid.*, **30**, 382 (1947).
(7) ———, *ibid.*, **32**, 455 (1949).
(8) BOLDINGH, J., and DROST, J. R., *J. Am. Oil Chemists' Soc.*, **28**, 480 (1951).
(9) BRAEKKAN, O., and LAMBERTSEN, G., *Tids. Kjemi, Bergvesen Met.*, **2**, 28 (1952).
(10) CAMA, H. R., COLLINS, F. D., and MORTON, R. A., *Biochem. J.*, **50**, 48 (1951).

REPORT ON CAROTENE

By F. W. QUACKENBUSH (Agricultural Experiment Station, Purdue University, Lafayette, Ind.), *Associate Referee*

A two-day workshop on carotene analysis was held at Purdue University on April 23 and 24, 1953. The purpose of the workshop, which was initiated at the request of industry, was to promote closer agreement in analytical procedure among the various laboratories. Invitations were sent to about 100 laboratories which had been participating in the monthly alfalfa meal check sample. Thirty-seven persons were enrolled at the workshop.

The program consisted of demonstration and discussion of each of the steps of analysis, together with laboratory work in which samples were carried through the entire A.O.A.C. procedure.

In the laboratory, portions of a single sample of alfalfa meal were extracted hot (one hour) and cold (overnight) by all participants, and the

* For report of Subcommittee A and action of the Association, see *This Journal*, **37**, 64 (1954).

TABLE 1.—*Carotene analysis of workshop samples of alfalfa meal*

COLLABORATOR NO.	RESULTS OBTAINED AT PURDUE U.		RESULTS OBTAINED IN ANALYSTS' OWN LAB. (4/28/53)		
	COLD EXT.	HOT EXT.	COLD EXT.	HOT EXT.	H_2O
1	80.6	76.6	81.3	76.8	
2	78.3	77.5	75.3	71.8	
3	77.6	74.6	75.3	72.5	4.03
4	76.0	77.1			
5	82.2	77.1	76.4	76.1	3.93
6	79.0	75.5	77.6	77.8	
7	78.5	75.2	77.6	77.8	
8	81.5	78.4	82.9	83.1 (Beckman)	
			81.7	81.7 (Evelyn)	
9	77.1	76.0	79.0	78.5	4.31
10	80.5	77.8	76.3	73.3	
11	78.3	76.2			
12	81.5	78.0	79.2	81.0	4.85
13	78.7	78.3	75.2	—	
14	78.7	78.3	82.0	—	4.9
15	81.8	77.6	73.9	72.2	5.11
16	80.1	81.1	79.9	77.0	4.28
17	80.3	76.6	79.7	77.4	4.5
18	84.1	77.4			
19	82.2	79.3	75.4	76.3	
20	81.1	79.0	80.3	76.7	
21	80.1	78.5	75.5	73.3 (Evelyn)	
			74.4	71.0 (Beckman)	
22	82.2	76.9	78.8		
23	75.3	76.2	81.5	74.8	4.18
24	—	79.9			
25	77.1	76.2	75.3	73.0	4.54
26	81.6	76.4			
Number not reported			78.6	77.1	
Number not reported			74.6	—	
Purdue		—	75.7	Not detd	
Av.	79.8 ±2.18	77.4 ±1.53	77.9 ±2.64	76.0 ±3.22	

extracts were then carried through all steps in the procedure. Each analyst also took with him a portion of the sample to be analyzed on a pre-arranged date (April 28, 1953) in his own laboratory. The results (Table 1) show very good agreement under all cases for most of the analysts. Results with overnight extraction averaged 2 to 3 per cent higher than those with the rapid extraction.

The participants agreed that two of the greatest sources of variation in carotene analysis were variability in the adsorbent and poor standardization of instruments. As a result of this discussion, efforts were made to

TABLE 2.—*Comparison of average carotene values (mg/lb) on monthly alfalfa meal check samples before and after workshop*

SAMPLE NO.	SENT OUT	ALL COLLABORATORS	THOSE WHO ATTENDED WORKSHOP
		Before Workshop	
40	Feb.	75.2±3.41	75.6±2.91
41	March	13.9±1.90	14.6±1.92
42	April	83.5±4.67	82.2±4.80
		After Workshop April 23 & 24	
43	May	55.4±3.8	53.6±4.3
44	June	114.0±5.26	115.1±4.34
45	July	60.1±7.03	60.1±5.96
46	August	33.2±3.37	32.0±2.70
47	Sept.	111.2±7.40	110.8±5.47

enlist the cooperation of the producer of the adsorbent in setting aside a homogeneous batch of magnesia sufficiently large to constitute a several years' supply for all laboratories engaged in carotene analysis. However, the producer expressed regret that he would be unable to reserve such a supply. To improve upon present practices in the photometry of carotene eluates, an effort is again being made to provide a standard carotene solution which can be made available to various laboratories. The problems of solubility and stability of such a carotene standard have not yet been solved.

To determine the degree to which the group discussion and laboratory at the workshop was effective in improving carotene analysis in the various laboratories, separate data were compiled for those collaborators who were in attendance (Table 2). The results seem to show somewhat better agreement resulting from the workshop. However, it is apparent that uncontrolled factors are still influencing the results to a considerable degree.

The Referee wishes to acknowledge the excellent cooperation of Mr. John Kephart, National Alfalfa Dehydrating and Milling Company, Lamar, Colorado, who prepared and mailed the alfalfa samples.

It is recommended* that the work on carotene be continued.

* For report of Subcommittee A and action of the Association, see *This Journal*, 37, 64 (1954).

REPORT ON XANTHOPHYLL IN MIXED FEEDS

By C. R. THOMPSON (Western Utilization Research Branch, Agricultural Research Service, U. S. Department of Agriculture, Albany 6, Calif.), *Associate Referee*

As the initial phase of this study, a literature search was conducted to determine what xanthophylls could be predicted to occur in mixed feeds. Results showed that about thirty separate pigments are present to some degree in the components of mixed feeds. Considering that a number of isomers of each pigment are known, it was indicated that literally hundreds of separate entities could be present. However, from a quantitative standpoint, a few xanthophylls (lutein, zeaxanthin, violaxanthin, cryptoxanthin, and neoxanthin) predominate in the feed ingredients that are richest, e.g. yellow corn, dehydrated alfalfa meal, dehydrated grass meal, etc. In any given feed it could be predicted that these xanthophylls would constitute over 90 per cent of the total.

A second phase of the study was to send an inquiry concerning methods presently in use for the determination of xanthophylls in mixed feeds to nineteen laboratories which are the source of publications dealing with either xanthophylls or closely related carotenoids. Replies were received from sixteen (4 foreign and 12 domestic). A few references to published methods were obtained, but only two laboratories submitted detailed methods which were being used. One method which utilized phasic distribution of carotenoids between 90 per cent methanol–petroleum ether was suggested, but it was further indicated that prior results, in which the determination of carotene had been studied, had shown that this procedure failed to separate xanthophylls quantitatively.

The other method was a chromatographic procedure somewhat similar to that presently used for the determination of carotene.[1] In this method, dried ground material was extracted with 30 per cent acetone–70 per cent hexane by refluxing for one hour. The sample was washed with hexane, evaporated to a small volume, and chromatographed on Magnesia #2641; carotene was eluted with 10 per cent acetone–90 per cent hexane. Xanthophylls were eluted with methanol.

Studies in this laboratory have shown that some modification of the chromatographic procedure described will probably be suitable, but that a number of questions should be answered. Of primary importance is quantitative extraction. Because xanthophylls exist as esters, it may be that saponification should precede extraction to liberate the pigment from any insoluble chemical combination. However, unless it is absolutely necessary, this step should be avoided because of the repeated operations

[1] *Official Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Box 540, Benjamin Franklin Station, Washington 4, D. C., 1950.

necessary to extract the xanthophylls from an aqueous mixture and also because of the corrosive nature of the reagents. Volumes should be adjusted prior to a given operation so that time-consuming evaporation can be avoided. The use of 100 per cent methanol has given indications of eluting yellow pigments other than xanthophylls. A detailed study should be made on this point, but experience in this laboratory favors the use of a less polar eluant such as 10 per cent methanol in hexane. Magnesia #2641 is still available, but equal success has been achieved with #2642 which is used for carotene analysis.

If possible, a method should be devised which would employ the basic equipment, chemicals, and procedure now used for carotene determination because of the similarity in the pigments.

The question of isomerization of all carotenoids during analysis has plagued many investigators. However, studies in these laboratories have indicated that, because of the extreme lability of these pigments, by the time a xanthophyll-rich constituent such as alfalfa meal or yellow corn is incorporated into a mixed feed, further manipulation will have little overall effect because of the number of isomers of each separate xanthophyll which are present. Because of this, some arbitrary standard such as lutein or even β-carotene, each of which can be obtained relatively pure, will probably have to be used.

RECOMMENDATIONS

It is recommended*—

(1) That studies on determination of xanthophyll in mixed feed be continued.

(2) That collaborative samples be submitted to a number of laboratories for analysis by a chromatographic method.

REPORT ON THE DETERMINATION OF THIAMINE IN ENRICHED FLOUR

COMPARISON OF FLUOROMETRIC METHODS

By LEWIS H. MCROBERTS (Food and Drug Administration, Department of Health, Education, and Welfare, San Francisco 2, Calif.), *Associate Referee*

This Associate Refereeship was established in 1951 to study thiochrome methods, with the view of developing a rapid procedure with official status. The 1952 report (1) contained results of a collaborative study designed to compare sample solution preparation by plain acid hydrolysis

* For report of Subcommittee A and action of the Association, see *This Journal*, 37, 64 (1954).

TABLE 1.—*Thiamine in enriched flour*[a]

EXPERIMENT REFERENCE	SAMPLE DESCRIPTION, ENRICHED FLOUR	DIGESTION ACID, CA 0.1 *N*	SALT DESCRIPTION	ACID DIGESTION TEMP., °C.	PHOTOFLUOROMETER READINGS								THIAMINE, MG/LB.	
					FILTER DESCRIPTION								FILTER DESCRIPTION	
					B1/PC1				B1/B2				B1/PC1	B1/B2
					STANDARD 1 MMG	STANDARD BLANK	SAMPLE	SAMPLE BLANK	STANDARD 1 MMG	STANDARD BLANK	SAMPLE	SAMPLE BLANK		
A–1				Water bath										
A–5	Check Sample #103	HCl	5% NaCl	95–100	74	2.0	80	3.5	—	—	—	—	2.12	—
A–2		HCl	15% NaCl	95–100	75	2.0	80	4.0	—	—	—	—	2.08	—
A–3		HCl	None	95–100	73	2.0	77	3.5	—	—	—	—	2.08	—
A–4		H_2SO_4	5% NaCl	95–100	74	2.0	80	4.0	—	—	—	—	2.10	—
		H_2SO_4	None	95–100	74	2.0	77	3.0	—	—	—	—	2.06	—
													Av. 2.09	
A–6	Check Sample #103	HCl	5% NaCl	Autoclaved	76	2.0	81	13.0	59	2.0	66	9.0	1.82	2.00
A–7		HCl	None		75	2.0	80	10.0	59	2.0	64	6.0	1.92	2.04
A–8		H_2SO_4	5% NaCl		76	2.0	80	7.0	60	2.0	64	4.5	1.98	2.06
A–9		H_2SO_4	None	(121–123)	76	2.0	80	6.0	59	2.0	62	3.5	2.00	2.05
													Av. 1.93	2.04
B–1	Check Sample #103	HCl	None	Autoclaved (121–123)	72	4.0	76	8.0	—	—	—	—	2.00	—
B–3		HCl	None	(108–109)	72	3.0	78	5.0	—	—	—	—	2.10	—
B–4		HCl	None	(108–109)	72	3.0	77	5.0	—	—	—	—	2.09	—
													Av. 2.06	—
C–1	Check Sample #103	HCl	5% NaCl[b]	Autoclaved 108–109	83.5	3.5	86.5	6.0	—	—	—	—	2.02	—
C–2		HCl	5% KCl[c]	108–109	84.5	4.0	86.0	6.0	—	—	—	—	1.98	—
C–3		HCl	5% NaCl[c]	108–109	84.0	2.5	87.5	6.0	—	—	—	—	2.00	—
C–4		HCl	5% KCl[c]	108–109	84.0	3.5	88.5	6.0	—	—	—	—	2.04	—
													Av. 2.01	—
D–3	Check Sample #104	HCl	5% NaCl	Water bath 95–100	60	2.0	72	3.0	60	2.0	71	2.5	2.38	2.38
D–4		HCl	5% KCl	95–100	60	2.0	70	3.0	60	2.0	70	2.5	2.31	2.36
D–6	41–041 L	HCl	5% NaCl	Autoclaved 108–109	60	2.0	68	4.0	59	2.0	68	3.0	2.21	2.28
D–1		HCl	5% NaCl	Water bath 95–100	60	2.0	90	3.0	60	2.0	89	2.5	3.00	2.98
D–2		HCl	5% KCl	95–100	60	2.0	92	3.0	60	2.0	91	2.5	3.07	3.06
D–5		HCl	5% NaCl	Autoclaved 108–10[illegible]	60	2.0	90	4.0	59	2.0	90	3.0	2.97	3.04

[a] Experimental studies: Acids comparison (A1–5); temperature comparisons (A1–9)(B1–4)(D1–6); salt comparison (C1–4). Rapid acid hydrolysis procedure. Analyses for comparison of rapid procedure with A.O.A.C. official method **40.18–40.22**: Check sample No. 103, thiamine 2.20 mg/lb; check sample No. 104, thiamine 2.31 mg/lb; sample No. 41–041 L, thiamine 3.03 mg/lb.

[b] Salt added previous to acid digestion.

[c] Salt added after acid digestion.

with the official method (2) which involves acid hydrolysis, enzyme digestion, and base exchange separation. Since the official method allows for acid digestion at either 95–100°C. or 121–123°C. (autoclaving), comparisons of the two methods were made at both temperatures. The results were in good agreement at the lower temperature but the majority of collaborators reported definitely low figures where autoclaving was employed for the rapid method. It was recommended that the study be continued.

This year after further experimental studies, the rapid procedure was revised to include the alternative of autoclaving at a lower temperature than that previously specified. The revised procedure was subjected to collaborative study. The results of this study and an outline of the procedure used are included in this report.

EXPERIMENTAL

In addition to the trial of a lower autoclaving temperature for the rapid method, other experiments were conducted on the comparison of various combinations of photofluorometer filters, the acids that are usually employed in the digestion (ca 0.1 *N* HCl *vs.* ca 0.1 *N* H_2SO_4), and the salts used for clarification (NaCl *vs.* KCl). The results are tabulated in Table 1. Experiments A, B, and C were all made on the basis of one sample of enriched flour, so that an over-all comparison of results may be made on this series of analyses.

(1) *Photofluorometer Filters.*—The photofluorometer used in this laboratory is equipped with five filters that are designated as B-1, B-1S, B-2, PC-1, and PC-2. The first three are primary filters to be inserted between the light source and the sample tube. The "PC" or secondary filters are inserted between the sample tube and the photocell and are for the purpose of screening the photocell from light other than the fluorescent light to be measured. The combinations of B-1/PC-1 or B-1S/PC-1 are recommended by the manufacturer for the determination of thiamine and the combination B-2/PC-2 for riboflavin. These filters have been analyzed for range by the Beckman spectrophotometer with the results shown in Table 2:

It appears that the PC-1 filter has a rather wide range for the secondary

TABLE 2.—*Analysis of filters for range*

FILTER	RANGE (mμ)	PEAK TRANSMITTANCE
		per cent
B-1	315–400	25
B-1S	315–400	53
B-2	400–475	26
PC-1	410–660	75
PC-2	500——	89

position for thiamine and that the B-2 filter would provide a more selective range for the fluorescent light from thiochrome. Apparently this is also the conclusion of the majority of collaborators reporting last year, in that four out of seven used either B-1S/B-2 or B-1/B-2 combinations. The filters used by each of the collaborators are listed, with the results tabulated in Table 5.

The Associate Referee has made frequent comparisons of the filter combinations B-1/PC-1 *vs.* B-1/B-2, and in general there has been no appreciable difference for the rapid method where the acid hydrolysis has been made at a water bath temperature of 95–100°C. However, where autoclaving is employed, there is some indication that the B-1/B-2 combination compensated for the resulting high sample blanks.

(2) *Autoclaving for Acid Hydrolysis.*—In the 1952 collaborative trial of rapid acid hydrolysis procedures, four of the seven collaborators reported definitely lower results by autoclaving at 15 lbs. pressure (121–123°C.) as compared to autoclaving at this same temperature for the official method. The following comparison analyses were therefore made to determine the cause of this variation.

(*a*) *Acid Hydrolysis at 95–100°C. vs. Autoclaving at 15 lbs. (121–123°C.), Table 1 (Exp. A, 1–9 inc.).*—Extremely high sample blanks are to be noted in autoclaving at 15 lbs. pressure with ca 0.1 *N* HCl (Determinations 6 and 7) with results from 6–14 per cent lower than with the digestion at 95–100°C. (filter combination B-1/PC-1.) In this experiment the low results seem to have been compensated by the use of the filter combination B-1/B-2.

(*b*) *Acid Hydrolysis at 5 lbs. Pressure (108–109°C.)*—In the report of his analysis of collaborative samples in 1952, Harold E. Theper made the following comments: " . . . Samples autoclaved at 15 lbs. gave higher blanks, and the longer the blank determination was allowed to stand after the addition of the 3 ml of NaOH and before extracting with isobutyl alcohol, the higher the blank. This was more pronounced upon the addition of KCl in the digestion. . . . " He recommended a trial of autoclaving at 5 lbs. pressure with a shorter heating time of twenty minutes which would be closer to the equivalent of one-half hour at water bath temperature. With this lead, the Associate Referee made the following analyses by the rapid acid hydrolysis procedure described in this report.

Five lbs. Pressure (108–109°C.) vs. 15 lbs. Pressure (121–123°C.), Table 1 (Experiment B, parts 1, 3, and 4).—Determination at 121–123°C. was about 5 per cent lower than the determinations at 108–109°C. A high sample blank is again to be noted for the higher temperature autoclaving. Determinations 3 and 4 with autoclaving at 5 lbs. pressure are considered to be check results with digestion at 95–100°C. (Experiment A, 1–5).

(3) *Digestion Acids Comparison* (ca 0.1 *N* HCl *vs.* ca 0.1 *N* H_2SO_4).—With water bath digestion at 95–100°C., the comparative data indicate

TABLE 3.—*Comparison of blanks from flour digestion*

TUBE NO.	BLANK DESCRIPTION	DIGESTION	TIME LAG, NaOH TO ISOBUTYL ALCOHOL	B-1/PC-1 PHOTOFLUOROMETER READING
1	Sample	Autoclaved	Immediate (10 sec.)	10
2	Sample	Autoclaved	15 min.	ε
3	Sample	Autoclaved	30 min.	9
4	Detn Standard	Autoclaved	Immediate (10 sec.)	3
5	Detn Standard	Autoclaved	15 min.	10
6	Detn Standard	Autoclaved	30 min.	10

no appreciable difference in the use of the two acids (Experiment A, 1–5). There is some indication that HCl may give lower results than H_2SO_4 where autoclaving at 15 lbs. pressure is employed (Experiment A, 6 and 7).

(4) *Salt as a Clarifying Agent (NaCl vs. KCl), Table 1 (Experiments C, 1–4, and D, 1–6).*—When salts were used as clarifying agents for efficient separation of the sample solution by filtration or centrifuging, no appreciable differences in results were noted in the use of NaCl and KCl with acid

TABLE 4.—*Comparisons of oxidation blanks from flour digestions by autoclaving at 108–109° and at 121–123° (ca 0.1 N HCl)*[a]

TUBE NO.	BLANK DESCRIPTION	TIME NaOH→ ISOBUTYL ALCOHOL	PHOTOFLUOROMETER READINGS B1/PC1	BLANK DESCRIPTION	TIME NaOH→ ISOBUTYL ALCOHOL	PHOTOFLUOROMETER READINGS B1/PC1
	Autoclaved at 15 lbs			Autoclaved at 5 lbs		
1	Sample	10 sec.	7.0	Sample	10 sec.	4.5
2	Sample	5 min.	7.0	Sample	5 min.	4.0
3	Sample	10 min.	7.0	Sample	10 min.	4.5
4	Sample	15 min.	7.0	Sample	15 min.	4.0
5	Sample	20 min.	6.8	Sample	20 min.	4.0
6	Sample	25 min.	6.8	Sample	25 min.	4.0
7	Sample	30 min.	6.5	Sample	30 min.	3.5
8	Sample	60 min.	6.5			
1	Detn Std (1 mmg)	10 sec.	2.5	Detn Std (1 mmg)	10 sec.	2.0
2	Detn Std (1 mmg)	5 min.	3.5	Detn Std (1 mmg)	5 min.	3.0
3	Detn Std (1 mmg)	10 min.	3.8	Detn Std (1 mmg)	10 min.	3.8
4	Detn Std (1 mmg)	15 min.	4.0	Detn Std (1 mmg)	15 min.	3.8
5	Detn Std (1 mmg)	20 min.	4.0	Detn Std (1 mmg)	20 min.	4.0
6	Detn Std (1 mmg)	25 min.	4.0	Detn Std (1 mmg)	25 min.	4.0
7	Detn Std (1 mmg)	30 min.	4.0	Detn Std (1 mmg)	30 min.	3.5
8	Detn Std (1 mmg)	60 min.	2.5			

[a] No salt added: basis check sample No. 103.

digestion at 95–100°C. There is some indication that salt causes the sample blank to be higher where autoclaving is used.

(5) *Sample Blanks and Standard Blanks.*—A study was made of sample blanks and standard blanks at the point of oxidation to thiochrome, from flour digestions at 95–100°C. and by autoclaving at 121–123°C. (5 per cent NaCl in ca 0.1 *N* HCl). The time lag between addition of 15 per cent NaOH and the isobutyl alcohol shakeout is shown in Table 3. Blank readings from solutions prepared by digestion at 95–100°C. were from 2 to 3 for the sample and the standard, and there was no change over a period of thirty minutes in the time lag between the additions of NaOH and isobutyl alcohol.

With digestion by autoclaving at 15 lbs. pressure, with immediate shakeout (10 seconds required to add alcohol), sample blanks show abnormally high readings that are constant up to fifteen to twenty minutes, followed by a progressive decrease. The behavior of the autoclaved standard blank is different from that of the sample blank. With immediate shakeout, the standard blank is normal but may increase with time up to fifteen minutes (Table 4).

With digestion by autoclaving at 5 lbs. pressure and with reduced total heating time, the sample blanks are close to those obtained in water bath digestion. There is, however, the phenomenon of increasing standard blank with time lag before the addition and shaking with isobutyl alcohol. This does not occur with determination of standard blanks with digestion in a water bath at 95–100°C.

Immediate shakeout is specified in the official procedure. This would probably also be the best procedure for the rapid method.

COLLABORATIVE STUDY

Three samples of enriched flour were submitted to collaborators with the following quoted instructions and outline of method:

"Sample No. 1 (Enriched Bromated Flour) (One 1 pt. jar, ca 200 g)
Sample No. 2 (Enriched Flour) (One 1 pt. jar, ca 200 g)
Sample No. 3 (Enriched Flour) (One 1 pt. jar, ca 200 g)

The jars are only partially filled to allow sufficient space for mixing the samples in the closed jars. (Roll the jars for about one minute previous to opening for sample weights.)

Following last year's collaborative study, further experimental work has been done with particular reference to preparation of the sample solution by autoclaving for the rapid procedure. In view of recommended changes, it is now necessary to make further comparisons between the official and the revised rapid procedures.

Analyze each sample by all of the following described methods:

(I) Official method, **40.18–40.22**, *Official Methods of Analysis*, 7th Ed. (Acid hydrolysis, enzyme digestion, and base exchange separation).
  (*a*) Acid hydrolysis at 95–100°C.
  (*b*) Acid hydrolysis at 121–123°C. (autoclaved).

(II) Rapid procedure (acid hydrolysis), described below.
  (*a*) Acid hydrolysis at 95–100°C.

(*b*) Acid hydrolysis at 108–109°C. (autoclaved).

(III) Report results as mg/lb, making use of both 'Determination' and 'Direct' standards.

NOTES

(1) Following acid hydrolysis in the rapid procedure, both centrifuging and filtration are now considered as alternative means of separation of the sample solution. Use the means of separation most often employed for this purpose in your laboratory and so indicate on the report form.

(2) There are differences of opinion as to the effectiveness of NaCl as a clarifying agent when added prior to the acid hydrolysis in the rapid procedure. Therefore its use has not been made mandatory. In the experience of the Associate Referee, the addition of salt to the flour is a definite aid in properly dispersing the flour in the acid and in providing clear solutions following filtration or centrifuging. Sodium chloride and potassium chloride are equally effective.

(3) Sodium chloride or potassium chloride will prevent clouding of the isobutyl alcohol in the final extraction of thiochrome. Sodium chloride has been specified in the method as the more economical reagent of the two.

(4) The previous collaborative trial of the rapid procedure indicated that autoclaving at 15 lbs. pressure may give results lower than those obtained by the official procedure. Extremely high blanks result when the sample is autoclaved at this pressure and when the complete official procedure is not employed. Lately it has been determined that autoclaving at 5 lbs. pressure with a controlled total heating time should give results that are comparable to the official procedure."

RAPID METHOD FOR THIAMINE IN ENRICHED FLOUR

REAGENTS

(a) *Thymol blue pH indicator.*—Triturate 0.1 g of indicator in mortar with 4.3 ml of 0.05 *N* NaOH and dil. to vol. of 200 ml with H_2O.

(b) *Sodium chloride.*—Reagent grade.

(c) *Isobutyl alcohol.*—B.p. 106°–108°C. Isobutyl alcohol may be recovered as follows: Sep. the alcohol from the alk. ferricyanide, wash with ⅓ vol. of ca 0.1 *N* HCl, and distil in all glass equipment. Reserve first portion of distillate (constant boiling mixt. of alcohol and H_2O at ca 90°C.) for subsequent recovery. The distillate recovered at 106–108°C. should have reading not exceeding 3% of total photofluorometer scale.

(d) *Sulfuric acid.*—Ca *N;* 57 ml of concd H_2SO_4 per 2000 ml of H_2O.

(e) *Sulfuric acid.*—Ca 0.1 *N;* 5.7 ml of concd H_2SO_4 per 2000 ml of H_2O.

(f) *Sodium hydroxide soln.*—15% w/v.

(g) *Potassium ferricyanide soln, alkaline.*—Dissolve 40–50 mg of $K_3Fe(CN)_6$ in 100 ml of the 15% NaOH. Use soln within 4 hrs.

(h) *Quinine sulfate stock soln and quinine sulfate standard soln.*—See **40.18 (m), (n)**.

(i) *Thiamine hydrochloride stock soln.*—Weigh accurately 22–25 mg of U.S.P. Thiamine Hydrochloride Reference Standard which has been kept in a desiccator over P_2O_5 at least 24 hrs. Keep stoppered during the weighing to avoid absorption of moisture. Dissolve in 20% alcohol adjusted to *p*H 3.5–4.3 with HCl and make to 1 l with the acidified alcohol. Add sufficient addnl acidified alcohol to bring the concn to exactly 20 mmg per ml. Store in a cool place in a glass-stoppered light resistant bottle.

(j) *Thiamine hydrochloride standard solns.*—(1) From a portion of the stock soln that has been warmed to the proper temp., pipet 10 ml into 200 ml volumetric flask

and dil. to vol. with the 0.1 *N* H_2SO_4 (1 ml=1 mmg). Make an addnl diln by pipetting 40 ml of the intermediate standard into 200 ml volumetric flask and dil. to vol. with the 0.1 *N* H_2SO_4 (1 ml=0.2) (direct standard soln).

(2) Add NaCl to the standards in amount to give final concn of ca 5% w/v if NaCl was added to the sample. (See note under "Extraction").

EXTRACTION

Weigh sufficient sample to give a final sample soln with a thiamine content of ca 0.2 mmg/ml (*i.e.*, 4.54 g of flour for 100 ml or 9.07 for 200 ml of final vol.) and proceed by one of the following methods:

(a) (95–100°C. *Digestion*).—Transfer weighed sample to a digestion bottle,[1] and add a vol. of ca 0.1 *N* H_2SO_4 that is ca 15:1, relating ml of acid to g of sample. The acid is added in 2 portions with vigorous stirring, using part of the acid to wash down sides of bottle. Place bottles in H_2O bath previously brought to 95–100°C. Stir at frequent intervals to keep solids in suspension during thickening stage (5–8 min.) and occasionally during the balance of the total heating time of 30 min. After hydrolysis has proceeded for ca 10 min., place a drop of the soln on spot plate and test with thymol blue. Soln should be distinctly red (*p*H 1–1.2). If not sufficiently acid (indicating presence of basic substances in sample) add ca *N* H_2SO_4 in 1.0 ml amounts until desired acidity is reached. Note amount of *N* acid required to supplement the 0.1 *N* acid and *repeat digestion* with new sample wt and the necessary mixture of *N* and 0.1 *N* acids. Cool to room temp. and make to required vol. with 0.1 *N* H_2SO_4.

Centrifuge mixt. until the supernatant liquid is clear or practically so, or filter thru paper. (Check filter paper for thiamine adsorption by comparing filtered and non-filtered thiamine standard soln.) Filtration may also be accomplished with suction, employing fritted glass funnels and analytical filter aid. Discard first part of filtrate in amount of ca 0.1 total vol.

(b) *Autoclaved digestion.*—Follow directions of method (a) without addn of NaCl, except to autoclave at 5 lb pressure (108–109°C.) for 20 min. with a total heating time of not more than 35 min. including 5–10 min. to attain the desired pressure and ca 5 min. to reduce pressure. (It may be necessary to preheat autoclave to ca 100°C. previous to introduction of samples. *Release pressure gradually to avoid foaming and resultant loss of soln.* Erlenmeyer flasks of ca 800 ml capacity are recommended.)

OXIDATION

Transfer 5 ml aliquots of prepd sample soln and of standard thiamine soln to glass-stoppered centrifuge tubes or glass-stoppered bottles of about 35 ml capacity contg ca 1.5 g of NaCl. (Where excessive amounts of thiamine are found, make sufficient diln with 0.01 *N* H_2SO_4 so that 5 ml will contain ca 1.0 mmg. Swirl tubes or bottles gently to dissolve part of the salt. (*The precision and accuracy of results depend upon a uniform technique in carrying out the oxidation.*) For addn of oxidizing agent, use pipet which will deliver 3 ml in 1 sec. Place tip of pipet holding the alk. ferricyanide soln in neck of tube and hold it so that stream of soln will not hit side of tube. Give tube a gentle swirl to impart rotational motion in liquid and immediately add 3.0 ml of alk. ferricyanide. Remove pipet and swirl tube once again to insure adequate mixing. *Immediately thereafter* add 13 ml of isobutyl alcohol; stopper, and shake tube for 10–15 sec. After alcohol has been added to all tubes, they should be given an addnl shaking for ca 2 min. (The tubes may be combined in a shaker box

[1] The addn of NaCl in amount to give a final concn of ca 5% w/v will aid in the subsequent sepn of the sample soln. The flour and salt should be thoroly mixed with a stirring rod previous to the addn of the 0.1 *N* H_2SO_4.

for this addnl shaking.) Centrifuge for 2–3 min. Pipet ca 10 ml of the alcohol layer into photofluorometer cuvettes, or, if small bottles have been employed, the alcohol can be poured off. The shoulder of bottle will aid in sepn of the two liquids.

BLANKS

Run blanks on 5 ml of standard and assay solns, adding 3 ml of 15% NaOH in place of the alk. ferricyanide soln and employing the same technique described above. (Avoid contaminating blanks with minute amounts of alk. ferricyanide soln that may be on the tip of the isobutyl alcohol buret or pipet.)

THIOCHROME FLUORESCENCE MEASUREMENT

Measure fluorescence with a photofluorometer fitted with an input filter with peak transmittance at 365 mμ and an output filter effective in screening out the incident light. The recommended range for output filter is 400–475 mμ with peak transmittance close to 435 mμ. Make a standard curve by plotting mmg of thiamine against readings corrected for blanks. By means of this curve, convert sample readings corrected for sample blanks to mmg of thiamine and calc. to mg per pound. Suggested amounts of final standard soln are 1, 3, and 5 ml representing 0.2, 0.6, and 1.0 mmg of thiamine, diluting all tubes to 5 ml with 0.1 *N* H_2SO_4. Use the quinine sulfate standard soln to govern reproducibility of the instrument.

SUMMARY

Experimental Study.—It was demonstrated that either approximately 0.1 *N* hydrochloric acid or approximately 0.1 *N* sulfuric acid could be used in acid hydrolysis conducted at the water bath temperature of 95–100°C. There was some evidence that hydrochloric acid contributed to high sample blanks in the alternative of autoclaving at 108–109°C. Therefore, approximately 0.1 *N* sulfuric acid was specified in the revised rapid method in the interest of uniformity.

When salt is added as a clarifying agent for the efficient separation of the sample solution, sodium chloride and potassium chloride are equally effective. There was some evidence that added sodium or potassium chlorides may contribute to high sample blanks in the alternative of acid digestion by autoclaving. This effect was not apparent in the acid digestion at 95–100°C.

It was demonstrated that autoclaving at 121–123°C. in the acid digestion is not applicable for the rapid procedure, in that correction for resulting high sample blanks may give low results. It was also found that acid digestion by autoclaving at 5 pounds pressure at 108–109°C. with controlled heating time gave results comparable with digestion at 95–100°C.

In the description of the rapid procedure for oxidation to thiochrome, submitted to collaborative study in 1952, a time lag of thirty minutes was allowed between the addition of alkaline ferricyanide or sodium hydroxide and the isobutyl alcohol shakeout. This is at variance with the directions in the official method in which immediate shakeout is specified. This year, with sample solutions prepared by autoclaving, it was demonstrated that standard blanks may become progressively higher with time lag after the

TABLE 5.—*Collaborative study of methods for thiamine in enriched flour, June–July 1953*

COLLABORATOR NO.	OFFICIAL A.O.A.C. METHOD[a]		RAPID PROCEDURE[a]				PHOTOFLUOROMETER FILTERS USED
	DIGESTION TEMPERATURE		DIGESTION TEMPERATURE				
	95–100°	121–123°	95–100°-WATER BATH		108–109°-AUTOCLAVED		
	WATER BATH	AUTOCLAVED	DETN STD[b]	DIR. STD[c]	DETN STD[b]	DIR. STD[c]	
			Sample No. 1				
	2.60	2.56	2.54	2.54	2.50	2.50	
1	2.54	2.52	2.54	2.54	2.50	2.50	B1/B2
	2.50	2.52	2.42	2.42	2.50	2.50	
	2.50	2.56	2.42	2.42	2.50	2.50	
Av.:	2.54	2.54	2.48	2.48	2.50	2.50	
2	2.60	2.72	2.54	2.54	2.46	2.46	B1/B2
	2.60	2.68	2.49	2.49	2.47	2.47	
Av.:	2.60	2.70	2.52	2.52	2.47	2.47	
	2.32	2.35	2.27	2.26	2.25	2.21	
3	2.37	2.38	2.30	2.28	2.25	2.21	B1/PC1
	2.34	2.33	2.30	2.28	2.21	2.19	
	2.30	2.38	2.31	2.30	2.20	2.19	
Av.:	2.33	2.36	2.30	2.28	2.23	2.20	
	2.80	2.52	2.10	2.07	2.19	2.15	
4	2.75	2.52	2.14	2.12	2.17	2.13	B1/PC1
	2.82	2.52	2.08	2.06	2.25	2.21	
	2.80	2.54	2.10	2.08	2.32	2.28	
Av.:	2.79	2.53	2.11	2.08	2.23	2.19	
5	2.60	2.64	2.74	2.82	2.64	2.66	B1/B2
	2.58	2.62	2.74	2.82	2.68	2.72	
Av.:	2.59	2.63	2.74	2.82	2.66	2.69	
	2.46	2.38	2.46	2.48	2.36	2.36	
6	2.46	2.43	2.35	2.36	2.31	2.31	B1-S/B2
	2.53	2.40	2.42	2.40	2.42	2.37	
	2.56	2.41	2.36	2.35	2.42	2.37	
Av.:	2.50	2.41	2.40	2.40	2.38	2.35	
Total Av.:	2.55	2.50	2.38	2.38	2.38	2.36	

[a] Results from duplicate sample weights.
[b] Results calculated on the basis of "Determination Standard."
[c] Results calculated on the basis of "Direct Standard."

TABLE 5—(*continued*)

COLLABORATOR NO.	OFFICIAL A.O.A.C. METHOD[a]		RAPID PROCEDURE[a]				PHOTOFLUOROMETER FILTERS USED
	DIGESTION TEMPERATURE		DIGESTION TEMPERATURE				
	95–100°	121–123°	95–100°-WATER BATH		108–109°-AUTOCLAVED		
	WATER BATH	AUTOCLAVED	DETN STD[b]	DIR. STD[c]	DETN STD[b]	DIR. STD[c]	
			Sample No. 2				
	1.72	1.72	1.68	1.68	1.62	1.62	
1	1.72	1.68	1.68	1.68	1.62	1.62	B1/B2
	1.72	1.68	1.62	1.62	1.60	1.60	
	1.72	1.68	1.62	1.62	1.60	1.60	
Av.:	1.72	1.69	1.65	1.65	1.61	1.61	
2	1.78	1.84	1.73	1.73	1.71	1.71	B1/B2
	1.77	1.84	1.69	1.69	1.68	1.68	
Av.:	1.78	1.84	1.71	1.71	1.70	1.70	
	1.58	1.60	1.52	1.51	1.51	1.49	
3	1.57	1.60	1.52	1.51	1.50	1.48	B1/PC1
	1.61	1.60	1.54	1.54	1.51	1.49	
	1.54	1.57	1.54	1.54	1.51	1.49	
Av.:	1.58	1.59	1.53	1.52	1.51	1.49	
	1.64	1.59	1.29	1.27	1.23	1.21	
4	1.65	1.63	1.26	1.25	1.23	1.21	B1/PC1
	1.64	1.60	1.40	1.38	1.45	1.43	
	1.65	1.59	1.39	1.37	1.47	1.45	
Av.:	1.65	1.60	1.34	1.32	1.35	1.33	
5	1.58	1.70	1.72	1.76	1.64	1.66	B1/B2
	1.58	1.66	1.66	1.70	1.64	1.66	
Av.:	1.58	1.68	1.69	1.73	1.64	1.66	
	1.71	1.71	1.60	1.61	1.63	1.63	
6	1.71	1.74	1.62	1.63	1.60	1.60	B1-S/B2
	1.66	1.68	1.61	1.60	1.63	1.60	
	1.63	1.72	1.58	1.57	1.61	1.57	
Av.:	1.68	1.71	1.60	1.60	1.62	1.60	
Total Av.:	1.65	1.67	1.56	1.56	1.55	1.54	

(*Table 5 continued on following page.*)

TABLE 5—(*continued*)

COLLABORATOR NO.	OFFICIAL A.O.A.C. METHOD[a]		RAPID PROCEDURE[a]				PHOTOFLUOROMETER FILTERS USED
	DIGESTION TEMPERATURE		DIGESTION TEMPERATURE				
	95–100°	121–123°	95–100°-WATER BATH		108–109°-AUTOCLAVED		
	WATER BATH	AUTOCLAVED	DETN STD[b]	DIR. STD[c]	DETN STD[b]	DIR. STD[c]	
			Sample No. 3				
	1.42	1.40	1.42	1.42	1.38	1.38	
1	1.46	1.40	1.38	1.38	1.38	1.38	B1/B2
	1.42	1.40	1.38	1.38	1.42	1.42	
	1.42	1.40	1.42	1.42	1.42	1.42	
Av.:	1.43	1.40	1.40	1.40	1.40	1.40	
2	1.51	1.57	1.43	1.43	1.40	1.40	B1/B2
	1.51	1.57	1.41	1.41	1.38	1.38	
Av.:	1.51	1.57	1.42	1.42	1.39	1.39	
	1.36	1.34	1.29	1.28	1.31	1.29	
3	1.35	1.39	1.27	1.27	1.30	1.28	B1/PC1
	1.35	1.34	1.29	1.29	1.31	1.29	
	1.33	1.33	1.30	1.29	1.28	1.26	
Av.:	1.35	1.35	1.29	1.28	1.30	1.28	
	1.49	1.44	1.18	1.17	1.16	1.15	
4	1.48	1.46	1.20	1.19	1.17	1.16	B1/PC1
	1.49	1.44	1.29	1.28	1.05	1.03	
	1.47	1.46	1.31	1.30	1.07	1.05	
Av.:	1.48	1.45	1.25	1.24	1.11	1.10	
5	1.34	1.30	1.40	1.42	1.34	1.36	B1/B2
	1.34	1.34	1.34	1.38	1.34	1.36	
Av.:	1.34	1.32	1.37	1.40	1.34	1.36	
	1.41	1.41	1.41	1.42	1.43	1.43	
6	1.42	1.44	1.43	1.44	1.41	1.41	B1-S/B2
	1.46	1.42	1.37	1.36	1.39	1.36	
	1.45	1.42	1.34	1.33	1.36	1.33	
Av.:	1.44	1.42	1.39	1.39	1.40	1.38	
Total Av.:	1.42	1.41	1.34	1.34	1.30	1.30	

addition of sodium hydroxide and before the isobutyl alcohol shakeout. Therefore, immediate shakeout was specified in the revised method submitted for collaborative study in 1953.

In the measurement of thiochrome fluorescence, the filters supplied with the photofluorometer used in this investigation were analyzed for range by the Beckman spectrophotometer. The primary filters that are inserted between the light source and the sample tube (B-1 or B1-S) were found to be within the range as specified in the official method. For secondary filters that are inserted between the sample tube and the photocell, it was found that filters PC-1 and B-2 were equally effective, for the most part. It is believed that the B-2 filter provides a more selective range for thiochrome fluorescent light.

Collaborative Study.—One sample of enriched bromated flour and two samples of plain enriched flour were submitted to collaborators, together with the instructions and outline of the revised rapid procedure for the determination of thiamine by rapid acid hydrolysis. The results are tabulated in Table 5. The following comments are applicable to all three samples.

The official method was used as a basis of comparison for the proposed rapid procedure. Results are in good agreement among collaborators, and excellent agreement is found in comparing results on the basis of the two alternatives of acid digestion at 95–100°C. or 121–123°C. by autoclaving. The method specifies that the standard be carried through the complete procedure.

In the rapid procedure, when acid digestion at 95–100°C. is compared with digestion at 108–109°C. by autoclaving, the results are considered to be in excellent agreement and provide evidence that either acid digestion procedure may be used. The range in results reported by each collaborator is similar to that found by the official method, and the results by the rapid procedure are considered to be in good agreement with the official method.

The collaborators were asked to calculate two sets of results on the basis of a "Determination Standard" that was carried through the entire procedure and on a "Direct Standard" containing the same amount of acid and salt. In all of the comparisons, the results calculated from both standards are considered to be equivalent, indicating no necessity of carrying the standard through the entire procedure.

Both filtration and centrifuging have been indicated as alternatives to accomplish separation of the sample solutions in the rapid procedure. Collaborators 1, 2, 4, and 6 reported using filtration; Collaborators 3 and 5 used centrifugation. The results reported in the collaborative study of 1952 and those of the present study have provided evidence that either means of separation is applicable.

The collaborators were asked to describe the photofluorometer filters used. The combinations of primary and secondary filters used have been

indicated in the table of collaborative results (Table 5). (See the above discussion, relative to the ranges of these filters.)

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RECOMMENDATIONS

It is recommended*—

(1) That the rapid acid hydrolysis procedure for thiamine in enriched flour be adopted, first action, with the two following minor changes indicated in footnotes: (*a*) The use of a "Determination Standard" is not necessary and may be deleted; and (*b*) added salt is not necessary for clarification when autoclaving is used.

(2) That the study of the thiochrome procedure be continued and extended to other cereal products.

REFERENCES

(1) McRoberts, L. H., *This Journal*, **36**, 837 (1953).
(2) *Official Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Box 540, Benjamin Franklin Station, Washington 4, D. C., 1950, Sections **40.18–40.22.**

REPORT ON RIBOFLAVIN

By Lawrence Rosner (Laboratory of Vitamin Technology, Chicago 19, Ill.), *Associate Referee*

The objective of the present investigation was to determine whether any change in directions in the method of extraction for the riboflavin assay described in *Official Methods of Analysis*, **40.30,** is advisable. Riboflavin supplements were studied in particular, since there were some reports to the effect that difficulties were encountered in obtaining interlaboratory agreement on this type of product.

* For report of Subcommittee A and action of the Association, see *This Journal*, **37**, 64 (1954).

Samples of riboflavin concentrates, which had been used as reference materials and had been repeatedly tested in the manufacturer's laboratories, were obtained from several manufacturers. The assay procedures found to give the best results were also submitted. This laboratory tested these samples fluorometrically, employing the extraction procedure described by the A.O.A.C. The results obtained by this laboratory, compared with those of the manufacturers, are shown in Table 1.

TABLE 1.—*Fluorometric assay of riboflavin concentrates*

SAMPLE	MANUFACTURER'S ASSAY VALUE	THIS LABORATORY'S ASSAY VALUE (A.O.A.C. EXTRACTION)
	mg/g	*mg/g*
A	13.0	13.7
B	46.0	46.7
C	8.45	8.35
D	27.5	27.7
E	18.25	18.1

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Biren Bass, Publicker Industries, Inc.
Steven Dereniuk, Fermentation Products, Inc.

RECOMMENDATION

It is recommended* that no change be made in directions for extraction in the official method for riboflavin.

REPORT ON THE EXTRACTION OF NICOTINIC ACID FROM NATURALLY-OCCURRING MATERIALS

By JAMES P. SWEENEY, *Associate Referee*, and WILLIAM P. PARRISH
(Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.)

A wide variety of procedures has been used in the extraction of nicotinic acid from natural materials for the purpose of its determination. Among other extractants, water, acid methanol, ethanol, and various concentrations of sulfuric acid, hydrochloric acid, and sodium hydroxide have been used for this purpose. The nicotinic acid values obtained have

* For report of Subcommittee A and action of the Association, see *This Journal*, 37, 64 (1954).

varied by as much as 500 per cent, depending upon the extraction procedure used. Consequently, there has existed considerable uncertainty regarding the true nicotinic acid content of many naturally-occurring materials. There arises also in this connection the question of biological availability of the vitamin.

In 1940, Kodicek (1) found that yellow cornmeal gave a rather low nicotinic acid value when extracted with water, a somewhat higher value when 4 per cent H_2SO_4 was used, and a still higher value when 8 per cent H_2SO_4 or 8 per cent NaOH was employed.

In spite of its high "apparent nicotinic acid content," yellow cornmeal has been found to be relatively inactive in the prevention of pellagra. It has, in fact, been a standard ingredient of pellagra-producing diets.

The findings of Kodicek have been confirmed by many other workers (2–5). Consequently, it was assumed that in plant material there existed certain unknown substances which gave a test for nicotinic acid—both chemical and microbiological—and yet have no pellagra-preventing activity.

However, it was subsequently demonstrated in 1944 by Krehl, *et al.*, (6) that this "precursor" when treated with normal NaOH or normal H_2SO_4, possessed biological activity for dogs. The compound was found in cereal products, especially in bran, and was also present in potatoes, but attempts to isolate it were unsuccessful.

In 1948 Kodicek, *et al.*, (7) made a study of extraction procedures, employing both chemical and microbiological methods for the nicotinic acid estimation. He concluded that extraction with either normal H_2SO_4 or normal NaOH was satisfactory. Good agreement between chemical and microbiological values was obtained.

Chaudhuri and Kodicek (8) in 1950 reported the purification of a precursor of nicotinic acid from wheat bran. This purification involved thirteen stages, including chromatography and precipitation with organic solvents. From this precursor they were able to split nicotinic acid by digestion for ten minutes at room temperature with 0.1 *N* NaOH, but not by boiling for thirty minutes with 0.1 *N* HCl. Strong acid hydrolysis was found necessary for the liberation of nicotinic acid. By various methods of hydrolysis they were able also to split glucose and several amino acids from the precursor.

In 1952 Reddi (9) reported the separation of the bound and free forms of nicotinic acid by means of paper chromatography. This separation was made possible because of the fact that the free form moved on the chromatogram while the bound form did not. The latter could be located on the chromatogram because of its bluish-white fluorescence under ultraviolet light. The bound form was removed from the paper by elution with normal NaOH and was subsequently digested on a water bath for thirty minutes. Reddi was able to demonstrate that the nicotinic acid of wheat bran

and rice bran is present entirely in the bound form.

Krehl, *et al.*, (6) have concluded that *Lactobacillus arabinosus* could utilize the precursor to a considerable extent, possibly as much as 80 per cent. Kodicek (7) has estimated about 45 per cent utilization.

The precursor is known also to give the Koenig reaction (10) but whether or not the result gives a true indication of the amount of nicotinic acid present is not known.

The Seventh Edition of *Official Methods of Analysis* contains both a microbiological and a chemical method for nicotinic acid. In both of these methods normal H_2SO_4 is recommended for sample extraction. In view of the uncertainty regarding the measurement of the precursor of nicotinic acid, brought out in the work reviewed here, a re-examination of the extraction step was carried out in the Associate Referee's laboratory.

In a preliminary study, use was made of water, acid methanol (160 ml of methanol+40 ml of 0.5 *N* HCl), 1 *N* and 0.1 *N* H_2SO_4, and 1 *N* and 0.1 *N* NaOH for the extraction of yellow and white corn meal. The acid-methanol extraction was carried out by refluxing on a water bath for one hour. In all other cases the extracts for the chemical and microbiological determinations were prepared by autoclaving in accordance with the respective A.O.A.C. procedures.

The results of this study, shown in Table 1, indicate that extraction with water, acid methanol, or 0.1 *N* H_2SO_4 will lead to low nicotinic acid values when compared with those obtained when normal H_2SO_4 or either 0.1 *N* NaOH or normal NaOH are used.

The effectiveness of normal H_2SO_4 and normal NaOH in the extraction of a number of other cereals was then tested. Results are given in Table 2. The value obtained for wheat bran, when the A.O.A.C. chemical method was used, appears to be low. Consequently, extractions were made in which the period of autoclaving was increased from thirty minutes (the period stipulated in the A.O.A.C. methods) to one hour. In addition, 2 *N* H_2SO_4 and 2 *N* HCl were tested in the extraction of wheat bran.

TABLE 1.—*Extraction studies with various solvents*

EXTRACTION	YELLOW CORN MEAL		WHITE CORN MEAL (ENRICHED)	
	CHEM.	MICRO.[a]	CHEM.	MICRO.
	mg/lb	*mg/lb*	*mg/lb*	*mg/lb*
1 *N* NaOH	10.3	8.94	23.60	22.68
1 *N* H_2SO_4	9.00	9.34	20.90	21.78
0.1 *N* NaOH	9.50	8.12	21.10	18.90
0.1 *N* H_2SO_4	6.75	7.64	19.50	19.98
Acid methanol (160 ml CH_3OH +40 ml 0.5 *N* HCl)	5.50	5.58	17.10	19.62
H_2O	4.35	5.08	14.00	14.40

[a] Microbiological.

TABLE 2.—*Effectiveness of 1 N H_2SO_4 and 1 N NaOH in cereal extraction*

EXTRACTION	BREAD		BARLEY CEREAL		FLOUR		FLOUR (ENRICHED)		WHEAT BRAN	
	CHEM.	MICRO.[a]	CHEM.	MICRO.	CHEM.	MICRO.	CHEM.	MICRO.	CHEM.	MICRO.
	mg/lb		*mg/lb*		*mg/lb*		*mg/lb*		*mg/lb*	
1 *N* NaOH	5.95	5.11	5.67	5.64	5.30	5.50	18.70	20.96	91.5	86.4
1 *N* H_2SO_4	5.59	6.48	5.69	5.00	4.95	5.60	18.20	19.52	71.9	84.5
0.1 *N* NaOH									89.5	94.1
0.1 *N* H_2SO_4									25.6	43.2
2 *N* HCl									118.0	89.3
1 *N* H_2SO_4 (1 hr)									77.7	104.3
2 *N* H_2SO_4									90.8	91.2

[a] Microbiological.

Although the increased autoclaving period produced a slightly increased chemical value in the case of wheat bran, the results still appeared low compared with those obtained when other extraction procedures were used.

While the use of 2 *N* H_2SO_4 resulted in values which appear satisfactory, the nicotinic acid color, produced by cyanogen bromide and sulfanilic acid, had a red-orange tinge, suggesting that some interfering material was being liberated by the 2 *N* acid. When 2 *N* HCl was used, even more of this interfering material was liberated and the "apparent" nicotinic acid value seemed to be too high.

As a result of the information gained in the preliminary study, further work was carried out in which normal, 2 *N*, and 3 *N* H_2SO_4 as well as nor-

TABLE 3.—*Results of extraction with NaOH and with three concentrations of H_2SO_4*

	1 *N* H_2SO_4		2*N* H_2SO_4		3 *N* H_2SO_4		1 *N* NaOH	
	CHEM.	MICRO.[a]	CHEM.	MICRO.	CHEM.	MICRO.	CHEM.	MICRO.
Yellow corn meal, mg/lb	9.0	9.3	11.0	9.4	11.93	10.8	10.3	8.9
Bread, mg/lb	5.59	6.50	5.55	5.30	5.57	5.70	5.95	5.10
Barley cereal, mg/4 oz	4.70	4.70	4.8	4.8	4.8	4.7	5.0	5.0
Rice bran, mg/oz	7.9	9.0	8.9	9.0	9.3	9.4	10.0	9.0
Corn bran, mg/lb	10.9	15.9	15.6	14.6	15.5	15.2	15.3	
Wheat bran, mg/lb	71.9	84.5	90.8	89.3	90.2	89.3	91.5	86.4
Wheat gluten, mg/lb	7.07	10.4	11.6	12.8	11.1	13.1	12.0	11.2

[a] Microbiological.

TABLE 4.—*Extraction on basis of total weight of H_2SO_4*

EXTRACTION	WHEAT BRAN		WHEAT GLUTEN	
	CHEM.	MICRO.[a]	CHEM.	MICRO.
	mg/lb		*mg/lb*	
450 ml 1 *N* H_2SO_4	70.0	89.3	11.7	12.2
225 ml 2 *N* H_2SO_4	93.0	89.3	11.2	13.7
150 ml 3 *N* H_2SO_4	90.0	91.2	11.7	14.4

[a] Microbiological.

mal NaOH were used for extraction purposes. Since the nicotinic acid from wheat bran appeared difficult to liberate, corn bran, rice bran, and wheat gluten were included in the investigation.

The results of this study, given in Table 3, indicate that the A.O.A.C. chemical method, using normal H_2SO_4, gives low nicotinic acid values for wheat bran, rice bran, and corn bran. The value for wheat gluten also appears low. When the microbiological method is used, low values are obtained only in the cases of wheat bran and wheat gluten.

At this point in the study it was suggested that perhaps the total weight of H_2SO_4 present was the significant factor rather than the concentration. Accordingly, extractions of wheat bran and wheat gluten were carried out as indicated in Table 4, using in each case a total weight of H_2SO_4 equivalent to that present in 150 ml of 3 *N* H_2SO_4.

The results obtained on wheat bran by the chemical method do not differ significantly from those obtained previously. It appears that normal H_2SO_4, as used in the A.O.A.C. extraction methods, does not liberate completely the bound nicotinic acid from wheat bran, rice bran, or corn bran. Since the microbiological values, obtained on these products when normal H_2SO_4 is used for extraction are uniformly higher than the chemical values, it would appear that *Lactobacillus arabinosus* is quite effective in the utilization of this bound nicotinic acid.

In the case of wheat gluten, higher values were obtained both chemically and microbiologically when a larger volume of normal H_2SO_4 was used. This is no doubt due to the fact that the larger volume of liquid gave more efficient mechanical extraction and not because of the liberation of bound nicotinic acid. Because of the sticky nature of wheat gluten it is necessary to exercise great care in addition of the extraction liquid in order to obtain uniform dispersion of the gluten.

Extraction of brans with NaOH, either normal or 0.1 *N*, gave satisfactory results (Table 3). However, the alkaline extracts are somewhat gelatinous in nature, and additional steps are consequently necessary for preparation of the clear extracts required for the chemical method.

The use of 2 or 3 *N* H_2SO_4 for extraction appeared to give satisfactory results. However, as previously mentioned, there were indications of the

TABLE 5.—*Nicotinic acid values for bran products obtained with modified procedure*

EXTRACTION	RICE BRAN		CORN BRAN		WHEAT BRAN	
	CHEM.	MICRO.[a]	CHEM.	MICRO.	CHEM.	MICRO.
	mg/oz		*mg/lb*		*mg/lb*	
1 *N* H_2SO_4	7.9	9.0	10.9	15.9	71.9	84.5
2 *N* H_2SO_4	8.9	9.0	15.6	14.6	90.8	89.3
3 *N* H_2SO_4	9.3	9.4	15.5	15.2	90.2	89.3
1 *N* NaOH	10.0	9.0	15.3		91.5	86.4
1 *N* H_2SO_4, followed by 0.1 *N* NaOH	9.4	9.0	15.4	16.0	90.0	84.5

[a] Microbiological.

liberation of interfering substances. Although the color produced by these substances, when reacted with CNBr and sulfanilic acid, appeared to give very little absorption at 450 mμ (the wavelength of maximum absorption for the nicotinic acid color), their presence was nevertheless believed to be a potential source of trouble.

Chadhuri and Kodicek (8) have reported that the precursor isolated from wheat bran readily yielded nicotinic acid when digested with 0.1 *N* NaOH for ten minutes at room temperature.

With the use of the above information, the following procedure was devised for the extraction and liberation of nicotinic acid from bran products:

The sample is autoclaved with 200 ml of normal H_2SO_4, as in the A.O.A.C. method. It is then cooled to room temperature and is brought to approximately *p*H 7 (green to bromothymol blue) by addition of 10 *N* NaOH. Then 2.2 ml of additional 10 *N* NaOH is added to make the solution approximately 0.1 *N* with respect to NaOH (*p*H 13). The material is mixed and allowed to stand for fifteen minutes. It is then adjusted to *p*H 4.5 (green to bromocresol green) by addition of 25 per cent HCl. The remainder of the analysis is carried out according to the A.O.A.C. method.

The nicotinic acid values obtained for rice bran, corn bran, and wheat bran are shown in Table 5. This modified procedure gave results which are in good agreement with those obtained by other methods of extraction. In addition it appears to have none of the shortcomings found in other extraction methods tried.

SUMMARY

A number of extractants were tested for the liberation of nicotinic acid from natural materials. Both chemical and microbiological determinations were carried out. Normal sulfuric acid did not give complete liberation of nicotinic acid from wheat bran, corn bran, and rice bran. The A.O.A.C. microbiological method gave higher values for these products than did the chemical procedure. It appears that *Lactobacillus arabinosus* can utilize

a high percentage of the bound nicotinic acid present in brans.

Material which might interfere in the chemical method was liberated from bran products by 2 *N* and 3 *N* sulfuric acid. The use of NaOH for extraction was found to be unsatisfactory because of the gelatinous nature of the extracts obtained.

It was found that nicotinic acid is apparently completely liberated from bran products if, after autoclaving with normal sulfuric acid, the sample is adjusted to *p*H 13 with 10 *N* sodium hydroxide and allowed to remain for fifteen minutes at room temperature before resumption of the A.O.A.C. method.

Difficulty was experienced in the analysis of wheat gluten by both chemical and microbiological methods. The use of a larger volume of extracting liquid appears desirable in the case of glutenous materials.

RECOMMENDATION

It is recommended* that in the chemical method for nicotinic acid given in *Official Methods of Analysis*, the following statement be added as a footnote to the extraction procedure:

For the extn of materials contg bran, after autoclaving with normal H_2SO_4 adjust the mixt. to ca *p*H 13 by addn of 10 *N* NaOH and allow to stand at room temp. for 15 min. Then adjust the *p*H to ca 4.5 by addn of (3+1) HCl and proceed as usual. For the extn of glutenous material with ca 1 oz. samples, use 400 ml of normal H_2SO_4.

REFERENCES

(1) KODICEK, E., *Biochem. J.*, **34**, 712 (1940).
(2) WAISMAN, H. A., and ELVEHJEM, C. A., *Ind. Eng. Chem., Anal. Ed.*, **13**, 221 (1941).
(3) OSER, B. L., and SIEGEL, I., *Food Ind.*, **13**, 66 (1941).
(4) SNELL, E. E. and WRIGHT, L. D., *J. Biol. Chem.*, **139**, 675 (1941).
(5) ANDREWS, J. S., BOYD, H., M., and GORTNER, W. A., *Ind. Eng. Chem., Anal. Ed.*, **14**, 663 (1942).
(6) KREHL, W. A., and STRONG, F. M., *J. Biol. Chem.*, **156**, 1 (1944).
(7) KODICEK, E., and PEPPER, C. R., *J. Gen. Microb.*, **2**, 306 (1948).
(8) CHAUDHURI, D. K., and KODICEK, E., *Nature*, **165**, 1022 (1950).
(9) REDDI, K. K., *ibid.*, **170**, 745 (1952).
(10) KOENIG, W. J., *J. prakt. chem.*, **69**, 105 (1904).

REPORT ON VITAMIN B_6 (CHEMICAL)

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More than ten years ago, P. György (1) stated that pyridoxine values for a number of products vary with the method of determination and that

* For report of Subcommittee A and action of the Association, see *This Journal*, **37**, 64 (1954).

the yeast growth method gives values "generally considerably lower than those obtained by bioassays and chemical methods." Much has been learned about vitamin B_6 chemistry since that statement was written. Melnick, *et al.*, (2) described the complexity of the situation as of 1945. The association of pyridoxine with naturally-occurring vitamin B_6, which includes pyridoxal and pyridoxamine, is of prime importance in analytical procedures, and the subject continues to be a problem. During the past year this laboratory had occasion to study the pyridoxine content of evaporated milk, determined by a chemical method. The vitamin B_6 values obtained for this product were considerably in excess of the values reported in the literature (3). Lack of agreement between chemical and biological results still presents difficulties. A reliable analysis is particularly desired today since the need for vitamin B_6 in human nutrition has been established (4).

A brief review of some of the pertinent background of vitamin B_6 studies should help in planning a future investigation of this problem.

The early microbiological assays for pyridoxine by Snell and co-workers (5) indicated the existence of a vitamin B_6 complex, but only after several years of careful research was the problem unraveled and clarified by Snell's research team (6). Using biological and microbiological methods, they demonstrated that pyridoxal and pyridoxamine constituted a considerable portion of the vitamin B_6 compounds present in many natural products, and that assay methods employing rats, molds, or yeasts resulted in an equal molar basis of vitamin activity for pyridoxal and pyridoxamine; however, only the rat and yeast (*Saccharomyces carlsbergenesis* 4228) growth methods gave equal response to pyridoxine, pyridoxal, and pyridoxamine. The three compounds were found to be present in plant tissues, but only two could be demonstrated to be in animal tissues and yeast; the presence of pyridoxine in the latter case was questioned. It is also known that pyridoxal in the presence of casein hydrolysate (amino acids) is partially converted to pyridoxamine and that *Lactobacillus casei* requires pyridoxal for growth, even failing to respond to pyridoxal phosphate, unless hydrolyzed by acid (7).

These selected phenomena are called to the reader's attention to arouse interest and to point out the complexity of the problem at hand. It is planned to carry out studies correlating a chemical method and a microbiological method of assay during the coming year.*

REFERENCES

(1) *Ann. Rev. Biochem.*, XI, 326 (1942).
(2) Melnick, D., Hochberg, M., Himes, M. W., and Oser, B. L., *J. Biol. Chem.*, 160, 1 (1945).
(3) Hodson, A. Z., *J. Nutrition*, 27, 415 (1944).
(4) *Ann. Rev. Biochem.*, XIX, 285 (1950); XX, 586 (1951).

* For report of Subcommittee A and action of the Association, see *This Journal*, 37, 64 (1954).

(5) SNELL, E. E., GUIRARD, B. M., and WILLIAMS, R. J., *J. Biol. Chem.*, **143**, 519 (1942).
(6) *Ann. Rev. Biochem.*, XV, 278 (1946); XIX, 284 (1950); RABINOWITZ, J. C., and SNELL, E. E., *J. Biol. Chem.*, **176**, 1157 (1948).
(7) SNELL, E. E., and RANNEFELD, A. N., *J. Biol. Chem.*, **157**, 475 (1945); *Ann. Rev. Biochem.*, XVIII, 450 (1949).

REPORT ON PANTOTHENIC ACID

MICROBIOLOGICAL METHOD

By HENRY W. LOY, JR. (Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.), *Associate Referee*

Many problems have been encountered in the microbiological assay for pantothenic acid. It was indicated from the results of an earlier study, reported in 1944 (1), that more than simple enzyme treatment or extraction is required in the determination of pantothenic acid in natural materials. Pantothenic acid, in the form of calcium pantothenate, is added to many feed supplements and pharmaceutical preparations. Until the total pantothenic acid content of natural materials can be determined accurately, a microbiological method that will measure added calcium pantothenate has been desirable. As a result of a collaborative study conducted in 1951 (2), a microbiological method of assay for the free form of pantothenic acid (3) was adopted, first action.

Through the work during the past several years, of Strong and his group (4–8), Lipmann and co-workers (9–18), Snell and co-workers (19–30), and others (31–40), the structure of the complex substances in which pantothenic acid is bound has been elucidated. Recent studies by Snell and co-workers have disclosed the identity of pantetheine as pantothenic acid combined with thiolethylamine, or pantethine as the corresponding disulfide. Coenzyme A was inactive as a growth factor for the test organism *Lactobacillus bulgaricus*. But when coenzyme A was treated with intestinal phosphatase, a fragment was released that had the same microbiological activity as the *Lactobacillus bulgaricus* factor. When either this fragment of coenzyme A or the *Lactobacillus bulgaricus* factor was treated with an enzyme from chicken liver, their *Lactobacillus bulgaricus* factor activity was destroyed and free pantothenic acid was released. It was concluded that the *Lactobacillus bulgaricus* factor was the same as, or similar to, the phosphorus-free conjugate of pantothenic acid that was liberated from coenzyme A treated with intestinal phosphatase. From the configuration of coenzyme A set forth by Lipmann and co-workers, it is indicated that pantothenic acid is linked to adenylic acid through a phosphate

bridge and to a sulfur-containing amino acid through a peptide linkage.

With the knowledge that the combined action of intestinal phosphatase and an enzyme extractable from bird liver is essential to free the pantothenic acid in natural materials, a specific detailed procedure for total pantothenic acid in natural substances can now be studied collaboratively. Enzyme preparations that are suitable for this purpose may be obtained commercially. Toepfer, *et al.*, (41) have reported values obtained by the two-enzyme system. It is apparent that higher values are obtained by this method for a large variety of food products.

Chemical methods recently developed for the determination of total pantothenic acid (42–44) also appear to be worthy of consideration. A careful comparison of these procedures with the two-enzyme microbiological method is desirable.

A comprehensive collaborative study of methods for determining total pantothenic acid is now feasible.

RECOMMENDATIONS

It is recommended*—

(1) That the microbiological method for the assay of total pantothenic acid, involving the two-enzyme system herein discussed, be studied collaboratively during the coming year.

(2) That consideration be given to chemical methods of assay for total pantothenic acid.

REFERENCES

(1) Loy, H. W., Jr., *This Journal*, **28**, 567 (1945).
(2) ———, *ibid.*, **35**, 722 (1952).
(3) Changes in Methods, *ibid.*, **35**, 103 (1952).
(4) Neilands, J. B., and Strong, F. M., *Arch. Biochem.*, **19**, 287 (1948).
(5) ———, Higgins, H., King, T. E., Handschumacher, R. E., and Strong, F. M., *J. Biol. Chem.*, **185**, 335 (1950).
(6) Lih, H., King, T. E., Higgins, H., Baumann, C. A., and Strong, F. M., *J. Nutrition*, **44**, 361 (1951).
(7) Beinert, H., Von Korff, R. W., Green, D. E., Buyske, D. A., Handschumacher, R. E., Higgins, H., and Strong, F. M., *J. Am. Chem. Soc.*, **74**, 854 (1952).
(8) Strong, F. M., private communication.
(9) Lipmann, F., Kaplan, N. O., Novelli, G. D., Tuttle, L. C., and Guirard, B. M., *J. Biol. Chem.*, **167**, 869 (1947).
(10) Kaplan, N. O., and Lipmann, F., *ibid.*, **174**, 37 (1948).
(11) Novelli, G. D., Kaplan, N. O., and Lipmann, F., *ibid.*, **177**, 97 (1949).
(12) Lipmann, F., Kaplan, N. O., Novelli, G. D., Tuttle, L. C., and Guirard, B. M., *ibid.*, **186**, 235 (1950).
(13) Novelli, G. D., Kaplan, N. O., and Lipmann, F., *Federation Proc.*, **9**, 209 (1950).
(14) DeVries, W. H., Govier, W. M., Evans, J. S., Gregory, J. D., Novelli, G. D., Soodak, M., and Lipmann, F., *J. Am. Chem. Soc.*, **72**, 4838 (1950).

* For report of Subcommittee A and action of the Association, see *This Journal*, **37**, 64 (1954).

(15) CHANTRENNE, H., and LIPMANN, F., *J. Biol. Chem.*, **187**, 757 (1950).
(16) STADTMAN, E. R., NOVELLI, G. D., and LIPMANN, F., *ibid.*, **191**, 365 (1951).
(17) ———, DOUDOROFF, M., and LIPMANN, F., *ibid.*, **191**, 377 (1951).
(18) GREGORY, J. D., NOVELLI, G. D., and LIPMANN, F., *J. Am. Chem. Soc.*, **74**, 854 (1952).
(19) WILLIAMS, W. L., HOFF-JØRGENSEN, E., and SNELL, E. E., *J. Biol. Chem.*, **177**, 933 (1949).
(20) BROWN, G. M., CRAIG, J. A., and SNELL, E. E., *Arch. Biochem.*, **27**, 473 (1950).
(21) SNELL, E. E., BROWN, G. M., PETERS, V. J., CRAIG, J. A., WITTLE, E. L. MOORE, J. A., MCGLOHON, V. M., and BIRD, O. D., *J. Am. Chem. Soc.*, **72**, 5349 (1950).
(22) RASMUSSEN, R. A., SMILEY, K. L., ANDERSON, J. B., VAN LANEN, J. M., WILLIAMS, W. L., and SNELL, E. E., *Proc. Soc. Exptl. Biol. Med.*, **73**, 658 (1950).
(23) BROWN, G. M., and SNELL, E. E., *ibid.*, **77**, 138 (1951).
(24) CRAIG, J. A., and SNELL, E. E., *J. Bact.*, **61**, 283 (1951).
(25) BROWN, G. M., and SNELL, E. E., *J. Biol. Chem.*, **198**, 375 (1952).
(26) PETERS, V. J., BROWN, G. M., WILLIAMS, W. L., and SNELL, E. E., *J. Am. Chem. Soc.*, **75**, 1688 (1953).
(27) BROWN, G. M., and SNELL, E. E., *ibid.*, **75**, 1691 (1953).
(28) WITTLE, E. L., MOORE, J. A., STIPEK, R. W., PETERSON, F. E., MCGLOHON, V. M., BIRD, O. D., BROWN, G. M., and SNELL, E. E., *ibid.*, **75**, 1694 (1953).
(29) BROWN, G. M., and SNELL, E. E., *ibid.*, **75**, 2782 (1953).
(30) SNELL, E. E., private communication.
(31) SARETT, H. P., and CHELDELIN, V. H., *J. Biol. Chem.*, **159**, 311 (1945).
(32) KING, T. E., LOCHER, L. M., and CHELDELIN, V. H., *Arch. Biochem.*, **17**, 483 (1948).
(33) MCRORIE, R. A., MASLEY, P. M., and WILLIAMS, W. L., *ibid.*, **27**, 471 (1950).
(34) NISHI, H., KING, T. E., and CHELDELIN, V. H., *J. Nutrition*, **41**, 279 (1950).
(35) LONG, C. L., and WILLIAMS, W. L., *J. Bact.*, **61**, 195 (1951).
(36) VITUCCI, J. C., BOHONOS, N., WIELAND, O. P., LEFEMINE, D. V., and HUTCHINGS, B. L., *Arch. Biochem. Biophys.*, **34**, 409 (1951).
(37) NOVELLI, G. D., and SCHMETZ, F. J., JR., *J. Biol. Chem.*, **192**, 181 (1951).
(38) STADTMAN, E. R., *ibid.*, **196**, 527 (1952).
(39) ———, *ibid.*, **196**, 535 (1952).
(40) ———, and KORNBERG, A., *ibid.*, **203**, 47 (1953).
(41) TOEPFER, E. W., ZOOK, E. G., and RICHARDSON, L. R., *This Journal*, **37**, 182 (1954).
(42) SZALKOWSKI, C. R., MADER, W. J., and FREDIANI, H. A., *Cereal Chem.*, **28**, 218 (1951).
(43) REFAI, F. Y., and MILLER, B. S., *ibid.*, **29**, 469 (1952).
(44) SZALKOWSKI, C. R., and DAVIDSON, J. H., JR., *Anal. Chem.*, **25**, 1192 (1953).

REPORT ON VITAMIN B_{12}

MICROBIOLOGICAL METHOD

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Previous A.O.A.C. vitamin B_{12} collaborative studies (1, 2) have revealed that: (*a*) Certain water-insoluble test materials require a buffered sodium

metabisulfite sample treatment for the stabilization of the naturally occurring analogues of vitamin B_{12}; (*b*) a sixteen to twenty-four hour turbidimetric evaluation appeared practicable; (*c*) far less variation was observed with the higher potency materials than with the low potency samples; and (*d*) more consideration must be given to the actual execution of the assay as written.

A limited number of laboratories, selected on the basis of their wide daily experience with vitamin B_{12} assays, participated in the 1953 A.O.A.C. study. The study was purposely designed to permit ascertaining what inter- and intralaboratory agreement could be obtained when experienced analysts executed the assay in detail.

The 1952 A.O.A.C. method, modified to include more specific instructions for assay procedure, was employed. Particular attention was given to the activity of the microorganism, the transmittance requirements of the inoculum culture, and the allowable transmittance range of the standard curve for a valid assay.

Each of the following samples was subjected to assay on three separate assay days. Sample A was a colored solution of U.S.P. Cyanocobalamin Reference Standard containing 13.7 micrograms of vitamin B_{12} per cc as measured spectrophotometrically (3). Sample B was a crude liver paste, sample C was a commercial grade of condensed fish solubles, and sample D was a crude vitamin B_{12} fermentation product. In addition to the samples subjected to assay by the prescribed procedure, certain samples were prepared in different ways to determine what effect, if any, the treatment had on the assay value.

METHOD

REAGENTS

(**a**) *Standard cyanocobalamin stock soln.*—To a suitable quantity of U.S.P. Cyanocobalamin Reference Standard, accurately weighed, add sufficient 25% alcohol to make a soln, each ml to contain 1.0 micrograms of cyanocobalamin. Store in a cool place and use no longer than 60 days.

(**b**) *Standard cyanocobalamin soln.*—To 10 ml of standard cyanocobalamin stock soln (**a**) add 25 ml of 1.0% $NaHSO_3$ in *p*H 4.5 phosphate-citrate buffer. Autoclave for 15 min. (121–123°C.) and dil. with H_2O to 500 ml. Dil. an aliquot of this soln 1000 times. Each ml represents 0.02 millimicrogram of cyanocobalamin. Prep. a fresh standard soln for each assay.

(**c**) *Basal medium stock soln.*—To prevent the possible formation of colloidal suspensions or ppts which may result in either slow growth or high blanks (or both), the following sequence of ingredient addns should be followed:

100 mg of *l*-cystine and 100 mg of *d,l*-tryptophane dissolved in ca 10 ml of *N* HCl; then add:	
Adenine-guanine-uracil soln, (**f**)	5 ml
Xanthine soln, (**g**)	5 ml
Vitamin soln I, (**h**)	10 ml
Vitamin soln II, (**i**)	10 ml
Salt soln A, (**j**)	5 ml
Salt soln B, (**k**)	5 ml

Asparagine soln, (e)	5 ml
Acid-hydrolyzed casein soln, (d)	25 ml
Dextrose, anhyd	10 g
5 g of sodium acetate, anhyd., and 1 g of ascorbic acid dissolved in 100 ml of H_2O	
Polysorbate 80 soln, (l)	5 ml

Adjust to *p*H 6.0 with NaOH soln, and finally add H_2O to 250 ml.

(d) *Acid-hydrolyzed casein soln.*[1]—Mix 100 g of vitamin-free casein with 500 ml of dil. HCl (1+1) and reflux the mixt. for 8–12 hrs. Remove the HCl by distn under reduced pressure to a thick paste. Redissolve paste in H_2O, adjust *p*H to 3.5 (±0.1) with NaOH soln, and add H_2O to 1000 ml. Add 20 g of activated charcoal, stir 1 hr, and filter. Repeat the treatment with activated charcoal. Store under toluene in refrigerator at temp. not below 10°C. Filter soln if ppt forms on storage.

(e) *Asparagine soln.*—Dissolve 2.0 g of *l*-asparagine in H_2O to make 200 ml. Store under toluene in refrigerator.

(f) *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% HCl; cool, and add H_2O to make 200 ml. Store under toluene in refrigerator.

(g) *Xanthine soln.*—Suspend 0.2 g of xanthine in 30–40 ml of H_2O, heat to ca 70°, add 6.0 ml of NH_4OH (40 ml dild to 100 ml with H_2O), and stir until solid is dissolved. Cool, and add H_2O to 200 ml. Store under toluene in refrigerator.

(h) *Vitamin soln I, riboflavin–thiamine–biotin–nicotinic acid soln.*—Dissolve 10 mg of riboflavin, 10 mg of thiamine hydrochloride, 0.1 mg of biotin, and 20 mg of nicotinic acid in 0.02 *N* acetic acid to make 400 ml. Store, protected from light, under toluene in refrigerator.

(i) *Vitamin soln II, p-aminobenzoic acid–calcium pantothenate–pyridoxine–pyridoxal–pyridoxamine–folic acid soln.*—Dissolve 20 mg of *p*-aminobenzoic acid, 10 mg of Ca pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in 25% neutralized alcohol to make 400 ml. Store, protected from light, in refrigerator.

(j) *Salt soln A.*—Dissolve 10 g of KH_2PO_4 and 10 g of K_2HPO_4 in H_2O to make 200 ml. Add 2 drops of HCl and store under toluene.

(k) *Salt soln B.*—Dissolve 4.0 g of $MgSO_4 \cdot 7H_2O$, 0.2 g of NaCl, 0.2 g of $FeSO_4 \cdot 7H_2O$, and 0.2 g of $MnSO_4 \cdot H_2O$ in H_2O to make 200 ml. Add 2 drops of HCl and store under toluene.

(l) *Polysorbate 80 soln.*—Dissolve 20 g of polysorbate 80 in sufficient alcohol to make 200 ml. Store in refrigerator.

(m) *Tomato juice preparation.*—Centrifuge commercially canned tomato juice so that most of pulp is removed. Suspend analytical filter aid (about 5 g/l) in the supernatant liquid and filter, with aid of reduced pressure, thru a layer of analytical filter aid. Repeat if necessary until a clear, straw-colored filtrate is obtained. Store under toluene in refrigerator.

(n) *Culture medium.*—Dissolve 0.75 g of water-soluble yeast ext., 0.75 g of peptone, 1 g of anhyd. dextrose, and 0.2 g of KH_2PO_4 in 60–70 ml of H_2O. Add 10 ml of tomato juice prepn (m), and 1 ml of polysorbate 80 soln (l). Adjust to *p*H 6.8 with NaOH soln, and add H_2O to 100 ml. Place 10 ml portions in test tubes, and plug with cotton. Sterilize tubes and contents in autoclave for 15 min. at 121–123°C. (exhaust line temp.). Cool as rapidly as possible to avoid color formation from overheating.

(o) *Suspension medium.*—Dil. measured vol. of basal medium stock soln with

[1] Commercial sources have been found satisfactory. If such a source is used, the suitability of each batch obtained should be demonstrated.

equal vol. of H_2O. Place 10 ml portions of the dild medium in test tubes. Sterilize, and cool as for culture mediun (n).

(p) *Stock culture of Lactobacillus leichmannii.*—To 100 ml of culture medium add 1.0–1.5 g of agar, and heat with stirring on steam bath until agar dissolves. Add ca 10 ml portions of the hot soln to test tubes, plug with cotton, sterilize for 15 min. in autoclave at 121°–123° (exhaust line temp.), and allow tubes to cool in upright position. Prep. stab cultures in 3 or more tubes, using pure cultures of *Lactobacillus leichmannii.*[2] (Before using a fresh culture in this assay, make at least 10 successive transfers of the culture in a two-week period.) Incubate 6–24 hrs at any selected temp. between 30° and 37° but held constant to within ±0.5°, and finally store in refrigerator.

Prepare fresh stab cultures thrice weekly and do not use them for preparing the inoculum if they are more than 4 days old.

The activity of the microorganism can be increased by daily or twice-daily transfer of the stab culture, and may be considered satisfactory when definite turbidity in the liquid inoculum can be observed 2–4 hrs after inoculation. With a slow growing culture a suitable response curve is seldom obtained, and may give rise to erratic results.

(q) *Inoculum.*—Make transfer of cells from stock culture of *Lactobacillus leichmannii* to sterile tube contg 10 ml of culture medium. Incubate for 6–24 hrs at any selected temp. between 30° and 37° but held constant to within ±0.5°. Under aseptic conditions, centrifuge culture and decant supernatant liquid. Suspend cells from culture in 10 ml of sterile suspension medium. Dil. an aliquot with sterile suspension medium to give a transmittance of about 85–90% (Evelyn and Lumetron),[3] 70–80% (Coleman), or 55–70% (Beckman Model B) when read against the suspension medium set at 100. The cell suspension so obtained is the inoculum.

TEST SOLUTION OF THE MATERIAL TO BE ASSAYED

Autoclave ca 1 g or 1 ml of sample, accurately measured, for 15 min. (121°–123°C.) in 25 ml of 0.1 *M* phosphate-citrate buffer at *p*H 4.5 (12.9 g of Na_2HPO_4 (anhyd.), 11.47 g of citric acid (monohydrate), and H_2O to make 1 l) contg 1.0% Na metabisulfite (the Na metabisulfite must be added to buffer just prior to use). Allow any undissolved particles to settle, or centrifuge if necessary. Dil. an aliquot of the clear soln with *water* so that the final test soln contains a vitamin B_{12} activity equivalent to ca 0.02 millimicrogram of cyanocobalamin. Addn of excess bisulfite to the assay tubes must be avoided to prevent inhibition of the test organism. This will not occur if amount of bisulfite in the assay soln does not exceed 0.025 mg/ml or if the assay tube does not contain more than 0.125 mg.

DETERMINATION

Cleanse hard glass test tubes, ca 20×150 mm, and other necessary glassware meticulously by suitable means because of the high sensitivity of the test organism to minute amounts of vitamin B_{12} activity and to traces of many cleansing agents.

To triplicate test tubes, add 1.0 ml, 2.0 ml, 3.0 ml, 4.0 ml, and 5.0 ml, respectively, of the standard cyanocobalamin soln. To each tube add 5.0 ml of basal medium stock soln and sufficient H_2O to make 10 ml. Provide 6 similar tubes contg no standard cyanocobalamin soln.

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[2] Pure cultures of *Lactobacillus leichmannii* may be obtained from the American Type Culture Collection, 2029 M Street, N.W., Washington, D. C. as No. 7830.

[3] Instruments vary in sensitivity and hence in % transmission. As a result, the assay range (from inoculated blank to highest level of standard) will vary accordingly. The Evelyn and Lumetron % transmission response is ca ½ to ¼ that of the Coleman and Beckman Model B, respectively. Because of this, % transmittances are expressed in terms of the instrument used.

To test similar test tubes add *in triplicate* respectively, 1.0 ml, 2.0 ml, 3.0 ml, 4.0 ml, and 5.0 ml of the test soln of material to be assayed. To each tube add 5.0 ml of basal medium stock soln and sufficient H_2O to make 10 ml.

Mix, cover the tubes suitably to prevent bacterial contamination, and sterilize tubes and contents in autoclave for 5 min. at 121–123°C. (exhaust line temp.), arranging to reach this temp. in not more than 10 min. Cool as rapidly as practicable to avoid color formation from overheating. Take precautions to maintain uniformity of sterilizing and cooling conditions thruout the assay. Too close packing of tubes in the autoclave, or overloading of it, may cause variation in heating rate.

Aseptically inoculate each tube (except 3 tubes contg no standard cyanocobalamin soln—uninoculated blank) with 1 drop of inoculum. Incubate for 16–24 hours at any temp. between 30° and 37°C., but held constant to within ±0.5°C., until maximum turbidity is obtained as demonstrated by a lack of significant change during a 2 hr period in the tubes contg the highest level of standard cyanocobalamin soln (0.1 millimicrogram). Read turbidity of tubes in a suitable instrument at a specific wavelength of between 540 and 660 mμ. In taking instrument reading, thoroly mix contents of each tube and transfer to optical glassware. Agitate each tube or cuvette to obtain a uniform suspension. A few sec. after agitation, a steady state is reached in which the galvanometer needle remains constant for 30 sec. or more, allowing sufficient time for an instrument reading. Allow ca the same time interval to elapse prior to each reading. A little practice will establish the proper time interval.

With uninoculated blank in instrument, set meter to read 100% transmittance and read transmittance of the inoculated blank (the inoculated tubes to which no standard cyanocobalamin soln has been added). Disregard results of an assay if contamination with a foreign organism is evident, or if the inoculated blank tubes give a reading of less than 90% transmission (Evelyn and Lumetron), 80% (Coleman), or 65% (Beckman), thereby indicating interference due to vitamin B_{12} activity in the basal medium stock soln or inoculum. Then with the inoculated blank in the instrument, set meter to read 100% transmittance. Read transmittance of tubes of the standard and sample series. Disregard results of an assay if transmittance of tubes contg the highest level of standard cyanocobalamin soln (0.1 millimicrogram) exceeds 65% (Evelyn and Lumetron), 45% (Coleman), or 30% (Beckman) of transmittance readings of inoculated blank, e.g., setting the % transmittance of inoculated blank at 100 (Beckman), then the 5 ml level of standard must have % transmittance of 30 or less.

CALCULATION

Prep. a standard concn response curve by plotting % transmittance readings for each level of the standard cyanocobalamin soln used, against millimicrograms of cyanocobalamin contained in the respective tubes. Draw the smooth curve which appears by visual inspection to fit the plotted points best. From this standard curve det. by interpolation for each tube the amount of cyanocobalamin equivalent to the vitamin B_{12} activity of each ml of the test soln of the material to be assayed.

Since in microbial assays occasional inexplicable aberrant values are obtained in individual tubes, inspect the series of values and set aside any which vary markedly from most of the series. Strike a provisional av. of the remaining values, and set aside any of the latter which are less than 90% and more than 110% of the provisional av. If less than 10 of the 15 original values remain, the data are insufficient for calcg the potency; if 10 or more values remain, calc. the potency from the av. Disregard any assay in which the calcd potency is less than 75% or more than 125% of the assumed potency.

COMMENTS AND SUGGESTIONS BY COLLABORATORS[4]

Collaborator No. 2.—" . . . We were very much pleased with the ease with which the assay handled and the results which we obtained. . . . I have no serious criticism of the method as it now stands, but I do believe more replicates should be suggested for each level of the standard curve (but not for the samples), since the entire calculation of samples is based on the accuracy of the levels of standard solutions used. In our lab we use four levels of standard and six replicates for each level. . . . "

Collaborator No. 4.—" . . . The method appears quite satisfactory, although we did have some difficulty with sample "C." However, we have several suggestions regarding the method. We found that assays may be valid even though the calculated potency differs from the assumed potency by more than ±25% (i.e., three levels are still readable and in good agreement). . . . We feel also that randomization of tubes is important, especially when an air incubator (instead of a water bath) is used. We have re-assayed the four samples (*with* the bisulfite treatment) by our current method, in which all tubes are randomized throughout the racks. A six-point design was also employed and eight replicate tubes were used at each of the 3 levels of the standard and four replicates at each of the three levels of the sample. The coefficient of variation does appear to be better on the average when using the design."

Collaborator No. 5.—" . . . We would like to point out that although the transmittance values of the high levels in these assays were below 65%, a good assay is possible with a smaller spread. We believe that 75% could be set as a minimum for a valid assay. Similarly, smaller spreads should be indicated for other instruments. We also feel that the lower limit for the calculated potency can be set at 50% rather than 75% of the assumed potency as stated in the procedure.

"It had been observed before this study, and was confirmed by it, that the use of a chloroform-toluene mixture for the prevention of bacterial growth in stock solutions, although common practice, must be avoided in connection with the phosphate-citrate buffer used for vitamin B_{12} extraction. Although toluene alone seems to be without effect, chloroform markedly lowers the detectable vitamin B_{12} content of extracts prepared with 'protected' buffer. This effect is noticeable, however, only with certain materials such as liver and fish solubles and not with others, notably pure cyanocobalamin and some fermentation products. Hence only five assay results are reported for samples B and C (with bisulfite) instead of eight. The lack of effect of the chloroform on the other samples is substantiated by the subsequent assays."

Collaborator No. 6.—" . . . One table contains the data obtained with the A.O.A.C. procedure as prescribed and the other the data from parallel assays on the same prepared samples by our version of the U.S.P. method. Incidently, this varies from the true U.S.P. method only in the use of a 40 hour incubation instead of a 72 hour, and a turbidity reading instead of a titration. . . . The recommended setup for the A.O.A.C. method calls for standard and unknown tubes to be set up at exactly the same levels, so that if the assumption of potency is at all inaccurate, at least three tubes will be off the end of the curve. This can be corrected by extending the standard curve over a greater range than the curve for samples. Our results with the A.O.A.C. method do not meet the requirements of a net range of 35 for the standard on the Evelyn. We think this is probably due to too short an incubation period."

Collaborator No. 7.—" . . . You will note that we have included one extra set of data in our report. We assayed each sample, with or without bisulfite as indicated, using the U.S.P. XIV medium and titrating at the end of 72 hours. The differences

[4] Only the comments and data of collaborators coincide, not the alphabetical listing.

found, although unexplained, are substantial. We have made similar observations in the past between turbidimetric and titrimetric values when the same medium was used for both. We have assumed, without making a thorough investigation of the point, that certain materials may contain inhibitory substances which depress the growth of the test organism relative to the reference standard during the first 16 to 18 hours of the incubation period. By the time full growth has taken place, these effects could become largely obliterated. If this effect has been experienced by other workers, it could constitute a serious problem in the desultory use of the turbidimetric 18 hour assay."

DISCUSSION OF COLLABORATIVE DATA

A summary of the vitamin B_{12} values obtained on the four samples subjected to study is given in Tables 1–4. Supplementary data obtained by methods other than the collaborative procedure are also included.

The results on sample A (U.S.P. Cyanocobalamin Reference Standard)

TABLE 1.—*Vitamin B-12 values for sample A (micrograms/ml)*

COLLABORATOR	WITH BISULFITE			WITHOUT BISULFITE		
	MEAN[a]	STANDARD DEVIATION	COEFFICIENT OF VARIATION	MEAN[a]	STANDARD DEVIATION	COEFFICIENT OF VARIATION
			per cent			*per cent*
Collaborative Method						
1	17.2	4.430	25.8	17.0	4.240	24.9
2	14.5	0.643	4.4	14.8	0.818	5.5
3	15.5	0.420	2.7	14.5	0.888	6.1
4	13.2	0.950	7.2	12.7	1.360	10.7
5	13.7	1.720	12.6	15.0	0.850	5.7
6	13.6	0.780	5.7	14.8	2.610	17.6
7	13.7	1.520	11.1	13.9	0.750	5.4
Mean	14.5			14.7		
Standard Deviation	1.420			1.300		
Coefficient of Variation, %	9.8			8.8		
Current Method						
1						
2						
3						
4	13.6	0.100	0.7			
5						
6	12.6	1.470	11.7	13.0	0.570	4.4
7	17.8[b]			17.1[b]		

[a] Each value represents the mean of at least three separate assays.
[b] Single assay value.

TABLE 2.—*Vitamin B_{12} values for sample B (micrograms/g)*

COLLABORATOR	WITH BISULFITE			WITHOUT BISULFITE		
	MEAN[a]	STANDARD DEVIATION	COEFFICIENT OF VARIATION	MEAN[a]	STANDARD DEVIATION	COEFFICIENT OF VARIATION
			per cent			*per cent*
Collaborative Method						
1	10.4	0.540	5.2	10.5	1.760	16.8
2	9.2	0.909	9.9	9.3	0.320	3.5
3	9.0	0.588	6.5	9.5	0.484	5.1
4	9.3	0.350	3.8	8.7	0.360	4.2
5	9.6	0.790	8.3	9.8	0.430	4.4
6	9.0	0.610	6.8	10.0	0.800	8.0
7	8.6	0.860	10.0	8.0	1.150	14.4
Mean	9.3			9.4		
Standard Deviation	0.570			0.840		
Coefficient of Variation, %	6.2			8.9		
Current Method						
1						
2						
3						
4	8.6	0.500	5.8			
5						
6	8.5	1.090	12.9	8.9	1.170	13.0
7	11.6[b]			9.8[b]		

[a] Each value represents the mean of at least three separate assays.
[b] Single assay value.

revealed that treatment of this solution with bisulfite had no effect on the final potency. Furthermore, with possibly one or two exceptions, good inter- and intralaboratory agreement was obtained.

Even better within and between laboratory agreement was observed with sample B which consisted of a crude liver paste. As with sample A, the bisulfite treatment had no effect on the assay value.

A low potency sample such as condensed fish solubles (sample C) also revealed reasonably good inter- and intralaboratory agreement, although the coefficient of variation between laboratories was slightly greater than that observed with samples A and B.

Sample D, a crude vitamin B_{12} fermentation product, was subjected to several different sample treatments. Regardless of the sample treatment, the data indicate that little or no difference existed within or between

TABLE 3.—*Vitamin B_{12} values for sample C (micrograms/g)*

	COLLABORATOR						
	1	2	3	4	5	6	7
Collaborative Method with Bisulfite							
Mean[a]	0.254	0.240	0.241	0.286	0.207	0.258	0.209
Standard Deviation	0.022	0.036	0.008	0.082	0.020	0.020	0.001
Coefficient of Variation, %	8.7	15.0	3.3	28.6	8.3	7.6	0.6
Total Mean:			0.242				
Total Standard Deviation:			0.030				
Total Coefficient of Variation, %:			11.5				
Current Method with Bisulfite							
Mean[a]				0.216		0.250	0.295[b]
Standard Deviation				0.025		0.050	
Coefficient of Variation, %				11.4		20.9	

[a] Each value represents the mean of at least three separate assays.
[b] Single assay value.

laboratories with respect to the final value. As with samples A and B, the bisulfite treatment had little effect. Similarly, the ratio of weight to volume of extracting solution had no marked bearing on the final value. However, experience has demonstrated that both of the latter are of importance with certain insoluble test materials. This is particularly true of the bisulfite treatment as has been demonstrated previously (1, 4, 5). In view of the fact that there is no evidence that the bisulfite treatment has a deleterious effect on the assay value but actually may have a stabilizing action on the naturally occurring analogues of vitamin B_{12}, particularly with certain samples, it appears highly desirable to retain this method of sample treatment.

The data (Tables 1–4) reveal that no definite correlation exists between potencies established and age of inoculum, instrument, wavelength, or transmittance of assay range (Table 5). Obviously some minor discrepancies still exist either within a laboratory or between laboratories, as was demonstrated with sample D. However, in general this study indicates that with careful assay execution, reasonably good inter- and intralaboratory agreement can be achieved. As was to be expected, the within laboratory agreement was better than that observed between laboratories. The bisulfite treatment seemingly presented no particular problem with the

TABLE 4.—*Vitamin B_{12} values for sample D (micrograms/g)*

	COLLABORATOR							
	1	2	3	4	5	6	7	AV. OF 7
Collaborative Method (1.0 g with Bisulfite)								
Mean [a]	15.4	13.7	13.8	13.5	10.7	13.9	9.4	12.9
Standard Deviation	2.700	0.985	0.159	0.800	1.320	1.130	0.290	1.950
Coefficient of Variation, %	17.5	7.2	1.2	6.0	12.4	8.1	3.1	15.1
Collaborative Method (1.0 g without Bisulfite)								
Mean [a]	15.0	12.3	14.3	12.1	10.8	14.4	9.8	12.7
Standard Deviation	3.740	1.249	0.361	1.260	1.640	1.820	0.076	1.870
Coefficient of Variation, %	24.9	10.2	2.5	10.4	15.2	12.6	0.8	14.8
Collaborative Method (0.1 g with Bisulfite)								
Mean [a]	16.7	13.1	14.2	13.1	11.3	15.2	9.9	13.4
Standard Deviation	5.360	2.425	0.550	1.010	1.700	1.020	0.630	2.290
Coefficient of Variation, %	32.1	18.5	3.9	7.7	15.0	6.7	6.4	17.1
Current Method (1.0 g with Bisulfite)								
Mean [a]				12.4		11.8	11.8 [b]	
Standard Deviation				0.780		1.310		
Coefficient of Variation, %				6.3		11.1		
Current Method (1.0 g without Bisulfite)								
Mean [a]						11.0	12.7 [b]	
Standard Deviation						1.030		
Coefficient of Variation, %						9.3		
Current Method (0.1 g with Bisulfite)								
Mean [a]						13.2	13.3 [b]	
Standard Deviation						1.990		
Coefficient of Variation, %						15.0		

[a] Each value represents the mean of at least three separate assays.
[b] Single assay value.

TABLE 5.—*Additional data relative to assay execution*

COLLABORATOR	INOCULUM AGE	INSTRUMENT	WAVELENGTH	ASSAY RANGE[a]
	hours		*mμ*	
1	7	Lumetron	580	35–37
2	6	Coleman	540	55.5–73
3	20	Coleman 6A, Jr.	660	45–53
4	24	Coleman No. 11	620	55–67
5	8	Lumetron	650	35–50
6	24	Evelyn	620	20–44
7	16	Evelyn	620	22–30

[a] Transmittance of inoculated blank minus transmittance of 0.1 microgram (minimum of at least three assays).

exception of the warning comment of collaborator No. 5. As in previous A.O.A.C. collaborative studies, the turbidimetric evaluation of the results continues to appear feasible (except for the comments of collaborator No. 7).

The comments of some of the collaborators (Nos. 4, 5, and 6) pertaining to transmittance limits of the permissible assay range or the validity range from the assumed potency, indicate that broader limits would allow for more valid assays with equally good results. In view of this, consideration should be given to making these limits less confining.

The over-all coefficient of variation on all the samples (regardless of sample treatment) ranged from approximately 6 to 17 per cent. Although at present this may appear reasonably satisfactory, room for improvement remains. This can be achieved only by continuing research efforts.

SUMMARY

A modification of the procedure employed in the 1952 A.O.A.C. microbiological assay of vitamin B_{12} was subjected to study by seven experienced laboratories. Four test materials (including variations in sample treatment) were assayed. In general, reasonably good inter- and intralaboratory agreement was obtained. Since the bisulfite treatment of the sample revealed no adverse effects, its general use is recommended as an aid in the stabilization of the naturally occurring analogues of vitamin B_{12}. The persistence of variations between laboratories indicates that further work is warranted.

LIST OF COLLABORATORS

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RECOMMENDATIONS

It is recommended*—

(1) That the method studied this year for the assay of vitamin B_{12} in materials containing approximately 0.1 microgram or more of vitamin B_{12} per gram or per ml be adopted, first action, replacing the first action method for vitamin B_{12} adopted last year, *This Journal*, **36**, 96 (1953).

(2) That the work on the microbiological assay for vitamin B_{12} be continued.

REFERENCES

(1) KRIEGER, C. H., *This Journal*, **35**, 726 (1952).
(2) ———, *ibid.*, **36**, 846 (1953).
(3) *United States Pharmacopeia*, Fourteenth Revision, Mack Publishing Co., Easton, Pa., 1950, p. 660.
(4) LOY, H. W., HAGGERTY, J. F., and KLINE, O. L., *This Journal*, **35**, 169 (1952).
(5) PRIER, R. F., DERSE, P. H., and KRIEGER, C. H., *Arch. Biochem. Biophys.*, **40**, 474 (1952).

No report was given on vitamin D—poultry feed supplements.

REPORT ON COSMETICS

By G. ROBERT CLARK (Division of Cosmetics, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.), *Referee*

The Referee recommends† that the following methods, now first action, be adopted as official:

Urea in deodorants, *This Journal*, **34**, 89 (1951).
Chlorides in deodorants, *ibid.*, **34**, 90 (1951).
Sulfates in deodorants, *ibid.*, **34**, 90 (1951).
Methenamine, *ibid.*, **35**, 90 (1952).
Phenolsulfonates, *ibid.*, **35**, 91 (1952).
Thioglycollate solutions, *ibid.*, **35**, 91 (1952).
Qualitative tests for potassium bromate and sodium perborate, *ibid.*, **35**, 92 (1952).

The Referee also recommends† that the following topics be continued:

Deodorants and anti-perspirants
Cold permanent wave preparations
Cosmetic creams
Mascara, eyebrow pencils, and eye shadow
Hair dyes and rinses

* For report of Subcommittee A and action of the Association, see *This Journal*, **37**, 64 (1954).
† For report of Subcommittee B and action of the Association, see *This Journal*, **37**, 67 (1954).

REPORT ON HAIR DYES AND RINSES

By S. H. NEWBURGER (Division of Cosmetics, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.), *Associate Referee*

In a recent issue of *This Journal,* Newburger and Jones (1) described an ultraviolet spectrophotometric technique for analyzing mixtures of *p*-aminophenol with 2,5-diaminotoluene or with *p*-phenylenediamine. The amines are extracted from the hair dye and converted into diacetyl derivatives, which are then dissolved in dilute alkaline-alcoholic solution and determined spectrophotometrically as a two-component system.

It was realized that it would be more desirable, particularly for positive identification of the amines, to separate the diacetyl derivatives before the spectrophotometric analysis was made. It has been found that the solubilities differ sufficiently to permit the desired separation.

METHOD

APPARATUS

(a) *Dean and Stark distilling trap.*—10 ml capacity.

(b) *Flask, trap, and condenser.*—Standard ground glass joints.

(c) *Spectrophotometer.*—Capable of isolating a wave band of 5 mμ, or less, in the region 220–325 mμ. (A Cary recording spectrophotometer, Model 11, was used.)

REAGENTS

(a) *Standard solutions of diacetyl derivatives of p-aminophenol, 2,5-diaminotoluene, and p-phenylenediamine.*—Dissolve 100 mg of the purified derivative in 100 ml of alcohol. These compounds were prepared as described in earlier papers (1, 2).

(b) *Alcohol, 95%.*—The alcohol used should have a high transmittance from 220 to 325 mμ.

(c) *Toluene.*—Reagent grade.

PROCEDURE

Dissolve the weighed, mixed diacetyl derivatives in known vol. of alcohol and pipet aliquot contg ca 25 mg of derivatives into 125 ml acetylation flask. Evap. alcohol on steam bath with aid of air jet and add 10 ml of H_2O and a few carborundum chips to residue. Place flask in transite board with 1.5 in. diam. circular opening, connect to water-cooled condenser, and heat with small gas flame until soln of material is complete. Disconnect condenser, insert Dean and Stark distn trap in flask, completely fill trap with toluene, add 5 ml in excess, and allow to overflow into acetylation flask. Connect trap to water-cooled condenser and heat flask until all the H_2O has distd. Disconnect flask, stopper, and allow to stand 2 hrs at room temp. Then filter contents of flask into beaker through quantitative filter paper previously moistened with toluene. Rinse flask and filter with two 5 ml portions of toluene. Evap. filtrate on steam bath under air jet and reserve residue (diacetyl-*p*-aminophenol). Return filter paper to acetylation flask and heat flask under jet of air until all toluene has evapd. The residue is the diacetyldiamine.

Dissolve each residue in hot alcohol and filter thru paper into 100 ml volumetric flasks. Cool flasks to room temp., fill to mark with alcohol, and mix. Dil. suitable

TABLE 1.—*Analysis of mixtures of diacetyl-diamines and diacetyl-p-aminophenol*

EXPT. NO.	DIACETYL-*p*-AMINOPHENOL		DIACETYL-*p*-PHENYLENEDIAMINE		DIACETYL-2,5-DIAMINOTOLUENE	
	ADDED	FOUND	ADDED	FOUND	ADDED	FOUND
	mg	*mg*	*mg*	*mg*	*mg*	*mg*
1	20	19.5	—	—	5	5.0
2	10	9.9	—	—	15	15.0
3	5	5.0	—	—	20	19.1
4	20	19.9	5	5.2	—	—
5	10	9.5	15	15.2	—	—
6	5	4.7	20	20.1	—	—

aliquots to concns of about 10 mg/l in alcohol. Measure the ultraviolet absorbance of the sample and of standards between 220 and 325 mμ.

Calc. the composition of the sample from the absorbances of the sample and the standards at the following wavelengths:

Diacetyl-*p*-aminophenol	245 mμ
Diacetyl-2,5-diaminotoluene	254 mμ
Diacetyl-*p*-phenylenediamine	265 mμ

Compute the diacetyl compounds as free amine bases:

1 mg diacetyl-*p*-aminophenol =0.565 mg *p*-aminophenol
1 mg diacetyl-*p*-phenylenediamine =0.563 mg *p*-phenylenediamine
1 mg diacetyl-2,5-diaminotoluene =0.592 mg 2,5-diaminotoluene

EXPERIMENTAL

Under the conditions of the proposed procedure, 0.04 mg of diacetyl-*p*-phenylenediamine and 0.2 mg of diacetyl-2,5-diaminotoluene were soluble in the toluene. A 25 mg sample of diacetyl-*p*-aminophenol was completely soluble.

Solutions of diacetyl-*p*-aminophenol in alcohol obey Beer's law to within ±1% in the concn range 5–15 mg/l.

A number of mixts of diacetyl derivatives were analyzed. The results are presented in Table 1.

A typical hair dye base of the following composition was prepared:

	per cent
Ethanol	10
Oleic acid	10
Ammonia	2
Sodium sulfite	0.2
Water	77.8

Known amounts of *p*-aminophenol and of either of the diamines were added to 5 ml portions of the base. The resulting solns were dild to 25 ml with H_2O, acidified with HCl, and extd with five 25 ml portions of water-washed ether. The ether exts were discarded. Five g of NaCl, 1 g of $NaHCO_3$ (sufficient to neutralize acid), and 50 mg of Na sulfite were dissolved in the remaining aq. soln which was then transferred to a continuous extractor with the aid of 25 ml of 20% NaCl soln. The extn of the amines and the subsequent prepn of the diacetyl derivatives were carried out as previously described.

The results obtained by the analysis of these derivatives are given in Table 2.

TABLE 2.—*Recoveries of p-aminophenol and diamine in hair dye*

EXPT. NO.	CONTAINED[a]		SPECTROPHOTOMETRIC RECOVERIES		WT OF DIACETYL DERIVATIVES	
			AS AMINE BASE	AS DIACETYL DERIVATIVES	RECOVERIES	CALCD
		mg	*mg*	*mg*	*mg*	*mg*
1	*p*-Aminophenol	100	92.5	163.7	212.4	221.4
	p-Phenylenediamine	25.0	24.7	43.8		
				207.5		
2	*p*-Aminophenol	24.9	22.4	39.7	215.5	221.7
	p-Phenylenediamine	100	97.4	173.0		
				212.7		
3	*p*-Aminophenol	100	88.7	157	205.4	218.9
	2,5-Diaminotoluene	24.8	23.3	39.4		
				196.4		
4	*p*-Aminophenol	24.9	23.3	41.2	208.2	210.6
	2,5-Diaminotoluene	98.6	95.6	161.5		
				202.7		

[a] The amine compounds were added as the hydrochlorides to 5 ml of the hair dye base.

DISCUSSION

A study of the ultraviolet curves used to calculate the data in Table 1 was informative. At first glance, the spectra seemed to represent pure diacetyl derivatives. However, closer examination of the curves showed that there was some cross contamination of the separated compounds.

The diacetyl-*p*-aminophenol isolated from admixture with diacetyl-*p*-phenylenediamine contained as much as 3 per cent of the diacetyldiamine. Conversely, the separated diacetyl-*p*-phenylenediamine was contaminated with some of the aminophenol compound. When the diamine derivatives were 80 per cent of the original mixture, the aminophenol contamination was 1 to 2 per cent; when the diamine compound was only 20 per cent of the mixture, the aminophenol contamination was about 10 per cent. Fortunately, the diacetyl-*p*-aminophenol has negligible absorption at the wavelength chosen for the determination of the diamine derivative.

The separated diacetyl derivatives of *p*-aminophenol and 2,5-diaminotoluene also exhibit cross contamination. Percentagewise, the contamination is manifested to a greater degree in the minor component. Isolated from 20 per cent mixtures, the contamination of the aminophenol derivative was 8 per cent and that of the diamine, 4 per cent.

The results in Table 1 are uncorrected. They are reasonably good, although dependent to a small degree on compensating errors. It is believed that the method is valid for mixtures containing as little as 10 per cent of any of the amines.

Although the number of experiments was small, a study of the hair dye amine recoveries reveals certain trends. About 98 per cent of the *p*-phenylenediamine, 95 per cent of the 2,5-diaminotoluene, and 91 per cent of the *p*-aminophenol were recovered.

The relatively low recovery of the aminophenol may be due to one or two factors: incomplete extraction or decomposition of the aminophenol or both. Unfortunately, the completeness of extraction was not checked in these experiments, since the conditions used had proved adequate in other work.

SUMMARY AND RECOMMENDATION

A modified procedure has been proposed for the analysis of hair dyes containing a mixture of *p*-aminophenol and 2,5-diaminotoluene or *p*-phenylenediamine. The diacetyl derivative of the diamine is separated from the diacetyl derivative of *p*-aminophenol, and each compound is determined individually by ultraviolet spectrophotometry. The method should be applicable for the determination of *p*-aminophenol in mixtures containing both diamines.

It is recommended* that work on hair dyes and rinses be continued.

REFERENCES

(1) NEWBURGER, S. H., and JONES, J. H., *This Journal*, **36**, 784 (1953).
(2) ———, *ibid.*, **33**, 374 (1950).

REPORT ON MASCARAS, EYEBROW PENCILS, AND EYE SHADOWS

By PAUL W. JEWEL (Max Factor & Co., Hollywood 28, Calif.),
Associate Referee

Methods reported earlier[1] proved to be reasonably satisfactory for the type of formulations on the market at the time. Recently, however, there have been appearing on the market several brands of mascara in which non-ionic emulsifiers are replacing the soap used previously.

The work done this year has been designed to develop a method which will enable the analyst to separate these products into various fractions for identification, and to determine the amounts of the fractions.

* For report of Subcommittee B and action of the Association, see *This Journal*, **37**, 67 (1954).
[1] *This Journal*, **31**, 585 (1948).

Complete separation of base from pigments in mascara made with non-ionic dispersing agents may be accomplished by the method previously described.[1] However, when the next step in the procedure is begun, the inadequacy of the original method quickly becomes apparent. The total triethanolamine determination is sometimes made difficult by the neutralization equivalents of the amides, if any, or by other alkaline-reacting substances. Also, the non-ionics keep the whole sample more or less emulsified and make separation very nearly hopeless. It will be necessary to do a great deal more work before this method will be ready, but it is hoped that this can be accomplished by next year.

A quick, easy, accurate method for the determination of pigments in mascara, eye-brow pencils, eye shadow, and lipsticks is frequently needed, and an attempt has accordingly been made to develop such a procedure. Chloroform is the solvent of choice for such a purpose, but its high specific gravity makes centrifuging impractical. Most of the other ordinary solvents will not hold the waxes in solution, especially in the cold. It has been found that a straight aliphatic cut with a boiling range of about 75–100°C. is very satisfactory, since the waxes melt at the boiling point of the solvent and are more or less miscible with it, even though they are not really soluble.

The following method is offered as a tentative one which should prove very satisfactory after some further refinement:

DETERMINATION

Weigh sample, ca 2.00 g, into tared centrifuge tube. Melt sample and add 5 ml of solvent.[2] Boil gently for 5 min., add 35 ml of addnl solvent, and heat just to boiling. Centrifuge immediately at fairly high speed so that solvent will not cool too much. Decant clear soln and repeat extn procedure until pigment residue loses no further weight. Dry in oven at 100°C. and weigh. Calc. weight of the residue as total pigment.

The extracted base may then be used for whatever analytical procedures are indicated. Due consideration should be given to nonvolatile residues possibly present in these solvents. It may prove to be desirable to redistil before using.

This method is reasonably practical for most of the pigmented wax-bearing mixtures. In the case of lipstick, however, certain complications occur. About half of the bromos will be found in the solution and about half will remain with the pigments. Also, in those lipsticks containing propylene glycol (which includes the majority), the glycol remains with the pigments, necessitating some type of further treatment. This method will be given more attention and it should be possible to develop it for practical use.

[2] Skellysolve "A" or "D" made by the Skelly Oil Co. or Amsco Special Textile Spirits made by American Mineral Spirits Co.

It is recommended* that this study be continued and that samples be submitted to collaborative study as soon as a suitable method is ready.

REPORT ON DEODORANTS AND ANTI-PERSPIRANTS

DETERMINATION OF PHENOLSULFONATES

By JOHN E. CLEMENTS (Division of Cosmetics, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.), *Associate Referee*

Kramer (1) applied Grant's modification (2) of the U.S.P. IX method for the determination of phenolsulfonates to anti-perspirants. It is a bromination procedure not applicable in the presence of other materials which brominate rapidly.

The characteristic ultraviolet absorbance of phenolsulfonic acid suggested an alternate procedure for its identification and determination in anti-perspirants. The following method was developed.

METHOD

APPARATUS

Spectrophotometer.—Capable of isolating a wave band of 5 mμ, or less, in the spectral region 220–350 mμ. (A Cary recording spectrophotometer, Model 11, was used.)

REAGENTS

(a) *Standard zinc phenolsulfonate solution (10 mg/l in ca 0.1 N NaOH).*—Dissolve 100 mg of zinc phenolsulfonate (equivalent to 62.66 mg of phenolsulfonic acid) in 100 ml of H_2O. Dil. a 10 ml aliquot of this soln to 100 ml with H_2O. Pipet a 10 ml aliquot of this soln into a 100 ml volumetric flask, add 4 ml of freshly prepd 10% NaOH soln, and dil. to vol. with H_2O. (The N.F. zinc phenolsulfonate used in this investigation analyzed 99.2% pure according to the method of Kramer.)

(b) *10% NaOH solution.*—Freshly prepd by dissolving 10 g of solid $NaOH$ in sufficient H_2O to make 100 ml of soln.

PROCEDURE

(a) *If a sulfated surface active agent is present*: Weigh accurately in a 250 ml Erlenmeyer flask a sample estimated to contain 5–10 mg of phenolsulfonic acid. Add 10 ml of H_2O and 2 ml of concd HCl to the flask, connect to a water-cooled condenser, and reflux for one-half hr. Cool the soln to room temp., transfer to a 100 ml separatory funnel with the aid of 20 ml of H_2O, and proceed as directed under (b), beginning with "ext. with three 30 ml portions of $CHCl_3$. . . ."

(b) *If a sulfated surface active agent is absent*: Weigh accurately in a weighing bottle a sample estimated to contain 5–10 mg of phenolsulfonic acid. Transfer the sample to a 100 ml separatory funnel with the aid of 30 ml of H_2O. Acidify the mixt. with concd HCl, and ext. with three 30 ml portions of $CHCl_3$. Discard the $CHCl_3$ extracts.

Filter the aq. soln thru a previously moistened quantitative filter paper into a 100 ml volumetric flask and dil. to vol. with H_2O. Pipet a 10 ml aliquot of this soln

* For report of Subcommittee B and action of the Association, see *This Journal*, 37, 67 (1954).

into a 100 ml volumetric flask, neutralize to litmus paper with 10% NaOH soln, add 4 ml in excess, and dil. to vol. with H_2O.

Det. the absorbance of the standard and sample solns at 253 mμ in 1 cm cells, using 0.1 *N* NaOH soln as the blank. Calc. the per cent of phenolsulfonic acid present in the sample.

$$\text{Per cent phenolsulfonic acid} = \frac{C_s}{10} \times \frac{A_{sple}\ 253\ m\mu}{A_{std}\ 253\ m\mu} \times \frac{1}{\text{Wt sample (g)}}$$

where:

C_s = concentration of phenolsulfonic acid (mg/l) in standard solution.
A_{sple} = absorbance of sample solution.
A_{std} = absorbance of standard solution.

Det. the absorbance of the sample soln at a sufficient number of points in the region 220 mμ to 300 mμ to enable plotting an absorbance *vs.* wavelength curve.

EXPERIMENTAL

Solutions of phenolsulfonic acid in 0.1 *N* HCl and 0.1 *N* NaOH, varying in concentration from 5 to 300 mg/l, obey Beer's law within ±1% at the absorbance

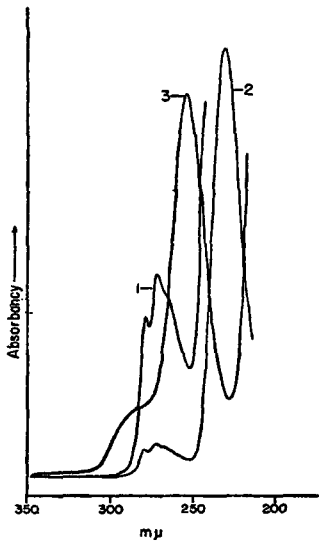

FIG. 1.—Absorption curves of zinc phenolsulfonate.
(1) 300 mg/l in 0.1 *N* HCl
(2) 50 mg/l in 0.1 *N* HCl
(3) 30 mg/l in 0.1 *N* NaOH

maxima. Fig. 1 shows the ultraviolet absorption curves of zinc phenolsulfonate.

The following solution, containing a number of ingredients likely to be found in anti-perspirants, was prepared:

TABLE 1.—*Recoveries of zinc phenolsulfonate from solution*

EXPT. NO.	PHENOLSULFONATE ADDED[a]	PHENOLSULFONATE FOUND	RECOVERY
	mg	*mg*	*per cent*
1	5	4.97	99.4
2	10	9.91	99.1
3	25	24.9	99.7
4	25	24.8	99.3
5	50	50.0	100.0
6	75	74.8	99.7
		Av.	99.6

[a] The phenolsulfonate was added to 0.5 ml of the prepared solution.

Glycerol	5 g
Urea	5 g
Calcium nitrate	5 g
Aluminum chloride	5 g
Zinc chloride	5 g
Magnesium chloride	5 g
Sodium borate	5 g
Hydrochloric acid, concd	25 ml
Water, q.s	500 ml

Known amounts of zinc phenolsulfonate were added to 0.5 ml aliquots of the above solution, and the solutions were analyzed by the proposed procedure. The results are given in Table 1.

An anti-perspirant cream containing the following ingredients was prepared:

Sodium lauryl sulfate	6 g
Glyceryl monostearate	18 g
Mineral oil	9 g
Spermaceti	15 g
Titanium dioxide	6 g
Glycerol	18 g
Water	161.5 ml
Aluminum sulfate	30 g
Urea	15 g

Known amounts of zinc phenolsulfonate were added to 0.5 g portions of the cream and the resulting mixtures were analyzed. The results are tabulated in Table 2.

DISCUSSION

The average recovery of zinc phenolsulfonate from the solution was 99.6 per cent; the largest deviations from this value were +0.4 per cent and −0.3 per cent. The average recovery of zinc phenolsulfonate from the cream was 98.9 per cent; the greatest deviations from this value were +1.4 per cent and −1.3 per cent.

The complete ultraviolet curves of the samples in both acid and alkali were plotted to establish the identity of the extracted material as phenolsulfonic acid.

TABLE 2.—*Recoveries of zinc phenolsulfonate from anti-perspirant cream*

EXPT. NO.	PHENOLSULFONATE ADDED[a]	PHENOLSULFONATE FOUND	RECOVERY
	mg	*mg*	*per cent*
1	5	4.98	99.6
2	25	24.5	98.0
3	25	24.7	98.8
4	25	25.1	100.3
5	50	48.8	97.6
6	75	74.3	99.1
		Av.	98.9

[a] The phenolsulfonate was added to 0.5 g of the prepared cream.

SUMMARY AND RECOMMENDATIONS

An ultraviolet spectrophotometric method has been presented for the determination of phenolsulfonates in anti-perspirants.

It is recommended*—

(1) That the method be submitted for collaborative study.

(2) That the subject of deodorants and anti-perspirants be continued.

REFERENCES

(1) KRAMER, H., *This Journal*, **35**, 279 (1952).
(2) GRANT, E. H., *ibid.*, **14**, 351 (1931).

No reports were given on cold permanent waves or cosmetic creams.

REPORT ON COAL-TAR COLORS

By KENNETH A. FREEMAN (Division of Cosmetics, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.), *Referee*

The Referee concurs in the recommendation† of the Associate Referee that the proposed chemical and spectrophotometric methods for the determination of 4-toluene-azo-2-naphthol in D&C Red No. 35 be submitted to collaborative study and that the topic, Subsidiary Dyes in D&C Colors, be continued.

The Referee concurs in the recommendation of the Associate Referee that the method for the determination of higher sulfonated dye in FD&C

* For report of Subcommittee B and action of the Association, see *This Journal*, **37**, 67 (1954).
† For report of Subcommittee B and action of the Association, see *This Journal*, **37**, 67, 68 (1954).

Yellow No. 6 be adopted, first action. He also concurs in the recommendation of the Associate Referee that the methods for the determination of subsidiary dyes in FD&C Red No. 2 be submitted to collaborative study, and that the topic, Subsidiary Dyes in FD&C Colors, be continued.

The Referee concurs with the recommendation of the Associate Referee on Paper Chromatography of Coal-Tar Colors that further collaborative study be undertaken and that the topic be continued.

The Referee recommends that the methods for the determination of intermediates derived from phthalic acid, lower sulfonated dyes in FD&C Yellow No. 5, and Lake Red C amine in D&C Red Nos. 8 and 9 be changed in classification from first action to official. These methods have been first action for at least two years. Experience has shown that they are reliable and worthy of adoption as official.

The Referee recommends that the method for the determination of boiling range of amines (pseudocumidine) derived from FD&C Red No. 1 be deleted. Recent investigations have disclosed that the boiling ranges of amines derived from this color do not give accurate indices of the composition of those amines. The Referee further recommends that the topic be discontinued.

The Referee recommends that all methods for the determination of ether extracts in coal-tar colors presently designated first action be changed in classification to official. Experience has shown that these methods are reliable for the determination of ether extracts in all coal-tar colors for which this determination is specified. It is further recommended that the topic Ether Extracts in Coal-tar Colors be discontinued.

The Referee recommends that the topic Halogens in Halogenated Fluoresceins be closed. All methods for these determinations are classified official. Experience has shown these methods to be accurate and reliable for the determination of halogens in all halogenated colors where the determination is specified.

The Referee recommends that the following topics be continued:

Heavy metals in coal-tar colors
Sulfonated amine intermediates in coal-tar colors
Volatile amine intermediates in coal-tar colors
Non-volatile unsulfonated amine intermediates in coal-tar colors
Inorganic salts in coal-tar colors
Sulfonated phenolic intermediates in coal-tar colors
Unsulfonated phenolic intermediates in coal-tar colors
Intermediates in triphenylmethane colors
Arsenic and antimony in coal-tar colors
Lakes and pigments.

REPORT ON SUBSIDIARY DYES IN D&C COLORS

4-TOLUENE-AZO-2-NAPHTHOL IN D&C RED NO. 35

By Louis Koch (H. Kohnstamm & Company, Inc., Brooklyn 31, N. Y.), *Associate Referee*

In 1952, collaborative studies were conducted on the estimation of 4-toluene-azo-2-naphthol in D&C Red No. 35 by reduction, followed by isolation of the primary unsulfonated amine and determination with bromate-bromide solution. Results indicated that a purely chemical method would yield recoveries of 75 to 85 per cent (Table 1).

Although such results would be useful in regulatory work, in which small quantities of subsidiary dye are involved, it was deemed advisable to investigate a spectrophotometric procedure before making a recommendation.

Graichen and Harrow[1] reported a spectrophotometric determination of this subsidiary pigment. They stated that recoveries averaged 88 per cent, a more accurate figure than that obtained by the proposed reduction-bromate method.

Studies were therefore initiated, employing essentially the procedure of Graichen and Harrow with minor modifications. Because of the slight solubility of D&C Red No. 35 in alcohol, a correction factor was required in the calculations.

The Associate Referee and a co-worker obtained results, given in Table 2, which tentatively confirmed that the spectrophotometric procedure was more accurate. An instrument capable of operating between 400 and 500 millimicrons was used.

Table 1.—*Collaborative results for estimation of 4-toluene-azo-2-naphthol with bromate-bromide solution*

Analyst	Subsidiary dye found			
	Sample A	Sample B	Sample C	Sample D
	per cent	*per cent*	*per cent*	*per cent*
1	0.41	0.59	1.07	1.98
2	0.40	0.46	1.03	1.70
3	0.12	0.55	1.01	2.09
4	0.38	0.52	1.07	2.10
Associate Referee	0.42	0.57	1.10	2.11
Calculated[a]	0.35	0.61	1.21	2.42
Calculated[b]	0.46	0.71	1.32	2.52
Average recovery, %	87	73	80	80

[a] Theoretical quantity of subsidiary dye formed by added 4-methyl aniline.
[b] Includes percentage of subsidiary dye found by proposed method in a sample of unadulterated primary dye.

[1] *This Journal*, 35, 754 (1952).

TABLE 2.—*Recovery of subsidiary dye by spectrophotometric method*

ADDED	FOUND	RECOVERY	
mg	*mg*[a]	*mg*[b]	*per cent*
0	2.96[c]	—	—
	3.00[c]	—	—
0	6.62[d]	—	—
	5.60[d]	—	—
5.0	11.58	4.96	99.2
	10.20	4.60	92.0
10.0	15.28	8.66	86.6
	14.43	8.83	88.3
20.0	24.11	17.49	87.5
	22.81	17.21	86.1
40.0	42.83	36.21	90.5
80.0	78.20	71.62	87.2
	78.72	73.12	91.4

[a] Total subsidiary dye.
[b] Net subsidiary dye.
[c] Assay of crystallized D&C Red No. 35, showing blank caused by solubility of the primary pigment.
[d] Assay of commercial certified sample of D&C Red No. 35, indicating a subsidiary dye content of 0.16%.

SPECTROPHOTOMETRIC METHOD

REAGENTS

(a) *Chloroform.*—Distd.

(b) *Alcohol.*—Special denatured No. 1.

(c) *4-Toluene-azo-2-naphthol.*—Crystd from alcohol. Dissolve 10 mg in alcohol and dil. to 1 l.

(d) *D&C Red. No. 35.*—Crystd from $CHCl_3$.

DETERMINATION

Ext. 2 g sample with $CHCl_3$ in Soxhlet app. until leachings are colorless. Cool, filter, and wash residue (crystd D&C Red. No. 35) and filter paper thoroly with alcohol. Evap. the $CHCl_3$-alcohol filtrate to dryness (avoid spattering), and heat residue with 25 ml of alcohol. Cool, transfer to 50 ml volumetric flask, and dil. to vol.

Remove insol. matter by filtration, and dil. 5 ml aliquot to 250 ml with alcohol. Read absorbance at 486 mμ.

Repeat procedure, substituting 2 g of crystd D&C Red No 35. Record absorbance of the standard 4-toluene-azo-2-naphthol soln.

$$\frac{A_{\text{Unknown}}}{A_{\text{Known}}} \times 25 = \text{Total mg subsidiary dye}$$

Total mg subsidiary dye − mg pseudo-subsidiary dye in crystd D&C Red No. 35 × 1/20 = % subsidiary dye.

RECOMMENDATION

It is recommended* that the proposed chemical and spectrophotometric analytical procedures for 4-toluene-azo-2-naphthol in D&C Red No. 35 be studied collaboratively.

REPORT ON SUBSIDIARY DYES IN FD&C COLORS

I. HIGHER SULFONATED DYES IN FD&C YELLOW NO. 6

By MEYER DOLINSKY (Division of Cosmetics, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.), *Associate Referee*

FD&C Yellow No. 6 is the disodium salt of 1-sulfophenylazo-2-naphthol-6-sulfonic acid and is prepared by coupling diazotized sulfanilic acid with 2-naphthol-6-sulfonate (Schaffer salt). The preparation of a purified sample of this color has been described (1). Previous work (1, 2) has shown the presence of small amounts of lower sulfonated dye in commercial samples of FD&C Yellow No. 6. This lower sulfonated material, which appears to be mainly D&C Orange No. 4, is readily determined by an extraction procedure (3).

In addition to lower sulfonated material, commercial FD&C Yellow No. 6 may contain appreciable amounts of higher sulfonated dye due to the presence of R salt (2-naphthol-3,6-disulfonate) or G salt (2-naphthol-6,8-disulfonate) in the Schaffer salt used to prepare the dye. This paper describes a method based on extraction and spectrophotometric measurement by which these higher sulfonated dyes may be quantitatively determined.

METHOD

REAGENTS

(a) *Isoamyl alcohol.*—Reagent grade.

(b) *Hydrochloric acid* (1+25).—Dil. 40 ml of concd HCl to one l with distd H_2O.

APPARATUS

(a) *Spectrophotometer.*—Suitable for measuring absorbance at 450–500 mμ.

(b) *Absorption cells.*—Preferably 5 cm path length.

EXTRACTION

Dissolve 100 mg of the FD&C Yellow No. 6 in 100 ml (1 + 25) HCl. Dil. 10 ml of this soln with 40 ml of (1+25) HCl and ext. by shaking the soln successively in 5 separatory funnels, each contg 50 ml of isoamyl alcohol. Transfer acid layer to a 100 ml volumetric flask. Wash amyl alcohol extracts with two 25 ml portions of (1 + 25) HCl, passing each portion thru the funnels in the same order as used for original extn. Add washings to the acid soln of subsidiary and dil. to 100 ml with

* For report of Subcommittee B and action of the Association, see *This Journal*, 37, 67 (1954).

distd H_2O. Det. absorbance of extd soln at the max. Det. absorbance per mg/l of a standard soln of the subsidiary in (1 + 25) HCl, at the same wavelength and in the same cell.

$$\textit{Calculation:}\ \frac{\text{Absorbance of extract}}{\text{Absorbance per mg/l of standard}} = \%\ \text{subsidiary}$$

EXPERIMENTAL

The recoveries obtained in the extraction of known samples by this procedure are shown in Table 1.

TABLE 1.—*Extraction data*

SAMPLE EXTRACTED	SUBSIDIARY RECOVERED	
	mg	*per cent*
R salt subsidiary (10 mg)	9.64	96.4
R salt subsidiary (0.5 mg)	0.46	92.0
G salt subsidiary (10 mg)	9.80	98.0
G salt subsidiary (0.5 mg)	0.47	94.0
FD&C Yellow No. 6 (10 mg)	0.01	0.1
FD&C Yellow No. 6 (10 mg) plus R salt subsidiary (0.5 mg)	0.48	94.0

The two higher sulfonated subsidiaries of FD&C Yellow No. 6[1] were prepared by coupling diazotized sulfanilic acid with commercial R salt and G salt, respectively. These dyes, plus a commercial sample of FD&C Yellow No. 6 of high purity, were used to develop the extraction procedure described above.

The visible absorption spectra of the two higher sulfonated subsidiaries, obtained on a Cary recording spectrophotometer, are shown in Figs. 1 and 2. Spectrophotometric data are summarized in Table 2.

TABLE 2.—*Spectrophotometric data*

	ABSORPTION MAXIMUM	
	NEUTRAL AND ACID SOLUTIONS	ALKALINE SOLUTION
	mμ	*mμ*
FD&C Yellow No. 6	482 ± 2	445 ± 2
R salt subsidiary	490 ± 2	466 ± 2
G salt subsidiary	476 ± 2	412 ± 2

A number of commercial samples of FD&C Yellow No. 6 were analyzed for higher sulfonated dye by the proposed method. The samples were from batches submitted for certification by seven color manufacturers.

[1] Prepared by Charles Stein, Division of Cosmetics, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.

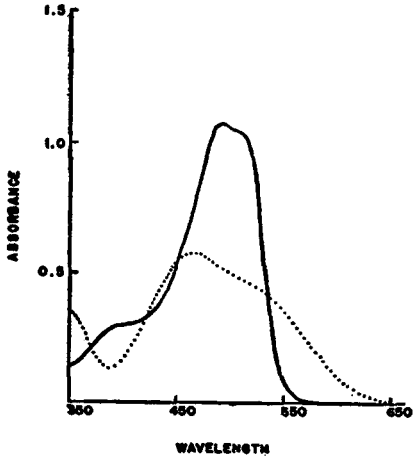

FIG. 1.—R salt subsidiary of FD&C Yellow No. 6.
Concn: 21.8 mg/l (pure dye); Cells: 1 cm.
Solvent:
———H_2O (neutral) and 0.1 *N* HCl
· · · · · ·0.1 *N* NaOH

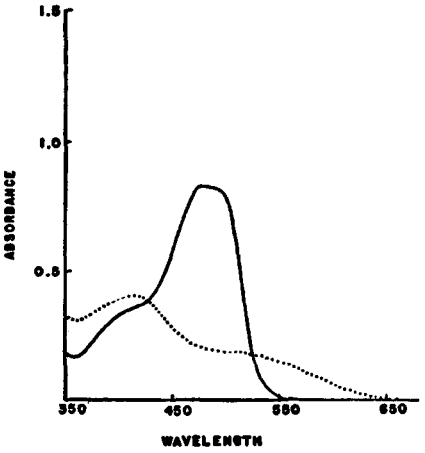

FIG. 2.—G salt subsidiary of FD&C Yellow No. 6.
Concn: 19.3 mg/l (pure dye); Cells: 1 cm.
Solvent:
———H_2O (neutral) and 0.1 *N* HCl
· · · · · ·0.1 *N* NaOH

The amount of subsidiary color found ranged from 0.1 to 6.0 per cent. In all cases, the color recovered appeared to be mainly, if not entirely, the R salt isomer.

COLLABORATIVE STUDY

A known sample was prepared by mechanically mixing R salt subsidiary with a relatively pure sample of FD&C Yellow No. 6. The subsidiary dye content of the mixture was calculated to be 3.45 per cent. This mixture was then analyzed by the Associate Referee and by the collaborators.

Results, given in Table 3, are tabulated in the order in which they were received.

TABLE 3.—*Collaborative results for mixture of R salt subsidiary and FD&C Yellow No. 6*

COLLABORATOR NO.	SUBSIDIARY DYE CONTENT FOUND	AVERAGE
	per cent	*per cent*
1	3.37, 3.40	3.38
2	3.48, 3.48	3.48
3	3.43, 3.37	3.40
4	3.77, 3.88, 3.43, 3.63	3.68
5	3.45, 3.50	3.48
6	3.3, 3.2, 3.2	3.23
7	3.34	3.34

Based on the average results reported, the maximum deviation from the calculated value is 6.7 per cent and the mean deviation is 3.1 per cent. This is considered satisfactory agreement for this type of analysis.

COLLABORATORS

John E. Clements, Division of Cosmetics, Food and Drug Administration, Washington 25, D. C.
John F. Walton, H. Kohnstamm & Company, Inc., New York, N. Y.
W. H. Kretlow, Wm. J. Stange Company, Chicago, Ill.
Robert Schumacher, The Hilton-Davis Chemical Company, Cincinnati, Ohio.
A. T. Schramm, National Aniline Division, Allied Chemical and Dye Corporation, Buffalo, N. Y.
James E. Noonan, Warner-Jenkinson Manufacturing Company, St. Louis, Mo.

RECOMMENDATIONS

It is recommended*—

(1) That the method for the determination of higher sulfonated dye in FD&C Yellow No. 6 be adopted, first action.

(2) That the topic be continued.

REFERENCES

(1) STEIN, C., *This Journal*, **32**, 672 (1949).
(2) DOLINSKY, M., unpublished data.
(3) *Official Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Box 540, Benjamin Franklin Station, Washington 4, D.C., 1950.

REPORT ON SUBSIDIARY DYES IN FD&C COLORS

II. SUBSIDIARY DYES IN FD&C RED NO. 2

By MEYER DOLINSKY (Division of Cosmetics, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.), *Associate Referee*

FD&C Red No. 2 is the trisodium salt of 1-(4-sulfo-1-naphthyl-azo)-2-naphthol-3,6-disulfonic acid and is prepared by coupling diazotized naphthionic acid with R salt (2-naphthol-3,6-disulfonate).

Commercial R salt may contain appreciable amounts of other sulfonated naphthols, particularly Schaffer salt (2-naphthol-6-sulfonate) which will couple with diazotized naphthionic acid to give a lower sulfonated dye (C.I. 182), and G salt (2-naphthol-6,8-disulfonate), which in turn will couple to give an isomeric dye (C.I. 185). The Coal-Tar Color Regulations (1) allow a maximum of 4.0 per cent subsidiary dye in certifiable samples of FD&C Red No. 2.

* For report of Subcommittee B and action of the Association, see *This Journal*, **37**, 67 (1954).

Methods for the determination of lower sulfonated and subsidiary dyes in FD&C Red No. 2 appear in *Official Methods of Analysis*, A.O.A.C., 6th Ed., 1945. At the 1950 meeting of the A.O.A.C., these methods were deleted, pending further study. During the past year both of these methods have been reinvestigated by the Associate Referee, using relatively pure samples of FD&C Red No. 2, C.I. 182, and C.I. 185. Results are presented in this paper.

C.I. 182 and C.I. 185 were prepared by diazotizing recrystallized naphthionic acid and coupling with purified Schaffer salt and G salt, respectively. The dyes were purified by crystallizing from alcohol-H_2O. The FD&C Red No. 2 was a commercial sample of high purity. Analytical data on the three colors (dried at 135°C.) are shown in Table 1.

TABLE 1.—*Analytical data*

SAMPLE	PURE DYE CONTENT (BY TITRATING WITH $TiCl_3$)	NaCl
	per cent	*per cent*
C.I. 182	98.5	0.2
C.I. 185	95.7	1.8
FD&C Red No. 2	95.3	4.3

The absorption spectra of C.I. 182 and C.I. 185, obtained on a Cary recording spectrophotometer, are shown in Figs. 1 and 2. Dilute solutions of the two compounds in neutral solution, 0.1 *N* HCl, and 0.1 *N* NaOH are stable, showing no change in spectrophotometric characteristics when re-run after an interval of two weeks. Spectrophotometric data are shown in Table 2.

TABLE 2.—*Spectrophotometric data*

COLOR	ABSORPTION MAXIMUM (NEUTRAL AND ACID SOLNS)	ABSORPTION MAXIMUM (ALKALINE SOLN)
	mμ	*mμ*
C.I. 182	505 ± 2	472 ± 2
C.I. 185	509 ± 2	440 ± 2
FD&C Red No. 2	520 ± 2	492 ± 2

LOWER SULFONATED DYE

In the former A.O.A.C. method, C.I. 182 is extracted from acid solution into amyl alcohol. The amyl alcohol extracts are washed with 0.25 *N* HCl and the residual lower sulfonated dye is determined colorimetrically or by titration with $TiCl_3$. This method was found to be satisfactory. Data obtained in the analysis of several samples of known composition are shown in Table 3.

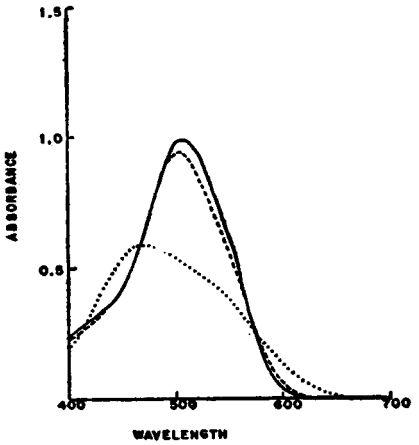

FIG. 1.—C.I. 182
Concentration: 21.2 mg/liter (pure dye); Cells: 1 cm.
Solvent:
———H_2O (neutral)
– – – 0.1 *N* HCl
· · · · · · 0.1 *N* NaOH

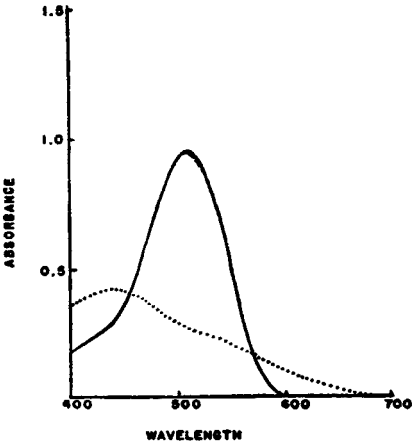

FIG. 2.—C.I. 185
Concentration: 24.2 mg/liter (pure dye); Cells: 1 cm.
Solvent:
———H_2O (neutral)
– – – 0.1 *N* HCl
· · · · · · 0.1 *N* NaOH

TABLE 3.—*Extraction of C.I. 182 by the former A.O.A.C. method (color determined spectrophotometrically)*

SAMPLE EXTRACTED	LOWER SULFONATED DYE	
	ADDED	FOUND
	per cent	*per cent*
C.I. 182 (200 mg)	100	97.8
C.I. 182 (10 mg)	100	98.7
C.I. 185 (100 mg)	0	0.2
FD&C Red No. 2 (200 mg)	0	0.4
FD&C Red No. 2 (100 mg) plus C.I. 182 (4 mg)	3.8	3.8

ISOMERIC DYE

In the former A.O.A.C. method, the FD&C Red No. 2 is precipitated as the benzidine salt and the residual color is determined colorimetrically. In the hands of the Associate Referee, this did not give quantitative results. However, the method does appear to be suitable for rapidly approximating the percentage of C.I. 185 in samples of FD&C Red No. 2. Data obtained in the analysis of known mixtures are shown in Table 4.

There appears to be a blank of 0.2–0.4 per cent in the isomeric dye

TABLE 4.—*Determination of isomeric dye by the former A.O.A.C. method*

FD&C RED NO. 2—C.I. 185 MIXTURES	
C.I. 185 ADDED	ISOMERIC DYE RECOVERED
per cent	*per cent*
0	0.2
1.0	0.9
2.0	1.4
2.9	2.0
4.8	3.3
9.1	7.1

determination by the former A.O.A.C. method which has been shown (2) to be due to FD&C Red No. 2 and not to traces of subsidiary color in the FD&C Red No. 2 standard. Subtracting this blank gives a subsidiary dye value which is approximately 60–70 per cent of that actually present. The presence of C.I. 182 does not interfere with the determination.

CHROMATOGRAPHIC DETERMINATION OF C.I. 185

C.I. 185 may be determined quantitatively by means of chromatography, using powdered cellulose as the adsorbent and 3 per cent NaCl as the eluant. The following procedure was found to be satisfactory.

Prep. chromatographic column ca 25 by 400 mm from a slurry of powdered cellulose in 3% NaCl. Dissolve 500 mg of color in 100 ml of 3% NaCl. Carefully pipet 2 ml of this soln onto top of column and elute with 3% NaCl. (C.I. 185, if present, moves rapidly down the column and can be observed as a distinct orange-red band preceding the intensely colored FD&C Red No. 2 band.) Elute the C.I. 185 and det. spectrophotometrically. Using this procedure, as little as 0.05 mg of C.I. 185 (0.5%) can be accurately detd. C.I. 182 does not interfere with the detn.

TABLE 5.—*Chromatographic determination of C.I. 185*

MIXTURE CHROMATOGRAPHED	C.I. 185 FOUND
mg	*mg*
FD&C Red No. 2, 1.0	
C.I. 182, 1.0	0.98
C.I. 185, 1.0	
FD&C Red No. 2, 12.5	
C.I. 182, 0.25	0.22
C.I. 185, 0.25	
FD&C Red No. 2, 10.0	
C.I. 182, 0.4	0.35
C.I. 185, 0.4	
FD&C Red No. 2, 9.5	0.06
C.I. 185, 0.05	

Analytical data obtained in the chromatographic determination of C.I. 185 in mixtures of known composition are shown in Table 5.

Five certifiable samples of FD&C Red No. 2, submitted for certification by five color manufacturers, were analyzed for lower sulfonated dye by the former A.O.A.C. procedure and for the isomeric dye by the chromatographic procedure. Results are shown in Table 6. In no case does the percentage of subsidiary dye approach the 4.0 per cent limit allowed by the Coal-Tar Color Regulations.

TABLE 6.—*Analysis of commercial samples of FD&C Red No. 2*

SAMPLE	LOWER SULFONATED DYE	ISOMERIC DYE
	per cent	*per cent*
1	0.2	<0.3
2	0.4	<0.3
3	0.5	<0.3
4	0.7	<0.3
5	0.7	<0.3

It is recommended* that the methods be submitted to collaborative study, and that the topic be continued.

ACKNOWLEDGMENT

The Associate Referee wishes to thank Dr. Charles Stein for preparing the sample of C.I. 182 used in this work.

REFERENCES

(1) S.R.A.F.D.C. 3, Coal-Tar Color Regulations.
(2) GORDON, N., unpublished data.

REPORT ON PAPER CHROMATOGRAPHY OF COAL TAR COLORS

By DORIS H. TILDEN (Food and Drug Administration, Department of Health, Education, and Welfare, San Francisco 2, Calif.), *Associate Referee*

The Associate Referee's report for 1952[1] consisted of additional material on color reactions produced by various reagents on paper swatches from chromatograms and on some chromatographic solvent systems for the migration or separation (or both) of certain coal tar dyes.

[1] TILDEN, D., *This Journal*, 36, 802 (1953).
* For report of Subcommittee B and action of the Association, see *This Journal*, 37, 67 (1954).

Collaborative work was designed for the study of a procedure for the separation of several food colors by paper partition chromatography. Instructions were given to collaborators for producing chromatograms separating given color mixtures under different *p*H conditions with three different solvent systems. Means were also outlined for identifying colors resolved on the paper strips. Results from two collaborators, received at that time, were reported.

This report includes a complete tabulation (Table 1) of the results of all collaborative work based on the studies conducted in 1952, together with a discussion of the work to date. Following are some pertinent suggestions made by collaborators.

COMMENTS OF COLLABORATORS

No. 1.—"I think spectrophotometric identification is best, provided collaborators are instructed to use a column to get a workable sample."

(The spot of color to be chromatographed may be "built up" on the paper by repeated addition of the sample solution (see "Instructions to Collaborators") so as to give a satisfactory amount of resolved dye material for a spectrophotometric determination. Also, several chromatograms of the same sample solution may be run simultaneously, and the color desired can be cut from the strips and combined to give a workable amount of color.)

No. 2.—"In my opinion the (chromatographic) method is a definite advance in the separation and identification of water-soluble coal-tar dyes. In this regard I would like to suggest that another column be added to the chart in section 34.9 of *Official Methods of Analysis*, giving the F&D color names and numbers specified in the Coal-Tar Color Regulations for certifiable dyes."

No. 3.—"In checking R_F values it was noted that for solvent systems 1 and 2, the values change radically with continued use of the solvent, while solvent system 11 gives relatively constant R_F values. It therefore seemed desirable to run the authentic dyes and the unknowns in solvent systems 1 and 2 simultaneously. However, the vessel in which solvent 2 was housed was too small to permit this. Solvent system 1 was housed in a vessel large enough to permit running 10 spots at one time.

"For running the authentic dyes, two solutions were made up, each containing 4 of the dyes under study. One solution contained Blue 1, Orange 1, Yellow 1, and Red 2; the other contained Green 2, Yellow 5, Yellow 6, and Red 1. Since the dyes in each solution can easily be distinguished from one another by their colors, it is possible to obtain the R_F values of all 8 dyes simply by running 2 spots.

"This was done in solvent system 1, and R_F values obtained from the unknowns agreed closely with the values of the authentics run at the same time."

No. 4.—"In my opinion this system of separating the colors is far superior to any I have tried or seen described to date. It is especially well adapted to the needs of field regulatory work. Even though colors are sometimes not completely resolved (and this is probably due to some variables such as acidity or alkalinity, solvent systems, or temperature variation), the resolution is sufficient to give clues to further separation or identification by spot tests or spectrophotometric curves. I found R_F values helpful, but only as an aid to deciding among several possibilities, and not in the absolute sense of using the R_F to identify the dye. The spot test seems the quickest means of narrowing down the identity, and the spectrophotometric curve is useful in checking or proving the identity. Preparation of this curve takes considerable time and manipulation, which is its only drawback."

TABLE 1.—*Collaborative results on three sample solutions of water-soluble coal-tar dyes*

Sample Solution: No. 1 Leaf Green Solvent System: No. 11 (Na_2CO_3 Spot) No. of Colors Resolved: 3

COLLABORATOR	NO. 2	NO. 3	NO. 4	NO. 5
APPROX. LENGTH OF RUN (HOURS)	15	18	16	12
Colors & R_F Values	FD&C Yellow 5 .01 FD&C Green 2 .12 FD&C Orange 1 .46	FD&C Yellow 5 0 FD&C Green 2 .10 FD&C Orange 1 .37	FD&C Yellow 5 .12 FD&C Green 2 .35 FD&C Orange 1 .65	FD&C Yellow 5 0 FD&C Green 2 .25 FD&C Orange 1 .60
Degree of Separation of Colors: (1) Poorly defined (2) Close, but satisfactory for identification (3) Widely spread	Yes	Degree of separation good	Yes	Yes
Identification of dye material: Is R_F value of authentic dye and corresponding one in sample solution (1) The same? (2) Proportional? (3) Helpful for use in identification?	No Somewhat Yes	Yes	Almost Yes Yes	No Yes Yes
Are spot tests (1) Unsatisfactory? (2) Characteristic? (3) Helpful for use in identification?	Yes	Yes Yes	Yes Yes	Yes
Are spectrophotometric curves from chromatographic spots of sample directly comparable to curve from authentic dyes?	Yes	Yes	Yes	Yes
Remarks:	Pale red spot resolved above Orange 1 spot, believed to be subsidiary of Orange 1. No attempt made to identify it		In sample solution, spot test and R_F probably enough to identify color from among authentic dyes furnished	

TABLE 1.—(*continued*)

Sample Solution: No. 2 Chocolate — Solvent Systems: No. 2 (HCl Spot) — No. 11 (Na_2CO_3 Spot)

COLLABORATOR	NO. 1		NO. 2		NO. 3		NO. 4		NO. 5	
SOLVENT SYSTEM	2	11	2	11	2	11	2	11	2	11
APPROX. LENGTH OF RUN (HOURS)	10	—	16	6.5	19	18	16	16	12	—
NO. OF COLORS RESOLVED	4	2	4	2	4	2	4	2	4	2
COLORS AND R_F VALUES	FD&C Red 2 FD&C Yellow 5 FD&C Blue 1 or FD&C Green 2 Possibly Yellow 6 or Orange 1		FD&C Red 2 .28 FD&C Yellow 5 .35 FD&C Blue 1 .11 FD&C Orange 1 .44		FD&C Red 2 .09 FD&C Yellow 3 .25 FD&C Blue 1 .60 FD&C Orange 1 .65		FD&C Red 2 .30 FD&C Yellow 5 .40 FD&C Blue 1 .35 FD&C Orange 1 .57		FD&C Red 2 .27 FD&C Yellow 5 .31 FD&C Blue 1 .25 FD&C Orange 1 .56	
Degree of separation of colors:										
(1) Poorly defined		Red 2 & Yellow 5			Orange 1 & Blue 1	Red & Yellow No separation		Red & Yellow No separation	Orange 1 & Blue 1	Red & Yellow
(2) Close but satisfactory for identification		Fair					Red & Yellow		Red 2 & Yellow 5	Blue 1 & Orange
(3) Widely spread	Yes		Yes	Yes	Red & Yellow Good	Orange & Blue Good		Orange & Blue		
Identification of dye material: Is R_F value of authentic dye and corresponding one in sample solution										
(1) The same?	No except in case of Yellow 5		No	No		Yes				
(2) Proportional?			No	Yes		Yes		Yes		Fairly
(3) Helpful for use in identification?			Yes	Yes				Yes		Yes
Are spot tests										
(1) Unsatisfactory?	Yes					Yes		Yes		
(2) Characteristic?										
(3) Helpful for use in identification?				Yes		Yes		Yes		Yes
Are spectrophotometric curves from chromatographic spots of sample directly comparable to curve from authentic dyes?	Too small amounts to get good curves		Yes		Yes		Yes		Yes	
Remarks:	Instructions ambiguous as to whether FD&C Blue #1 or FD&C Blue #2 should be spotted		See remarks on Orange 1 in "Leaf Green." Subsidiary spot not very apparent when authentic Orange 1 was developed in Sol. system #1				Only point of confusion: topmost red spot obtained when using solvent system #11 (basic). Spot proved to be secondary spot of Red 2, shown by spot test and spectrophotometric curve			

TABLE 1.—(*continued*)

Sample Solution: No. 3 Raspberry. Solvent System: No. 1 (HCl Spot)

COLLABORATOR	NO. 1	NO. 2	NO. 3	NO. 4	NO. 5
APPROX. LENGTH OF RUN (HOURS)	10	23	18	16	12
NO. OF COLORS RESOLVED	4	5	5	5	5
Colors and R_F values	FD&C Red 2 FD&C Yellow 6 or FD&C Red 1 FD&C Yellow 5 FD&C Orange 1	FD&C Red 2 .36 FD&C Red 1 .43 FD&C Yellow 6 .45 FD&C Yellow 1 .53 FD&C Orange 1 .68	FD&C Red 2 .42 FD&C Red 1 .54 FD&C Yellow 6 .60 FD&C Yellow 1 .70 FD&C Orange 1 .76	FD&C Red 2 .45 FD&C Red 1 .50 FD&C Yellow 6 .60 FD&C Yellow 1 .65 FD&C Orange 1 .85	FD&C Red 2 .25 FD&C Red 1 .31 FD&C Yellow 6 .41 FD&C Yellow 1 .69 FD&C Orange 1 .70
Degree of Separation of Colors: (1) Poorly defined (2) Close, but satisfactory for identification		Red 1 and Yellow 6	Degree of separation good; complete separation of all colors	Red 1 and Yellow 6	Red 1 and Yellow 6
(3) Widely spread	Yes	Red 2, Yellow 1, and Orange 1		Red 2, Yellow 1, and Orange 1	Red 2, Yellow 1, and Orange 1
Identification of dye material: Is R_F value of authentic dye and corresponding one in sample solution (1) The same?	Yes	No	Yes	No	No
(2) Proportional?		Yes		Yes	Yes
(3) Helpful for use in identification?		Yes		Yes	Yes
Are spot tests (1) Unsatisfactory?	Yes				
(2) Characteristic?			Yes	Yes	Yes
(3) Helpful for use in identification?		Yes	Yes	Yes	Yes
Are spectrophotometric curves from chromatographic spots of sample directly comparable to curve from authentic dyes?		Yes	Yes	Yes	Yes
Remarks:	Yellow 1 trailed considerably. Unknown yellow spot between Y 1 and Y 5 but closer to Y 5. Small amount of sample did not allow differentiation between R 1 and Y 6. Looked more like Y 6	Very satisfactory			

DISCUSSION

Collaborative work was designed to study the possibilities of paper partition chromatography technique as an aid in separating and identifying some water-soluble coal-tar dyes found in routine food samples (see 1952 report). Three sample solutions of authentic dye material were sent out, containing three, four, and five colors, respectively. Directions given included three different solvent systems and two *p*H levels. Results received from four collaborators, together with results obtained by the Associate Referee, are reported in Table 1.

Solution No. 1, containing FD&C dyes Yellow 5, Green 2, and Orange 1, was correctly resolved by all collaborators who used solvent system No. 11[2] and spotted from the solution made alkaline with sodium carbonate.

Solution No. 2, containing the FD&C dyes Red 2, Yellow 5, Blue 1, and Orange 1, was resolved with little difficulty by all collaborators, using solvent systems No. 2 and No. 11 and spotting from hydrochloric acid and sodium carbonate, respectively.

Solution No. 3, containing the FD&C dyes Red 1, Red 2, Yellow 6, Yellow 1, and Orange 1, was resolved satisfactorily by four collaborators. Collaborator No. 1 experienced some difficulty, although he identified three of the dyes present. Solvent system No. 1 was used, and the dye solution was made acid with hydrochloric acid.

Collaborative results on the three sample solutions were very promising on the whole. The reported R_F values are, as anticipated, only proportional figures, at least at the present stage of development of the technique, and perhaps may never be anything more.

The majority of the collaborators found that spot tests applied to paper swatches of chromatographed dyes are helpful for use in identifying the colors. They also report that coloring material removed from developed chromatograms gives characteristic curves with the Beckman spectrophotometer which are directly comparable to those obtained with similarly produced material from authentic dyes.

RECOMMENDATIONS

It is recommended*—

(1) That the study of the application of paper partition chromatography techniques to the separation and identification of coal-tar colors be continued.

(2) That further collaborative work be undertaken.

No reports were given on arsenic and antimony in coal-tar colors; boiling range of amines derived from coal-tar colors; ether extract in coal-tar colors; halogens in halogenated fluoresceins; heavy metals in coal-tar colors; identification of coal-tar colors; inorganic salts in coal-tar colors;

[2] TILDEN, D., *This Journal*, **35**, 423 (1952).

intermediates derived from phthalic acid; intermediates in triphenylmethane dyes; lakes and pigments; non-volatile unsulfonated amine intermediates in coal-tar colors; spectrophotometric testing of coal-tar colors; sulfonated amine intermediates in coal-tar colors; unsulfonated phenolic intermediates in coal-tar colors; and volatile amine intermediates in coal-tar colors.

REPORT ON EGGS AND EGG PRODUCTS

By F. J. McNALL (Food and Drug Administration, Department of Health, Education, and Welfare, Cincinnati 2, Ohio), *Referee*

No report was received from the Associate Referee on ammonia nitrogen.

V. E. Munsey, Referee for Cereal Foods, has suggested that several minor changes be made in the method for sterols. The Referee concurs in these recommendations.

RECOMMENDATIONS

It is recommended* that the following changes be made in the method for sterols:

(1) That **16.13(c)** be changed to read "*Ether.*—U.S.P. or A.C.S. free of peroxides."

(2) That **16.13(d)** be changed to read "*Dried ether.*—Immediately before use shake peroxide-free ether with anhyd. $CaCl_2$ equal to 10% of the vol. of the ether and filter."

(3) That in **16.15**, the word "Approx." in seventh line from bottom of page 278 be deleted.

(4) That in **16.16(b)**, the phrase "Add 4–5 g of Br" be deleted and that the phrase "Add 0.6 ml Br from a graduated 1.0 ml pipet," be substituted.

(5) That in **16.16(f)**, *Sodium hypochlorite soln*, the following be added at end of paragraph: "Reagent or commercial NaOCl soln, 5%, checked for concn as above, is also satisfactory."

It is also recommended that the first action method for glycerol in eggs (*This Journal*, **36**, 77 (1953)) be made official.

No report was given on ammonia nitrogen.

* For report of Subcommittee C and action of the Association, see *This Journal*, **37**, 69, 70 (1954).

REPORT ON MICROBIOLOGICAL METHODS

By GLENN G. SLOCUM (Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.), *Referee*

The Referee is unable to report substantial progress in the further development of microbiological methods for the examination of foods. Progress reports received from the Associate Referees on Sugar, Canned Vegetables, and Frozen Fruits and Vegetables do not form a basis for recommending a change of status for the respective methods. The method for frozen fruits and vegetables has been considerably revised, but the changes are largely in organization to make it conform more closely to a corresponding method under consideration by the American Public Health Association.

As reported last year, the latter organization (A.P.H.A.) has made substantial progress in the development of a manual of microbiological methods for the examination of a variety of foods in addition to water, shellfish, milk, and other dairy products. The Referee recommends* that the role of these two organizations (A.O.A.C. and A.P.H.A.) in the development of microbiological methods be carefully evaluated by Committee C at this time and that the Referee be instructed with respect to future activities in this field.

It is pertinent to point out that *Standard Methods for the Examination of Dairy Products* of the A.P.H.A. contains methods for the microbiological examination of eggs and egg products, fruits, and nuts. These methods have received wide recognition and are employed by regulatory officials in determining compliance with local laws or regulations. Obviously, methods sponsored by the two organizations must be essentially identical and, if this is true, unnecessary duplication must be eliminated.

Microbiological examination of canned foods consists primarily of qualitative procedures designed to detect the presence of specific spoilage organisms, and thus has little forensic application. The same is true of the sugar methods which are used primarily to determine compliance with commercial specifications. Ultimately all of these products will be included in the A.P.H.A. *Manual of Recommended Procedures.*

Pending instructions from Committee C, it is recommended* that work be continued on eggs and egg products, frozen fruits and vegetables, sugar, canned vegetables, canned fruits, other canned foods, fish and fishery products, and nuts and nut products.

No reports were given on canned acid foods; canned meats; canned vegetables; eggs and egg products; fish and fishery products; frozen fruits and vegetables; nuts and nut products; and sugar.

* For report of Subcommittee C and action of the Association, see *This Journal*, 37, 72, 73 (1954).

REPORT ON PROCESSED VEGETABLE PRODUCTS

By L. M. BEACHAM (Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.), *Referee*

The Associate Referee on Quality Factors in Processed Vegetable Products did not submit a report this year. Under his direction, work was continued on the study of acetaldehyde in frozen vegetables, but in his opinion sufficient progress was not made to warrant a report at this time. The same is true with respect to peroxidase in frozen vegetables, according to the Associate Referee for that subject, who likewise did not submit a report.

The Associate Referee on Catalase in Frozen Vegetables has developed a rapid method for measuring catalase activity in such foods at ordinary laboratory temperatures. By means of a curve showing changes in activity with changes in temperature, results obtained at room temperatures can be corrected to standard temperature conditions. This is important because his results show that temperature has a pronounced effect upon the reaction of catalase with hydrogen peroxide. The proposed method has given good results in the hands of the Associate Referee and others working in the same laboratory but has not been subjected to wider collaborative study. The Referee recommends* that the rapid method for determining catalase activity reported by the Associate Referee be submitted to comprehensive collaborative study during the coming year. The Referee also recommends* that continued study be given to the method for detecting acetaldehyde in frozen vegetables, and that the ascorbic acid oxidation method for measuring peroxidase reported by the Associate Referee last year likewise be the object of continued study during the coming year.

REPORT ON CATALASE IN FROZEN VEGETABLES

A RAPID METHOD OF DETERMINATION

By BENJAMIN M. GUTTERMAN (Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.) *Associate Referee*

In the last report on this subject[1] it was stated that the rapid method for determining catalase activity in frozen vegetables could not be evaluated because not all collaborators had had sufficient time to complete

* For report of Subcommittee C and action of the Association, see *This Journal*, **37**, 68 (1954).
[1] *This Journal*, **35**, 181 (1952).

their analyses. Their results have since been received and are listed in Table 1. In the hands of any one collaborator, using a specific apparatus on any one package, the method gives rather good reproducibility. However, the agreement between collaborators is not as good.

All samples coded SB-9 were prepared from the same batch of peas, all those coded SB-10 were prepared from another, etc. At this time the Associate Referee can only suggest possible causes for this lack of agreement.

Difficulty has always been encountered in preparing a homogeneous

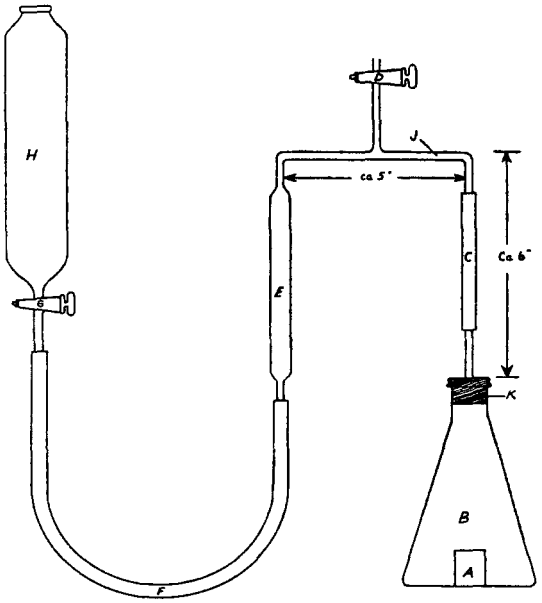

FIG. 1.—Apparatus for determining catalase activity. A. Vial approximately 1½" high and ⅜" in diameter. B. Erlenmeyer flask, 300 ml. C. Rubber pressure tubing, approximately ¼" inside diameter. D. Stopcock. E. 25 ml graduated pipet. F. Rubber tubing. G. Stopcock. H. Unstoppered straight-sided separatory funnel (leveling bulb). K. One-hole rubber stopper.

batch of under-blanched peas on a small laboratory scale; perhaps the samples studied here were not homogeneous, either. A second cause of variation may have been the differences in atmospheric pressure in the several laboratories. A third cause could have been the varying heights of the column of water in the graduated tube in reference to the height of the orifice of the gas delivery tube. Suggested additional factors are: the temperature of the filtrate, room temperature, and the time lapse between comminution, filtration, and actual analysis. All of these suggestions may be pertinent and they are in line with findings of Joslyn[2] in his work on

[2] JOSLYN, M. A., *This Journal*, 36, 161 (1953).

TABLE 1.—*Collaborative results*

SAMPLE	SUB	ANALYST	ML O_3	AV.	SAMPLE	SUB	ANALYST	ML O_3	AV.
SB-9	1	A	31.5, 32.2, 31.8	31.8	SB-11	1	A	7.6, 4.6, 6.3, 7.5	6.5
	2	A	30.7, 30.4, 31.0	30.7		2	A	5.5, 5.9, 6.0	5.8
	3	B	16.8, 16.5, 17.3	16.9		3	B	2.8, 2.7, 2.6	2.7
	4	B	14.2, 13.8, 14.0	14.0		4	B	4.4, 4.1, 4.1	4.2
	5	C	26.8, 26.6, 26.6	26.7		5	C	7.3, 6.7, 7.2	7.1
	6	C	32.6, 32.1, 32.9	32.5		6	C	5.2, 4.7, 5.2	5.0
	7	D	24.8, 24.0, 24.1	24.3		7	D	8.9, 9.1, 9.4	9.1
	8	D	33.2, 33.6, 34.0	33.6		8	D	8.8, 8.7, 8.5	8.7
	9	D	39.0, 38.0, 38.6	38.5		9	E	7.2, 8.0, 7.6	7.6
	10	E	27.8, 29.0, 29.4	28.7		10	E	8.2, 8.0, 7.8	8.0
	11	E	33.6, 32.4, 32.8	32.9		11a	F	6.8, 6.8, 5.6	6.4
	12a	F	26.6, 25.8, 25.0	25.8		11b[a]	F	8.2, 7.8, 8.0	8.0
	12b[a]	F	34.0, 38.4, 38.8	37.1					
SB-10	1	A	11.2, 10.4, 10.3, 11.2	10.8	SB-12	1	A	44.2, 43.9, 44.2	44.1
	2	A	11.4, 11.5, 11.2	11.4		2	A	46.5, 48.9, 50.5	48.6
	3	B	4.8, 4.8, 4.5	4.7		3	B	22.0, 22.5, 23.1	22.5
	4	B	4.7, 4.5, 4.4	4.5		4	B	18.9, 19.1, 18.8	18.9
	5	C	8.2, 7.4, 7.2	7.6		5	C	36.8, 36.5, 36.3	36.5
	6	C	9.5, 8.8, 9.2	9.2		6	C	40.2, 39.9, 39.2	39.8
	7	D	10.5, 10.2, 10.4	10.4		7	D	52.1, 51.2, 50.6	51.3
	8	D	11.8, 12.1, 11.7	11.9		8	D	45.6, 46.5, 47.5	46.5
	9	E	9.0, 9.4, 9.8	9.4		9	E	44.8, 44.2, 45.4	44.8
	10	E	9.4, 9.8, 9.4	9.5		10	E	52.8, 52.6, 54.0	53.1
	11a	F	8.4, 8.0, 9.0	8.5		11a	F	53.0, 52.8, 52.0	52.6
	11b[a]	F	11.2, 11.0, 11.0	11.1		11b[a]	F	48.6, 50.6, 50.4	49.9

[a] Results were obtained on peas permitted to thaw for 5 hours at room temperature before blending. The (b) subs were obtained from the same package as (a).

TABLE 2.—*Effect of varying amounts of H_2O_2*

SAMPLE	ALIQUOT	FILTRATE	H_2O_2	GAS	SAMPLE	ALIQUOT	FILTRATE	H_2O_2	GAS
	No.	*ml*	*ml*	*ml*		*No.*	*ml*	*ml*	*ml*
A	1	8	10.0	21.6	C	4	8	4	5.6
	2	8	8.0	23.6		5	8	10	5.6
	3	8	6.0	24.0		6	8	2	5.0
	4	8	4.0	23.4		7	4	10	2.3
	5	8	10.0	23.6		8	4	8	2.4
	6	8	2.0	18.4		9	4	6	2.6
	7	8	2.0	18.6		10	4	4	3.0
						11	4	2	2.6
B	1	8	10.0	39.8		12	4	10	2.4
	2	8	8.0	41.0		13	4	1	2.3
	3	8	6.0	42.2		14	4	4	3.3
	4	8	4.0	39.6					
	5	8	10.0	42.0	D	1	8	10	7.0
	6	8	2.0	24.0		2	8	8	7.5
	7	8	4.0	38.6		3	8	6	7.2
						4	8	4	7.6
C	1	8	10.0	5.8		5	8	2	7.0
	2	8	8.0	5.2		6	8	10	7.1
	3	8	6.0	5.8					

peroxidase activity of frozen vegetables.

Another important point raised by one of the collaborators was the possibility of "poisoning" the catalase by an excess of hydrogen peroxide. In the determinations reported in Table 2, experimental packs of frozen peas were used. The volume of filtrate was kept constant at 8 ml (except for sample C, where only 4 ml of filtrate was sometimes used); and the amount of hydrogen peroxide was varied. When necessary the total volume was adjusted to 18 ml with water. The results indicate that the ratio of 8 ml of filtrate to 10 ml of hydrogen peroxide is satisfactory.

The comments of the collaborators indicated the need for a more precise control of the sample and a better source of catalase. Therefore a commercial preparation of pure catalase was secured. In an attempt to refine the method, the apparatus (Figure 1), which permits the operator to bring the system to atmospheric pressure before beginning and after finishing the reaction, was used. Results are obtained in ml of oxygen at atmospheric pressure. Basically, it consists of a manometer attached to a reaction flask, in which catalase and hydrogen peroxide are allowed to react.

Several protein substances were studied in an attempt to find one which would stabilize the catalase preparation. A papaic extract of soya protein was found to be the most efficient of those tried.

Variations in temperature had a noticeable effect on the results. The reaction flask was immersed in an ice bath in order to study that effect.

Reproducibility could not be obtained because of continued fluctuations in the height of the water column. Another approach was to chill the soya extract and stock solution in an ice bath, but again the results were unacceptable. During the early stage of the study, small aliquots were used for diluting the commercial preparation of pure catalase. However, due to the very strong activity of our supply, small errors in transfer were greatly magnified, and dilutions were made to reduce these errors.

The effects of conducting the analysis while maintaining all solutions and equipment at 20°C. and of shaking the reaction flask manually were then studied. Table 3 illustrates this work by two analysts with two different sets of apparatus.

TABLE 3.—*Results of shaking procedure*

ANALYST	APPARATUS	WORKING SOLN	O_2
	No.	*ml*	*ml*
BMG	1	6	8.5, 8.2, 9.5, 8.7
CJH	2	6	6.1, 6.8, 6.6, 7.1
BMG	2	7	8.6, 8.1, 8.8, 8.2
CJH	1	7	7.8, 7.7, 8.1, 7.5
BMG	2	8	9.0, 8.7, 9.0, 7.4,[a] 8.4
CJH	1	8	7.9, 7.6, 7.9, 9.2,[a] 7.2

[a] BMG shook apparatus 1 and CJH shook apparatus 2. Reactants added by individual listed under "Analyst."

The effect of different techniques of shaking is immediately apparent in Table 3. We can conclude that shaking has two functions; the first is to mix the reactants and the second is to release the oxygen produced by the action of the catalase on the peroxide. Some of the difference in results between analysts can safely be attributed to the vigor used in manual shaking when all other factors are constant.

A shaking machine was next utilized in an attempt to standardize the shaking procedure. All other preparations and procedures were as described for Table 3. A preliminary run showed that the type of shaking machine available produced a swirling rather than a shaking action. Therefore the analyst occasionally broke the rhythm during a continuous 3.5 minutes on the machine. Results were 6.8 and 6.7 ml. Continuing with the same working solution, 3.5 minutes of uninterrupted swirling was allowed. The results were 5.5, 5.5, 5.9, 5.9, 5.5, and 5.5 ml of oxygen. However, in spite of the good reproducibility of results, it was necessary to abandon this type of shaker because of the excessive strain it placed on the apparatus (one unit was broken during the shaking) and because of the inconvenience of the hook-up.

A further modification was the use of a magnetic stirrer set for a fixed

speed. Agitation was expedited by selecting a magnetic stirrer bar of such length, and a cup of such diameter, that as the bar rotated within a 300 ml flask it would gently strike the cup. (In order to reduce breakage the bar was padded with 3 narrow circular ribbons of rubber tubing, placed one on each end and one in the middle, all at right angles to the long axis of the bar; the cup was constructed of flexible plastic.) The previous 8 to 10 ratio of catalase to hydrogen peroxide was changed to a 5 to 6 ratio to permit the use of a pipet for the accurate addition of catalase and thereby to eliminate errors in transfer.

The method and reagents found to give most readily reproducible results are as follows:

REAGENTS

(a) *Papaic extract of soya protein.*—("Phytone," supplied by Baltimore Biological Laboratories, is suitable.) 4% in water. One drop of capryl alcohol may be used to reduce foaming.

(b) *Hydrogen peroxide soln.*—A 3% soln freshly prepd from 30% H_2O_2.

(c) *Catalase.*—Stock soln prepd from a commercial supply of catalase. (Product supplied by Paul Lewis Laboratories, Inc., of Milwaukee, Wis. has been found satisfactory.) Dil. 10 ml of commercial supply to 100 ml with the 4% papaic ext. of soya protein. Working soln (used to test method and apparatus) is prepd by dilg proper aliquot of the stock soln.

Adjust all solns and equipment to room temp. before use.

DETERMINATION

(1) Add 5.0 ml of sample soln,[3] or (to test method) 5.0 ml of catalase working soln, to reaction flask. (2) Place magnetic stirrer bar in flask. (3) Place flask on magnetic stirrer base. (4) Add 6 ml of 3% H_2O_2 to vial. (5) Carefully place vial in flask with the aid of long forceps. (6) Record temp. of reactants in flask. (7) Using a rubber stopper, attach flask very tightly to system. (8) Open stopcock D and permit system to come to atmospheric pressure (be certain stopcock G in leveling bulb is always open). (9) Record level of liquid in graduated tube. (10) Close stopcock D. Wait 2–3 min. to observe whether any change in height develops in measuring tube. If so, reopen stopcock D to permit water level recorded to return to that recorded in step 9 and close again. Repeat if necessary. If a continual positive pressure is exerted, then disassemble flask, clean equipment, and repeat steps 1 thru 9. (11) Start magnetic stirrer and then start stopwatch as soon as the vial is overturned. Stir for 3.5 min. at a constant rate. (12) Keeping stopcocks closed, return system to atmospheric pressure by use of leveling bulb. (13) Record difference between first reading and final reading. (14) Record temp. of reactants and det. average temp. (Analyses should be started within 10 to 30 minutes of preparation of working soln.)

Table 4 illustrates the reproducibility of the method in the hands of two analysts. Working solution "B" was double the strength of "A." Each subdivision was freshly prepared from the stock solution. The analysts used the same supply of solutions A 3 and A 4 for individual alternate analyses.

[3] See footnote 1.

TABLE 4.—*Reproducibility of results*

ANALYST	WORKING SOLN	SUB.	YIELD (ML O_2)
BMG	A	1	7.0, 7.3, 7.9, 7.8, 8.1, 8.0
BMG	A	2	7.6, 7.5, 7.2, 7.0, 6.7, 7.2, 7.7
BMG	A	3	7.2, 8.0, 8.1, 7.6
JWC	A	3	7.2, 7.5, 7.5
JWC	A	4	8.1, 7.3, 7.4
BMG	A	4	7.3, 8.3, 7.7
BMG	B	5	14.9, 13.9, 14.1, 14.2, 14.9, 14.3
BMG	B	6	15.7, 15.1, 15.0, 15.4, 15.4

As stated previously, temperature has a pronounced effect upon results. The results in Table 5 and Figure 2 demonstrate the temperature effect. Two different dilutions of the stock were selected, and each was analyzed at different temperatures.

SUMMARY

Collaborative results have been studied and it is noted that an in-

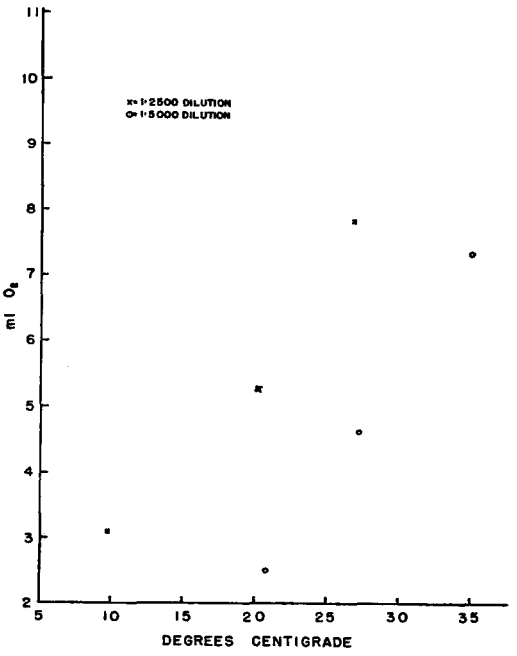

FIG. 2.—Effect of temperature on yield of oxygen.

TABLE 5.—*Results at different temperatures*

1:2500 DILUTION		1:5000 DILUTION	
AV. TEMP.	AV. YIELD	AV. TEMP.	AV. YIELD
(°C.)	(ml O_2)	(°C.)	(ml O_2)
9.8	3.12	20.8	2.50
20.3	5.38	27.2	4.62
26.8	7.83	34.9	7.30
33.3	10.75		

dividual operator, using a specific apparatus, is able to demonstrate good reproducibility. Over-all collaborative results were more divergent. Therefore, the apparatus was modified so that adjustments to atmospheric pressure and elimination of pressure of varying columns of water may be accomplished. In addition, modifications of the method were studied. A commercial supply of pure catalase was used and the effects of vigor of shaking and of temperature were evaluated. Both had a pronounced effect. However, with controlled agitation and temperature, variations between operators and equipment have been reduced.

The method and equipment are quite sensitive and it is possible they may be successfully used as a semi-quantitative analytical procedure for catalase activity. It is therefore recommended* that this method be subjected to collaborative study.

No reports were given on moisture in dried vegetables, peroxidase in frozen vegetables, and quality factors.

REPORT ON FATS, OILS, AND WAXES

By G. KIRSTEN (Food and Drug Administration, Department of Health, Education, and Welfare, New York 14, N. Y.), *Referee*

The Associate Referee for spectrophotometric methods reported on the determination of polyunsaturated acids in fats and oils. This year's work was largely exploratory since different methods, or rather, variations of the same basic method, have been reported in the literature. These variations mainly involve the use of glycerol or ethylene glycol in the isomerization reagent, and heating for different lengths of time at 180°C. The Associate Referee reports that the different methods appear to give agreeing and reproducible results. No specific method is being recommended, pending further study.

The Associate Referee made a number of determinations on various

* For report of Subcommittee C and action of the Association, see *This Journal*, 37, 68 (1954).

vegetable oils and animal fats which confirm published reports that the spectrophotometric method can be used for the detection of soya bean oil in admixture with other vegetable oils, and for the detection of horse meat when mixed with beef, pork, or lamb. It appears that the method may be useful in distinguishing animal fats from vegetable fats because of the presence of more highly unsaturated acids in the former.

The Associate Referee for antioxidants conducted a collaborative study of methods for butylated hydroxyanisole (BHA) and nordihydroguaiaretic acid (NDGA) and for combinations containing these and also propyl gallate for which a method has been previously adopted by the Association. In general the collaborative results indicate that the methods as submitted are not yet entirely satisfactory. In two of the samples NDGA was reported where none was present. The methods for BHA and NDGA are similar, and depend upon differing rates of reaction to distinguish between these antioxidants. It is hoped that refinement of the method and clarification of the directions will eliminate the difficulties.

No report was received from the Associate Referee for peanut oil.

RECOMMENDATIONS

It is recommended*—

(1) That studies on spectrophotometric methods for the analysis of fats and oils be continued.

(2) That further work be done on methods for antioxidants in fats and oils.

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

REPORT ON ANTIOXIDANTS IN OILS, FATS, AND WAXES

DETERMINATION OF NORDIHYDROGUAIARETIC ACID AND BUTYLATED HYDROXYANISOLE

By S. KAHAN (Food and Drug Administration, Department of Health, Education, and Welfare, New York 14, N. Y.), *Associate Referee*

The three most important antioxidants in use today are propyl gallate (PG), butylated hydroxyanisole (BHA), and nordihydroguaiaretic acid (NDGA). Since a first action method for PG had been accepted by the Association in 1952 (1), it was decided to proceed with collaborative testing of methods for NDGA and BHA. The current trend is to use combinations of antioxidants, usually with a synergist, and therefore methods for each in the presence of the others are as necessary as a method for each alone. The most favored combination at the present time is a mixture of

* For report of Subcommittee C and action of the Association, see *This Journal*, 37, 74 (1954).

PG with BHA; the combination of BHA and NDGA finds slight commercial acceptance while the combination NDGA and PG is quite rare in this country and Canada. The use of all three antioxidants in a single product has not yet been reported.

Mahon and Chapman (2) have already published methods for each of the antioxidants and these were taken as starting points, with the intention of adapting them for routine use where indicated.

BUTYLATED HYDROXYANISOLE

If BHA alone is present, it can be determined quickly and accurately by means of the ferric chloride–α,α'-diperidyl reaction. The method as sent to collaborators is as follows:—

METHOD

REAGENTS

(a) *Ethyl alcohol.*—To absolute alcohol add ca 0.1% KOH and 0.1% $KMnO_4$. Distill in an all-glass app. Dil. to 72% by vol. with distd H_2O.

(b) *Ferric chloride.*—0.2% soln of $FeCl_3 \cdot 6H_2O$ in purified absolute ethyl alcohol. This reagent should be freshly prepd.

(c) *α,α'-Dipyridyl.*—0.2% soln in purified absolute ethyl alcohol.

DETERMINATION

Dissolve 40 g of the fat or oil in the purified petr. ether reagent (see reference 1) and make up to 250 ml. Pipet 100 ml of the fat soln into a 250 ml separatory funnel, and ext. with 25 ml of 72% ethyl alcohol by shaking for 3 min. Repeat with 3 more 25 ml portions of 72% alcohol. Finally ext. for one min. with 60 ml of 72% alcohol. Combine all exts in a 200 ml volumetric flask and make up to vol. with 72% alcohol. If soln is cloudy, filter through dry, rapid paper.

Depending on the amount of antioxidant expected, place 3 different aliquots of from 1 to 5 ml from the BHA extract into 50 ml glass-stoppered Erlenmeyer flasks. These flasks must be rendered impervious to light with black paint or tape. (Corning "Lifetime Red" flasks have been found convenient). Dil. the aliquots in all flasks to 5 ml with 72% alcohol, and add 3 ml of absolute alcohol, 2 ml of the 0.2% $FeCl_3$ reagent, and 2 ml of the 0.2% dipyridyl reagent.

Stopper flasks immediately and mix gently. Exactly 30 min. after adding the $FeCl_3$ reagent, measure the absorbance of the soln at 515 mμ relative to a soln contg 5 ml of 72% alcohol, 3 ml of absolute alcohol, and 2 ml each of reagents (b) and (c).

Prepare a standard curve over the range 10–50 mmg of BHA, using the same spectrophotometer and the same sized cells.

NORDIHYDROGUAIARETIC ACID

If NDGA is present alone, use the method outlined for BHA as given above, except measure the absorbance after 3 minutes instead of after 30 minutes.

PROPYL GALLATE AND BUTYLATED HYDROXYANISOLE

Ext. PG according to the official method (see reference 1), using 1.67% aq. ammonium acetate soln and det. PG. Ext. fat soln remaining after removal of PG with 72% alcohol and det. BHA as under DETERMINATION (above).

TABLE 1.—*Collaborative results*[a]

SAMPLE	ADDED	LABORATORY NO.									
		1	2	3	4	5	6	7	8	9	10
A (oil)	.0192% BHA	.0081% BHA	.0181% NDGA .0068% BHA	.0021% NDGA .0171% BHA	.0079% NDGA	.0183% BHA	.0035% BHA .0124% NDGA	.0189% BHA	.0160% BHA	.0130% BHA .0063% NDGA	BHA & NDGA present
B (oil)	blank	0	0	0	0	0	0	.0011% NDGA	0	0	0
C (lard)	.01% PG .033% BHA	.0073% PG .0141% BHA	.0077% PG .0362% BHA	.0091% PG .0303% BHA	.0071% PG	.0088% PG .0256% BHA	.0096% BHA .0293% NDGA	.0044% PG .0287% BHA	.0032% NDGA .0074% PG .0260% BHA	.0106% PG .0233% BHA NDGA: present	PG & NDGA present
D (lard)	.02% PG .01% BHA .02% NDGA	.0234% PG NDGA present	.0237% PG .0282% BHA	.0235% PG	.0230% PG	.0508% NDGA	.0507% NDGA	.0204% PG .0247% BHA	.0120% NDGA .0108% BHA .0218% PG	.0244% PG NDGA: present	PG present
E (shortening)	.015% NDGA	.0148% NDGA	.0185% NDGA	.0154% NDGA	.0159% NDGA	.0148% NDGA	.0121% NDGA	.0182% NDGA	.0103% NDGA	.0180% NDGA	NDGA present

[a] Laboratories:
Cincinnati District, Food and Drug Admin.
General Foods, Hoboken, N.J.
Canadian Dept. of National Health & Welfare, Ottawa.
Connecticut Agricultural Expt. Sta., New Haven, Conn.
Baltimore District, Food and Drug Admin.
Tennessee Eastman, Kingsport, Tenn.
American Meat Institute, Chicago, Ill.
San Francisco District, Food and Drug Admin.
Swift and Co., Chicago, Ill.
Army Medical Service Graduate School (Qualitative only).

PROPYL GALLATE AND NORDIHYDROGUAIARETIC ACID

NDGA will be partially extd by the 1.67% aq. ammonium acetate soln used for extracting PG and therefore these two antioxidants cannot be satisfactorily sepd at the present time. Fortunately, however, this combination is quite rare.

BUTYLATED HYDROXYANISOLE AND NORDIHYDROGUAIARETIC ACID

Ext. with 72% alcohol as for the detn of BHA alone, but set up a duplicate series of aliquots for color development. Det. the absorbance at 1 min. after adding the reagents, and again at 30 min. after adding the reagents. The absorbance measured after 1 min. is due to the reaction of 90% of the NDGA and 10% of the BHA. Similarly the absorbance measured after 30 min. is due to the reaction of 100% of the NDGA and 100% of the BHA. The respective absorbances can therefore be calcd by the following equations:

$$L \text{ at } 1 \text{ min.} = 0.1\ B + 0.9\ N$$
$$L \text{ at } 30 \text{ min.} = 1.0\ B + 1.0\ N$$

where L = observed absorbance; B = absorbance due to BHA; and N = absorbance due to NDGA.

QUALITATIVE TESTS

In order to determine which, if any, of the three antioxidants are present in a sample, the following qualitative tests are applied:

Place ca 10 g of fat in a separatory funnel and dissolve in 50 ml of petr. ether. Ext. the fat soln by shaking 3 min. with 20 ml of 72% ethyl alcohol.

PG.—To 5 ml of the alcoholic ext. in a test tube add 2 drops of concd NH_4OH. The appearance of a pink to red color indicates the presence of PG.

BHA.—To 5 ml of the ext. add 1 ml of 2% aq. borax soln and a few small crystals of 2,6-dichloroquinonechloroimide. The appearance of a blue color indicates the presence of BHA. Disregard any brown or grey-brown colors that may appear.

NDGA.—If the above qualitative tests indicate the absence of BHA and PG, add 2 ml of $FeCl_3$ reagent and 2 ml of 0.2% dipyridyl reagent to 5 ml of the ext. A red color indicates the presence of NDGA.

NDGA in the presence of BHA.—If the qualitative test for BHA is positive, then proceed with the quantitative sepn and detn of BHA in the presence of NDGA as given above.

Table 1 indicates the results obtained by 10 collaborators on a series of 5 samples sent out by the Associate Referee.

DISCUSSION

Despite the wide variations in results, several signs of progress can be noted. First, sample B had no antioxidant added, and 9 of the 10 collaborators reported it as blank. The tenth reported 0.001 per cent NDGA, which is less than would be used commercially and is at the lower limit of sensitivity of the method. Secondly, sample E, a shortening to which 0.015 per cent NDGA had been added, was correctly reported by all 10 collaborators as having only NDGA. Results varied from 0.010 to 0.018 per cent, with an average of 0.0153 per cent. However, the range of results indicates that further refinements in this method are needed.

Sample A was an oil to which 0.0192 per cent BHA had been added. Four analysts reported only BHA present, one reported only NDGA present, and 5 found both BHA and NDGA. The amounts of BHA found by the analysts reporting only BHA were 0.0081, 0.0183, 0.0189, and 0.0160, with three of the four coming within 15 per cent of the amount added. The amounts of NDGA reported varied from 0.0021 to 0.0181 per cent, which is in the range of commercial practice and well within the limits of accuracy of the method. An interesting observation is that the average value of the sum of all antioxidants reported in Sample A is 0.0165 per cent, or 86 per cent of the amount of antioxidant present.

A possible cause of the discrepancies noted in this sample may have been the wording of the directions. Seven of the 10 reported positive qualitative tests for NDGA when in fact none had been added. Similarly, in the case of Sample C, 5 reported positive tests for NDGA with none present. The directions to the collaborators included a rapid qualitative test for NDGA in the presence of BHA in which the colors produced by the antioxidant–ferric chloride–dipyridyl reaction are read after 1 minute and after 10 minutes.

The reaction between NDGA and the color reagent is virtually complete in one minute, whereas the reaction with BHA is much slower. A small variation from the one-minute time interval will produce a large change in the one minute–10 minute ratio, since the curve has such a steep slope at that point. Also, the directions were at fault since they read merely " . . . determine the absorbance at one minute after adding the reagents, and at 30 minutes after adding the reagents." It was not specified which of the ferric chloride or the α, α'-dipyridyl reagents should be added first, and this could be decisive as to how much color is developed in the first minute after adding the second reagent. Also, the fact that an exactly one minute interval was desired was not emphasized.

It is therefore believed that the difficulties encountered in Sample A can be overcome by more specific directions. Also, absorbance values at one minute and at 30 minute intervals will be re-checked, since they seem to be somewhat at variance with the values obtained. One of the collaborators suggested that readings be taken at 3 minutes and 30 minutes, since the one minute interval was too small to permit accurate manipulation of the solutions. The feasibility of this suggestion will also be investigated.

Sample C was a lard to which 0.167 per cent of Tenox II had been added, and therefore contained 0.01 per cent PG and 0.033 per cent BHA. Nine of the 10 collaborators reported PG present, with values ranging from 0.0044 to 0.0106 per cent for a numerical average of 0.0078 per cent. Disregarding one low value, the numerical average of the other 8 is 0.0083 or 83 per cent recovery, which compares with about 90 per cent recovery reported in the previous collaborative study (1). It is felt that as the PG method is now written, the size of sample is a little too large to permit quantitative extraction of PG with the 1.67 per cent aqueous ammonium

acetate solution. Therefore, in future collaborative studies the size of the original fat sample taken for analysis will be reduced.

With further reference to Sample C, 8 of the 10 collaborators reported BHA present and 4 reported NDGA which had not been added. The values of BHA ranged from 0.0096 to 0.0362 per cent with a numerical average of 0.0242 per cent. The average of the total antioxidants reported for Sample C is 0.0321 per cent, somewhat short of the 0.043 per cent present; this again indicates incomplete extraction where large amounts of antioxidants are present.

Sample D contained a mixture of all 3 antioxidants, a situation unlikely to be met commercially and one incapable of resolution with present methods. Only one collaborator correctly reported all three, but 5 analysts reported a total quantity of antioxidants quite close to the .05 per cent actually present. Here again, further evaluation of the extraction and color development techniques is needed.

SUMMARY AND RECOMMENDATION

Methods for the determination of propyl gallate, butylated hydroxyanisole, and nordihydroguaiaretic acid, singly and in combinations, are given. Collaborative testing of 5 samples indicates that where each of the antioxidants is present alone, the methods are fairly reliable and accurate. Where combinations of two or more antioxidants occur, difficulties in separation and identification make the methods less reliable and accurate. It is believed, however, that some of the difficulty can be reduced by rewriting the directions and rechecking the absorbances of the solutions at certain critical points and times. Where no antioxidant is present at all, the methods give a clear indication of the fact.

It is recommended* that further work be done on these methods and that they be subjected to further collaborative study next year.

REFERENCES

(1) KAHAN, S., *This Journal*, **35**, 84, 186 (1952).
(2) MAHON, J. H., and CHAPMAN, R. A., *Anal. Chem.*, **23**, 1116 (1951).

REPORT ON SPECTROPHOTOMETRIC METHODS FOR OILS AND FATS

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Associate Referee

Ultraviolet spectrophotometry has recently become the most important tool for the determination of polyunsaturated constituents of fats and

* For report of Subcommittee C and action of the Association, see *This Journal*, **37**, 74 (1954).

oils. Unconjugated, unsaturated acids can be determined by catalytic isomerization into their conjugated, absorbing forms by means of alkali and heat.

Since publication in 1943 of a quantitative method for the spectrophotometric determination of linoleic and linolenic acids in fats and oils (18), the method has been extended to include arachidonic acid (2) and pentaenoic acids (13, 14) and has been modified to increase the transparency of the isomerization medium (4, 20). Efforts to improve the sensitivity and accuracy of the method by applying correction factors (4, 5, 25) or by varying the conditions of isomerization (1, 3, 13–16) have been described. Recently, new and more accurate constants for use in spectrophotometric analyses of common natural fats and oils have been published (6, 13, 14). The new constants were determined on acids isolated by physical means in their natural geometric configuration (11, 12, 23), whereas the earlier constants were determined on chemically prepared bromination-debromination acids. The latter acids contain a large proportion of geometrical isomers other than the natural all-*cis* type (17). The geometrical isomers vary in their rates of conjugation during treatment with alkali; this results in significant differences in their observed absorptivities (11, 19). The Spectroscopy Committee of the American Oil Chemists' Society (22), in an effort to establish a suitable standard method of analysis, has been conducting collaborative tests for determining polyunsaturated acids in fats and oils by spectrophotometric analysis. The American Oil Chemists' Society, as a result of its November 15, 1948 report, which recommended a detailed spectrophotometric method for the analysis of fats and oils, issued A.O.C.S. Tentative Method Cd 7-48 (revised in May, 1951) as part of A.O.C.S. Methods (22).

OUTLINE OF METHOD

According to the A.O.C.S. Tentative Method Cd 7-48 (Rev. May, 1951), the conjugated constituents of a fat or oil are determined by measuring the ultraviolet absorption in a purified solvent. The non-conjugated polyunsaturated constituents (such as linoleic and linolenic acids) are partially conjugated by heating in KOH–glycol solution and the absorption of each of the conjugated constituents is measured with an ultraviolet spectrophotometer (e.g., the Beckman Model DU). The percentages of conjugated diene, triene, and tetraene acids, and of linoleic, linolenic, and arachidonic acids are calculated from these measurements. The method specifies the reagents and their preparation, the isomerization apparatus, and the techniques for isomerizing the sample and making the spectral absorbance measurements. Detailed directions are necessary because of the empirical nature of the isomerization. Up to the present time, two alkali-isomerizing reagents have been used by workers in this field. The KOH–ethylene glycol reagent is described in the A.O.C.S. Tentative

Method Cd 7-48. The KOH–glycerol reagent is described by Brice, *et al.* (5).

Isomerization Apparatus.—The 1948 Report of the A.O.C.S. Spectroscopy Committee (22) gives a description of suitable isomerization equipment. A constant temperature bath, operating at 180° ± 0.5°C., was built in this laboratory similarly to the Rubber Reserve model described in the report. A nitrogen purifying train was found to be unnecessary if the nitrogen supply contains less than 0.01 per cent of oxygen. The gas manifold and distributing heads used in this laboratory are similar to those described in the report. For the glycerol–air method, the test tubes were covered with flask covers.

EXPERIMENTAL

A number of vegetable oils were subjected to alkali isomerization for various lengths of time by the glycerol–air method of Brice, *et al.*, (4, 5) and by the ethylene glycol–nitrogen method of the A.O.C.S. (Tentative Method Cd 7-48, as revised). The standardized conditions of both methods include addition of an accurately weighed sample (approximately 0.1 gram) of the oil to 11 grams of the alkali reagent and heating at 180°C. in a covered 10×1 inch test tube. After isomerization, the cooled solutions are diluted with solvent and the absorbance is read at appropriate wavelengths with a Beckman Model DU spectrophotometer. The equations and methods of calculation used here are those of Brice, *et al.* (6).

A number of animal fats were also analyzed. The fats were rendered by heating three hours in an 80°C. oven and filtering the melted fat through filter paper in an oven at 100°C. The rendered fats were isomerized by the KOH–glycerol–air technique.

Several horse meat samples were obtained and were analyzed by the KOH–glycerol–air technique. The fat from each cut and a portion of the meat were run separately. The fat was first rendered by the method described above. The meat samples were then ground and extracted by the Soxhlet extraction procedure of Crouse and Leffler (7).

The pentaenoic acid content of the butterfat and horse meat samples were calculated as 50 per cent C_{20}–50 per cent C_{22} acids from absorptivities published by Herb and Riemenschneider (13, 14). The arachidonic acid content of these samples was corrected for interference from pentaene acids by using pertinent data found in the above references (13, 14).

All fats and oils studied here were also analyzed for conjugated polyunsaturated acids by the A.O.C.S. Tentative Method Cd 7-48 (Rev. May, 1951).

RESULTS AND DISCUSSION

The composition of a variety of vegetable oils, isomerized at 180°C. under varying conditions, is given in Table 1. Individual analyses are recorded to give an indication of the precision attainable with the methods.

TABLE 1.—*Spectrophotometric analysis of vegetable oils for polyunsaturated acids. Comparison of results using natural fatty acid standards and various conditions of isomerization*

SAMPLES	CONJ. DIENE	CONJ. TRIENE	CONJ. TETRAENE	ISOMERIZATION METHOD[a]	LINOLEIC	LINOLENIC	ARACHIDONIC
	per cent	*per cent*	*per cent*		*per cent*	*per cent*	*per cent*
Cottonseed Oil I	0.26	0.028	0.0000	45′ EGN	55.5	0.30[b]	—
					54.3	0.30	—
				25′ EGN	54.1	0.29[b]	—
					54.4	0.30	—
				30′ GA	52.2	0.27[b]	—
					52.1	0.23	—
				45′ GA	53.8	0.26[b]	0.00
					53.6	0.26	0.00
Cottonseed Oil II	2.77	0.041	0.0000	45′ EGN	52.0	0.13[b]	—
					52.0	—	—
				30′ GA	51.7	0.13[b]	—
					51.7	0.08	—
Cottonseed Oil III	0.83	0.009	0.0000	25′ EGN	48.9	0.00	0.00
					48.7	0.00	0.00
				30′ GA	47.1	0.00	0.00
					47.5	0.00	0.00
				45′ GA	48.1	0.00	0.00
					48.4	0.00	0.00
Cottonseed Oil IV	0.65	0.034	0.0000	25′ EGN	56.0	0.00	0.00
					56.4	0.00	0.00
				30′ GA	54.9	0.00	0.00
					57.4	0.00	0.00
Soybean Oil I	0.78	0.000	0.0000	45′ EGN	51.2	9.07	—
					51.2	9.29	—
					50.6	9.25	—
				25′ EGN	51.0	9.15	0.00
					51.4	9.23	0.00
					51.6	9.21	0.00
				30′ GA	50.6	8.82	—
					51.2	8.72	—
					50.8	8.92	—
				45′ GA	51.0	9.16	—
					51.6	9.12	—
					50.9	9.08	—
Soybean Oil II	0.56	0.046	0.0045	25′ EGN	50.4	8.58	0.00
					51.5	8.56	0.00
				30′ GA	49.7	7.75	0.00
					49.8	7.77	0.00
Soybean Oil III	0.74	0.000	0.0000	25′ EGN	50.3	7.53	0.00
					50.3	7.45	0.00
				30′ GA	46.8	7.22	0.00
					47.5	6.85	0.00
Soybean Oil IV	0.49	0.013	0.0009	25′ EGN	49.5	7.36	0.00
					50.3	7.45	0.00
				30′ GA	47.7	7.26	0.00
					47.1	6.90	0.00
				45′ GA	50.3	7.12	0.00
					49.7	7.12	0.00
Sesame Seed Oil I	0.60	—	0.0000	25′ EGN	44.2	0.22	0.00
					44.8	0.21	0.00
				30′ GA	43.6	0.21	0.00
					44.1	0.17	0.00
Sesame Seed Oil II	0.57	—	0.0000	25′ EGN	43.5	0.20	0.00
					43.7	0.20	0.00
				30′ GA	44.8	0.24	0.00
					45.3	0.22	0.00

TABLE 1.—(*continued*)

SAMPLES	CONJ. DIENE	CONJ. TRIENE	CONJ. TETRAENE	ISOMERIZATION METHOD[a]	LINOLEIC	LINOLENIC	ARACHIDONIC
	per cent	*per cent*	*per cent*		*per cent*	*per cent*	*per cent*
Olive Oil I (Spanish)	0.16	0.012	0.0009	25′ EGN	8.71 8.60	0.69 0.75	— 0.02
				45′ EGN	7.78 7.76	0.63 —	— —
				30′ GA	8.22 8.17	0.59 0.59	— —
Olive Oil II (Moroccan)	0.50	0.029	0.0004	25′ EGN	12.5 12.8	0.93 0.91	0.00 0.00
				45′ EGN	13.0 12.8	0.91 —	— —
				30′ GA	12.2 12.2	0.97 0.93	— —
Olive Oil III (French)	0.57	0.043	0.0004	25′ EGN	7.45 7.24	0.57 0.57	0.02 0.01
				45′ EGN	6.51 6.58	0.57 —	— —
				30′ GA	6.94 6.76	0.55 0.52	— —
Olive Oil IV (Italian)	0.67	0.007	0.0000	25′ EGN	8.16 8.22	0.54 0.54	0.00 0.00
				30′ GA	7.90 7.99	0.48 0.48	0.00 0.00
Corn Oil I	0.67	0.082	0.0000	45′ EGN	54.5 54.9	0.83 —	— —
				30 GA	55.2 53.4	0.83 0.83	— —
Corn Oil II	0.55	0.120	0.0000	25′ EGN	55.4 55.0	0.79 0.75	0.00 0.00
				30′ GA	55.5 55.2	0.75 0.73	0.00 0.00
Corn Oil III	0.42	0.051	0.0000	25′ EGN	54.4 54.5	0.61 0.65	0.00 0.00
				30′ GA	53.7 54.3	0.55 0.64	0.00 0.00
Peanut Oil I	0.20	0.016	0.0000	25′ EGN	22.7 22.7	0.02[b] 0.02	0.00 0.00
				30′ GA	22.5 22.6	0.00 0.00	0.00 0.00
Peanut Oil II	0.26	0.061	0.0000	25′ EGN	29.1 28.9	0.00 0.00	0.00 0.00
				30′ GA	29.6 29.8	0.00 0.00	0.00 0.00
Peanut Oil III	0.27	0.013	0.0000	25′ EGN	25.0 25.3	0.09[b] 0.11	0.00 0.00
				30′ GA	25.1 25.2	0.09[b] 0.11	0.00 0.00
Sunflower Seed Oil I	2.06	0.008	0.0000	45′ EGN	59.5 59.4	1.40 —	— —
				30′ GA	57.6 58.0	1.48 1.40	— —
Teaseed Oil I	0.67	0.197	0.0000	30′ GA	9.88 9.83	2.07 1.99	0.00 0.00
				45′ GA	10.4 10.1	1.93 1.97	0.00 0.00
Oleomargarine I	0.82	0.012	0.0000	45′ GA	4.49	0.05	0.00

[a] EGN: Ethylene glycol–nitrogen; GA: Glycerol–air.
[b] Apparent per cent linolenic acid.

TABLE 2.—*Spectrophotometric analysis of rendered or extracted animal fats for polyunsaturated acids*[a]

SAMPLES	CONJ. DIENE	CONJ. TRIENE	CONJ. TETRAENE	CONJ. PENTAENE	LINOLEIC	LINOLENIC	ARACHIDONIC	PENTAENOIC[b]
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Beef flank fat	0.47	0.013	0.0035	0.0000	1.18	0.81	0.05	0.00
					1.17	0.79	0.05	0.00
Beef kidney fat	0.37	0.011	0.0019	0.0000	1.02	0.44	0.02	0.00
					0.98	0.47	0.00	0.00
Veal fat	0.45	0.006	0.0038	0.0000	0.94	0.79	0.22	0.00
					0.88	0.78	0.23	0.00
Lamb kidney fat	1.97	0.007	0.0005	0.0000	1.10	1.29	0.01	0.00
					1.08	1.23	0.04	0.00
Pork loin fat	0.22	0.001	0.0000	0.0000	11.91	0.66	0.15	0.00
					11.86	0.73	0.18	0.00
Lard	0.24	0.001	0.0000	0.0000	8.04	0.34	0.12	0.00
Bacon drippings	0.22	0.0003	0.0000	0.0000	11.76	1.75	0.16	0.00
					11.77	1.75	0.16	0.00
Chicken fat	0.72	0.006	0.0000	0.0000	14.6	0.80	0.14	0.00
					14.3	0.82	0.14	0.00
Butter fat	0.66	0.014	0.0018	0.0000	1.64	0.71	0.19[c]	0.09
					1.64	0.66	0.21	0.06
Horse fat, fore quarter over ribs	0.19	0.002	0.0008	0.0000	9.58	12.1	0.12[c]	0.10
Horse fat, flank	0.22	0.004	0.0008	0.0000	13.0	10.5	0.12[c]	0.11
Horse fat, round	0.18	0.005	0.0006	0.0000	9.73	9.39	0.20[c]	0.14
Horse fat, kidney	0.15	0.002	0.0017	0.0000	5.87	16.4	0.11[c]	0.08
Horse fat, heart	0.22	0.004	0.0008	0.0000	10.8	18.1	0.36[c]	0.12
Horse meat, fore quarter over ribs	0.21	—	—	—	8.51	9.84	0.09[c]	0.10
Horse meat, flank	0.19	0.0005	0.0000	0.0000	8.98	9.46	0.16[c]	0.05
Horse meat, round	0.13	—	—	—	9.59	8.37	0.13[c]	0.15
Horse meat, kidney	—	—	—	—	8.29	10.9	—	—
Horse meat, heart	—	—	—	—	9.00	9.67	—	—

[a] Samples isomerized in air for 45 minutes with 11% KOH-glycerol.
[b] Calculated as 50% C_{21}-50% C_{23} from coefficients reported by Herb and Riemenschneider, *J. Am. Oil Chem. Soc.*, 29, 456 (1952), Table 1.
[c] Corrected for interference by pentaene acids, using data referred to in note *b*.

Results obtained with different conditions of isomerization are in good agreement and no single method is recommended by the author at this time. The equations of Brice, *et al.* (6), obtained from natural fatty acid standards, were used here to determine the percentages of linoleic, linolenic, and arachidonic acids. These authors analyzed a number of animal and vegetable fats and oils. The results, based on natural and on bromination-debromination fatty acid standards, were compared. The natural fatty acid standards were found to lead to results of substantially higher accuracy. The forty-five minute glycerol–air technique was recommended because of its simplicity and high precision, and the forty-five minute glycol–nitrogen technique was recommended as a second choice because of the greater transparency of reagent blanks. The 1953 A.O.C.S. Spectroscopy Committee Report (22) finds that no particular increase in either precision or accuracy is obtained with the ethylene glycol–nitrogen method by using a forty-five minute isomerization time instead of the twenty-five minute isomerization.

It is generally agreed that normal vegetable oils contain no arachidonic acid (22). However, Table 1 indicates that olive oil may be an exception and may contain small amounts of arachidonic acid.

Of the common vegetable oils, soybean oil is the only one that contains substantial amounts of linolenic acid (Table 1). Cottonseed and peanut oils contain no linolenic acid (20, 22). Thus, a quantitative spectrophotometric determination of soybean oil in admixtures with cottonseed, peanut, and similar vegetable oils can be made (20). Swain and Brice (25) have shown that low-intensity absorption bands characteristic of conjugated trienoic and tetraenoic fatty acids, which may be found in the ultraviolet spectra of alkali-isomerized vegetable oils prepared by ordinary extraction methods, have their probable origin in oxidation products of linoleic and linolenic acid, respectively.

This might account for the apparent linolenic acid content of two of the cottonseed oil samples and for the arachidonic acid found in two of the olive oil samples listed in Table 1.

A number of animal fats, including fat from pork, beef, mutton, and horse, were also analyzed by the spectrophotometric method. The results are tabulated in Table 2. The large difference in the linolenic acid content of horse fat from that of the other common animal fats is clearly demonstrated. It has been known for many years (9, 24) that the fatty acid composition of horse meat differs from that for pork, beef, or mutton. In 1938, Paschke (21) developed a method for the identification of horse fat in admixture with beef, mutton, or pork, based on the observation that horse fat is richer in linolenic acid than are the depot fats of other large domestic animals. The hexabromide number is used as a measure of linolenic acid. However, poor reproducibility limits the effective use of this method. Recently, precise spectrophotometric methods based on alkali isomeriza-

tion of the animal fats have been published (7, 8). Crouse and Leffler (7) include a table of published linolenic acid analyses of normal domestic animals. It is also of interest to note that of the common animal depot fats studied, horse fat is the only fat that contains a detectable amount of pentaenoic acids (Table 2). As far as the author knows, there are no published data on the presence of non-conjugated pentaenoic acids in horsefat. However, in a private communication, S. F. Herb (10) states that an analysis of a horse oil gave a value of 0.28 per cent non-conjugated pentaene, calculated as 50 per cent C_{20}–50 per cent C_{22} acids. The Associate Referee also found a small amount of pentaene acid in a butterfat sample (Table 2). Although no detectable pentaenoic acid was found in a sample of lard run in this laboratory, Herb and Riemenschneider (13) present evidence of the presence of small amounts of pentaene acids in lard. These authors examined two concentrates of lard methyl esters and obtained definite peaks in the pentaene region.

Herb and Riemenschneider (13) recently have developed a method of isomerization which greatly increases the sensitivity of spectrophotometric analysis of all polyunsaturated acids except linoleic, for which the sensitivity is unchanged from that of the ordinary methods. The procedure comprises heating the sample in 21 per cent KOH-glycol for fifteen minutes at 180°C. This technique is especially applicable to the determination of micro amounts of fats and oils (14) and to the determination of small amounts of non-conjugated tetraene and pentaene acids in fat and oil samples. The Associate Referee intends to investigate this procedure.

Tables 1 and 2 also indicate the possibility of distinguishing lard and butterfat from oleomargarine derived from vegetable fat. The method might be useful for differentiating animal from vegetable fats, since most animal fats contain tetraenoic acids which are not generally present in vegetable fats.

The total composition of a fat and oil can be determined by determining the iodine number of the sample in addition to the polyunsaturated acid

TABLE 3.—*Fatty acid composition of several vegetable oils*

SAMPLE	IODINE NUMBER (HANUS)	CONJ. DIENE	CONJ. TRIENE	LINOLEIC	LINOLENIC	ARACHIDONIC	OLEIC[a]	SATURATED[a]
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Soybean oil I	134.2	0.78	0.000	51.1	9.09	0.00	17.1	17.5
Cottonseed oil I	113.8	0.26	0.028	53.8	0.28[b]	0.00	16.7	24.5
Olive oil I	86.30	0.16	0.012	8.21	0.65	0.02	77.1	9.4
Peanut oil I	90.55	0.20	0.016	22.6	0.01[b]	0.00	54.7	18.1

[a] Calculated by difference. See Am. Oil Chem. Soc. Tentative Method Cd 7-48 (Rev. May 1951), section E(e).
[b] Apparent % linolenic acid.

content (A.O.C.S. Tent. Method Cd 7-48 (Rev. May, 1951), section E(e)). The fatty acid composition of 4 vegetable oils is given in Table 3.

SUMMARY AND RECOMMENDATION

The ultraviolet spectrophotometric method for determining polyunsaturated acids in oils and fats was investigated. Both the ethylene glycol and the glycerol isomerization reagents gave similar and reproducible results. Either the twenty-five or thirty minute isomerization, using ethylene glycol or glycerol, or the forty-five minute isomerization, using either reagent, gave satisfactory results.

The alkali isomerization–spectrophotometric technique provides a rapid method for determining soybean oil in admixture with cottonseed and other oils which contain little or no linolenic acid, and for detecting adulteration of ground beef, pork, or lamb with horse meat. Since most animal fats contain tetraenoic acids which are not generally present in vegetable fats, the spectrophotometric method might be useful for distinguishing animal from vegetable fats.

The method can also be used to develop other procedures for identifying fats and oils, and for detecting the admixtures of fats and oils through adulteration or other means, provided the polyunsaturated constituents of each of the fats (or oils) are sufficiently different.

By determining the iodine number of the sample in addition to the polyunsaturated acid content, the total fatty acid content of a fat or oil can be calculated.

It is recommended* that study on this subject be continued, both on the method and on its applications.

ACKNOWLEDGMENT

The author gratefully acknowledges the generous advice of Samuel F. Herb of the Eastern Utilization Research Branch, U. S. Department of Agriculture, Philadelphia, Pa.

REFERENCES

(1) BALDWIN, A. R., and LONGENECKER, H. E., *Oil and Soap*, **22**, 151 (1945).
(2) BEADLE, B. W., and KRAYBILL, H. R., *J. Am. Chem. Soc.*, **66**, 1232 (1944).
(3) BERK, L. C., KRETCHMER, N., HOLMAN, R. T., and BURR, G. O., *Anal. Chem.*, **22**, 718 (1950).
(4) BRICE, B. A., and SWAIN, M. L., *J. Opt. Soc. Am.*, **35**, 532 (1945).
(5) ———, ———, SCHAEFFER, B. B., and AULT, W. C., *Oil and Soap*, **22**, 219 (1945).
(6) ———, ———, HERB, S. F., NICHOLS, P. L., JR., and RIEMENSCHNEIDER, R. W., *J. Am. Oil Chemists Soc.*, **29**, 279 (1952).
(7) CROUSE, R. H., and LEFFLER, I. D., *Food Research*, **18**, 253 (1953).
(8) DUGAN, L. R., JR., and PETHERAM, M., *This Journal*, **35**, 767 (1952).
(9) HEIDUSCHKA, A., and STEINRUCK, A., *J. pract. Chem.*, **102**, 241 (1921).

* For report of Subcommittee C and action of the Association, see *This Journal*, **37**, 74 (1954).

(10) HERB, S. F., *private communication*, 1953, Eastern Utilization Research Branch, U. S. Department of Agriculture, Phila., Pa.
(11) ———, RIEMENSCHNEIDER, R. W., and DONALDSON, J., *J. Am. Oil Chem. Soc.*, **28**, 55 (1951).
(12) ———, WITNAUER, L. P., and RIEMENSCHNEIDER, R. W., *ibid.*, **28**, 505 (1951).
(13) ———, and RIEMENSCHNEIDER, R. W., *ibid.*, **29**, 456 (1952).
(14) ———, and ———, *Anal. Chem.*, **25**, 953 (1953).
(15) HILDITCH, T. P., MORTON, R. A., and RILEY, J. P., *The Analyst*, **70**, 68 (1945).
(16) HOLMAN, R. T., and BURR, G. O., *Arch. Biochem.*, **19**, 474 (1948).
(17) MATTHEWS, N. L., BRODE, W. R., and BROWN, J. B., *J. Am. Chem. Soc.*, **63**, 1064 (1941).
(18) MITCHELL, J. H., JR., KRAYBILL, H. R., and ZSCHEILE, F. P., *Ind. Eng. Chem., Anal. Ed.*, **15**, 1 (1943).
(19) NICHOLS, P. L., JR., RIEMENSCHNEIDER, R. W., and HERB, S. F., *J. Am. Oil Chemists Soc.*, **27**, 329 (1950).
(20) O'CONNER, R. T., HEINZELMAN, D. C., and DOLLEAR, F. G., *Oil and Soap*, **22**, 257 (1945).
(21) PASCHKE, B., *Untersuch. Lebenm.*, **76**, 476 (1938).
(22) Report of the Spectroscopy Committee, *J. Am. Oil. Chem. Soc.*, **26**, 399 (1949); **28**, 331 (1951); Report presented at the 44th annual meeting of the American Oil Chemists' Society, May 4, 1953 (not published to date).
(23) RIEMENSCHNEIDER, R. W., HERB, S. F., and NICHOLS, P. L., JR., *J. Am. Oil Chemists Soc.*, **26**, 371 (1949).
(24) SCHUETTE, H. A., GARWIN, T. M., and SCHWAEGLER, E. J., *J. Biol. Chem.*, **107**, 635 (1937).
(25) SWAIN, M. L., and BRICE, B. A., *J. Am. Oil Chemists Soc.*, **26**, 272 (1949).

No report was given on peanut oil.

REPORT ON MEAT AND MEAT PRODUCTS

By R. M. MEHURIN (Livestock Regulatory Programs, Meat Inspection Branch, U. S. Department of Agriculture, Washington 25, D. C.), *Referee*

No report was received from the Associate Referee on Chemical Methods for the Detection of Horse Meat in Ground Meat. However, an Associate Referee was appointed to present at the 1953 meeting a serological method for the detection of horse meat which employs generally accepted procedures used by well-known control agencies. The chemical test based on the formation of hexabromides yields low results and is inconclusive in cases where the horse meat is roughly around one-third of the mixture or less, but the serological method is conclusive when the horse meat is only 10 per cent or less, depending on the titer and specificity of the anti-horse serum and the experience of the operator. This procedure is not applicable to cooked meat, although the center of large sausages will sometimes yield sufficient unaltered protein for a satisfactory test.

The detection of horse meat by the spectrophotometric determination of linolenic acid was briefly described in last year's report. Results obtained by others using this procedure have been published (1, 2).

The first action method for lactose (dried skim milk) in meat products is not applicable in the presence of maltose. Since maltose constitutes approximately 20 to 40 per cent of dried corn syrup and since this substance is now being used in meat products in amounts up to 3 per cent, it appears necessary to devise a method to separate these two sugars in comminuted meat products containing dried skim milk (non-fat dry milk solids). It is believed that this may be accomplished by the use of special types of yeast which have been obtained from the Eastern Utilization Research Branch of the Department of Agriculture.

RECOMMENDATION

It is recommended*—

(1) That collaborative work be carried out on the serological method for the detection of horse meat.

(2) That work be continued on the chemical detection of horse meat, including spectrophotometric procedures.

(3) That work be continued on the determination of starch in meat products.

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

(5) That an Associate Referee be appointed for the determination of lactose in meat products in the presence of maltose.

REFERENCES

(1) DUGAN, L. R., JR., and PETHERAM, M., *This Journal*, **35**, 767 (1952).
(2) MITCHELL, J. H., JR., KRAYBILL, H. R., and ZSCHEILE, F. P., *Ind. Eng. Chem., Anal. Ed.*, **15**, 1 (1943).

REPORT ON SEROLOGICAL TESTS FOR IDENTIFICATION OF MEATS

By PAUL J. BRANDLY (Meat Inspection Branch, Agricultural Research Service, Beltsville, Md.), *Associate Referee*

The serological laboratory of the Federal Meat Inspection Service has found the Proom method (1) to be satisfactory for the serological identification of meat. However, attempts have been made to improve the method by increasing the yield, the specificity, and the stability of the antiserum.

In an attempt to increase the amount of blood recovered from sacrificed

* For report of Subcommittee C and action of the Association, see *This Journal*, **37**, 72 (1954).

hyperimmune rabbits, 100 to 300 ml of physiological saline was injected intraperitoneally just prior to total bleeding. This modification did not appreciably affect the yield of recovered blood. Heparin was also injected intravenously just prior to sacrifice. No increase in recovered blood was observed, but the serum harvest was markedly reduced because the clot did not shrink. When the blood was centrifuged prior to clotting, the serum later clotted.

It has been the experience of this laboratory that rabbit serum will occasionally be cloudy even though feed has been withheld from the animals for forty-eight hours prior to sacrifice. In an attempt at salvage, this serum was precipitated with 20 volumes of cold anhydrous acetone, and was then washed with cold absolute ether, as suggested by Merrill and Fleisher (2). The dried precipitate was clear when it was reconstituted, and the accurate reading of the test was thus assured.

It was believed that by keeping the serum in the dried state, the problem of storage maintenance of titer and specificity could be solved. On the basis of preliminary findings, the Associate Referee was hopeful that the procedure would be successful and accordingly attempted to set up a collaborative study. By the time arrangements could be completed, however, eight weeks had elapsed, and the previously precipitated serum, when tested again, was found to have become so sensitive that it had lost its specificity. Further work was done in an attempt to stabilize the precipitated antiserum but with no success.

Another phase of the problem which was investigated was the antigenic potential of serum protein compared to that of meat protein. As a rule, an antiserum which will react with a serum (containing 6.25 per cent protein) in a saline dilution of 1:4000 (1 part serum protein in 64,000) will not react with a homologous meat protein in a dilution of 1:6400, but will react in a 1:3200 dilution. This suggests that the meat proteins and the serum proteins from the same animal may be significantly different and that an immunizing antigen made from meat protein might produce better antiserum for meat identification than an immunizing antigen made of serum protein. It was been found, however, that it is much more difficult to prepare a satisfactory antiserum with meat protein as the hyperimmunizing antigen than when serum protein is used. Judging from the results of the investigations conducted in this laboratory during the past year, no improvement in the Proom method along these lines can be foreseen.

It is recommended* that collaborative work be continued on a method for the serological identification of meat, based upon the Proom procedure.

REFERENCES

(1) PROOM, H., *J. Path. Bact.*, **55**, 419 (1943).
(2) MERRILL, M. H., and FLEISCHER, M. S., *J. Gen. Physiol.*, **16**, 243 (1932).

* For report of Subcommittee C and action of the Association, see *This Journal*, **37**, 72 (1954).

REPORT ON STARCH IN MEAT PRODUCTS

By Ross A. CHAPMAN (Department of National Health and Welfare, Food and Drug Divisions, Ottawa, Canada), *Associate Referee*

The collaborative study conducted on the proposed method for starch in meat products,[1] carried out in 1952, indicated average recoveries of over 100 per cent. It was felt that these high values may have been caused by the production of non-sugar reducing substances produced during the hydrolysis of a sample, which reacted with the modified Fehling's reagent. In an attempt to overcome this difficulty, the use of the anthrone reagent for the determintaion of the starch has been investigated.

Extraction of the soluble carbohydrates was carried out as previously described.[1] The starch was then solubilized with 0.5 *N* sulfuric acid in a boiling water bath for one hour. After filtration and dilution, an appropriate aliquot was heated for ten minutes in a boiling water bath with 10 ml of a 0.2 per cent anthrone solution. The anthrone reagent was prepared by dissolving 0.2 grams of anthrone in a solution of 75 ml of concentrated sulfuric acid and 25 ml of water. It was found that this anthrone solution did not darken appreciably over a period of weeks. The method has now been applied with satisfactory results to a number of meat samples containing various carbohydrates. However, this investigational work was not completed in time to permit a collaborative study of the modified procedure. It is recommended* that the study on the method for starch in meat products be continued.

No reports were given on chemical tests for the identification of meats, creatin in meat products, or moisture and fat in meat products.

REPORT ON NUTS AND NUT PRODUCTS

By A. M. HENRY (Food and Drug Administration, Department of Health, Education, and Welfare, Atlanta 3, Ga.), *Referee*

Methods for moisture, crude fat, crude protein, crude fiber, and ash were studied collaboratively this year. Results are given in Table 1.

A study of methods for glycerine and propylene glycol in shredded coconut has been made by J. C. M. Griffin of Atlanta District. He has developed a method that appears to be satisfactory not only for shredded

[1] STEVENS, F. J., and CHAPMAN, R. A., *This Journal*, **36**, 292 (1953).
* For report of Subcommittee C and action of the Association, see *This Journal*, **37**, 72 (1954).

TABLE 1.—*General methods for nuts and nut products*

DETERMINATION	BRAZIL NUTS					CHESTNUTS				
	1	2	3	4	5	1	2	3	4	5
Moisture	2.92	3.05	3.22	2.90	3.23	49.8	50.0	50.0	49.4	49.1
Crude fat	68.2	69.0	68.3	68.2	68.6	1.36	1.30	1.30	1.40	1.36
Crude fiber	1.84	2.23	2.31	2.05	2.02	2.00	1.78	2.17	1.70	1.78
Crude protein	16.9	16.4	16.8	16.6	16.5	3.41	3.32	3.72	3.50	3.70
Ash	3.44	3.37	3.30	3.26	3.27	1.63	1.27	1.44	1.50	1.30

TABLE 2.—*Determination of starch in peanuts by three methods*

PRODUCT	ROASTING LOT	MODIFIED RASK METHOD				ORIGINAL RASK METHOD			ACID HYDROLYSIS	
		1	2	3	4	1	2	3	1	2
		per cent				*per cent*			*per cent*	
Raw runners	0	3.81				3.36			6.72	6.68
Lightly roasted runners	0	2.71				2.80			6.71	
Fully roasted runners	0	1.15	1.32	2.21	0.15	0.94	1.02	0.40	4.86	5.04
Raw Spanish	1	3.02				3.13			5.56	
Lightly roasted Spanish	1	2.39	2.72			2.80			5.05	
Fully roasted Spanish	1	1.50	2.10	1.46	0.11	1.41	1.00		4.64	4.70
Raw runners	2	4.62	3.39			3.80				
Lightly roasted runners	2	2.88				2.44				
Fully roasted runners	2	1.32	0.56			0.96				
Lightly roasted runners	3	2.99				2.77				
Fully roasted runners	3	1.15				0.90				

coconut but for other food products such as peanut butter, wine, and vinegar. Details are given in another article.[1]

A study has been made of the first action method for starch by George L. Patrick and Robert E. O'Neill of Atlanta District. They have reviewed a number of starch methods and have attempted to fit them to the determination of added starch in peanut butter.

A number of investigators (1–6) have studied this problem. The method of Rask (1) was tentatively adopted by this Association in 1943 and was given first action status in 1950. Work done by Munsey (4) and confirmed by Alfend (7), however, indicated that the Rask procedure does not give results comparable to those obtained with other methods for products that have been heated. It was also indicated that in high protein products, the Rask procedure does not give comparable results even for raw products.

Patrick and O'Neill examined two series of peanuts, runners and Spanish, by the Rask procedure (1), the modified Rask method (8), and the acid hydrolysis method (9). Results are shown in Table 2.

The Rask procedure is lengthy and tedious, and much work has been done in attempts to modify and shorten it. A number of improvements were made but are not discussed at this time since neither the method nor its modifications gave results for peanuts that could be duplicated or compared with those by other methods. It should be noted that the Rask method indicated that the degree of roasting influenced the percentage of starch in peanuts (see Table 2).

No work was done on the other recommendations of the committee.

RECOMMENDATIONS

It is recommended*—

(1) That first action methods for moisture, crude fat, crude protein, crude fiber, and ash be made official.

(2) That methods for sucrose, reducing sugar, and sodium chloride be studied further.

(3) That the method for starch, **25.13,** be deleted, first action.

(4) That methods for hydrogenated oil in nut products be studied.

(5) That methods for sorbitol in shredded coconut be studied.

(6) That the method for glycerine and propylene glycol be studied collaboratively.

ACKNOWLEDGMENT

The Referee wishes to thank the employees of the following organizations for their collaboration in this work:

Department of Agriculture and Industry, Montgomery, Ala.
Board of Agriculture, Dover, Del.

[1] GRIFFIN, J. C. M., *This Journal*, **37**, 874 (1954).
* For report of Subcommittee C and action of the Association, see *This Journal*, **37**, 72 (1954).

Department of Agriculture, Atlanta, Ga.
Law & Company, Atlanta, Ga.
Agricultural Experiment Station, Amherst, Mass.
State College, Miss.
Department of Agriculture, Columbia, S. C.

REFERENCES

(1) RASK, O. S., *This Journal*, **10**, 108 (1927).
(2) ———, *ibid.*, **11**, 33 (1928).
(3) ETHEREDGE, M. P., and SULLIVAN, J. T., *ibid.*, **18**, 621 (1935).
(4) MUNSEY, V. E., *ibid.*, **20**, 547 (1937).
(5) ———, *ibid.*, **21**, 394 (1938).
(6) ETHEREDGE, M. P., *ibid.*, **24**, 113 (1940).
(7) ALFEND, S., unpublished data.
(8) *Official Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Box 540, Benjamin Franklin Station, Washington 4, D. C., 1950, section **25.13.**
(9) *Ibid.*, section **22.34.**

No report was given on shredded coconut (glycols and glycerol).

ANNOUNCEMENTS

REFEREE ASSIGNMENTS

PESTICIDES:

Dr. Kenneth Helrich, New Jersey Agricultural Experiment Station, New Brunswick, N. J., has been appointed Associate Referee on Parathion.

ERRATA

This Journal, **37**, 521 (1954)	Under (2), fifth line from bottom of page, after "ca 0.1 *N* with NaOH," add " . . . and 1% with Na_2SO_3." Under (3), third line from bottom of page, after "ca 0.1 *N* with NaOH," add " . . . and 1% with Na_2SO_3."
Ibid., **37**, 89 (1954)	Under **9.** Beverages: Distilled Liquors, (3), change *This Journal*, **36**, 70 (1953) to *This Journal*, **35**, 70 (1952).

CONTRIBUTED PAPERS

LOSS OF FAT DURING SOURING OF CREAM

By JESSE E. ROE and HOWARD EDELSON (Food and Drug Administration, Department of Health, Education, and Welfare, Kansas City 6, Mo. and Washington 25, D. C.) and WILLIAM E. POLZEN (Dairy Division, Colorado State Department of Agriculture, Denver, Colo).

INTRODUCTION

Recently, a dairy firm was prosecuted for shipping two lots of whipping cream which contained less than the required 30 per cent of butterfat. Three subdivisions of the first shipment showed 26.33, 26.52, and 27.38 per cent butterfat by the Roese-Gottlieb method (1), and three subdivisions of the second (shipped about one month later) showed 28.46, 28.56, and 28.54 per cent. In both cases, the samples were sent without refrigeration from the sampling point to the testing laboratory, and in the first case, five days, and in the second case, two days, elapsed between times of collection and analysis.

The case was successfully contested by the firm, mainly due to the testimony of a defense witness who claimed that the fat content of cream, as measured by the Babcock method (2), is diminished by souring.

No well-correlated or published figures on the change of fat content of cream during progressive souring were on hand to refute such testimony, and it is the purpose of this paper to provide them.

SAMPLES AND ANALYTICAL PROCEDURES

A total of 12 coffee cream (18 per cent fat) and 12 whipping cream (30 per cent fat) samples were analyzed in January and February, 1952. Each sample consisted of six half-pint cartons of cream which were composited in a half-gallon fruit jar. Fat was determined by the Roese-Gottlieb method (1) in the Denver Laboratory of the Food and Drug Administration and by the Babcock method (2) in the Dairy Division laboratory of the Colorado State Department of Agriculture on the day purchased and on each day thereafter until representative samples could no longer be obtained from the half-gallon jars. The cream was kept at room temperature throughout the course of the experiment. In addition to fat determinations by the two methods, *p*H and per cent acid as lactic acid were obtained. Data are summarized in Table 1.

Statistical analysis of the data obtained by the Roese-Gottlieb method shows a statistically significant decrease in the fat content of the cream as it ages. The estimate of this decrease is 0.2 per cent per day. (This is 0.2 per cent of the average per cent fat; thus for a cream that has 30 per

TABLE 1.—*Changes in cream during souring*

SAMPLE NO.	DATES EXAMINED	AGE OF CREAM	FAT, ROESE-GOTTLIEB		FAT, BABCOCK		pH	ACID AS LACTIC
		days	*per cent*		*per cent*			*per cent*
INV 49-354 K	1/28/52	0	36.70	36.71	36.75	36.75	6.65	0.08
	to	1	36.73	36.63	36.75	36.75	6.62	0.11
	1/31/52	2	36.79	36.76	36.75	36.50	4.49	0.51
		3	36.34	36.42	36.00	36.50	4.42	0.57
INV 49-355 K	1/28/52	0	35.22	35.22	35.25	35.50	6.47	0.09
	to	1	35.25	35.22	35.50	35.50	6.68	0.10
	1/31/52	2	36.17	35.57	36.00	35.50	4.51	0.47
		3	32.66	34.39	36.00	37.00	4.32	0.57
INV 49-356 K	1/28/52	0	29.01	28.94	29.00	29.00	6.76	0.10
	to	1	28.67	28.64	29.00	28.75	6.20	0.17
	1/31/52	2	28.33	28.46	29.50	29.50	4.33	0.62
		3	28.77	28.52	29.00	28.50	4.38	0.67
INV 49-357 K	1/28/52	0	35.06	35.13	34.75	34.75	6.43	0.10
	to	1	35.06	—	35.00	35.00	6.48	0.12
	1/31/52	2	34.82	34.92	35.00	34.75	4.46	0.51
		3	35.02	34.79	34.50	34.00	4.51	0.62
INV 49-358 K	1/28/52	0	35.54	35.45	35.50	35.00	6.75	0.10
	to	1	35.71	35.54	35.00	35.00	6.52	0.15
	1/31/52	2	35.23	35.33	35.50	35.50	4.48	0.54
		3	35.45	35.41	35.00	34.50	4.36	0.50
INV 49-359 K	1/28/52	0	34.85	34.81	34.50	34.50	6.78	0.10
	to	1	34.82	34.83	34.00	34.25	6.68	0.10
	1/31/52	2	33.38	34.28	36.00	36.25	4.62	0.48
		3	*Could not sample*					
INV 49-360 K	2/ 4/52	0	18.33	18.33	17.50	17.50	6.78	0.09
	to	1	18.33	18.33	17.75	17.50	6.60	0.12
	2/ 6/52	2	*Sample lost due to gas formation*					
INV 49-361 K	2/ 4/52	0	11.76	11.75	11.25	11.25	6.50	0.11
	to	1	11.77	11.78	11.50	11.50	4.82	0.58
	2/ 7/52	2	11.68	11.70	11.50	11.50	4.12	0.78
		3	11.63	11.65	—	—	4.15	0.77
INV 49-362 K	2/ 4/52	0	22.60	22.61	22.00	22.00	6.60	0.09
	to	1	22.44	22.36	22.25	22.00	4.95	0.47
	2/ 7/52	2	22.57	22.52	22.00	22.00	4.30	0.60
		3	22.51	22.52	—	—	4.20	0.66
INV 49-363 K	2/ 4/52	0	18.40	18.41	18.00	18.00	6.45	0.09
	to	1	18.39	18.42	18.25	18.25	4.62	0.57
	2/ 7/52	2	18.32	18.32	18.50	18.00	4.15	0.71
		3	18.27	18.30	—	—	4.12	0.71
INV 49-364 K	2/ 4/52	0	18.54	18.48	18.00	17.75	6.60	0.16
	to	1	18.37	18.54	18.00	18.00	6.55	0.12
	2/ 7/52	2	18.48	18.54	18.00	18.00	4.35	0.77
		3	18.36	18.44	—	—	4.12	0.89
INV 49-365 K	2/ 4/52	0	18.56	18.56	18.00	18.00	6.50	0.11
	to	1	18.58	18.58	18.00	18.00	6.70	0.11
	2/ 7/52	2	18.50	18.57	17.75	18.00	4.25	0.66
		3	18.52	18.51	—	—	4.12	0.74
INV 49-347 K	1/21/52	0	16.86	16.90	16.75	16.75	6.81	0.07
	to	1	16.82	16.84	16.75	16.50	5.66	0.29
	1/25/52	2	16.88	16.91	16.75	—	4.62	0.62
		3	16.86	16.91	16.75	—	4.41	0.71
		4	16.81	16.91	16.50	—	4.49	0.74

TABLE 1.—(*continued*)

SAMPLE NO.	DATES EXAMINED	AGE OF CREAM	FAT, ROESE-GOTTLIEB		FAT, BABCOCK		pH	ACID AS LACTIC
		days	*per cent*		*per cent*			*per cent*
INV 49-348 K	1/21/52	0	20.01	19.98	20.00	19.75	6.60	0.09
	to	1	20.04	19.72	20.00	20.00	6.48	0.09
	1/25/52	2	19.98	20.05	19.25	—	4.40	0.66
		3	19.99	19.95	19.25	—	4.27	0.73
		4	19.97	20.01	19.50	—	4.28	0.78
INV 49-349 K	1/21/52	0	17.85	17.85	18.00	18.00	6.48	0.12
	to	1	17.64	17.68	18.00	17.75	6.56	0.14
	1/25/52	2	17.96	17.99	18.00	—	4.43	0.69
		3	17.82	17.72	17.50	—	4.22	0.77
		4	17.70	17.89	18.00	—	4.20	0.78
INV 49-350 K	1/21/52	0	18.51	18.48	18.50	18.50	6.69	0.11
	to	1	18.31	18.39	18.25	18.25	6.61	0.11
	1/25/52	2	18.47	18.47	18.50	—	4.70	0.51
		3	18.36	18.47	18.50	—	4.37	0.69
		4	18.49	18.45	18.25	—	4.26	0.73
INV 49-351 K	1/21/52	0	19.41	19.39	19.50	19.50	6.52	0.12
	to	1	19.13	19.19	19.00	19.50	6.70	0.13
	1/25/52	2	19.42	19.42	19.00	—	4.51	0.68
		3	19.31	19.52	18.50	—	4.35	0.85
		4	19.37	19.37	18.75	—	4.33	0.91
INV 49-352 K	1/21/52	0	20.06	20.03	19.75	19.75	6.58	0.10
	to	1	19.72	20.02	19.75	19.75	6.55	0.12
	1/25/52	2	19.97	20.00	19.75	—	4.48	0.65
		3	20.06	20.04	19.50	—	4.28	0.69
		4	19.03	18.96	19.75	—	4.27	0.74
INV 49-341 K	1/14/52	0	34.89	34.88	35.00	35.00	6.65	0.09
	to	1	34.87	34.85	34.50	34.50	6.45	0.11
	1/17/52	2	34.39	34.36	33.50	34.00	4.70	0.44
		3	*Could not sample*				4.51	0.67
INV 49-342 K	1/14/52	0	33.73	33.70	33.50	33.50	6.62	0.10
	to	1[a]	33.97	33.94	33.50	33.50	5.51	0.25
	1/17/52	2	33.47	33.63	34.50	33.50	4.30	0.57
		3	33.70	33.68	33.50	33.50	4.40	0.63
INV 49-343 K	1/14/52	0	34.20	34.17	34.00	33.75	6.70	0.10
	to	1	34.17	34.08	34.25	34.00	6.28	0.13
	1/17/52	2	34.38	34.28	34.75	34.75	4.38	0.50
		3	34.32	34.20	35.00	35.00	4.27	0.56
INV 49-344 K	1/14/52	0	37.06	37.06	36.50	36.25	6.70	0.07
	to	1	36.99	37.31	37.00	37.00	6.28	0.09
	1/17/52	2	37.31	37.04	37.25	37.25	4.23	0.45
		3	36.76	36.58	37.50	37.00	4.23	0.48
INV 49-345 K	1/14/52	0	36.99	36.96	36.00	36.25	6.70	0.09
	to	1	36.75	37.05	36.75	36.75	5.80	0.19
	1/17/52	2	36.98	37.02	37.00	37.50	4.22	0.56
		3	36.86	37.19	*No sample*		4.30	0.48
INV 49-346 K	1/14/52	0	30.72	30.72	30.00	30.00	6.39	0.13
	to	1	30.68	30.61	30.75	31.00	5.10	0.39
	1/17/52	2	30.51	30.60	30.50	30.50	4.39	0.58
		3	30.34	30.41	30.75	31.00	4.40	0.60

[a] After drying ether extract of fat, a white solid material was noted in bottom of each beaker.

TABLE 2.—*Changes in cream during souring*

SAMPLE NO.	DATES EXAMINED	AGE OF CREAM	FAT, ROESE-GOTTLIEB			pH	ACID AS LACTIC
		days	*per cent*				*per cent*
INV 88-527 K	7/28/52	0	29.79	29.84	—	6.60	—
	to	1	29.80	29.79	—	5.31	—
	8/ 4/52	2	29.78	29.82	29.85	4.54	—
		3	29.82	29.80	29.82	4.51	—
		4	29.84	29.81	29.80	4.50	—
		7	29.79	29.82	—	4.95	—
INV 88-528 K	7/28/52	0	34.23	34.37	—	6.40	—
	to	1	34.32	34.37	—	4.55	—
	8/ 4/52	2	34.28	34.29	34.31	4.36	—
		3	34.26	34.25	34.28	4.37	—
		4	34.32	34.24	34.24	4.38	—
		7	34.10	34.15	—	4.31	—
INV 88-529 K	7/28/52	0	32.94	32.84	—	5.20	—
	to	1	32.92	32.89	—	4.42	—
	8/ 4/52	2	32.90	32.84	32.93	4.28	—
		3	32.91	32.89	32.91	4.30	—
		4	32.89	32.91	32.91	4.79	—
		7	32.86	32.89	32.85	4.62	—
INV 88-565 K	1/19/53	0	32.99	32.92	—	6.65	—
	to	1	32.80	32.83	—	4.81	0.39
	1/26/53	2	32.81	32.79	32.78	4.45	0.52
		3	32.82	32.80	32.79	4.37	0.60
		4	32.79	32.78	32.76	4.30	0.61
		7	32.75	32.75	—	4.36	0.61
INV 88-566 K	1/19/53	0	24.75	24.73	—	6.68	—
	to	1	24.60	24.59	—	5.72	0.22
	1/26/53	2	24.62	24.59	24.62	4.38	0.64
		3	24.59	24.62	24.63	4.32	0.69
		4	24.54	24.55	24.54	4.40	0.72
		7	24.47	24.49	24.52	4.70	0.65
INV 88-567 K	1/19/53	0	33.82	33.83	—	6.62	—
	to	1	33.84	33.84	—	6.45	0.12
	1/26/53	2	33.83	33.83	33.82	5.08	0.44
		3	33.86	33.94	33.78	4.61	0.53
		4	33.85	33.84	33.83	4.51	0.62
		7	33.85	33.83	33.85	4.72	0.56
INV 94-967 K	2/ 2/53	0	31.55	31.59	—	6.65	—
	to	1	31.52	31.53	—	4.40	0.51
	2/ 9/53	2	31.51	31.48	31.48	4.35	0.59
		3	31.47	31.44	31.46	4.32	0.59
		4	31.45	31.46	31.51	4.32	0.58
		7	31.42	31.44	31.53	4.46	0.58
INV 94-968 K	2/ 2/53	0	35.91	35.89	—	6.38	—
	to	1	35.88	35.86	—	5.03	0.28
	2/ 9/53	2	35.86	35.85	—	4.19	0.48
		3	35.77	35.83	35.81	4.22	0.48
		4	35.87	35.85	35.87	4.28	0.47
		7	35.83	35.61	35.77	4.38	0.52
INV 94-969 K	2/ 2/53	0	34.58	34.56	—	6.59	—
	to	1	34.35	34.42	—	5.38	0.31
	2/ 9/53	2	34.25	34.24	34.28	4.52	0.61
		3	34.23	34.27	34.25	4.40	0.65
		4	34.25	34.23	34.10	4.33	0.71
		7	34.08	34.07	34.12	4.98	0.59

TABLE 2.—(*continued*)

SAMPLE NO.	DATES EXAMINED	AGE OF CREAM	FAT, ROESE-GOTTLIEB			pH	ACID AS LACTIC
		days		*per cent*			*per cent*
INV 88-579 K	3/ 2/53	0	37.48	37.44	—	6.71	—
	to	1	37.44	37.44	—	6.70	0.11
	3/ 9/53	2	37.44	37.46	37.44	4.71	0.47
		3	37.47	37.31	37.46	4.33	0.53
		4	37.47	37.48	37.35	4.35	0.56
		7	37.47	37.45	37.48	4.28	0.62
INV 88-580 K	3/ 2/53	0	33.86	33.83	—	6.72	—
	to	1	33.82	33.83	—	6.62	0.11
	3/ 9/53	2	33.66	33.68	33.67	4.41	0.47
		3	33.61	33.67	33.61	4.22	0.53
		4	33.48	33.50	33.65	4.28	0.55
		7	33.35	33.46	33.48	4.31	0.64
INV 88-583 K	3/16/53	0	33.79	33.77	—	6.71	—
	to	1	33.75	33.77	—	6.50	0.11
	3/23/53	2	33.77	33.76	33.79	4.75	0.45
		3	33.78	33.77	33.80	4.33	0.53
		4	33.72	33.76	33.77	4.31	0.58
		7	33.75	33.80	—	4.41	0.55
*INV 88-584 K	3/16/53	0	37.64	37.63	—	6.74	—
	to	1	37.60	37.62	—	6.50	0.11
	3/23/53	2	37.59	37.59	37.67	4.52	0.46
		3	37.52	37.52	37.52	4.39	0.51
		4	37.61	37.63	37.47	4.41	0.51
		7	37.49	37.56	37.52	4.38	0.55
INV 88-585 K	3/16/53	0	36.21	36.21	—	6.34	—
	to	1	36.12	36.09	—	4.49	0.47
	3/23/53	2	35.97	36.01	36.00	4.42	0.56
		3	35.90	35.98	35.96	4.28	0.59
		4	35.92	35.92	35.93	4.31	0.64
		7	35.87	35.95	35.87	4.30	0.71
INV 88-562 K	1/ 5/53	0	17.08	17.08	—	6.50	—
	to	1	17.09	17.12	—	6.10	—
	1/12/53	2	17.05	17.07	17.08	5.60	—
		3	17.09	17.08	17.08	4.60	0.57
		4	17.09	17.08	17.07	4.38	0.64
		7	17.05	17.06	17.05	4.26	0.74
INV 88-563 K	1/ 5/53	0	16.91	16.90	—	6.50	—
	to	1	16.90	16.90	—	6.27	—
	1/12/53	2	16.90	16.91	16.91	4.52	—
		3	16.89	16.93	16.92	4.32	0.70
		4	16.90	16.91	16.90	4.26	0.72
		7	16.87	16.91	16.87	4.24	0.74
INV 88-564 K	1/ 5/53	0	18.70	18.68	—	6.45	—
	to	1	18.62	18.62	—	6.00	—
	1/12/53	2	18.52	18.54	18.58	4.43	—
		3	18.59	18.57	18.45	4.35	0.89
		4	18.45	18.35	18.52	4.30	0.93
		7	18.36	18.39	18.40	4.29	1.01
INV 88-586 K	3/30/53	0	20.06	20.04	—	6.84	—
	to	1	20.05	20.05	—	5.45	0.43
	4/ 6/53	2	20.01	20.01	20.02	4.69	0.64
		3	20.02	20.03	20.02	4.87	0.77
		4	20.02	20.02	20.06	4.84	0.78
		7	20.00	19.94	19.94	4.55	0.87

TABLE 2.—(*continued*)

SAMPLE NO.	DATES EXAMINED	AGE OF CREAM	FAT, ROESE-GOTTLIEB			pH	ACID AS LACTIC
		days	*per cent*				*per cent*
INV 88-587 K	3/30/53	0	16.74	16.76	—	6.71	—
	to	1	16.75	16.75	—	4.82	0.55
	4/ 6/53	2	16.72	16.71	16.72	4.28	0.71
		3	16.73	16.76	16.75	4.24	0.76
		4	16.73	16.70	16.71	4.18	0.79
		7	16.67	16.71	16.67	4.17	0.86
INV 88-588 K	3/30/53	0	18.65	18.62	—	6.78	—
	to	1	18.70	18.65	—	6.03	0.22
	4/ 6/53	2	18.62	18.63	18.63	5.62	0.37
		3	18.63	18.62	18.63	4.75	0.63
		4	18.67	18.62	18.63	4.42	0.92
		7	18.62	18.61	18.59	4.40	0.90
INV 88-593 K	4/13/53	0	18.09	18.06	—	6.70	—
	to	1	18.08	18.09	—	6.32	0.14
	4/20/53	2	18.05	18.05	18.05	5.02	0.48
		3	18.07	18.06	18.05	4.50	0.71
		4	18.06	18.07	18.06	4.29	0.74
		7	18.10	18.07	18.10	4.29	0.78
INV 88-594 K	4/13/53	0	20.13	20.12	—	6.82	—
	to	1	20.09	20.11	—	6.69	0.16
	4/20/53	2	20.05	20.04	20.10	5.16	0.55
		3	19.99	19.97	19.96	4.72	0.80
		4	19.94	19.97	—	4.64	0.84
		7	19.84	19.79	19.80	4.53	0.93
INV 88-595 K	4/13/53	0	17.42	17.43	—	6.70	—
	to	1	17.30	17.27	—	5.08	0.44
	4/20/53	2	17.30	17.29	17.35	4.30	0.76
		3	17.24	17.28	17.25	4.31	0.82
		4	17.31	17.25	17.25	4.35	0.82
		7	17.19	17.20	17.20	4.32	1.09
INV 100-361 K	4/27/53	0	16.04	16.03	—	6.55	—
	to	1	16.03	16.04	—	4.82	0.56
	5/ 4/53	2	15.98	15.99	15.99	4.45	0.73
		3	15.99	15.98	15.97	4.41	0.80
		4	15.97	15.99	15.99	4.41	0.80
		7	15.90	15.90	15.89	4.25	0.80
INV 100-362 K	4/27/53	0	18.13	18.14	—	6.70	—
	to	1	18.16	18.13	—	6.52	0.21
	5/ 4/53	2	18.16	18.10	18.09	4.62	0.83
		3	18.08	18.09	18.07	4.50	0.97
		4	18.09	18.09	18.08	4.50	0.97
		7	18.01	18.03	18.02	4.35	1.00
INV 100-363 K	4/27/53	0	17.37	17.36	—	6.60	—
	to	1	17.37	17.38	—	6.58	0.13
	5/ 4/53	2	17.32	17.35	17.35	4.75	0.33
		3	17.27	17.38	17.36	5.15	0.49
		4	17.34	17.36	17.37	4.50	0.70
		7	17.31	17.30	17.30	4.25	0.78

cent fat, it would mean a decrease of 0.06 per cent of fat per day.) There were four extremely low observations in this set of data and the analysis was recalculated, omitting these four figures. The decrease was still statistically significant, but the estimate of the decrease was only 0.1 instead of 0.2 per cent.

In addition to the formal analysis, a quick but less sensitive test revealed that 22 of the 24 samples showed less fat by the Roese-Gottlieb method on the last day of analysis than on the first. If there is no drop in fat, the chances of getting a result this extreme are 2 in 100,000.

The analysis of the data obtained by the Babcock method shows no evidence of a decrease in the fat content of cream as it ages.

The failure of the Babcock method to confirm the conclusions reached by using the Roese-Gottlieb method is probably due to the lack of sensitivity of the Babcock method. The estimates of the decrease from the Roese-Gottlieb data are only 0.03 to 0.06 per cent fat per day for cream containing 30 per cent fat. Since the Babcock results are reported only to the nearest 0.25 per cent (the Babcock flasks are graduated only to 0.5 per cent), it is quite evident that they would not reveal a decrease of this magnitude.

Analysis revealed a significant increase in the difference between duplicates as the cream ages, confirming the laboratory observation that representative samples become more difficult to obtain with increasing age of the cream in the half-gallon jars. In order to check on this point, a second series of determinations was made by the Roese-Gottlieb method. A total of 14 whipping cream (30 per cent fat) and 12 coffee cream (18 per cent fat) samples were analyzed. Each sample consisted of six half-pint cartons of cream which were composited in a half-gallon fruit jar. However, subdivisions for all succeeding determinations were weighed out into individual Mojonnier flasks on the day of sample receipt. This obviated the concern about representative sampling. The flasks containing the cream were held at room temperature. The *p*H and per cent acid as lactic were determined in the half-gallon jar on the days the fat was extracted from the Mojonnier flasks. Data are summarized in Table 2.

Analysis again shows a statistically significant decrease in the fat content as the cream ages. The estimate of this decrease obtained from these data is 0.075 per cent per day. (This is 0.075 per cent of the average per cent fat; thus for a cream that has 30 per cent fat, it would mean a decrease of 0.0225 per cent of fat per day.) The figure of 0.075 per cent compares with the values of 0.2 and 0.1 per cent obtained in the analysis of the data reported in Table 1 (with and without extreme observations).

CONCLUSIONS

Loss of fat in cream during souring is not measurable by the Babcock method (2) over a 4-day period. Losses are detectable by the Roese-

Gottlieb method (1) but, over a 7-day period, amount to only about 0.1 per cent of total per cent fat per day. This represents about 0.03 per cent per day for a whipping cream containing 30 per cent fat.

REFERENCES

(1) *Official Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Box 540, Benjamin Franklin Station, Washington 4, D. C., 1950, p. 246, section **15.61.**

(2) *Ibid.*, p. 246, section **15.62—15.63.**

SEMI-MICRO ORSAT GAS ANALYZER*

By JOHN J. MCMULLEN and OTTO J. STARK (Quartermaster Food and Container Institute for the Armed Forces,† Chicago 9, Ill.)

An oxygen content of 3 per cent or less by volume in headspace gas (0.03 ml per gram of dried milk powder) is essential to the protection against oxidative off-flavors in dehydrated milk products during long storage (1–5). The military specifications for sweetened dry milk products (6) and dry whole milk and non-fat solids (7) set 2 per cent by volume at atmospheric pressure as the maximum allowable amount of headspace oxygen in these products.

The inclusion as a component of operational military rations of the individual dried milk or cream packet, a laminated cellulose acetate-aluminum foil packet, $2\frac{5}{8} \times 2\frac{5}{8} \times 3/16$ inches, required the development for control purposes of a simple but accurate gas analyzer. Due to the small amount of headspace in these packets, the analyzer had to be capable of analyzing for oxygen and carbon dioxide in gas volumes no greater than 4 ml.

The problem of analyzing gases in vacuum packed cans of various sizes generally may be accomplished by the use of an apparatus which can analyze the 1–10 ml of headspace gas (at atmospheric pressure) which is available.

There are a number of well-designed micro-volumetric gas analyzers (8–12), none of which was found to be completely or economically adaptable to the particular problem of packet analyses.

Orsat gas analyzers (13) with many and varied modifications have enjoyed wide application, but mainly in the macro range. The apparatus

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† This paper reports research undertaken at the Quartermaster Food and Container Institute for the Armed Forces and has been assigned No. 447 in the series of papers approved. The views or conclusions contained in this paper are those of the authors; they are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

described here (Figure 1) is an adaptation from the fundamental Orsat design, but is modified so as to permit the analysis of less than 10 ml of gas with an accuracy of ± 0.2 per cent.

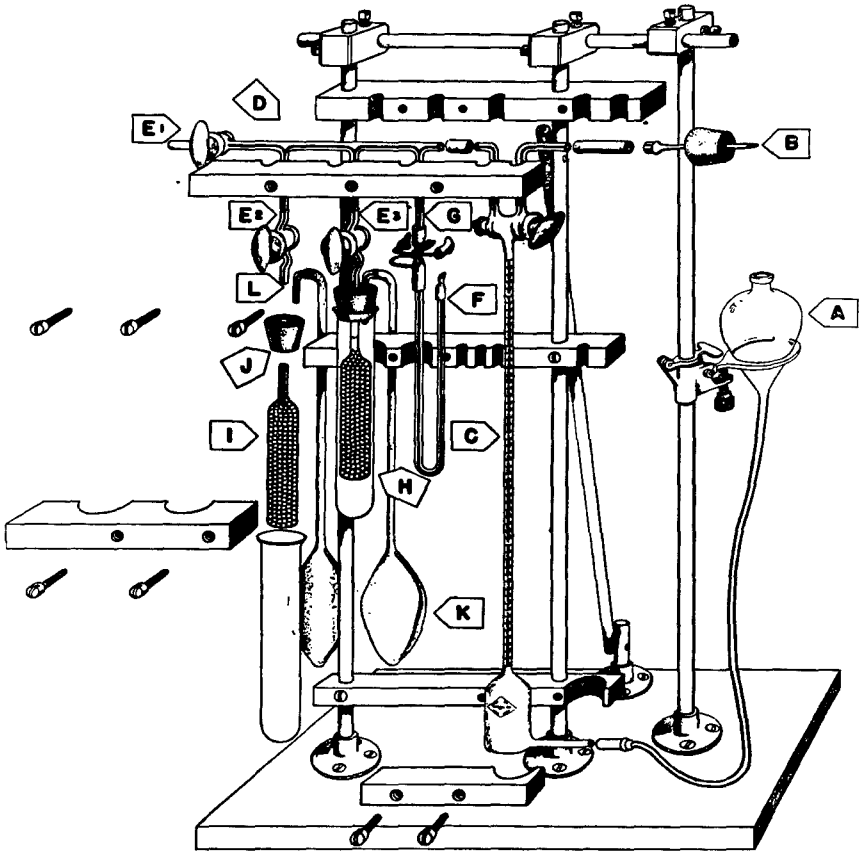

FIG. 1.—Semi-micro Orsat gas analyzer.

DESCRIPTION OF APPARATUS

The assembled apparatus, as shown in Figure 1, is mounted on a plywood base; the framework is constructed from Flexiframe. Alkali-resistant glass is used throughout.

(A) *Leveling bulb.*—125 ml capacity, connected to gas buret (C) with Tygon tubing.

(B) *Sampling device.*—A No. 20 hypodermic needle is inserted thru a No. 6 rubber stopper so that the point extends ca 0.5 inches. The shank of the needle is attached to the gas buret by means of Tygon tubing.

(C) *Gas buret.*—Van Slyke type, over-all length 56 cm, 5 ml capacity with 0.01 ml divisions, or 10 ml capacity with 0.02 ml divisions.

(D) *Manifold.*—25 cm length of 1 mm capillary tubing to which are perpendicularly sealed, at 5 cm intervals, 3 Pyrex No. 3360 stopcocks E_1, E_2, E_3.

(F) *Manometer.*—1 mm capillary tubing, bent as pictured, 18 cm over-all, connected to the manifold (D) with rubber tubing either at a stopcock or at the single capillary opening (G) next to the gas buret. A pinch clamp on the rubber tubing affords quick and more positive control of the manometer during pressure adjustment.

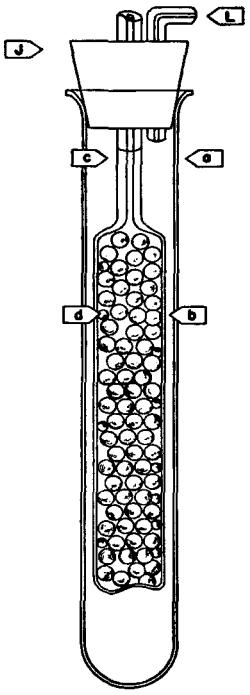

FIG. 2.—Detail of absorber (see "H," Fig. 1).

(H) *Carbon dioxide absorber.*—(Supplementary Figure 2): (*a*) an outer test tube, 22×175 mm; (*b*) an inner test tube, 18×105 mm, modified by sealing 70 mm of 1 mm capillary tubing (*c*) to the bottom, scratching a meniscus mark on the capillary tubing (*c*) ca 2 cm from the seal, and reducing the length of the test tube from 150 mm to 105 mm. In order to afford greater surface absorption of gas, the inner tube (*b*), is filled with 4 mm glass beads (*d*), which are held in place either by glass wool or by crimping.

(I) *Oxygen absorber.*—Identical in construction with (H).

(J) *Rubber stoppers.*—No. 4, two-hole.

(K) *Gas bags.*—Connect to absorbers by 1 mm capillary tubing (L) inserted thru (J).

REAGENTS

(a) *Potassium hydroxide soln.*—Dissolve 60 g of KOH, C. P., in 60 ml of distd water.

(b) *Alkaline pyrogallol soln.*—Dissolve 1.5 g of pyrogallol, C. P. in 30 ml of (a).

(c) *Mercury.*—C. P., ca 4 lbs.

(d) *Manometer soln.*—Dissolve 0.01 g of amaranth in 7.6 ml of 1% Tergitol; dil. to 100 ml with distd water.

PREPARATION FOR ANALYSIS

With any good household or rubber cement, seal one rubber disk ($\frac{3}{4}\times\frac{3}{4}\times 3/16$ inch) to each side of laminated foil packets so that the two disks are in the same relative position. (One rubber disk is sufficient for cans or rigid containers.) Assemble apparatus with 5 ml gas buret as shown in Figure 1. Fill the manometer (F) with soln (a), then fill the leveling bulb (A) with mercury, introducing ca 0.1 ml of water or glycerine, so that it floats above the mercury in the gas buret (C). (The purpose of these liquids is to maintain a constant humidity in gas samples.) Then fill absorber (H) with ca 30 ml of reagent (a), so that after inner tube (*b*) is filled to the meniscus mark and inserted in the outer tube (*a*) (Fig. 2), the reagent level will be ca 2 cm below the seal. Fill absorber (I) with reagent (b).

Saturate absorbers (H) and (I) by passing in four 4 ml air samples without recording results. This operation also provides inert gas for the manifold, manometer, absorber system. Check reagents and apparatus by analyzing several samples of air, using the technique outlined below, until the theoretical carbon dioxide (0.1%) and oxygen (20.94%) content of air is attained (14).

DETERMINATION

Close all stopcocks and the manometer pinch clamp. Open buret stopcock and pump mercury until liquid just reaches the tip of the sampling device (B). Close the stopcock, insert the needle by forcing one rubber disk against the needle, so that the needle just penetrates the packet interior. Open the buret stopcock and withdraw the max. gas sample (ca 3–5 ml in laminated foil packages) by lowering the leveling bulb (A). Close the stopcock to the mercury and open it to the manifold. Approximate atmospheric pressure by means of the leveling bulb (A) and finally adjust exactly to atmospheric pressure by means of the manometer (F) and leveling bulb (A). Record initial volume V_1.

Attach manometer pinch clamp, open stopcock (E_1) of absorber (G), and pump gas sample in and out to constant vol. (ca 5 times). Level liquid to meniscus mark, adjust to atmospheric pressure, and record value V_2. % $CO_2 = (V_1 - V_2) \times 100/V_1$. Repeat operation in absorber (H) and record value V_3. % $O_2 = (V_2 - V_3) \times 100/V_1$.

Table 1 demonstrates the accuracy of the apparatus when air samples were analyzed by a chemist who had used the apparatus on only one occasion prior to collecting these data.

TABLE 1.—*Analyses of air*

RUN	PER CENT CO_2	PER CENT O_2
1	none found	20.92
2	none found	20.97
3	none found	20.95
4	none found	20.91
Av.	none found	20.94
Theory	0.1	20.94±0.2%

In order to check the relative accuracy of the micro Orsat, an operator analyzed the gas in the headspace of a No. 10 can of dry whole milk powder with both types of apparatus. The results are given in Table 2.

TABLE 2.—*Comparison of macro and semi-micro Orsat*

MACRO DATA			SEMI-MICRO DATA		
RUN	PER CENT CO_2	PER CENT O_2	RUN	PER CENT CO_2	PER CENT O_2
1	1.2	0.6	1	1.2	0.6
2	1.4	0.5	2	1.1	0.6
3	1.2	0.6	3	1.3	0.5
Av.	1.3	0.6	Av.	1.2	0.6

Table 3 demonstrates the need for close control of flexible packets if they are to meet military specifications. The data indicate the divergence which may crop up in one series of packets, all of which were packaged at the same time with the same dried cream product.

TABLE 3.—*Control packet analyses*

CODE NO.	PER CENT CO_2	PER CENT O_2
1	1.5	13.6
2	0.8	0.4
3	0.9	6.6
4	none found	16.3
5	0.7	2.1
6	0.7	1.1
7	1.8	0.7
8	0.4	2.0
9	1.4	0.4
10	1.2	0.4

Military specification (6), stipulates that if more than 5 per cent of the packets in a lot indicate leakage by the vacuum test, the entire lot shall be rejected. In the lot tested (Table 3) four packets exceeded the 2 per cent oxygen maximum. Hence, the lot would have to be rejected.

The optimum oxygen level and percentage of deviation from this undetermined level is at present under study.

SUMMARY

A semi-micro gas analyzer, which utilizes the Orsat principle, is described. With a minimum of training, an operator can analyze headspace gases for carbon dioxide and oxygen with an accuracy of ± 0.2 per cent on samples ranging from 1 to 10 ml of gas. The apparatus is simple in construction, rapid in operation, and inexpensive in initial cost. The design is primarily for the analysis of the small flexible packets used by the Armed Forces, but may be modified for other containers where only a small volume of headspace gas is available.

REFERENCES

(1) LEA, C. M., MORAN, T., and SMITH, J. A., *J. Dairy Research*, **13**, 162 (1943).
(2) TORASSUK, N. P., and JACK, E. L., *J. Dairy Sci.*, **31**, 255 (1948).
(3) COULTER, S. T., JENNESS, R., and CROWE, L., *ibid.*, **30**, 995 (1947).
(4) ———, and JENNESS, R., "Packing Dry Whole Milk in Inert Gas," *Technical Bulletin* **167**, p. 4, University of Minnesota Agricultural Experiment Station, June, 1945.
(5) REMALEY, R. J., STOLTZ, P. C., and HENING, J. C., *Dairy Products*, **7**, Operation Studies Number One, QMF&CI, 1949.
(6) MIL-M-3260A, 21 August 1951.
(7) MIL-M-1459A, 31 July 1952.
(8) BLACET, F. E., and LEIGHTON, P. A., *Ind. Eng. Chem., Anal. Ed.*, **3**, 266 (1931).
(9) NASH, L. K., *Anal. Chem.*, **18**, 505 (1946).
(10) CARTWRIGHT, L. C., *ibid.*, **18**, 779 (1946).
(11) LEWIS, V. M., *ibid.*, **21**, 635 (1949).
(12) PITTS, J. N., DEFORD, D. D., and RECKTENWALD, G. W., *ibid.*, **24**, 1566 (1952).
(13) SCOTT, W. W., *Standard Methods of Analysis*, **5**, Vol. 2, D. Van Nostrand Co., New York, 1939, p. 2349.
(14) DENNIS, L. M., *Gas Analysis*, Macmillan Co., New York, 1920, p. 371.

DETERMINATION OF FURFURAL, PENTOSES, AND PENTOSANS IN DISTILLED SPIRITS*

By ALEX P. MATHERS and JOHN E. BECK (Alcohol and Tobacco Tax Division, Laboratory Section, Internal Revenue Service, Washington 25, D. C.)

The pentose and pentosan content of distilled spirits is derived from the wooden containers in which the products are stored. The furfural usually comes from the same source, but may have its origin in the distillate, particularly when pot stills are employed. Colorimetric, gravimetric, polarographic, spectrophotometric, and titrimetric procedures have been used for furfural determinations. Analytical methods for pentoses and pentosans are based on hydrolysis of the pentosans to pentoses, and dehydration of the pentoses to furfural, followed by a determination of the furfural produced.

The A.O.A.C. method (1) for pentosan determination in dry wines, grain, and stock feed utilizes 12 per cent HCl in the reaction vessel with continuing distillation during the reaction period to bring about the formation and recovery of furfural. The furfural in the distillate is precipitated with phloroglucin and weighed. Krober (2, 3), in rather extensive work on the pentosan determination, found that arabinose and xylose were converted into furfural in about 74 and 88 per cent yields, respectively. The formula for calculating the pentose or pentosan content indicates that about 80 to 81 per cent of the theoretical amount of furfural is recovered. This is based on the presence of equal parts of xylan and arban.

A method for the quantitative estimation of furfural with bromine at 0°C. (4), and procedures for the quantitative formation of furfural and methyl furfural from pentoses and methyl pentoses (5, 6), have been presented by Hughes and Acree.

A special type of reaction and distillation apparatus was designed for the present study. Figure 1 represents the essential components, which consist of a reaction chamber "a" with attached water-cooled condenser and steam inlet tube; flask "b" with attached water-cooled reflux condenser, into which toluene, xylene, or other solvent may be added for maintaining constant temperature in the reaction chamber; and a steam generator "c" for passing either steam or acid vapors into the reaction chamber.

The temperature in the reaction chamber can be controlled quite accurately with no possibility of superheating the sample by choice of proper solvent mixture in flask "b." Homogeneity of the solution in the reaction chamber can be maintained by addition of a suitable salt or acid to the sample. Boiling xylene (137°–140°C.) gives about the maximum tempera-

* Presented at the Sixty-seventh Annual Meeting of the Association of Official Agricultural Chemists, held at Washington, D. C., Oct. 12, 13, and 14, 1953.

ture which can be advantageously employed in the reaction. Above this temperature, considerable decomposition of pentosans and pentoses takes place with production of products other than furfural. The externally located and separately heated steam generator permits the use of water or volatile acid solutions in furnishing steam for distilling the furfural as rapidly as it is formed. Additional water or volatile acid is added to the generator as required through a dropping funnel at approximately the rate of distillation. At 137°C. the rate of distillation must be at least

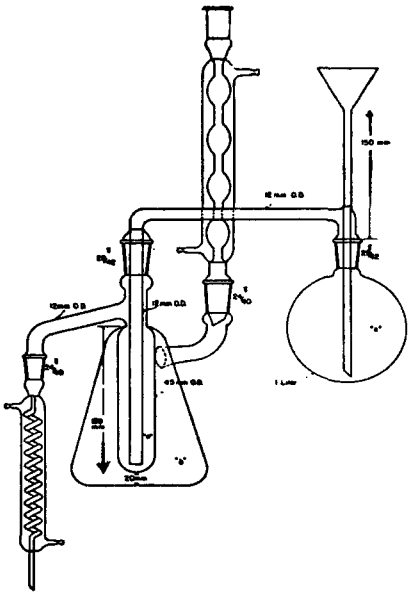

FIG. 1.—Controlled temperature still.

100 ml in 15 minutes, and the furfural is usually recovered in 500 to 600 ml of distillate. At 110°C. the distillation rate need not be as rapid but the total amount of distillate required is about 2,000 ml—more if much arabinose is present.

The furfural was determined spectrophotometrically, utilizing its absorption at 277 mμ. This method is more rapid than either the gravimetric procedure or the bromine absorption method. The three methods suffer from a common fault—substances other than furfural can give the characteristic reaction measured, *viz.*, a precipitate with phloroglucin, absorption of bromine, or absorption of light at 277 mμ. In this work, the A.O.A.C. method was not examined exhaustively, but the bromine absorption and spectrophotometric methods were usually compared. The

bromine method was inapplicable if diethylamine hydrochloride was employed in the reaction chamber, as an excessive quantity of bromine was consumed. When pure sugars were used, results obtained by the two procedures agreed quite closely but the bromine absorption method gave much higher results with impure sugars or distilled spirits. A critical examination of both furfural methods indicated that either will give the total furfural content but certain impurities may cause positive errors in either or both cases. In this work, the spectrophotometric results were never greater than those obtained by bromine absorption.

Several acids, including sulfuric, *p*-toluene sulfonic, phosphoric, oxalic, and hydrochloric, were investigated for the conversion of pentoses and pentosans to furfural. The most quantitative results were obtained with hydrochloric acid or a combination of hydrochloric acid with diethylamine. Phosphoric or *p*-toluene sulfonic acids gave lower but reproducible yields of furfural. Sodium chloride, calcium chloride, and a number of salts were suitable for use with hydrochloric acid at 110°C., but at 137°C. the common mineral salts became dehydrated. At this temperature it was necessary to use salts of the diethylamine hydrochloride type and phosphoric or *p*-toluene sulfonic acid with hydrochloric acid. Sulfuric acid always gave poor yields of furfural.

Eight reaction systems are described in Table 1. Numerous other suitable systems could be devised. The hydrochloric acid initially placed in the steam generator is prepared by addition of three volumes of water to

TABLE 1.—*Reaction systems for converting pentoses and pentosans to furfural*

SYSTEM NO.	REACTION CHAMBER "a"	FLASK "b"	STEAM GENERATOR "c"
1	85% Phosphoric acid, 20 ml	Xylene	Water
2	85% Phosphoric acid, 20 ml	Toluene	
3	*p*-Toluene sulfonic acid, 20 g	Xylene	
4	*p*-Toluene sulfonic acid, 20 g	Toluene	
5	85% Phosphoric acid, 20 ml; Hydrochloric acid, concd, 5 ml	Xylene	9% HCl
6	*p*-Toluene sulfonic acid, 20 g; Hydrochloric acid, concd, 5 ml	Xylene	
7	Diethylamine, 15 ml; Hydrochloric acid, concd, 20 ml	Xylene	
8	Sodium chloride, 15 g; Hydrochloric acid, concd., 10 ml	Toluene	

one of concentrated hydrochloric acid, but additional acid added to the generator has the composition of nine volumes of water to one of concentrated acid.

EXPERIMENTAL

Ultraviolet spectrum of furfural.—Furfural was redistilled through a short fractionating column at atmospheric pressure and the center fraction was used. One ml of furfural was accurately weighed into a 100 ml volumetric flask, 25 ml of 95% ethyl alcohol was added, and the solution was made to mark with distilled water. Successive dilutions were made with water to give a final solution containing 5.8 mg of furfural per liter. Figure 2 illustrates the characteristic ultraviolet absorption spectrum of furfural in aqueous solution.

Preparation of standard curve for furfural.—The absorption maximum at 277 mμ

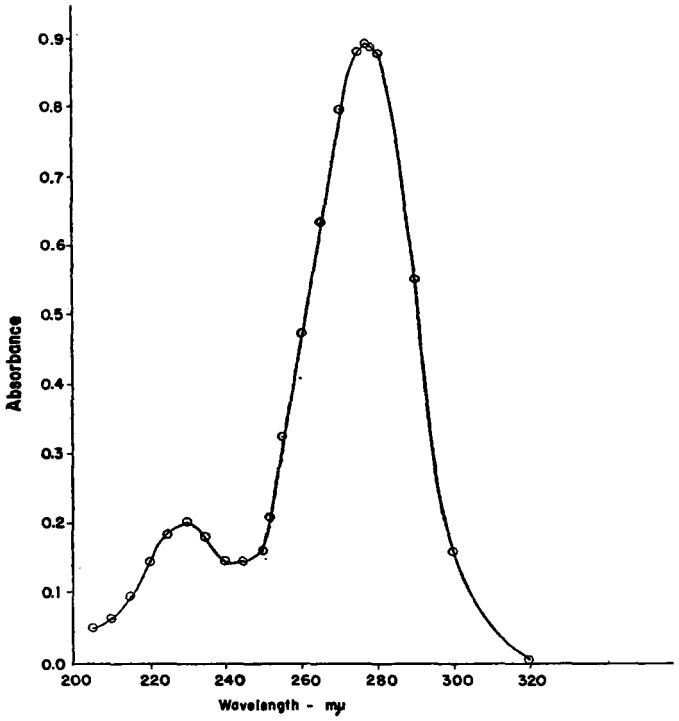

FIG. 2.—Ultraviolet absorption spectrum of furfural (5.804 mg/liter).

was chosen in the preparation of a calibration curve for 1, 2, 3, 4, and 5 mg of furfural per liter. Figure 3 demonstrates that the absorbance at this wavelength is proportional to the concentration of furfural. The presence of ethyl alcohol or hydrochloric acid has little or no effect on the position or height of the absorption peak.

Determination of furfural.—Pipet 25 ml of distilled spirits into a volatile acid distillation flask (7) and steam distil until 150 to 200 ml of distillate is collected. Determine the absorbance of the distillate at 277 mμ. Calculate the furfural value of the sample by the following formula:

$$\text{Furfural (mg)} = \frac{A_{(obs)}}{A_{(mg/l)}} \times V \times F,$$

where:

$A_{(obs)}$ is observed absorbance

$A_{(mg/l)}$ is the absorbance of a solution containing 1 mg of furfural per liter (0.154 in this work)

V is the volume of distillate in liters

F is the dilution factor (volume of final solution on which absorbance is determined divided by volume of sample).

Pentose or pentosan determination.—(*System No. 1.*)—Introduce 20 ml of phosphoric acid (85%) into reaction chamber, and add 25 ml of distilled spirits. Connect

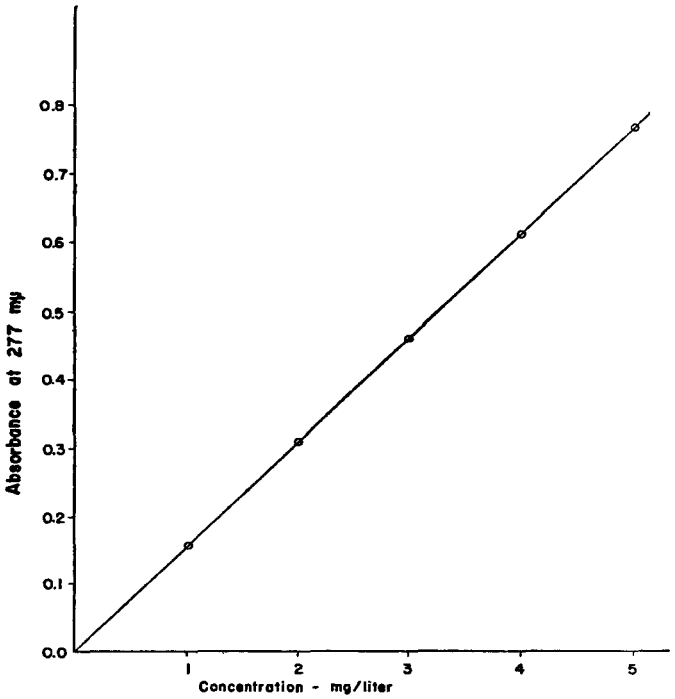

FIG. 3.—Absorbance-concentration curve for furfural.

vessel to steam generator and bring water almost to boiling before applying heat to flask "b" containing xylene. Bring xylene to reflux temperature rapidly and at the same time admit steam. (Distillation rate should be not less than 100 ml in 15 minutes.) Collect 500 ml of distillate, and then additional 100 ml in portions, testing for completion of reaction by absorption at 277 mμ. When no additional furfural is found, combine the distillates and read the absorbance at 277 mμ, diluting if necessary. Make calculations as above for furfural. Subtract the furfural found by preceding steam distillation to obtain that produced from wood sugars. Divide this value by 0.81 to correct for loss during conversion. The corrected furfural value multiplied by 1.5626 gives the mg of pentose per 100 ml of sample. The corrected furfural value multiplied by 1.3750 gives the mg of pentosans per 100 ml of sample.

Conversion of sugars to furfural and allied products.—Aqueous solutions of ribose, xylose, arabinose, dextrose, sucrose, and caramel were prepared and 10 ml portions of these solutions and 25 ml portions of distilled spirits were added to the reaction vessel. Table 2, Part A, shows the data obtained when the tests were run with system No. 1; Part B with system No. 8; and Part C with system No. 5. The results were calculated as mg of furfural, even though the substance giving the reaction may have been methyl furfural, hydroxymethyl furfural, or other breakdown products of the reaction.

Rate of formation of furfural.—Figure 4 shows the rate at which furfural is recovered in the distillate, using system No. 1 with xylose, ribose, arabinose, and whisky. The volume of distillate is plotted against mg of furfural recovered rather than

TABLE 2.—*Yields of furfural from sugars plus distilled spirits*

SAMPLE	WEIGHT (MG) OR VOL. (ML)	VOLUME OF DISTILLATE (ML)	FURFURAL (MG)			PER CENT YIELD (SPECTROPHOTOMETER)
			SPECTROPHOTOMETER	BROMINE TITRATION	THEORETICAL	
A. Reaction system No. 1. Approx. temp. = 137°C.						
Xylose	22.77	800	11.69	14.74	14.57	80.23
Ribose	13.13	800	6.40	7.06	8.40	76.19
Arabinose	22.36	800	8.35	11.12	14.31	58.35
Dextrose	41.10	600	3.94	5.47		
Sucrose	43.69	600	0.10	0.14		
Caramel	68.55	600	2.63	3.55		
4 yr old Whisky	25 ml	600	4.00	15.55		
4 yr old Brandy	25 ml	600	5.43	8.86		
4 yr old Rum	25 ml	600	2.73	5.90		
B. Reaction system No. 8. Approx. temp. = 110°C.						
Xylose	22.77	1400	13.35	14.11	14.57	91.63
Ribose	18.30	1000	10.01	11.98	11.71	85.48
Arabinose	23.90	2000	11.47	14.10	15.30	74.97
Dextrose	33.03	2000	2.21	2.24		
Sucrose	43.69	600	0.20	0.00		
4 yr old Whisky	25 ml	1200	4.50	7.20		
4 yr old Brandy	25 ml	1400	5.73	8.28		
4 yr old Rum	25 ml	1200	3.31	6.44		
C. Reaction system No. 5. Approx. temp. = 137°C.						
Xylose	22.77	800	12.92	14.11	14.57	88.68
Ribose	18.30	800	9.08	10.61	11.71	77.54
Arabinose	22.36	800	8.73	10.46	14.31	61.01
Dextrose	41.10	600	3.73	6.34		
Sucrose	43.69	600	0.01	0.00		
Caramel	137.10	800	5.30	7.20		
4 yr old Whisky	25 ml	600	4.54	8.35		
4 yr old Brandy	25 ml	600	5.37	8.82		
4 yr old Rum	25 ml	600	3.36	6.00		

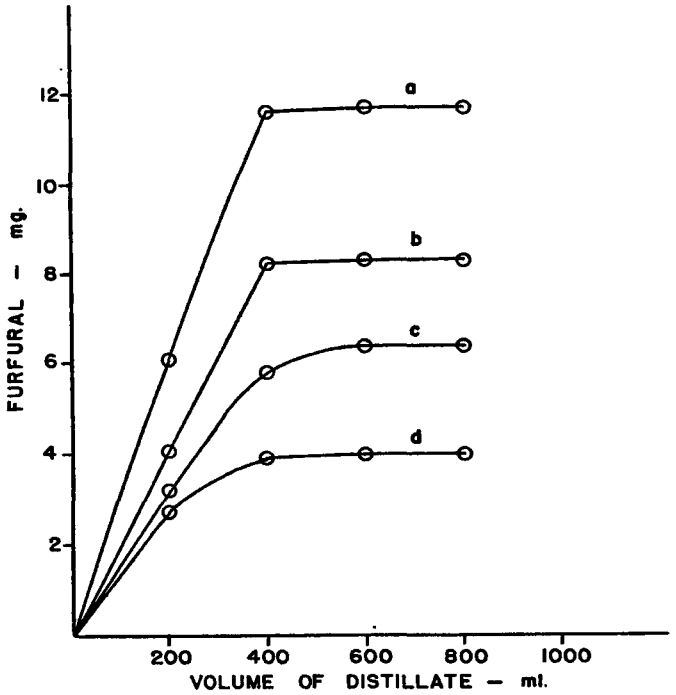

FIG. 4.—Recovery of furfural from sugars by distillation system No. 1.
a. Xylose b. Arabinose c. Ribose d. Whisky Sugars

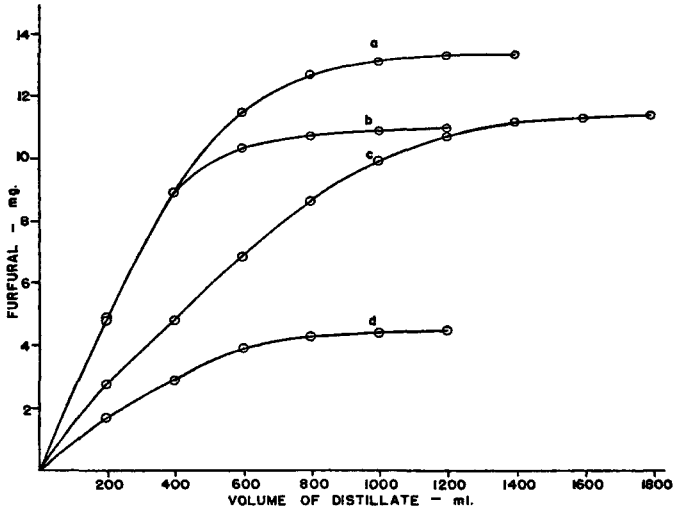

FIG. 5.—Recovery of furfural from sugars by distillation system No. 8.
a. Xylose b. Ribose c. Arabinose d. Whisky Sugars

per cent yield, because the theoretical yield is based on gravimetric values without taking into consideration the purity of the sugars. Similar data are shown in Figure 5 for system No. 8.

DISCUSSION

The sugars used in these experiments were labeled C.P., but their degree of purity was unknown. Their melting points were considerably below the literature values. Arabinose gave a slightly yellow solution in water, and during the reaction turned almost black. Xylose, which was very nearly white, gave a colorless solution, and during the reaction became very light tan. Ribose (which was a little off-color) produced a very slightly colored solution and a tan color during the reaction. Attempts were made to recrystallize the arabinose as it apparently contained considerable impurities.

One fraction of recrystallized material from the arabinose gave furfural values between 74 and 75 per cent of theoretical when distilled from any of the eight reaction systems of Table 1. Another fraction gave zero recovery of furfural with systems No. 1 and No. 8, which were the only ones tested. A third fraction gave 79.35 per cent recovery of furfural with system No. 8, and 60.5 per cent with No. 1. Surprisingly, sucrose gave almost no detectable degradation products, even though it must have been inverted in the course of the reactions. Dextrose, c.p., varied from bottle to bottle.

Results from any one of the systems were highly reproducible, even though the analytically recovered amount of furfural varied from system to system. Because of the simplicity of system No. 1, and since no acid distils during the reaction, it was considered preferable for routine analysis. System No. 7 was not extensively investigated but had several points of superiority; the most important was that it consistently gave high recovery of furfural and could be operated at high temperature, and thus shortened the distillation period.

The fact that 100 per cent recovery of furfural from any of the pentoses was not achieved by the spectrophotometric determination does not necessarily reflect on the quantitative conversions reported by Hughes and Acree. It may merely indicate that the sugars employed here were not analytically pure. However, in a number of tests where the spectrophotometer indicated between 75 and 95 per cent recovery of furfural, the bromine absorption method gave values of from 95 to 101 per cent. This work does not throw any new light on the possibility of 100 per cent conversion of pentose to furfural. Until further work has been done, the 81 per cent average conversion of pentose to furfural, as used in the A.O.A.C. method, seems a desirable figure to use.

SUMMARY

A design for a reaction apparatus for use in pentose and pentosan determinations is shown.

Reaction systems are described for the conversion of pentoses and pentosans to furfural.

A spectrophotometric method is presented for determining furfural, pentoses, and pentosans in distilled spirits or aqueous solution.

ACKNOWLEDGMENT

The authors are indebted to Arthur D. Etienne of this laboratory for the construction of the apparatus and his aid in its design.

REFERENCES

(1) *Official Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Box 540, Benjamin Franklin Station, Washington 4, D. C., 1950, p. 350.
(2) KROBER, E., *J. Landw.*, **48**, 357 (1900).
(3) *Official Methods of Analysis*, 7th Ed., p. 833.
(4) HUGHES, E. E., and ACREE, S. F., *Ind. Eng. Chem., Anal. Ed.*, **6**, 123 (1934).
(5) ———, and ———, *J. Research Natl. Bur. Standards*, **21**, 327 (1938).
(6) ———, and ———, *ibid.*, **23**, 293 (1939).
(7) *Official Methods of Analysis*, 7th Ed., p. 173.

THE DETERMINATION OF PROPYLENE GLYCOL AND GLYCEROL IN FOODS AND MEDICINALS

By ALEX P. MATHERS and MAYNARD J. PRO (Alcohol and Tobacco Tax Division, Laboratory Section, Internal Revenue Service, Washington 25, D. C.)

Both glycerol and propylene glycol have wide use in the manufacture of numerous commercial products. Elving (1) discusses some of the inherent difficulties in glycerol determinations which pertain to fermentation residues. These problems are increased in attempting to devise a procedure of universal applicability. The experimental work forming the basis of this paper indicates that the procedure described may have wider applicability than previously published analytical methods.

Analytical methods in the past have been developed for the determination of these compounds in specific products. Koppe (2), Lawrie (3), and *Official Methods of Analyses* (4) describe a number of useful analytical procedures for glycerol. Malaprade (5, 6) introduced periodic acid as a reagent for glycols after noting its specificity on 1,2-glycols and related compounds. Shupe (7) used potassium periodate and described procedures for the determination of glycerol, ethylene glycol, and propylene glycol in certain cosmetic preparations. Métayer (8) investigated methods of codistillation and entrainment of glycols and showed that they could be quantitatively distilled. Isacoff (9–12) and Bruening (13), in rather extensive investigations, showed the applicability of the distillation technique

combined with periodate oxidation for the quantitative determination of propylene glycol. Thaler (14) and Keppel (15) removed sugars, proteins, and other substances by precipitation; the remaining glycerol was then determined by periodate oxidation.

The procedures described in this paper utilize precipitation of interfering substances and steam distillation of propylene glycol and glycerol at elevated temperatures from a special still (Fig. 1). The periodic acid oxidations are carried out in a manner similar to those described in the references.

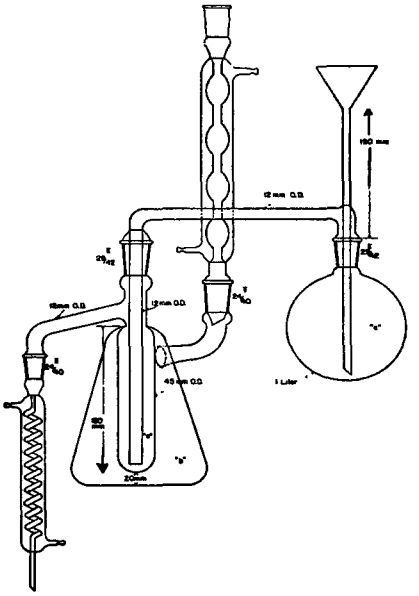

FIG. 1.—Controlled temperature still.

METHOD

APPARATUS

(a) *Steam distillation app.*—Described by Mathers and Beck (16).[1]

REAGENTS

(a) *Arsenious oxide.*—0.1 *N*, *Official Methods of Analysis*, par. **39.5–39.6.**

(b) *Starch indicator.*—0.5% soln of sol. starch in water.

(c) *Bromcresol purple indicator.*—50 mg per 100 ml ethanol.

(d) *Sodium hydroxide.*—0.02 *N* soln.

(e) *Sodium periodate.*—Dissolve 10 g of periodic acid in 500 ml of water. Add bromcresol purple indicator, neutralize with NaOH, and make to 1000 ml. The soln is ca 0.05 *M*.

[1] Available from the Ace Glass Co., Vineland, N. J.

PRECIPITATION OF INTERFERING SUBSTANCES

Pipet a sample contg 0.05 to 1 g of propylene gycol and glycerol into a glass-stoppered flask; add 20 ml of anhyd. ethanol, 1 g of Filter-cel, and 50 ml of anhyd. ether; shake thoroughly and decant through a filter paper, using suction. To the residue add 10 ml of ethanol, warm, and shake to effect disintegration; add 25 ml of ether, shake, and decant through the same filter. Repeat twice. Reduce the vol. of filtrate to about 50 ml on a steam-bath, using a short air condenser to prevent any loss of the glycols.

PROPYLENE GLYCOL

Distillation.—Propylene glycol alone is detd by using xylene (b. p. 135–137°) in the outer chamber of the distn app., with 15 g of sodium formate and 10 g of KOH in the inner chamber. Add the filtrate from the preceding step to the inner chamber. Initially, heat the distn flask quite strongly, while steam is admitted slowly, to drive over most of the remaining alcohol and reduce the vol. of liquid in the inner chamber. Heat sufficiently thereafter to maintain a xylene reflux, and a distn rate of about 100 ml per 15 min. Collect 400 ml of distillate. (In most cases the propylene glycol is quantitatively distd in the first 200 ml.)

Oxidation.—Transfer an aliquot of the distillate contg about 50 mg of propylene glycol to a flask (a trial oxidation may be necessary to select proper sample size, as an excess of periodate must be present. This also applies to the glycerol determination). Add 25 ml of periodate soln and allow to stand in the dark for one hr. Add 1 g of sodium bicarbonate and 0.5 g of KI, and titrate at once with the arsenite soln, using starch indicator. The difference between the arsenite titration obtained from the sample and a 25 ml periodate blank soln measures the periodate reduced by the propylene glycol. (1 ml of 0.1 *N* arsenite = 3.805 mg of propylene glycol.)

GLYCEROL

Distillation.—The distn is the same as for propylene glycol with the exception that 20 ml of mineral oil (U.S.P. light) is placed in the inner chamber of the app. instead of the sodium formate–KOH. About 1400 to 1500 ml of distillate must be collected to obtain a quantitative yield of glycerol.

Oxidation.—Neutralize an aliquot of the above distillate, contg not more than 25 mg of glycerol and 25 mg of other glycols, to bromcresol purple and add 25 ml of neutral periodate soln. Oxidize for one hr in the dark and then titrate to the bromcresol purple endpoint with 0.02 *N* NaOH. 1 ml of 0.02 *N* NaOH = 1.84 mg glycerol.

PROPYLENE GLYCOL AND GLYCEROL SIMULTANEOUSLY

Distillation.—The distn with mineral oil is used. Collect the distillate in two portions consisting of 400 ml and 1000 ml.

Oxidation.—Oxidations are carried out as previously indicated for propylene glycol and glycerol. In the first fraction of distillate both propylene glycol and glycerol are detd so that the periodate consumed by glycerol can be subtracted from the total periodate consumed. The difference is due to the propylene glycol. 1 ml of 0.02 *N* NaOH = 0.8 ml of 0.1 *N* arsenite.

Combine aliquots of the two portions of distillate in the ratio of 4:10 for the glycerol determination.

EXPERIMENTAL

These methods have been applied to medicinal elixirs containing propylene glycol and glycerol prepared according to Isacoff (11), wines with and without added increments of glycerol, an egg mixture as prepared by

Keppel (15), and several commercial preparations. Results are given in Table 1.

DISCUSSION

Propylene glycol recovery was in the range of 98 to 101 per cent whether distilled in the presence of mineral oil or sodium formate–potassium hydroxide. The latter medium has the advantage of preventing the distillation of glycerol. Samples tested by this procedure showed that propylene glycol was usually recovered in the first 200 ml of distillate. Distillation from mineral oil required 300 to 400 ml of distillate in order to recover propylene glycol quantitatively. A small amount of mineral oil distills but does not interfere with either the propylene glycol or glycerol determinations.

The addition of sorbitol and dextrose in the amount of 10 g per 100 ml of sample caused no interference in the tests. Apparently these materials were removed by the precipitation. A number of commercial products not reported in this paper were tested with excellent results.

Using sodium formate and potassium hydroxide in the inner chamber of the distillation apparatus, tests were made on several types of samples for propylene glycol in the presence of sugars and glycerol without the precipitation steps with alcohol and ether. No glycerol or other interfering material distilled; however, insufficient work was done to establish this as a satisfactory procedure in all cases.

Glycerol recovery was in the range of 97 to 101 per cent of theory. In distilling the egg product there was some foaming, and it was found necessary to add "Anti-Foam A" to prevent the material from frothing over into the receiver. The anti-foam compound had no adverse effects on the determination, and its use permitted a smooth distillation.

The addition of the sample to the distillation flasks, without added material to retain a liquid medium, caused some charring on the sides of the inner tube. Various materials which would maintain a liquid in the inner chamber of the distillation apparatus were evaluated. It was found that salts inhibited the distillation of glycerol to some degree. Likewise, those salts which dehydrated and formed solids made the propylene glycol distillations more difficult. Sodium formate and potassium hydroxide melted very readily to form a homogeneous solution which was maintained throughout the distillation. Potassium sodium tartrate acted similarly but some glycerol was found in the distillate. Mineral acids were not found desirable. Tetralin and decalin steam-distilled more rapidly than mineral oil and were less desirable. A more extensive search may reveal a better material than mineral oil for the glycerol distillation.

Higher boiling solvents than xylene were tested in the glycerol distillations. These brought about a slightly more rapid distillation of the glycerol, but the tendency of some sample material to char on the sides of the inner

TABLE 1.—*Recovery of propylene glycol and glycerol*

MATERIAL TESTED	QUANTITY ADDED (G/100 ML)		RECOVERY (G/100 ML)		RECOVERY PER CENT	
	PROPYLENE GLYCOL	GLYCEROL	PROPYLENE GLYCOL	GLYCEROL	PROPYLENE GLYCOL	GLYCEROL
U.S.P. light liquid petrolatum in inner chamber of distillation apparatus						
Aqueous Solution	9.96	0.00	9.98	0.00	100.2	
Aqueous Solution	0.00	11.08	0.00	11.04		99.6
Aqueous Solution	9.96	11.08	9.96	11.04	100.0	99.6
Elixir No. 1	0.00	0.00	0.00	0.00		
Elixir No. 2	0.498	0.554	0.502	0.546	100.8	98.6
Elixir No. 3	4.98	5.54	5.00	5.40	100.4	97.5
Elixir No. 4	9.96	11.08	9.80	11.40	98.4	102.9
Wine A		0.00		.38		
Wine A		1.04		1.43		100.6
Wine B		0.00		.72		
Wine B		1.04		1.70		98.1
Cough Syrup[a]			0.00	0.00		
Cough Syrup[b]			0.00	18.40		
Tonic[c]			15.30	0.00		
Egg Product				0.00		
Egg Product		11.08		11.20		101.1
Sodium formate-potassium hydroxide in inner chamber of distillation apparatus						
Aqueous Solution	9.96	0.00	9.90	0.00	99.4	
Aqueous Solution	9.96	11.08	10.00	0.01	100.4	0.1
Elixir No. 1	0.00	0.00	0.00	0.00		0.0
Elixir No. 2	0.498	0.554	0.49	0.00	98.4	0.0
Elixir No. 3	4.98	5.54	5.02	0.02	100.8	0.4
Elixir No. 4	9.96	11.08	10.05	0.00	100.9	
Cough Syrup[a]			0.00	0.00		
Cough Syrup[b]			0.00	0.01		
Tonic[c]			14.96	0.00		

[a] This commercial syrup had been difficult to analyze by a number of other methods; according to formula it contained no glycerol or propylene glycol but did contain a number of natural extractives.

[b] This commercial syrup, according to formula, had been prepared with some natural extractives percolated with glycerol and should contain 20% glycerol if none was lost in the processes.

[c] This commercial tonic contained 15% propylene glycol according to formula and a number of natural extractives but no glycerol.

tube was also greater. This charring apparently had no adverse effects on either propylene glycol or glycerol determinations but did make it more difficult to clean the distillation apparatus. Ordinarily, sufficient cleansing was provided by pouring water or an organic solvent into the inner chamber of the distilling apparatus and removing it with an aspirator. Use of a larger steam generator to double the rate of distillation appears to offer more advantage than higher boiling solvents, although a complete study of this problem was not made.

The necessity for oxidation of either compound in the dark was not substantiated, but a number of references indicate a photochemical effect of periodates on formaldehyde and formic acid.

SUMMARY

Quantitative steam distillation procedures are described for propylene glycol and glycerol. It is shown that propylene glycol can be quantitatively separated from glycerol by distillation in the presence of sodium formate and potassium hydroxide. Methods are presented for both propylene glycol and glycerol determinations in several types of products.

REFERENCES

(1) ELVING, P. J., *et al.*, *Anal. Chem.*, **20**, 25 (1948).
(2) KOPPE, S. W., *Glycerine*, 2nd Ed., Scott, Greenwood & Son, London, 1915, pp. 209–231.
(3) LAWRIE, J. W., *Glycerol and the Glycols*, The Chemical Catalog Co., Inc., 1928, pp. 208–260.
(4) *Official Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Box 540, Benjamin Franklin Station, Washington 4, D. C., 1950, p. 892
(5) MALAPRADE, L., *Bull. Soc. Chim.* (4), **43**, 683 (1928).
(6) ———, *Compt. rend.*, **186**, 382 (1928).
(7) SHUPE, I. S., *This Journal*, **26**, 249 (1943).
(8) MÉTAYER, G. L., *Ann. Chim.* (12), **2**, 790 (1947).
(9) ISACOFF, H., *This Journal*, **30**, 484 (1947).
(10) ———, *ibid.*, **33**, 218 (1950).
(11) ———, *ibid.*, **35**, 579 (1952).
(12) ———, *ibid.*, **36**, 734 (1953).
(13) BRUENING, C. F., *ibid.*, **33**, 103 (1950).
(14) THALER, H., and ROOS, W., *Z. Anal. Chem.*, **131**, 24 (1950).
(15) KEPPEL, G. E., *This Journal*, **36**, 195 (1953).
(16) MATHERS, A. P., and BECK, J. E., *ibid.*, **37**, 861 (1954).

THE DETERMINATION OF GLYCEROL AND PROPYLENE GLYCOL IN DESICCATED COCONUT BY DISTILLATION WITH DECALIN*

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Propylene glycol and glycerol are used singly and in combination in foods, drugs, and cosmetics as sweeteners, humectants, solvents, or emollients. Such a variety of uses indicates the need for a method by which these two chemicals may be determined quantitatively.

Malaprade (1, 2) studied the selective oxidation of glycols by periodic

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acid. From his general formula the following reactions for glycerol and propylene glycol may be derived:

$$CH_2OHCHOHCH_2OH + 2KIO_4 \rightarrow 2HCHO + HCOOH + 2KIO_3 + H_2O$$

$$CH_2OHCHOHCH_3 + KIO_4 \rightarrow HCHO + CH_3CHO + KIO_3 + H_2O$$

Shupe (3) used the potassium salt since it is neutral to methyl red indicator and permits the direct titration of formic acid, the key oxidation product in the determination of glycerol. Bruening (4) reported an appreciable volatility for glycerol at 100°C., a fact which made the choice of decalin desirable as the co-distillation agent, since it boils at 197°C. (pract. grade). Isacoff (5) had found the direct distillation of propylene glycol–glycerol solutions in sugar-containing pharmaceutical preparations impractical because of charring. Newburger and Bruening (6) substituted bromocresol purple for methyl red as indicator in the formic acid titration. They also investigated the error due to periodate acidity. Isacoff (10) proposed a correction for periodate acidity in determining propylene glycol in the presence of glycerol. Bruening (7) and Métayer (9) investigated the efficiency and distillation times of these chemicals with several co-distillation agents, and reported further experimentation with decalin.

In adapting a method specifically for the determination of glycerol and propylene glycol in desiccated coconut, the following procedure was finally developed.

METHOD

APPARATUS

(a) *Modified Dean and Stark type distilling tube.*—10–20 ml capacity, with glass joints and stopcock in receiver arm.

(b) *Florence or Erlenmeyer flasks.*—500 ml, with glass joints.

(c) *Condenser.*—Straight tube with glass joints.

(d) *Two microburets.*—0.05 ml graduations (at least 10 ml capacity).

REAGENTS

(a) *Decalin (decahydronaphthalene).*—Practical grade, b.p. 197°C.

(b) *Potassium periodate soln.*—0.02 *M* (0.04 *N*). Dissolve 4.6 g of KIO_4 in ca 500 ml of hot water. Dil. to ca 900 ml with water, cool to room temp., and make to 1 l. Store in brown glass bottle.

(c) *Potassium arsenite soln.*—0.02 *N*. Dil. 1 vol. of U.S.P. 0.1 *N* potassium arsenite soln to 5 vols.

(d) *Sodium hydroxide soln.*—0.02 *N*.

(e) *Bromocresol purple indicator.*—Dissolve 0.1 g of indicator in 100 ml of alcohol.

(f) *Starch indicator.*—*Official Methods of Analysis*, 7th Ed., 17.5 (f).

PREPARATION OF THE SAMPLE SOLUTION

Weigh 100 g of desiccated coconut (may or may not be ground) into 500 ml volumetric flask; add 10 g of lime and 250 ml of water. Warm on steam-bath with occasional shaking for 30 min. Cool to room temp., dil. to vol., and filter.

Pipet an aliquot of ca 25 ml of the prepd soln (contg not more than 50 mg propylene glycol and 35 mg glycerol) into a 500 ml flask, and add a few glass beads.

Add 50–60 ml decalin and connect flask, lubricated with a silicone type grease, to the distg tube of the receiver which has been previously connected to a water-cooled condenser. Using a Meker burner and a sand bath, distil *slowly* until most of the water has been removed, as shown by sharp rise of temp. to ca 200°C. (Water in excess of the amount the receiver holds may be drawn off into a 100 ml volumetric flask.) Continue distn for 2 hrs. Cool, transfer the aq. and decalin layers from Dean-Stark tube into the 100 ml volumetric flask, remove the distn flask, and wash the condenser and receiver with three 10 ml portions of warm water. Add the wash water to the 100 ml volumetric flask, cool, and make the water layer to volume with distd water. There should be very little decalin in the layer (about 4 or 5 ml). Remove decalin by filtering thru a previously moistened filter paper.

Pipet two aliquots of 40 ml (aliquots should contain no more than 20 mg propylene glycol and 15 mg glycerol) into 300 ml Erlenmeyer flasks. To one aliquot add 35 ml 0.02 *M* KIO_4 soln; dil. to about 100 ml and allow to stand one hour. Add 1 g of $NaHCO_3$, 0.5 g of KI, and 5 ml of starch soln. Titrate to a disappearance of the blue color with 0.02 *N* $KAsO_2$. By the same method standardize 35 ml of the 0.02 *M* KIO_4 against the 0.02 *N* $KAsO_2$. The difference between the titrations is the amount of 0.02 *N* $KAsO_2$ used by the combined glycols in the aliquot.

To the other aliquot add a drop of bromocresol purple and 0.02 *N* NaOH until a light purple soln is obtained. (This destroys the residual acidity of the distillate and provides a null point for the following oxidation step.)

Add 35 ml of 0.02 *M* KIO_4 soln, dil. to about 100 ml, and let stand one hr. Add 0.5 ml propylene glycol and allow to stand one-half hr to destroy excess KIO_4. Add 6 drops of bromocresol purple and titrate to a light purple endpoint with 0.02 *N* NaOH.

Determine the acidity of the periodate soln by titration of 10 ml to a bromocresol purple endpoint. Calc. the results to a 35 ml aliquot, as taken for the oxidation, and apply the correction as in the equation below:

Mg glycerol = (total ml 0.02 *N* NaOH) − (ml 0.02 NaOH periodate acidity) × 1.84

Mg propylene glycol = (ml 0.02 *N* $KAsO_2$) − [4 (total ml 0.02 *N* NaOH)] × 0.76.

DISCUSSION

Glycerol and propylene glycol are miscible with water but not with decalin. Glycerol, and more especially propylene glycol, have very considerable volatility at the boiling point of decalin. Hence separation from coconut is best accomplished by extraction with water and co-distillation of this solution with decalin for the exclusion of sugars, fats, and other food elements.

A half hour extraction of the coconut with water at elevated temperatures was found to be sufficient. Distillation of this solution for two hours, as measured from the point at which the water was removed, was found to give quantitative recoveries of both glycerol and propylene glycol. A shorter period may fail to remove both glycols completely. It is essential to time the distillation from that moment at which water is completely removed. This is the point at which the temperature of the mixture rises to about 200°C., which is the approximate boiling point of decalin.

Intense foaming and poor separations occurred when this method was applied to commercial desiccated coconut or moistened coconut. It was believed this foaming was due to the presence of free fatty acids in the

water extract. Antifoams such as the silicones and heavy alcohols did not depress the foaming, but the addition of lime and use of a larger distilling flask helped. The lime appears to form insoluble soaps which in the larger flask do not produce a lasting foam. The addition of lime does not interfere with the distillation rates or efficiency of the separation and improves the clarity of the distillate.

The method provides for a correction due to the acidity of the periodate added in the oxidizing reaction. The change of indicators from methyl red to bromocresol purple resulted from the realization that the periodate acidity must be known in order to compute correctly the results of the reaction. The acidity correction is used when propylene glycol and glycerol are both present in the sample and may also be used when glycerol alone is present. When propylene glycol is alone in the sample solution it is determined by oxidation with periodate by the method outlined by Bruening (7).

Preliminary to the analysis of authentic samples designed to test the method, the laboratory stocks of glycerol and propylene glycol were checked. It was found that the propylene glycol gave a 100.2 per cent recovery and that the glycerol gave 92.8 per cent. The unrecovered 7.2 per cent of the glycerol was shown to consist of 5.8 per cent of propylene glycol (based on the whole); the other 1.4 per cent was undetermined inert material, probably water.

Authentic samples containing known amounts of propylene glycol and glycerol were analyzed. The first of these consisted of the glycols in water, and were designed to test the efficiency of the distillation, particularly with reference to the recommended two-hour period. These tests determined that the period should be timed from the point at which the water was almost expelled, or the point where the temperature rises to about 200°C. (Table 1).

In the next series, known amounts of propylene glycol and glycerol

TABLE 1.—*Determination of the distillation time*

TIME	PROPYLENE GLYCOL			GLYCEROL		
	TAKEN	RECOVERED	RECOVERY	TAKEN	RECOVERED	RECOVERY
hr	*mg*	*mg*	*per cent*	*mg*	*mg*	*per cent*
1	10.53	10.04	95.3	7.89	7.47	94.7
2	4.97	5.11	102.8	2.95	2.96	100.3
2	4.95	4.92	99.4	2.95	2.88	97.6
2	10.53	10.71	101.7	7.89	7.76	98.4
2	10.53	10.79	102.5	7.89	7.73	98.0
2	10.53[a]	10.67	101.3	7.89	7.62	96.6[a]
2	10.53[a]	10.70	101.6	7.89	7.58	94.7[a]

[a] Decalin was used a second time without repurification by distillation. Apparently recovery is diminished.

were added to 50 g samples of fresh coconut meats. (The computed amounts of each in the distillation aliquot are given under the heading "Amount taken for distillation aliquot" (Table 2)).

TABLE 2.—*Results on authentic samples prepared using fresh coconut*

	AMOUNT TAKEN PER DISTILLATION ALIQUOT	AMOUNT RECOVERED (UNCORRECTED)	RECOVERY	CORRECTED FOR PERIODATE ACIDITY	RECOVERY
	mg	*mg*	*per cent*	*mg*	*per cent*
Propylene Glycol	21.1	21.6	102.4	20.6	92.6
	21.1	20.7	98.5	19.7	93.4
	21.1	20.8	98.6	19.7	93.4
Glycerol	15.5	16.3	105.2	15.4	99.4
	15.5	16.1	103.9	15.2	98.1
	15.5	16.3	105.2	15.2	98.1

It may be noted from this table that the average uncorrected value for glycerol is about 5 per cent too high which suggests that a negative 5 per cent correction might be applied.

The next series of authentics were prepared as above except that all corrections are itemized (Table 3) and the result of applying this 5 per cent correction is shown.

Analysis of two samples of commercial moistened coconut by this method showed the absence of propylene glycol, but showed glycerol to be present to the extent of 0.23 and 0.26 per cent. Two further samples, No. 1 (declaring glycerol) and No. 2 (declaring either glycerol or propylene glycol), showed: No. 1, 3.02 per cent propylene glycol and 0.64 per cent glycerol, and No. 2, 3.07 per cent propylene glycol and 0.74 per cent glycerol. Blanks on commercial desiccated coconut showed small amounts of propylene glycol (0.19 per cent) to be present, but no glycerol was indicated.

Application of this method to the determination of glycerol in wine and vinegar gave results similar to those quoted in the literature. Wine and vinegar samples were first treated by adding one gram of lime per 100 ml, and 25 ml of the filtrate was taken for analysis. No propylene glycol was found in either case; results for glycerol were, for wine: 0.44 and 0.48 per cent; for vinegar: 0.39 and 0.39 per cent.

The method evidently is versatile but it has not been yet subjected to collaborative study. However, it is promising, and appears to be a shorter method than any of those now proposed for the determination of propylene glycol and glycerol in desiccated coconut.

REFERENCES

(1) MALAPRADE, L., *Bull. soc. chim.*, **43**, 683 (1928).

TABLE 3.—*Itemized results showing the five per cent correction*

	AMOUNT TAKEN	AMOUNT RECOVERED UNCORRECTED		CORRECTED FOR COCONUT BLANK	CORRECTED FOR PERIODATE ACIDITY	CORRECTED FOR BLANK AND PERIODATE		5% CORRECTION SUBTRACTED	
	mg	*mg*	*per cent*	*mg*	*mg*	*mg*	*per cent*	*mg*	*per cent*
Propylene Glycol	10.54	11.05	104.8	10.92	10.15	10.02	95.1	—	—
	10.54	11.15	105.8	10.98	10.24	10.11	95.9	—	—
	21.08	23.81	112.95	22.12	22.75	21.06	99.9	—	—
	21.08	23.93	108.8	21.24	21.87	20.18	95.7	—	—
	21.08	23.05	109.4	21.36	21.99	20.30	96.3	—	—
Glycerol	7.76	8.09	109.3	7.72	7.54	7.17	—	7.66	98.7
	7.76	8.06	103.9	7.69	7.51	7.14	—	7.69	99.1
	15.52	16.33	105.2	16.05	15.70	15.42	—	15.53	100.0
	15.52	16.12	103.9	15.84	15.47	15.19	—	15.31	98.8
	15.52	16.26	104.8	15.98	15.62	15.24	—	15.45	99.7

(2) ———, *ibid.*, [5] **1**, 833 (1934).
(3) SHUPE, I. S., *This Journal*, **26**, 249 (1943).
(4) BRUENING, C. F., *ibid.*, **29**, 29 (1946).
(5) ISACOFF, H., *ibid.*, **30**, 484 (1947).
(6) NEWBURGER, S. H., and BRUENING, C. F., **30**, 651 (1947).
(7) BRUENING, C. F., *This Journal*, **33**, 103 (1950).
(8) NEWBURGER, S. H., *ibid.*, **30**, 683 (1947).
(9) MÉTAYER, G. L., *Ann. Chim.*, [12] **2**, 790 (1952).
(10) ISACOFF, H., *This Journal*, **35**, 579 (1952).

STANDARDIZATION OF ALUMINA ADSORBENTS FOR VITAMIN A CHROMATOGRAPHY*

By J. B. WILKIE and S. W. JONES (Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.)

Past experience with chromatography has shown that the strength or activity of the adsorbent may have an important bearing on the successful application of the chromatographic method. Last year a method for the standardization of MgO and its mixtures, using the dye FD&C Yellow No. 4, was reported from this laboratory (1).

In our studies this year, that were a part of the A.O.A.C. collaborative study on vitamin A in margarine, directed by Dr. Morgareidge (2), it became evident that the alumina and alkaline alumina used in the chromatographic column as described by Boldingh and Drost (3) varied greatly in adsorptive activity. The work reported here involved our attempts to measure this activity, to correlate it with chromatographic performance, and to standardize it.

The technique for the actual procedure was patterned after the successful method used for the MgO and the MgO mixtures. However, it was found necessary to modify the detailed procedure used previously since the relative retention of FD&C Yellow No. 4 by alumina is much smaller (of the order of 5 per cent) than that by the MgO mixtures. In the case of the MgO it had been found that the amount of dye adsorbed was uniform and was held strongly, so that it resisted thorough rinsing. In contrast, rinsing of the alumina which contained adsorbed dye resulted in significant and variable wash-offs with each successive rinse. However, if the rinsing part of the procedure was confined to one rinse of definite size (25 ml) of petroleum ether, duplicate readings within 10 per cent could be obtained for each different strength alumina or alkaline alumina. As soon as this characteristic was established, alumina adsorbents were evaluated as they became available.

The details of the procedure follow.

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METHOD

Weigh 1 g sample of adsorbent in a 50 ml beaker. Add 5 ml petr. ether. Stir. Add 1 ml of the dye FD&C Yellow No. 4 soln contg 0.5 mg per ml petr. ether. Stir thoroly for 1 min., transfer all of the soln quickly to a small fritted filter chromatographic tube (ca 12 mm diam.), using just 10 ml of petr. ether in the process. Apply 20 in. of vacuum to the column until just dry. Pour just 25 ml more of petr. ether thru the column. Make the entire filtrate to vol. and measure the absorbance at 438 mμ on the spectrophotometer. Calc. % of total dye retained by 1 g of adsorbent and use this figure as the relative index of strength of the adsorbent. (The g of dye retained per g of adsorbent corresponds to the absorption index reported in the work with MgO (1). It is not used in this procedure with alumina because of the relatively low retaining capacity of the alumina for the dye with the consequent need for a more appropriate and somewhat independent unit of designation.

CALCULATIONS

5×10^{-4} = g of dye per ml of stock solution actually used.

1.71×10^{-5} = g of dye per unit of spectrophotometric absorbance at 438 mμ. (Each analyst should determine this value independently.)

V_f = final vol. as made for spectrophotometric measurement.

V_t = total vol. of unadsorbed dye.

V_{alq} = vol. aliquot of total unadsorbed dye.

A = absorbance of final unadsorbed dye in 1 cm cell.

$$([5\times10^{-4}-1.71\times10^{-5}\times A(V_t/V_{alq})]\times100)/5\times10^{-4} = \% \text{ dye retained by adsorbent.}$$

% dye retained $\times(5\times10^{-4})$ = Index − g dye retained per g adsorbent.

The strengths of various aluminas, used in a series of about 100 separate chromatographings in which general performance and activity were correlated, are presented in Tables 1 and 2. For this purpose, a single tube 13 cm long and 5 mm in diameter containing both alumina and alkaline alumina was used.

Table 1 gives the strengths of aluminas from various sources. Experiment No. 1 used the Merck chromatographic alumina passing a 160 mesh screen, as recommended by Morgareidge. With this alumina, heated at 750°C. and subsequently treated with 2 per cent water, the dye retained

TABLE 1.—*Strengths of adsorbents from different sources measured by the F&D Yellow #4 method*

DETERMINATION NO.	TYPE, SOURCE, AND TREATMENT OF ADSORBENT	MESH	DYE RETAINED
			per cent
1	Merck alumina: heated 750°C. +2% H_2O	<160	11.2
2	Merck alumina: NaOH treated, vacuum oven dried	<160	3.5
3	Food research alumina: heated 750°C. +2% H_2O	<160	7.2
4	Food research alumina: NaOH treated	<160	0.0
5	FDA alumina: untreated	80–200	92.5
6	FDA alumina: NaOH treated, vacuum oven dried	80–200	81.0

amounted to 11.2 per cent of the total added. Determination No. 3 refers to a sample similarly prepared in the Food Research Laboratory that had a dye retention of 7.2 per cent. For practical purposes, these two samples are in the same range of activity. Similarly, the Merck, and the Food Research alkaline aluminas, (Nos. 2 and 4) showed a dye retention strength of 3.5 per cent and 0.0 per cent. Again the strengths are relatively weak but are in the same range.

Determinations Nos. 5 and 6 were made with a product of the Aluminum Ore Company of America, labeled 80 to 200 mesh. Determination No. 5 used the material as received with no additional heating or treatment of any kind. This material is relatively very active as indicated by the 95.5 per cent dye retention. The usefulness of this material is limited, however, by its intense fluorescence, which can be removed with HCl

TABLE 2.—*Variation in strength of adsorbent with heat and moisture modification*

DETERMINATION NO.	TYPE, SOURCE, AND TREATMENT OF ADSORBENT	MESH	DYE RETAINED
			per cent
7	FDA alumina: heated >800°C.	80–200	0
8	FDA alumina: heated at 620°C.; no H_2O	80–200	96.0
9	FDA alumina: heated at 620°C.+2% H_2O	80–200	89.0
10	FDA alumina: heated at 620°C.+5% H_2O	80–200	5.3
11	Mixture of 9 and 10, 1:1	80–200	28.0
12	FDA alumina: heated at 620°C., no H_2O[a]	80–200	97.5
13	FDA alumina: heated at 620°C.+3.5% H_2O[a]	80–200	34.0
14	Mixture of No. 6 of Table 1 and No. 13 of this Table, 1:1	80–200	53.0

[a] Rejuvenated by treating used activated alumina with excess HCl for 15 min., followed by H_2O rinsing and drying at 100°C.

and heat treatment. The FDA alkaline alumina examined in determination No. 6 has a very high dye retention figure of 81 per cent in comparison with the corresponding Merck 160-mesh products of 3.5 per cent and 0 per cent, respectively.

Determination No. 7, Table 2, was a test of the 60–200 mesh alumina that had been heated to more than 800°C. This resulted in a complete loss of dye retention. Nor did the addition of 2 per cent water or an attempted rejuvenation by saturating with water and heating at a lower temperature affect the lost retention. It appears that the Alorco product is irreversibly and completely de-activated by too high a temperature; a result that is compatible with the published technology (4) of alumina which states that such alumina may be converted to inactive (gamma and alpha) forms at 900° and 1000°C., respectively.

As a consequence of this experience, further attempts to activate the alumina by heat were conducted at a temperature of 620°C. Time of heating was not critical, but a period of 7 hours was used.

Determination No. 8 indicated that alumina heated to such a temperature with no subsequent water treatment has a high dye retention figure of 95.5 per cent. Such a material would have a very limited usefulness in the chromatographic column since smooth control by usual elution techniques is difficult, if not impossible. This material was then treated with 2 per cent water according to the procedure recommended by Morgreidge. This resulted in a dye retention of 89 per cent which is still too high an activity for useful chromatography.

The material heated at 620°C. was then treated with 5 per cent water. The dye retention dropped to 5.3 per cent by this treatment and the alumina was weak but still usable if due regard for its weakness was observed.

In determination No. 11 the activity of a 1:1 mixture of Samples 9 and 10 was tested and found to have a dye retention of 28 per cent. Since this mixture contained 3.5 per cent water, an alumina was prepared with this amount of directly added water to demonstrate that the water content is a controlling factor in adsorptive activity. In determinations Nos. 12 and 13 an alumina that had been used previously was rejuvenated with HCl treatment, rinsed with water, dried, then heated at 620°C. Without added water, the dye retention was 97.5 per cent, but with 3.5 per cent added water the activity dropped to 34 per cent. The samples having a retention of 28 per cent and 34 per cent were found to give optimum performance in the chromatographic column. This trial demonstrated not only the importance of water content, but the feasibility of re-use of the alumina after rejuvenation with HCl and heating at 620°C.

Although the mere non-critical addition of water appears to be the simplest and most effective means of de-activation of a too-active alumina, it appears that its effectiveness may depend to some extent upon the manner of addition of the water. At least the precaution of adding the water dropwise, between shakings, should prove helpful.

Determination No. 14 of Table 2 represents a rather special product—a 1:1 mixture of alumina No. 13 having a dye retention of 34 per cent and the No. 6 alkaline alumina having a dye retention figure of 81 per cent. The attempt in this manner to decrease the caking characteristics of the alkaline alumina was successful. (In the practical chromatographic trials of the No. 6 alkaline alumina it was noted that it had a tendency to cake at the top, even to the point of plugging the column completely in some of the trials.) The use of the 1:1 mixture (activity 53 per cent) led to a reduced caking effect and gave a column of greatly improved performance. This observation indicated that the alkaline alumina adsorbents of weaker retention need further study.

Table 3 gives the results obtained with the various aluminas standardized as indicated in the previous two tables. A single sample of margarine was used for the chromatographings. No doubt, some variation in the prechromatographic processing resulted in experimental variations that may

TABLE 3.—*Chromatographic results with various alumina–alkaline alumina combinations*

IDENTIFICATION COLUMN AND ADSORBENT TYPE M=MESH, #1=ALUMINA, #2=ALKALINE ALUMINA	WATER AND ADSORBENT STRENGTH			MARGARINE VITAMIN A RESULTS		
	H_2O IN ALUMINA	DYE RETAINED ALUMINA	DYE RETAINED ALKALINE ALUMINA	UNCORR. VIT. A u/lb×10^{-3}	OP. NEUT. VIT. A u/lb×10^{-3}	RATIO A_{328}/A_{328}
	per cent	*per cent*	*per cent*			
(1) Boldingh & Drost, two columns Food Research (Merck 160 M #1) (Merck 160 M #2)	2	11.2	3.5	9.5	8.9	0.85
(2) (Merck 160 M #1) (F&D 80 M #2)[a]	2	11.2	81.0	9.6	9.1	0.89
(3) (F&D 80 M #1) (F&D 80 M #2)[a]	3½	34.0	52.0	9.3	8.2	1.00
(4) (F&D 80 M #1) (F&D 80 M #2)[a]	3½	34.0	81.0	8.8	8.3	0.945
(5) No alkaline alumina used (80 M #1)[a]	3½	34.0	—	11.0	9.9	1.52
(6) Merck 160 M #1 and #2[a]	(No results possible—plugged)					
(7) Too weak combined adsorbents (F&D 80 M #1) (Merck 160 M #2)[a]	3½	26.0	3.5	6.7	5.2	0.95
(8) Too strong eluent. 50% ether in petroleum ether (F&D 80 M #1) (80 M #2)[a]	3½	34.0	81.0	13.2	13.6	1.27
(9) Alkaline alumina on top of column (F&D 80 M #2)[a]	3½	—	81.0	(No results—blocked by caking)		
(10) Alumina heated 800°C.–1000°C. (F&D 80 M #1) (F&D 80 M #2)[a]	0 or 2	0.0	(No retention of vitamin A or dye—worthless)			

[a] Single short column.

account for small differences (of the order of 10 per cent) in vitamin A potency values. The larger differences and enhanced A_{310}/A_{325} ratios, however, may be attributed to differences in adsorbent activity of the column that are responsible for lack of elution control.

In the first determination in this table the Morgareidge-Boldingh and Drost method was followed as closely as possible. The first adsorption column contained Merck alumina with 2 per cent water added as recommended, and the second column contained the Merck alkaline alumina. As shown in the table the strengths of these adsorbents were relatively weak. The vitamin A potency based on the 325 mμ absorption, uncorrected, was 9,500 units vitamin A per pound, and by the optical neutralization method, i.e., by the per cent of vitamin A standard neutralized, the results calculated to 8,900 units per pound. The latter value may be more accurate by virtue of the corrective nature of the optical neutralization process. At any rate, these differences are small and of the same order of magnitude as obtained by other types of chromatography under control believed to be satisfactory. The absorbance ratio A_{310}/A_{325} is very low (0.85), indicating a good clean-up of possible interference at 325 mμ. Last year, the A_{310}/A_{325} ratio was recommended in the Associate Referee's report as a useful criteria for gauging the effectiveness of the MgO chromatography. Further work with MgO has supported this recommendation, and also has served to increase the quality of the MgO results as indicated by the lower values of this ratio obtained during the past year. Our present belief is that there will be very little error by interference at 325 mμ even if the 310/325 ratio is as large as 1:1, especially if the optical neutralization evaluation technique is used. This criteria is now believed to hold for both the magnesium oxide and alumina adsorbents.

For experiment No. 2 in Table 3, the results were obtained with a single 13 cm column using the Merck alumina adsorbent (the same as that used in the first column where the Boldingh and Drost procedure was followed) in conjunction with the stronger FDA 81 per cent retentive alkaline alumina. It will be seen that the results obtained with the single column compare favorably with those from the dual column system. This indicates the suitability of the adsorbents as well as the feasibility of substituting a simpler single column for the Boldingh and Drost apparatus.

The next two results, Nos. 3 and 4 (Table 3), are further examples of what can be accomplished with a single column. It will be noted that medium strength adsorbents were used (34 to 52 per cent retention) and that the potencies are within an acceptable range even though the A_{310}/A_{325} ratios appear somewhat larger than those for experiments Nos. 1 and 2. The next experiment, No. 5, indicates the best that could be done with an optimum strength 13 cm alumina column when the alkaline alumina was omitted. The results are approximately 20 per cent higher, and the A_{310}/A_{325} ratio of 1.52 is consistent with this erroneously high result.

Following the original instructions that the alumina should pass the 160 mesh screen, the Merck alumina that did not pass this sieve was ground with mortar and pestle until it did. It was then activated as recommended, and the column for experiment No. 6 was prepared. No results were obtainable, since the material was ground too fine to be of any use whatever. It formed a non-percolating column and demonstrated in no uncertain terms the importance of the granular size of the adsorbent.

After this experience, most of the further experiments were made without percolation difficulties with the FDA activated or alkaline material. Some of this material was sieve graded, and it was found that 16.7 per cent passed the 60–100 mesh sieve, 50 per cent passed the 100–160 mesh sieve, and 33.3 per cent passed the 160 mesh sieve. It appears that some kind of grading specification is necessary for adsorbent alumina, mainly to avoid the use of excessive amounts of fine material which prevent adequate percolation through the column.

Experiment No. 7 represents a sample in which both adsorbents were relatively weak but had previously given satisfactory results. However, chromatography provided results much too low (by about 30 per cent). This is an example of improper eluent control, probably caused by the preliminary use of an eluent too strong to match the adsorbent. When this happens, the vitamin A band becomes diffuse and this makes it much more difficult to get a sharp separation, or complete collection of the vitamin coming off the column.

Experiment No. 8 represents another extreme that will cause trouble. In this case the adsorbents were also of adequate strength but a solution of 50 per cent diethyl ether in petroleum ether was used as the final eluent. This solution simply swept through some of the interfering material normally held back by the alkaline alumina sufficiently fast to cause interference at 325 mμ and beyond. The 1.27 A_{310}/A_{325} ratio would automatically invalidate such a result.

Experiment No. 9 demonstrated the caking effect of a too strong alkaline alumina. Here the alkaline alumina was placed at the top of the column. The caking was so complete that no results were obtainable. Experiment No. 10 shows the effect of overheating alumina as previously mentioned. It seems possible that a temperature of 750°C. may be marginal, since a portion of the product may become inactive and irreversible under such conditions. This may render subsequent tempering or de-activation with water highly indefinite, and quite likely would confuse any attempts at subsequent rejuvenation of the used alumina. Heating to approximately 600°C. can be recommended on the basis of the results presented here.

CONCLUSION

On the basis of the above findings, alumina for chromatography should be quantitatively evaluated with respect to its particle size and adsorptive

activity if optimum control of the column during elution is to be attained. A procedure involving the retention of FD & C Yellow is suitable for measuring adsorptive activity. Provided the elution technique is properly matched, a fairly wide range of adsorptive activity is acceptable.

With the present information, the authors believe that a 20–50 per cent dye retention will provide an adequate working range of adsorbent activity. Further studies may provide a basis for narrowing or modifying this range.

REFERENCES

(1) WILKIE, J. B., and JONES, S. W., *Anal. Chem.*, **24**, 1409 (1952).
(2) MORGAREIDGE, K., *This Journal*, **37**, 748 (1953).
(3) BOLDINGH, A. J., and DROST, J. R., *J. Am. Oil. Chemists Soc.*, **28**, 480 (1951).
(4) KIRK, R. E., and OTHMER, D. F., *Encyclopedia of Chemical Technology*, Vol. I, Interscience Publishers, New York, p. 642 (1947).

A CHROMATOGRAPHIC-SPECTROPHOTOMETRIC METHOD FOR DETERMINATION OF VITAMIN A IN MARGARINE*

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The problem of the determination of the vitamin A content of margarine has been before us for several years. Until now the only official method is the biological assay, and for obvious reasons this procedure is unsuitable for day-to-day control or research. For its own control purposes the margarine industry has largely relied upon the antimony chloride blue-color method. However, it is generally recognized that the blue-color method may give vitamin A results slightly to substantially different from those indicated by bioassay or the U.S.P. spectrophotometric test. Moreover, inasmuch as the A.O.A.C. and U.S.P. prescribe a spectrophotometric method for the vitamin A concentrates used to fortify margarine, it would be desirable to conduct the vitamin A assay on margarine by such a method.

Attempts to develop a spectrophotometric procedure for vitamin A in margarine have been made. Melnick and coworkers have published a series of papers (1) describing the use of spectrophotometry in plant control. However, the procedures of this group depend upon having available the blank oils which are used in preparing each batch of margarine tested. Thus from an enforcement point of view these methods are not acceptable.

Wilkie (2–4) turned to chromatography with magnesia in an attempt

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to purify margarine extracts sufficiently to enable spectrophotometry to be employed. Collaborators testing Wilkie's method were not able to obtain curves typical of vitamin A. This made necessary the measurement of absorption at wavelengths other than the maximum and the application of arbitrary factors. However, Wilkie's work pointed the direction to be taken for development of a successful spectrophotometric margarine vitamin A method.

Boldingh and Drost (5) carried chromatography further in making a two-step process of it, employing alumina in the first column, followed by an alkali treated alumina in the second. In the work of Boldingh and Drost, eluates were obtained which gave spectrophotometric curves typical of vitamin A. An objection to the published procedure of these workers is its complexity, which makes it cumbersome for routine use. Certain short-cuts have been introduced but it still remains a double-column procedure. Braekkan and Lambertsen (6) combined the two adsorbents of Boldingh and Drost into a single column and reported satisfactory results.

About a year ago our laboratory undertook to study methods for spectrophotometric margarine vitamin A assay. The procedure of Boldingh and Drost was first examined. The experimental work with their adsorbents showed that we could obtain no better purification of vitamin A by passing the extract containing the unsaponifiable fraction of margarine through alumina, followed by alkaline alumina, than through the same length of alkaline alumina alone.

Experiments with chromatography on alkaline alumina, following saponification, showed that we could (*a*) completely recover pure vitamin A from the column, (*b*) recover vitamin A added to blank uncolored margarine, and (*c*) recover vitamin A added to blank colored margarine colored either with FDC 3 & 4 or carotene. The recoveries in the margarine experiments were in the range of 95–105 per cent, based upon $E^{1\%}_{1cm}$ at 325 mμ multiplied by 1900.

The following procedure was developed. Margarine is saponified and extracted in the usual manner according to the method of Rice, Primm, and Coombes (7). The residue is taken up in petroleum ether and passed through a column of alkali-treated alumina. In earlier experiments suction was used to draw the eluate through the column; however, we later found pressure to be more satisfactory. Elution of the vitamin A is accomplished by the use of ethyl ether dissolved in petroleum ether; successive portions of eluant contain increasing concentrations of ethyl ether, up to 36 per cent. The course of the vitamin A down the column is followed by the use of the $SbCl_3$ reaction or by weak ultraviolet radiation in a darkened room. When colored margarines containing either FDC dyes or carotene are being tested, the coloring materials precede vitamin A down the column and make a convenient indicator. The vitamin A fraction is

collected, the solvent is evaporated off with nitrogen, and the residue is taken up with isopropanol for spectrophotometric measurement. A detailed description of the procedure follows:

METHOD

REAGENTS

(a) *Ethanol.*— 95% distd from NaOH and $KMnO_4$.

(b) *Potassium hydroxide.*—90%.

(c) *Ether.*—Peroxide free and redistd, first and last 10% discarded.

(d) *Petroleum ether or Skellysolve F.*—Boiling range between 37–60°C. Reflux with 1+5 H_2SO_4 for 6 hrs. Separate from acid, and wash acid-free. Distill and collect 37°–60° portion. Filter thru anhyd. Na_2SO_4.

(e) *Alkaline alumina.*—Merck chromatographic grade, 160–200 mesh. Incinerate at 600°C. for 5–6 hrs. Mix equal parts of alumina with 10% NaOH (w/v) and allow to stand at room temp. for 1 hr with occasional shaking. Dry in vacuum oven at 30–40°C. until most of H_2O is removed, then at 100–105°C. for 1 hr. Allow to cool. Add 2% H_2O and mix thoroly in tightly stoppered bottle.

(f) *Antimony trichloride.*—25% soln in $CHCl_3$.

(g) *Isopropanol.*—Adequate for spectrophotometric readings.

(h) *Sodium sulfate.*—Anhyd., c.p. All reagents used in chromatography must be completely dry. It is advisable to shake solvents with anhydrous Na_2SO_4 and filter or allow Na_2SO_4 to settle before use.

APPARATUS

(a) *Chromatographic column.*—Stem: 6 mm inner diam.; 200 mm long with constriction of 3 mm inner diam. at bottom. Funnel attached at top, 25 mm inner diam., 65 mm long.

(b) *Spectrophotometer.*—Model DU Beckman spectrophotometer.

DETERMINATION

Saponify and ext. a 10 g sample of margarine by the method of Primm, Coombes, and Rice (7). Evap. ether by means of mild heat and use a stream of N or CO_2 to drive off the last 20 ml of ether. Make residue up to 25 ml with petr. ether, add anhyd. Na_2SO_4 to *completely dry* the sample, and filter, keeping funnel covered with watch glass to avoid loss of solvent by evapn. Alternatively, allow all Na_2SO_4 to settle to bottom of flask and pipet *only clear* supernatant liquid.

Preparation of chromatographic column.—Insert a small plug of glass wool in the bottom of the chromatographic column, and close bottom of column by means of rubber tubing and clamp. Fill column with petr. ether to ca 20 mm in the upper funnel. Pour the alk. alumina into the column and allow to settle. Use glass stirring rod to brush particles of adsorbent down into column, but *do not apply pressure.* Fill column with adsorbent to a height of 180 mm. Top with small amount of anhyd. Na_2SO_4.

Chromatography.—Place 10 ml of petr. ether on column and force solvent down column by pressure. Leave ca 10 mm of solvent on top of column (never let column go dry). Pipet 10 ml of filtered sample ext. onto top of column and apply pressure. Follow this with portions of petr. ether. The progress of the vitamin A is followed by fluorescing the column with ultraviolet light (a Black Light Products Lamp Model No. Bl. 6C–36–60 is satisfactory for this purpose) in a darkened area.

If the color is FD&C 3 or 4, the vitamin A is usually 3–4 cm behind the color on the column. Elute all of the portion preceding and contg color with petr. ether

and collect in first receiving flask. When the color is completely eluted, place diethyl ether on column (about 10 ml) and continue to elute, collecting eluate in first receiving flask until the vitamin A is at the tip of the column.

Place a fresh flask under the column and continue to elute with diethyl ether until all vitamin A is collected. This elution usually requires 7–15 ml ether. A double check for the appearance of vitamin A may be made by allowing one drop of eluate to fall onto a spot plate and adding a drop of $SbCl_3$ in chloroform. A blue color indicates the presence of vitamin A. If the color is carotene, the colored fraction is collected separately from the preceding eluate and held for spectrophotometric carotene determination. After complete elution of the color, add enough petr. ether to bring the vitamin A band to within 2 cm of the column tip. Then elute with diethyl ether and follow the procedure described in the preceding paragraph. If the margarine is colorless, elute with petr. ether until the vitamin A band is within 2 cm of the tip of the column and then proceed as above.

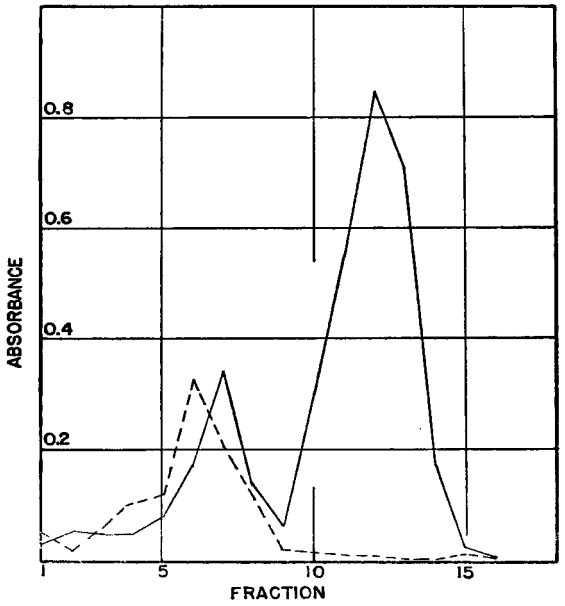

FIG. 1. Comparison of 5 ml eluate fractions from fortified and unfortified colored (FDC 3 & 4) margarines. Solid line—fortified; dashed line—unfortified.

Evap. petr. ether of the vitamin A fraction under a stream of N or CO_2 with mild heat. Make the residue to 15 ml with isopropanol (make to 10 ml if margarine contains carotene) and read in a Beckman spectrophotometer, Model DU, at 325 mμ.

Determine the absorptivity ($E^{1\%}_{325}$) and multiply by 1900 to convert to U.S.P. units vitamin A per g.

The adsorbent itself is probably the most critical factor of the assay. The procedure of Boldingh and Drost was followed in the preparation of the

alkali-treated alumina. The resulting dry adsorbent, however, was too retentive. Experiments to determine the effect of additions of water demonstrated that 2 per cent water gave optimum adsorbent qualities to the alumina.

Fractionation of the eluates from blank colored margarine, and from the same margarine fortified with vitamin A, by collection of successive 5 ml portions followed by spectrophotometric measurement in isopropanol at 325 mμ, gave the curves shown in Fig. 1. Note that absorption from the extraneous material is essentially negligible before the vitamin A is eluted.

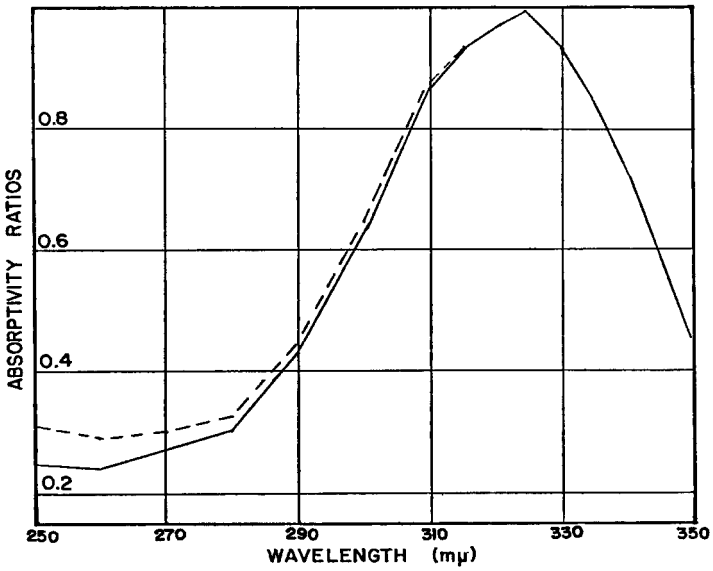

FIG. 2.—Comparison of absorption curves following chromatography of vitamin A alone, and in margarine. Solid line—vitamin A alone; dashed line—vitamin A in margarine.

Curves illustrating the absorption of U.S.P. Reference vitamin A and that of a vitamin A-colored margarine mixture, both from application of the method described, are shown in Fig. 2. The chromatographed Reference Oil showed a slight rise at wavelengths below the maximum, compared with non-chromatographed Reference Oil. Thus the 6/7 fixation point occurs at 309.5, rather than at 310 mμ. This rise is not due to chromatography *per se*, but rather to the effect of absorbing substances in the solvents, since it can be duplicated simply by adding the solvents without chromatography.

The absorption of chromatographed margarine appears to be identical

with U.S.P. Reference vitamin A on the upper side of 325 mμ. However, below this wavelength the absorption of fortified margarine is consistently higher than that of Reference vitamin A. In any case this affects the absorption at 325 mμ very little.

TABLE 1.—*Recovery of vitamin A added to unfortified colored (F.D.C. 3 & 4) margarine*

VITAMIN A ADDED	VITAMIN A RECOVERED	RECOVERY
U.S.P. units per pound		*per cent*
14,800	14,300	97
13,700	13,700	100
16,200	16,500	102
17,500	17,000	97
12,100	12,700	105

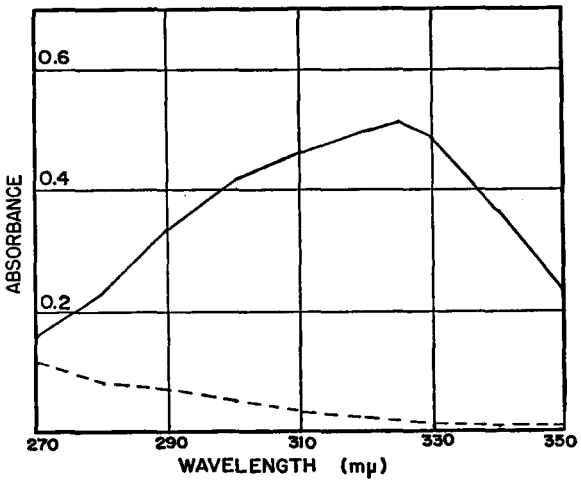

FIG. 3.—Comparison of spectrophotometric absorption curves of fortified and unfortified margarine after chromatography. Solid line—fortified; dashed line—unfortified.

Unfortified colored margarine was saponified and chromatographed, and the fraction in which the vitamin A would normally occur was collected. The spectrophotometric absorption characteristics of this fraction were compared with those of the same margarine fortified with vitamin A. The curves are shown in Fig. 3.

Recoveries of vitamin A and carotene from fortified margarines are shown in Tables 1 and 2. Carotene presents no problem in separation and

TABLE 2.—*Recovery of vitamin A and carotene added to unfortified margarine*

VITAMIN A			CAROTENE		
ADDED	RECOVERED	RECOVERY	ADDED	RECOVERED	RECOVERY
U.S.P. units per pound		*per cent*	*units per pound*		*per cent*
10,670	10,410	98	6,050	6,270	103
11,040	10,630	96	6,050	6,220	103
10,780	10,260	95	6,000	6,500	108
12,130	12,090	100	5,900	6,350	108
10,700	10,800	101	5,520	5,650	102

estimation, since it precedes vitamin A down the column by an even greater margin than do the FD & C dyes.

Results of one collaborative test on margarine where the blue color method was used, are shown in Table 3, together with the result on the same margarine by the method described.

TABLE 3.—*Collaborative vitamin A assay on margarine (N.A.M.M. check Sample No. 6, April 1953)*

COLLABORATOR	VITAMIN A CONTENT	
	$SbCl_3$ METHOD	PRESENT METHOD
	U.S.P. units per pound	
1	22,300	
2	23,800	
3	19,100	
4	22,400	
6	22,300	
7	22,000	20,200
12	22,200	
13	22,100	
14	21,900	

Theoretical: 20,600 units per pound (spectrophotometric basis).

SUMMARY

The method described is an acceptable spectrophotometric procedure for determination of vitamin A in oleomargarine in that it meets the following criteria: 1. Curves approaching those of true vitamin A are obtained; 2. At 325 mμ the spectrophotometric absorption is substantially due to vitamin A only, and requires no correction factors; 3. Approximately complete recovery of vitamin A and/or carotene is obtained. Further, this procedure has an advantage over certain others in that a single adsorbent and column are employed.

ACKNOWLEDGMENT

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REFERENCES

(1) LUCKMAN, F. H., MELNICK, D., and VAHLTEICH, H. W., *J. Am. Oil Chemists Soc.*, 29, 104, 121, 174, 321 (1952).
(2) WILKIE, J. B., *This Journal*, 30, 382 (1947).
(3) ———, and DEWITT, J. B., *ibid.*, 32, 455 (1949).
(4) ———, and JONES, S. W., *Anal. Chem.*, 24, 1409 (1952).
(5) BOLDINGH, J., and DROST, J. R., *J. Am. Oil Chemists Soc.*, 28, 480 (1951).
(6) BRAEKKAN, O. R., and LAMBERTSEN, G., *Tids. Kjemi. Bergresen Met.*, 12, 29 (1952).
(7) RICE, E. E., PRIMM, E., and COOMBES, A. I., *This Journal*, 31, 621 (1948).

XANTHOPHYLL DETERMINATION IN DEHYDRATED ALFALFA MEAL

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The increasing demand for a mixed-feed ingredient to impart uniform yellow color to poultry has stimulated interest in a simplified xanthophyll assay. Since many laboratories also analyze for carotene, a method that permits assay for both pigments on the same extract is desirable. Two methods employing differential adsorption on chromatographic columns have been reported (8, 9). They specify saponification of the sample (8) or the use of ether as a solvent (9); both methods require much manipulation and transfer of solutions (8, 9, 10), and are not readily adaptable to a large number of routine analyses.

In the following report, two methods for carotene (5, 13) are examined with regard to their suitability, also, for xanthophylls. The methods proposed are relatively rapid and suitable for routine use.

PROPOSED METHODS

(1) *Rehydration technique.*—The following procedure, based on a recently published rapid method for the determination of carotene (5), was found to be applicable with no modification to the determination of xanthophylls.

A 2.00 g sample of meal is placed on a No. 1 Whatman filter paper and washed with warm water (500 ml at 50°C.) Vacuum is used to move the water through the meal (5, 8). The volume of water absorbed by the filter paper and the re-hydrated meal is ascertained after first removing the excess water with suction. The filter paper containing the sample is transferred to a one-quart blendor jar and disinte-

grated at high speed for one minute[1] in the presence of 100 ml of acetone. (The lid of the blendor jar should carry a form-fitting aluminum or stainless steel baffle about 6 cm below the top of the jar, to avoid splashing losses.) The jar is allowed to stand one minute and a 5 ml aliquot of the cloudy supernatant acetone extract is removed and added to 5 ml of hexane plus 3 ml of water, contained in a 50 ml separatory funnel. Gently swirling the funnel for one minute transfers most of the acetone into the lower water layer, which is then withdrawn and discarded. The entire hyperphase is chromatogaphed on a column (12 mm inside diameter) packed to 70 mm in height with a mixture of equal parts by weight of magnesium oxide (No. 2642, Westvaco) and filter-aid. The carotene is eluted with a mixture of hexane and acetone (9+1) until 25 ml is collected in a volumetric flask. Elution is temporarily discontinued, the flask is removed, and a second 25 ml is eluted with a mixture of hexane and alcohol (9+1) for the xanthophyll fraction. The absorbance of each of the solutions is read in a colorimeter, employing a 440 mμ filter. The xanthophyll concentration is found by multiplying the value determined from the carotene calibration curve by 1.06. Alternately, a spectrophotometer is employed at wavelength 451 mμ for carotene, and 445 mμ for xanthophyll. The concentrations are determined by employing the absorptivities of 251 for carotene, and 231 for xanthophyll.

(2) *Overnight soaking technique.*—The following procedure, based on a modification of the A.O.A.C. method for carotene (13), is proposed. Experimental work on this method is included below. The method follows:

A 2.00 g sample of meal, ground to pass 40 mesh, is transferred to a 100 ml volumetric flask together with 30 ml of a mixture of hexane-acetone (7+3). After adding 0.50 ml of water to the contents of the flask, it is stoppered, swirled gently for ½ minute, and allowed to stand in the dark for 16 hours. The mixture is then diluted to 100 ml with hexane, and a 5.00 ml aliquot is chromatographed on a column similar to that described above. The carotene is eluted with hexane-acetone (9+1) until 25 ml of solution is collected; the xanthophyll is eluted with hexane-alcohol (1+1) to 25 ml volume. The concentrations of each of the solutions are determined as described above.

EXPERIMENTAL WORK

The technique for accurately estimating the quantity of each pigment in alfalfa meal may be conveniently divided into three stages: (a) quantitatively extracting the pigments into a suitable solvent for chromatography; (b) chromatographically separating the pigments from each other and from other associated pigments such as the chlorophylls and their degradation products; and (c) measurement in a suitable colorimeter or spectrophotometer.

QUANTITATIVE EXTRACTION OF THE PIGMENTS

The A.O.A.C. procedure for carotene in alfalfa meal offers as alternate extraction procedures either hot (one-hour reflux) or cold (overnight soaking) extraction, employing as extracting solvent a 7+3 mixture of hexane and acetone (1). Cold extraction is the preferred procedure, since hot reflux may result in oxidation, isomerization, or less complete extrac-

[1] This has been found to be the optimum time to quantitatively extract the pigments and avoid acetone loss due to overheating.

tion of the pigments (6). However, even when cold extraction is employed, the pigments are not completely removed from the meal as demonstrated by the following experiment:

Duplicate 2 g portions of 5 different alfalfa meals were extracted by the cold A.O.A.C. procedure (overnight soaking with 30 ml of 7+3 hexane-acetone.) Following extraction of carotene and xanthophyll, the meal residues were filtered from the extract, washed with 7+3 hexane-acetone until the washings were colorless, and air-dried. Each meal residue was then re-analyzed for both pigments by the rehydration technique described under *Proposed Method 1.* Additional carotene and xanthophyll were obtained in every case (Table 1). The total amount of unextracted pigment varied with each meal and was not correlated with the initial pigment content of the meal.

TABLE 1.—*Extraction of carotene and xanthophyll from dehydrated alfalfa meal by A.O.A.C. overnight extraction*

SAMPLE	A.O.A.C.		ADDITIONAL PIGMENTS IN RESIDUE[a]			
	CAROTENE	XANTHOPYLL	CAROTENE		XANTHOPYLL	
	mg/lb	*mg/lb*	*mg/lb*	*%*	*mg/lb*	*%*
A	37	118	2.0	5.4	7.5	6.4
	38	112	1.4	3.7	12.9	11.5
B	58	107	4.4	7.6	24.3	22.7
	60	112	3.6	6.0	17.4	15.5
C	74	157	0.8	1.1	18.7	11.9
	75	156	1.0	1.3	27.6	17.7
D	94	167	9.3	9.9	31.6	18.9
	89	169	7.2	8.1	29.8	17.6
E	123	233	5.3	4.3	18.7	8.0
	118	227	5.2	4.4	25.8	11.4

[a] Residue from 30% acetone extraction re-extracted by rehydration and acetone extraction (Method 1).

Zscheile and Whitmore (14) attempted without success to extract this additional pigment by adding small amounts of water (2.5 per cent of the acetone concentration) with the acetone-hexane mixture. It has since been found[2] that if the water content of the extractant is increased to about 5–6 per cent of the acetone concentration, additional carotene and xanthophyll can be extracted. However, the amount extracted is variable and extraction is never complete. Columns 1 and 5 of Table 2 present the amount of carotene and xanthophyll extracted by the A.O.A.C. extractant on 4 representative meals. Columns 2 and 6 of Table 2 present comparable data except that a half ml of water has been added with the 30 ml of extractant in each case.

[2] Carl A. Taylor, Salinas Laboratories, Calif., *private communication.*

TABLE 2.—*Comparison of several methods for the determination of carotene and xanthophyll in dehydrated alfalfa meal*

SAMPLE	CAROTENE CONTENT				XANTHOPYLL CONTENT			
	A.O.A.C.	MODIFIED A.O.A.C.	PROPOSED METHOD 1	PROPOSED METHOD 2	A.O.A.C.[a]	MODIFIED[a] A.O.A.C.	PROPOSED METHOD 1	PROPOSED METHOD 2
	mg/lb	*mg/lb*	*mg/lb*	*mg/lb*	*mg/lb*	*mg/lb*	*mg/lb*	*mg/lb*
Old alfalfa meal (stored 6 months)	40	36	44	36	108	111	134	131
Collab. carotene sample No. 48	44	47	52	47	91	121	137	138
Collab. carotene sample No. 49	78	83	89	83	141	165	182	187
Dehydrated meal (freshly prepared)	120	120	130	120	211	214	257	241

[a] 25 ml of 10% alcohol in hexane used to elute the xanthophyll.

Dutton and co-workers (8) showed that some dehydrated materials require rehydration before the pigments can be extracted quantitatively. Rehydration results in imbibition and swelling of the cell walls and cell constituents. This treatment facilitates the entry of water-miscible solvents and the extraction of pigments otherwise "sealed off" or too firmly absorbed. When 25 ml of hexane-alcohol (9+1) is used to elute the xanthophylls, the rehydration technique (*Proposed Method 1*) yields higher values for xanthophyll than does the 16-hour cold soaking technique even when some water is added to facilitate extraction (compare modified A.O.A.C. with *Proposed Method 1*, Table 2).

CHROMATOGRAPHIC SEPARATION OF THE PIGMENTS

The developed column from which the carotene has been eluted with hexane-acetone (9+1) contains most of the xanthophylls tightly adsorbed near the top. Although the xanthophylls can be eluted readily with alcohol (9) the proper concentration is critical since too high a concentration will elute oxidation products of the xanthophylls as well as some green pigments, and too low a concentration will not elute all of the xanthophylls.

Of the various xanthophylls present in dehydrated alfalfa, neoxanthin is most strongly adsorbed on a magnesia column (7). The minimum concentration of alcohol in hexane which would quantitatively elute purified neoxanthin from a magnesia-filter aid column (12 mm×70 mm) with 25 ml of eluant was ascertained. It was found that 10 per cent alcohol in hexane would accomplish this with the adsorbent presently being used in this laboratory, and that lower concentrations of alcohol required more eluant for quantitative elution. Testing this concentration of eluant on a highly purified preparation of crystalline xanthophylls from alfalfa and on an extract from fresh alfalfa verified the conclusion that this concen-

tration of alcohol would quantitatively elute all of the non-oxidized xanthophylls.

When 10 per cent alcohol in hexane is employed as xanthophyll eluant in *Proposed Method 1*, most of the colored oxidation products are retained on the column. However, in *Proposed Method 2*, 50 per cent alcohol in hexane is employed in order to elute the oxidation products with the xanthophyll fraction.

The accuracy of *Proposed Method 2* depends on the fact that in most of the cases tested, the amount of additional color contributed to the xanthophyll fraction by these yellow-colored oxidation products compensates very closely for the incomplete extraction of the xanthophylls from the meal by the soaking method of extraction (Table 1). Consequently, the "Total Apparent Xanthophyll" value so obtained is very similar to the "Total Xanthophyll" value obtained by the rehydration technique, wherein the xanthophyll is quantitatively extracted from the meal and wherein most of the oxidation products remain on the column (compare *Proposed Methods 1* and *2*, Table 2). *Method 1* is basically more accurate, and individual assays may be carried out in less than one hour. *Method 2*, however, has the advantage of being more adaptable to the routine assay of large numbers of samples.

MEASUREMENT OF THE CONCENTRATION OF THE PURIFIED PIGMENT FRACTIONS

The measurement of carotenes and xanthophylls is only approximate because of the presence in each fraction of several pigment bands. Carotene exists in at least three stereoisomeric forms; all have different absorptivities (2). Although routine methods exist for the determination of the individual stereoisomers (3, 4, 12), they have not been adopted by the A.O.A.C., and approximate values for "Total Carotene" are accepted.

The situation with the xanthophylls is considerably more complicated, since at least two major xanthophylls and several minor ones, each existing in several isomeric forms, are present in dehydrated alfalfa (7). For this reason, it has been the practice to utilize a carotene calibration curve for estimation of xanthophylls. To obtain some measure of the error that is involved when xanthophylls are thus determined, calibration curves were prepared, employing a filter type colorimeter with the 440 mμ filter, from the five major xanthophylls which together constitute about 99 per cent of the total xanthophylls of fresh alfalfa (7). The constants, taken from the literature (11), were used to obtain the concentration of each solution spectrophotometrically. The calibration curves for lutein, cryptoxanthin, and zeaxanthin were very similar to that of carotene. The absorption spectra for violaxanthin and neoxanthin differ from carotene to the extent that assays based on a carotene calibration curve for these two pigments must be multiplied by 120 per cent or 125 per cent, respectively, to obtain the xanthophyll concentration. Since the relative

amounts of the individual xanthophylls present in the xanthophyll fraction of fresh alfalfa are known (7), it is possible to calculate the total error involved in the use of a carotene calibration curve for estimation of xanthophyll. For fresh alfalfa the error is 11 per cent. The xanthophyll fraction of dehydrated alfalfa is more complex and variable (7). However, because the violaxanthin content is lower in dehydrated alfalfa than in the fresh (7), six per cent is found to be a reasonable compromise correction to apply in order to compensate for the decreased absorption of certain of the xanthophylls and their stereoisomers relative to carotene. In the absence of experimental work showing the value of the xantho-

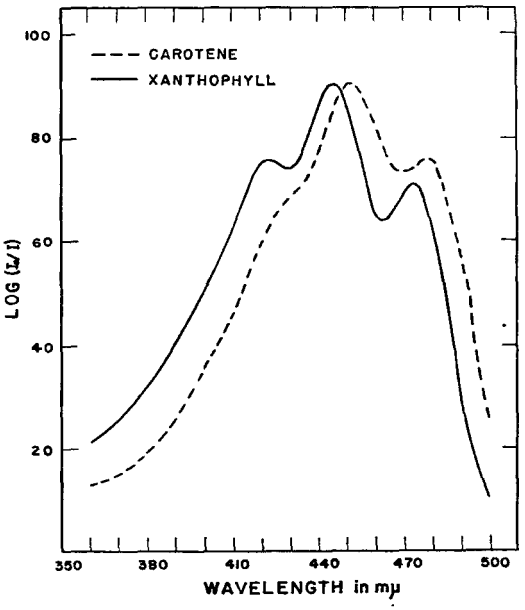

FIG. 1.—Spectral absorption curves of carotene and xanthophyll fractions from dehydrated alfalfa meal.

phyll oxidation products for pigmenting poultry, this laboratory preferred to omit them from the analysis (except as a compensatory factor for incomplete xanthophyll extraction in *Proposed Method 2*.)

A study of the xanthophyll fraction of a number of dehydrated meals indicates that 445 mμ is a close approximation to the absorption maximum (Figure 1). In order to compare the relative values obtained colorimetrically (including application of the correction factor 1.06 to the concentration obtained from a carotene curve) with spectophotometric values, a number of representative commercially prepared alfalfa meals were as-

TABLE 3.—*Comparison of a spectrophotometer with a photoelectric colorimeter for xanthophyll assay*

MEAL SAMPLE	COLORIMETRIC ASSAY[a]	SPECTROPHOTOMETRIC ASSAY[b]
	mg/lb	*mg/lb*
1	125	123
2	119	119
3	111	110
4	133	130
5	84	83
6	93	97
7	96	97
8	122	121
9	168	165
10	123	124
11	241	247
12	207	215

[a] 1.06 ×absorbance (based on carotene calibration curve).
[b] Measured in a Cary automatic recording spectrophotometer, using the absorptivity 231.

TABLE 4.—*Precision of proposed methods for xanthophyll in dehydrated alfalfa meal*

REPLICATES	PROPOSED METHOD 1		PROPOSED METHOD 2	
	CAROTENE	XANTHOPHYLL	CAROTENE	XANTHOPHYLL
	mg/lb	*mg/lb*	*mg/lb*	*mg/lb*
1	129	254	119	236
2	131	238	116	241
3	130	265	117	240
4	127	262	118	241
5	130	250	117	242
6	130	260	118	242
7	121	249	118	245
8	120	226	118	245
9	132	253	117	245
10	136	260	119	241
11	132	267	125	242
12	132	267	123	242
13	129	268	123	241
14	131	269	125	242
15	129	259	122	242
16	130	259	120	241
17	132	260	121	241
18	133	261	122	241
19	132	246	122	241
20	134	252	123	240
21	132	253	122	241
22	128	261	121	240
23	129	268	121	240
24	130	257	118	241
Average	130	257	120	241
Standard deviation	2.97	10.14	2.65	1.90

sayed. Comparison of xanthophyll concentration, determined colorimetrically and spectrophotometrically, showed that the results are comparable in most cases if an absorptivity of 231 at wavelength 445 mμ is employed for the spectrophotometric determination (Table 3). This value is similar to that given for violaxanthin (11). The greatest deviations occurred with newly dehydrated meals, in which the xanthophyll fraction approached most closely in composition to that of fresh alfalfa.

PRECISION OF THE METHODS

The results of replicate analyses of a commercially prepared alfalfa meal are presented in Table 4. All these analyses were run the same day by both proposed methods. The apparent concentrations of the purified xanthophyll solutions were determined colorimetrically. The standard deviations (Table 4) indicate that under favorable circumstances the reproducibility of the procedures is probably better than their accuracy, in view of the large number of pigment bands with unknown absorptivities included in the xanthophyll fraction. The precision of *Proposed Method 2* is better than that of *Proposed Method 1*. The xanthophyll fraction collected in *Method 2* contains most of the oxidation products of the xanthophylls as well as those of carotenes.

SUMMARY

Two alternate methods for the determination of xanthophyll in dehydrated alfalfa are described. Each method permits the determination of both carotene and xanthophyll in a single extract. Both methods are adaptations of published procedures for carotene analysis. The pigments are either extracted by 16-hour soaking of the sample of alfalfa meal in a mixture of hexane, acetone, and water, or they are extracted rapidly in a blendor after a prior rehydration of the meal. In either case, the pigments are separated by chromatography. The xanthophyll concentration can be determined either colorimetrically or spectrophotometrically. Data on reproducibility of results by the two methods are presented.

ACKNOWLEDGMENT

The authors wish to acknowledge the technical assistance of Mr. I. V. Ford in the large number of assays required. We are also grateful to Dr. Carl A. Taylor of Salinas Laboratories for his suggestion of adding water to the xanthophyll extractant and to American Chlorophyll Division, Strong Cobb and Co., Inc., for furnishing us with their procedure for assay of xanthophyll in feed products.

REFERENCES

(1) *Official Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Box 540, Benjamin Franklin Station, Wash. 4, D. C., 1950, p. 769.

(2) BICKOFF, E. M., WHITE, L. M., BEVENUE, A., and WILLIAMS, K. T., *This Journal*, 31, 633 (1948).
(3) ———, ATKINS, M. E., BAILEY, G. F., and STITT, FRED, *ibid.*, 32, 766 (1949).
(4) ———, and THOMPSON, C. R., *ibid.*, 32, 775 (1949).
(5) ———, LIVINGSTON, A. L., and VAN ATTA, G. R., *ibid.*, 35, 826 (1952).
(6) ———, LIVINGSTON, A. L., and BAILEY, G. F., *ibid.*, 37, 509 (1954).
(7) ———, LIVINGSTON, A. L., BAILEY, G. F., and THOMPSON, C. R., *J. Agr. Food Chem.* (in press).
(8) DUTTON, H. J., BAILEY, G. F., and KOHAKE, E., *Ind. Eng. Chem.*, 35, 1173 (1943).
(9) GRIFFITH, R. B., and JEFFREY, R. N., *Ind. Eng. Chem., Anal. Ed.*, 16, 438 (1944).
(10) ———, and ———, *ibid.*, 17, 448 (1945).
(11) MOSTER, J. B., and QUACKENBUSH, F. W., *Arch. Biochem. and Biophys.*, 38, 297 (1952).
(12) STITT, FRED, BICKOFF, E. M., BAILEY, G. F., THOMPSON, C. R., and FRIEDLANDER, S., *This Journal*, 34, 460 (1951).
(13) THOMPSON, C. R., and BICKOFF, E. M., *ibid.*, 34, 219 (1951).
(14) ZSCHEILE, F. P., and WHITMORE, R. A., *Anal. Chem.*, 19, 170 (1947).

BIOLOGICAL SCREENING TEST FOR CHLORINATED INSECTICIDES*

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In the course of their work, regulatory chemists are confronted with the problem of assaying a potentially large number of food samples for numerous insecticidal residues. Since the insecticidal spray histories of the samples are frequently unavailable, and because many of the analytical methods are time-consuming and sometimes cumbersome, it is extremely difficult to make a comprehensive analysis for the many possible insecticides currently employed without an expenditure of an exorbitant amount of time and effort. Therefore, a laboratory technique which would rapidly segregate the contaminated samples would effect a substantial saving in time and money.

Because insecticides owe their activity to an effect on a biological system, a pharmacological approach to a screening technique was investigated. Although methods employing flies (1) or mosquito larvae (2) were available, the use of insects as test organisms was rejected as unsuitable for laboratories concurrently analyzing samples for filth.

Literature reports (3) indicate that lindane is toxic to goldfish at a concentration of 1 p.p.m. Since common goldfish are readily available,

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inexpensive, require little care, and are easily handled, their sensitivity to other insecticides was investigated. The insecticides selected for trial were those belonging to the chlorinated hydrocarbon group.

Tests consisted of adding two 2-inch goldfish to each of a series of solutions containing increasing quantities of the various insecticides. Solution of the insecticide in water was accomplished by previously dissolving the insecticide in acetone and then adding 0.1 ml of the acetone solution to 250 ml of water. The concentration of 0.04 per cent acetone has no adverse effect upon the fish.

The symptoms of a strong positive reaction are, first, hyperexcitability; then convulsions, loss of equilibrium, and death within 3 hours. On lower doses the fish may not succumb, even though they exhibit the other symptoms of poisoning. Since it is often difficult to distinguish the hyperactivity from the normal movements of control fish, and since death of the fish does not always occur, convulsions and loss of equilibrium were therefore chosen as the most suitable indicators of a positive reaction. The convulsions are generally intermittent with apparent rest periods between the extremely rapid and eccentric swimming seizures. During the rest periods the fish frequently assume an almost vertical position with their mouths close to the surface.

TABLE 1.—*Sensitivity[a] of goldfish to various chlorinated insecticides*

	MICROGRAMS/250 ML WATER						
	1000	500	300	200	100	50	10
DDT				+	+	±	−
Lindane				+	+	+	−
Heptachlor				+	±	−	
Toxaphene				+	+	−	
Dieldrin				+	+	±	
Aldrin	+	+	−	−	−		
Chlordane	+	+	−	−	−		
Methoxychlor				+	+	−	
Dilan				+	−		

[a] (Convulsions and loss of equilibrium.)
+ All positive. ± Some positive. − All negative.

Table 1 shows the sensitivity of the fish to the various insecticides. Under the described conditions, the dose required for a toxic response in goldfish, on a weight basis, is of the same order of magnitude as a toxic intravenous dose in mammals, and a toxic contact dose in insects. The reason for this sensitivity is attributed to the high systemic concentration achieved by absorption of the insecticide through the gill system.

Fig. 1 shows that the various insecticides had a rather characteristic time pattern with regard to the onset of convulsions, and presents the extremes in time for a fish to react to a particular insecticide. In the case

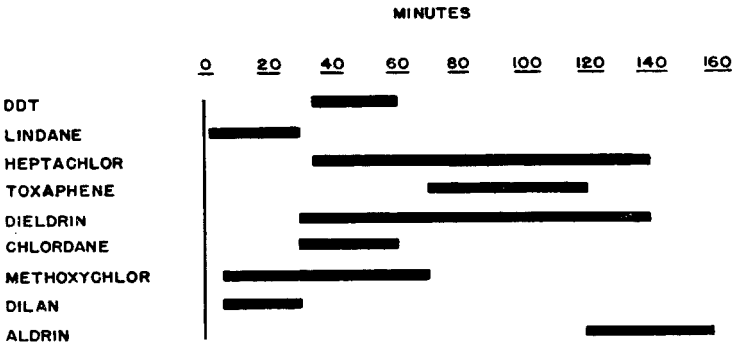

FIG. 1.—Time pattern of convulsions.

of DDT, no fish responded in less than 35 minutes nor more than 60 minutes, despite wide variations in dose.

Undoubtedly, as the number of such assays increases, a fish whose response to a particular insecticide exceeds this time pattern may occasionally be found. Nevertheless, the characteristic delay in the onset of symptoms may serve as a clue to the identity of an unknown insecticide. Thus, if a fish exhibited convulsions at 20 minutes, the insecticide present would be lindane, methoxychlor, or Dilan.

A study of techniques for separating the insecticide from the sample in a form suitable for administration to goldfish was undertaken. It was found that micro-quantities of these chlorinated insecticides could be steam-distilled and that essentially all of the insecticide was present in the first 200 ml of distillate. Furthermore, it was found that the distillate from 100 gram samples of carrots, apples, lima beans, grapefruit and orange juice, milk, and alfalfa had no toxic effect upon the fish during the 3 hour observation time.

Therefore, the following procedure was used in the assay of foods:

METHOD

Puree 100 g of sample in Waring blendor and transfer with 100 ml of H_2O to 500 ml flask of the steam-distn equipment (Scientific Glass Co. No. J1356 or equivalent). Add a drop of DC Antifoam A. Heat flask with a flame to near boiling, attach boiler tube, and collect 250 ml of distillate. Cool the distillate to room temp. and transfer to a 50×100 mm crystg dish. Add two goldfish and observe for 3 hrs for convulsions. (It was found that about 20 distillates can be observed conveniently at one time.)

The steam distillation method was tested by means of recovery runs on various foods. Positive results were obtained with food containing 7 p.p.m. DDT, 5 p.p.m. lindane, 7 p.p.m. toxaphene, 14 p.p.m. methoxychlor, and 7 p.p.m. Dilan. These values must not be taken as the lower

limits of sensitivity for these five insecticides. In the case of heptachlor, chlordane, aldrin, and dieldrin, the fish failed to respond positively to levels of 0.1 p.p.m.

To overcome the difficulties presented by the lack of sensitivity with the latter group of insecticides, further investigation will be required. Approaches which have been considered are: (*a*) increase in sample size, which has obvious limitations; (*b*) the addition of a sub-convulsive amount of insecticide (sufficient to trigger the reaction) to the distillate; and (*c*) use of a more sensitive test animal. The use of the small crustacean, *Daphnia Magna*, has been explored, and preliminary studies indicate that it may be a suitable test organism.

SUMMARY

The approximate sensitivity of goldfish to DDT, lindane, heptachlor, toxaphene, dieldrin, chlordane, methoxychlor, Dilan, and aldrin has been established. A steam distillation method for isolating the insecticide from the sample in a form suitable for administration to goldfish is presented. Goldfish are suggested as a test animal for the rapid sorting of foods which may be contaminated with excessive amounts of DDT, lindane, toxaphene, methoxychlor, and Dilan.

REFERENCES

(1) LAUG, E. P., *J. Pharmacol. Exptl. Therap.*, **86**, 324 (1946).
(2) HARTZELL, A., and STORRS, E. E., *Contrib. Boyce Thompson Inst.*, **16**, 47 (1950).
(3) SLADE, R. E., *Chemistry and Industry*, **64**, 314 (1945).

STUDIES ON COAL-TAR COLORS. XVI. FD&C RED NO. 4*

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The di-sodium salt of 2-(5-sulfo-2,4-xylylazo)-1-naphthol-4-sulfonic acid (Ponceau SX) is certifiable as FD&C Red No. 4 (1). This paper describes the preparation of a purified sample of FD&C Red No. 4 and of its isomeric compound, 2-(6-sulfo-2,4-xylylazo)-1-naphthol-4-sulfonic acid. The purified FD&C Red No. 4, its isomer, and the purified intermediates used in preparing them were used as standards in gathering data by chromatography and by infrared, ultraviolet, and visible spectrophotometry. These data were in turn applied to the determination of the qualitative and quantitative composition of six commercial samples

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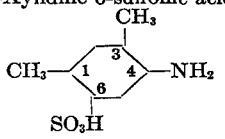
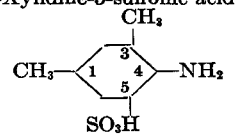
of FD&C Red No. 4. The purified FD&C Red No. 4 was used to determine the validity of the titanium trichloride titration procedure for the determination of the dye. The spectrophotometric properties of solutions of the pure dye were also determined.

The following nomenclature will be used to describe the two *m*-xylidine sulfonic acids:

(1)
m-Xylidine-6-sulfonic acid



(2)
m-Xylidine-5-sulfonic acid



PURIFICATION AND ANALYSIS OF INTERMEDIATES

1-Naphthol-4-sulfonic acid.—One hundred g of commercial 1-naphthol-4-sulfonic acid was dissolved in approximately 800 ml of hot water. Fifty g of *p*-toluidine was dissolved in approximately 200 ml of hot water which contained 43 ml of concentrated HCl. The two solutions were mixed, approximately 5 g of decolorizing carbon was added, and the mixture was filtered while hot. The filtrate was cooled in an ice bath, and the resultant crystals were filtered off and recrystallized twice from approximately 600 ml of 1 per cent acetic acid. (Yield: 49.7 g after drying at 80°C.) Several melting point determinations varied over a range of 6°C.; the highest was 190–192°C. (literature (3) 195°C.) Additional recrystallizations failed to raise the melting point. Analysis of the purified *p*-toluidine salt of 1-naphthol-4-sulfonic acid is summarized in Table 1.

TABLE 1.—*Analysis of purified p-toluidine salt of 1-naphthol-4-sulfonic acid*

	SULFUR	NITROGEN
	(*per cent*)	(*per cent*)
Calculated	9.67	4.23
Found	9.32	4.07
Calculated purity	96.4	96.2
(Volatile matter at 135°C. = 3.15 per cent.)		

m-Xylidine-6-sulfonic acid.—Fifty-six g of commercial *m*-xylidine-6-sulfonic acid was dissolved in approximately 800 ml of hot water, 5 g of decolorizing carbon was added, and the solution was filtered while hot. Upon cooling, the resulting crystals were collected and recrystallized from water. The yield was 19 g. Results of the analysis of this material are shown in Table 2.

m-Xylidine-5-sulfonic acid.—Fifty g of commercial *m*-xylidine-5-sulfonic acid was dissolved in 500 ml of boiling water, a few g of decolorizing carbon was added, and the mixture was filtered. The filtrate was cooled, and the resultant crystals were collected on a Büchner funnel. This procedure was repeated twice. The yield was 15 g.

An attempt was made to isolate isomeric sulfonic acids or impurities by fractional recrystallization of commercial *m*-xylidine-6-sulfonic acid. The ultraviolet absorption curves of all fractions obtained were identical. Dyes were prepared from each fraction by diazotizing the amine sulfonic acid and coupling with β-naphthol.

TABLE 2.—*Analysis of purified m-xylidine-6-sulfonic acid*

	EQUIVALENT WEIGHT	NITROGEN
		per cent
Calculated	201.1	6.96
Found	202.7	6.91
Calculated purity	99.3%	99.3
(Volatile matter at 135°C. =0.2 per cent)		

Solutions of the colors prepared from the different fractions had identical visible and ultraviolet absorption spectra, and no evidence of isomers in the amine-sulfonic acid could be found by this procedure.

Attempts to separate known mixtures of the isomeric *m*-xylidine sulfonic acids by chromatography on wood cellulose columns (Solka-Floc BW 40)[1] from 0.1 *N* HCl, distilled water, and 20 per cent sodium sulfate solution resulted in failure.

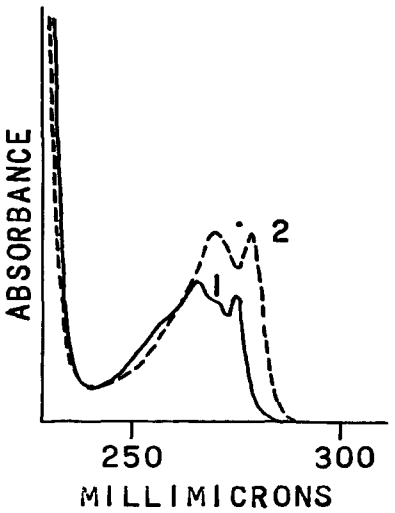


FIG. 1.—Absorption curves of *m*-xylidine-6-sulfonic acid and *m*-xylidine-5-sulfonic acid. Curve 1.—*m*-Xylidine-6-sulfonic acid (118 mg/l in 0.1 *N* HCl.) Curve 2.—*m*-Xylidine-5-sulfonic acid (100 mg/l in 0.1 *N* HCl.)

Figure 1 shows that there is a marked difference in the ultraviolet spectra of *m*-xylidine-6-sulfonic acid and *m*-xylidine-5-sulfonic acid in 0.1 *N* HCl. However, with each of these compounds, at least 1 per cent of its isomer must be present before the absorption spectrum is noticeably changed.

[1] Brown Company, 500 Fifth Avenue, New York 18, New York.

Armstrong and Wilson (2) state that baking *m*-xylidine with one mole of 100 per cent sulfuric acid yields *m*-xylidine-5-sulfonic acid, while simple sulfonation of *m*-xylidine with an excess of fuming sulfuric acid yields mainly *m*-xylidine-6-sulfonic acid. For purposes of establishing the identity of the *m*-xylidine sulfonic acids used in this work, small amounts of *m*-xylidine (Eastman "White Label" 2,4-dimethylaniline) were sulfonated by each of the above methods. In each instance the sulfonated material, both in the crude and recrystallized forms and in solution in the filtrates, appeared from ultraviolet absorption spectra (220 to 350 mμ) to contain no more than traces of impurities. In all cases the ultraviolet absorption spectra were identical with those of the corresponding *m*-xylidine sulfonic acids obtained by recrystallizing the commercial materials.

PREPARATION OF COLORS

FD&C Red No. 4.—One-tenth mole (34.8 g) of the purified *p*-toluidine salt of 1-naphthol-4-sulfonic acid was dissolved in approximately 300 ml of water which contained 0.2 mole of NaOH. Approximately 0.5 g of sodium sulfite was added to prevent oxidation. The *p*-toluidine was extracted from this solution with three 50-ml portions of petroleum ether, and the aqueous layer was boiled for 15 minutes to remove traces of petroleum ether and p-toluidine. Thirty-two g of sodium bicarbonate (0.38 mole) was added and the solution was cooled to 0–5°C.

One-tenth mole (20.1 g) of *m*-xylidine-6-sulfonic acid was dissolved in approximately 300 ml of water which contained 0.1 mole of NaOH. Thirty-two ml of concentrated HCl (0.37 mole) was added with rapid mixing to obtain a finely divided precipitate. The mixture was cooled to 0–5°C., 76 ml of cold 10% sodium nitrite (0.11 mole) was added, and the stirring was continued for 15 minutes after the precipitate dissolved. Cold 10% sulfamic acid solution was added until a negative test was obtained with starch iodide paper. The solution of the diazonium compound was added slowly with stirring to the solution containing the 1-naphthol-4-sulfonic acid, and the stirring was continued for one-half hour while the temperature was held at 0–5°C. The mixture was allowed to warm to room temperature, and was then heated on the steam bath for 1 hr, cooled in an ice bath, and filtered with suction. The yield was 48.0 g (dried at 135°C.) The crude dye was dissolved in ca 1200 ml of hot 1+1 HCl. The mixture was cooled, the crystals were separated on a Büchner funnel, and the crystallization was repeated. The recovered material was dissolved in ca 450 ml of water, and the solution was adjusted to about *p*H 8 with 30% NaOH. The solution was heated to approximately 80°C, 450 ml of hot alcohol was added, and the resulting solution was cooled in an ice-bath. The precipitate was filtered with suction, dried in air, ground, and then dried at 135°C. (The yield was 40.0 g.)

Analytical data for the purified FD&C Red No. 4 is summarized in Table 3.

2-(6-Sulfo-2,4-xylylazo)-1-naphthol-4-sulfonic acid.—The crude dye was prepared in exactly the same way as FD&C Red No. 4 except that 7.88 g of *p*-toluidine salt of 1-naphthol-4-sulfonic acid and 4.55 g of *m*-xylidine-5-sulfonic acid were used. Although the diazonium compound obtained from the *m*-xylidine-5-sulfonic acid was insoluble, the coupling reaction proceeded satisfactorily.

The crude dye was dissolved in 100 ml of water, and 50 ml of concentrated HCl was added. A fine crystalline precipitate formed. The mixture was cooled in an ice bath and the dye was collected on a Büchner funnel. The precipitate was redissolved in 100 ml of hot water and re-precipitated by adding 50 ml of concentrated HCl. The mixture was cooled in an ice-bath and the precipitate was again collected on a Büchner funnel. This recrystallized material was dried at 80°C. for 2 hrs and then at 140°C. for 16 hrs. Figure 2 shows the absorption spectra of solutions of the above compound between 400 and 500 mμ.

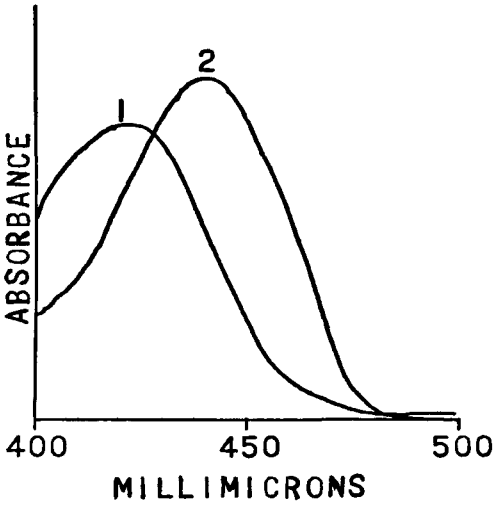


FIG. 2.—Absorption curves of 2-(6-sulfo-2,4-xylylazo)-1-naphthol-4-sulfonic acid. Curve 1.—Alkaline solution. Curve 2.—Acid solution.

COMPOSITION OF COMMERCIAL SAMPLES OF FD&C RED NO. 4

Hydrosulfite reduction of FD&C Red No. 4.—Six samples, obtained from six different manufacturers, were treated in the following manner: A twenty gram sample was dissolved in approximately 300 ml of boiling water. To the boiling solution, solid sodium hydrosulfite was added in small quantities until the solution was colorless. When the solution had cooled to room temperature, concentrated HCl was added dropwise until the addition of several drops produced no further precipitate. The precipitate was collected on a Büchner funnel and more HCl was added to the filtrate. The first few ml gave no change, but on further addition of HCl a second precipitate formed. The second precipitate was collected on a separate Büchner funnel.

The first precipitate was re-dissolved in a minimum amount of boiling water, decolorizing carbon was added, the mixture was filtered, and the filtrate was allowed to cool. On refrigeration, there was a slight increase in the yield of crystals. In each of the six commercial samples examined, the ultraviolet spectra of solutions of these crystals corresponded both qualitatively and quantitatively with those obtained from the *m*-xylidine-6-sulfonic acid.

The second precipitate was dissolved in boiling water, filtered, and cooled. A small amount (approximately 3 g) of needle-like crystals formed slowly. The ultraviolet spectra of the material collected in the second fraction is quite different from that of the material in the first fraction. The spectra showed that the material was the same as that obtained when the purified FD&C Red No. 4 was subjected to similar treatment. It was assumed that this compound was 2-amino-1-naphthol-4-sulfonic acid.

Variable reference titration.—Additional evidence as to the composition of the commercial samples was obtained by variable reference titration (4) in both the visible and ultraviolet regions. With each of the commercial samples a straight line was readily obtained by titration against the purified sample of FD&C Red No. 4.

The six commercial samples were titrated against each other and again straight lines were obtained.

Infrared data.—Samples of FD&C Red No. 4 may be divided into two groups according to their physical appearance and bulk density. In the solid form one group has a deep red color, a very small particle size, and a relatively high bulk density, while the other group is lighter in color and has a larger particle size and lower bulk density.

The six commercial samples of FD&C Red No. 4 used for analysis included members from both of these groups. Infrared spectra of suspensions of each of these samples in a carbon disulfide-aluminum stearate mixture were obtained (5). Two characteristic but quite similar forms of spectra were obtained, one for each of the two forms of the dye noted. However, when the supposedly different samples were recrystallized under identical conditions, the infrared spectra of all samples were identical. It was found that one or the other of the infrared spectra could be obtained from any of the dye samples tested, depending entirely upon the method of recrystallization. It appears, therefore, that the physical differences noted in the original samples were due to differences in crystalline form.

Chromatography of FD&C Red No. 4.—Portions of the purified FD&C Red No. 4 and of the 2-(6-sulfo-2,4-xylylazo)-1-naphthol-4-sulfonic acid were chromatographed individually on purified wood cellulose (Solka-Floc BW 40) with 20 per cent sodium sulfate solution as the eluant. Each compound appeared homogenous. A mixture of the two compounds, however, was readily separated by chromatography, since the FD&C Red No. 4 is held up quantitatively with 20 per cent sodium sulfate solution, while the isomer flows through quite readily. Known amounts of the isomer were added to the FD&C Red No. 4 and the following recoveries were obtained using the chromatographic method of separation:

At a level of 0.2 per cent of the isomer, the recovery was 90.5 per cent.
At a level of 0.5 per cent of the isomer, the recovery was 86.0 per cent.
At a level of 2.0 per cent of the isomer, the recovery was 98.6 per cent.
At a level of 5.0 per cent of the isomer, the recovery was 95.0 per cent.

Qualitative results similar to those obtained by column chromatography were obtained when the individual compounds and their mixtures were subjected to paper chromatography. Two solvents found useful were:

(1)	(2)
200 parts butyl alcohol	300 parts butyl alcohol
88 parts water	150 parts water
2 parts concd ammonium hydroxide	5 parts acetic acid
40 parts ethyl alcohol	80 parts ethyl alcohol

With paper chromatography, as with the column chromatography, it was found that the isomer moved more rapidly than the FD&C Red No. 4. Using paper chromatography, the presence of as little as one per cent of the isomer in the FD&C Red No. 4 could be detected.

When paper chromatography was applied to the purified FD&C Red No. 4 and representative commercial samples, and either water or 1 per cent ammonium hydroxide was used as a developer, there was an apparent initial separation into two distinct bands. However, as the chromatogram progressed the small leading band became larger and the trailing band smaller, until all of the less mobile form had been converted to the more mobile form. When extracted from the paper, solutions of these two fractions showed the same visible and ultraviolet spectra. No further investigation of this phenomenon was made; it was assumed that the results were due to a change in cis-trans isomerism at the azo linkage.

Spectrophotometric data.—Spectrophotometric measurements were made with a General Electric recording spectrophotometer with an effective slit width of 8 mμ and matched 1 cm Pyrex cells.

A weighed sample of dried FD&C Red No. 4 was dissolved in a definite volume of water. Suitable aliquots of this solution were buffered with Clark and Lubb's buffer mixture and diluted to volume. The spectrophotometric data obtained from these solutions is summarized below. The *p*H values were obtained with a glass electrode *p*H meter.

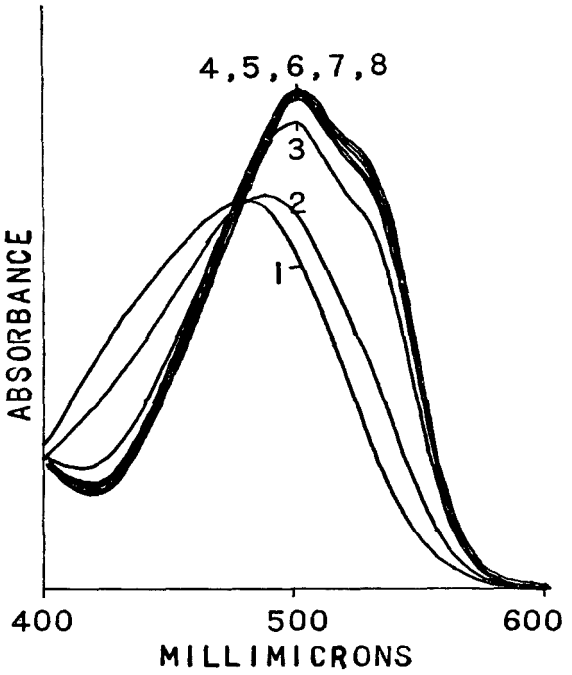


FIG. 3.—Absorption curves of FD&C Red No. 4 in water at various *p*H levels (concn 15.98 mg per liter). Curve 1.—*p*H 13 (calcd). Curve 2.—*p*H 9. Curve 3.—*p*H 8. Curve 4.—*p*H 7. Curve 5.—*p*H 6. Curve 6.—*p*H 5.1. Curve 7.—*p*H 3.0. Curve 8.—*p*H 1.1.

As may be seen from Fig. 3, the visible spectrum of FD&C Red No. 4 varies little over the *p*H range 1.1 to 7.0. In more alkaline solution there is a marked shift toward the yellow and a loss in absorbance.

Solutions of FD&C Red No. 4 were buffered to a *p*H of 6.7±0.1 with ammonium acetate. The average absorbance, based on twenty determinations at concentrations from 2.8 to 16.4 mg/l was 0.0542/mg/l at 502 mμ. The average deviation of these measurements was 0.31%.

Intermediates in FD&C Red No. 4.—Attempts were made to recover known amounts of both 1-naphthol-4-sulfonic acid and *m*-xylidine-6-sulfonic acid from FD&C Red No. 4 by column chromatography, using purified wood cellulose with

TABLE 3.—*Analysis of purified FD&C Red No. 4*

	NITROGEN	SULFUR	TITRATION WITH 0.1 *N* $TiCl_3$[a]	SULFATED ASH	Na_2SO_4
	(*per cent*)	(*per cent*)	(*ml/g*)	(*per cent*)	
Calculated for pure dye	5.83	13.35	83.3	29.57	
Found	5.81	13.30	83.4	29.55	nil
Calculated Pure Dye	99.7	99.6	100.1%	99.9	

(Volatile matter at 138°C. and 1 mm Hg = nil.)

[a] See *Official Methods of Analysis*, A.O.A.C., section 34.21(c).

20 per cent sodium sulfate solution as eluant. Approximately 10 ml fractions were collected from the column and the absorption spectrum of each fraction was observed with an ultraviolet spectrophotometer. Known amounts of both intermediates were added to a sample of purified FD&C Red No. 4 and the mixture was subjected to column chromatography. It was found that, while both intermediates were eluted much more rapidly than the FD&C Red No. 4, they could not be fully separated from each other. The first band obtained was rich in *m*-xylidine-6-sulfonic acid; however, this band trailed into the second band which contained mainly 1-naphthol-4-sulfonic acid.

Average recoveries were only 75% at levels of 0.5% and 1.0% for each intermediate.

Using this semi-quantitative method, six commercial samples of FD&C Red No. 4 were examined for intermediates. In five instances, no intermediates were found; in the sixth, a maximum of 0.1% of 1-naphthol-4-sulfonic acid was detected.

SUMMARY

A purified sample of FD&C Red No. 4 has been prepared from the purified intermediates, 1-naphthol-4-sulfonic acid and *m*-xylidine-6-sulfonic acid.

Six commercial samples of FD&C Red No. 4 were established as authentic by comparison with the purified FD&C Red No. 4 and the most likely contaminant, 2-(6-sulfo-2,4-xylylazo)-1-napththol-4-sulfonic acid. Procedures used in this comparison included hydrosulfite reduction of FD&C Red No. 4 followed by isolation of the reduction products, variable reference titration, infrared analysis, and chromatographic analysis.

Both the spectrophotometric and titanium trichloride titration procedures give good results in the quantitative determination of the dye.

Data on the visible spectra of FD&C Red No. 4 are presented.

REFERENCES

(1) S.R.A., F.D.C. 3, Food and Drug Administration, *Coal-Tar Color Regulations.*
(2) ARMSTRONG, H. E., and WILSON, L. P., *Chem. News*, **83**, 46 (1901).
(3) DERMER, O. C., and DERMER, V. H., *Org. Chem.* **7**, 582 (1942).
(4) CLARK, G. R., JONES, J. H., and HARROW, L. S., *This Journal*, **34**, 135 (1951).
(5) DOLINSKY, M., *ibid.*, **34**, 750 (1951).

STUDIES ON COAL-TAR COLORS. XVII. EXT. D&C RED NO. 11*

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Ext. D&C Red No. 11 is similar to D&C Red No. 33. D&C Red No. 33 is prepared by coupling diazotized aniline with H acid (1-naphthol-8-amino-3,6-disulfonic acid), while Ext. D&C Red No. 11 is prepared by coupling diazotized aniline with acetylated H acid.

A previous paper (1) described the preparation, analysis, and optical properties of D&C Red No. 33 and of Chromotrope 2R, a subsidiary color present in commercial samples of D&C Red No. 33. This paper describes the preparation, analysis, and optical properties of purified Ext. D&C Red No. 11, as well as procedures for the determination of the colored impurities ordinarily present in commercial samples of the dye.

EXPERIMENTAL

PURIFICATION OF INTERMEDIATES

Aniline was purified by distillation over zinc dust.

H acid was purified by recrystallizing three times from water. Small quantities of sodium hydrosulfite were added to the water to reduce oxidation. Charcoal was used in the first recrystallization.

Acetyl H acid (1-naphthol-8-acetylamino-3,6-disulfonic acid) was prepared directly before use by acetylating purified H acid in the manner described by Fierz-David (2).

A chromatographic procedure was devised for determining the presence of unacetylated H acid and of diacetylated H acid in a solution of acetylated H acid. One drop of the soda solution of the batch of acetylated H acid is withdrawn and spotted about 2 cm from the edge of a strip of Whatman No. 1 filter paper approximately 24 cm long. The chromatogram is developed with an aqueous solution of 5% NaCl and 1% NH_4OH by ascending chromatography. The completed chromatogram is examined under ultraviolet light. Mono- and diacetylated H acids fluoresce green, and unacetylated H acid fluoresces blue. When a known mixture of the three compounds is chromatographed, three distinct spots are formed by the time the solvent has ascended two-thirds of the strip. Unacetylated H acid is the upper spot, mono-acetylated H acid is the middle spot, and diacetylated H acid is the lower spot. It takes about one hour to develop such a chromatogram.

A chromatogram of a sample of the batch of acetylated H acid used in this work showed only a trace of unacetylated H acid and no diacetylated H acid to be present.

PREPARATION OF EXT. D&C RED NO. 11

Aniline (0.1 mole) was diazotized in the manner described by Fierz-David (3). When diazotization was complete, the excess nitrous acid was destroyed with sulfamic acid. The diazotized aniline solution was poured into an ice-cold alkaline solution

* Presented at the Sixty-seventh Annual Meeting of the Association of Official Agricultural Chemists, held Oct. 12, 13, and 14, 1953, at Washington, D. C.

of acetyl H acid (0.1 mole) with vigorous stirring. The solution was kept slightly alkaline to indicator paper by the addition of small amounts of Na_2SO_3 solution as required. The mixture was stirred for 2.5 hours, at 0–5°C., and was then heated to 70°C. Hydrated sodium acetate, 27 g for each 100 ml of solution, was added slowly and with vigorous stirring. The dye precipitated in an easily filterable form. The warm mixture was filtered with suction on a Büchner funnel and most of the mother liquor was pressed out with the aid of a rubber dam. The product was redissolved in water and was again salted out with sodium acetate. The process was repeated a third time.

Chromatographic analysis of this material as described below showed that no unacetylated dye (Acid Fuchsin D) was present.

The product was dried on a Büchner funnel with suction, ground in a mortar, and passed through a 40-mesh sieve. The sodium acetate was then extracted by digesting the sifted dye with successive 100 ml portions of boiling methanol, filtering after each extraction, and washing the filter cake with 50 ml of hot methanol in essentially the manner described by Hartwell and Fieser (4). The alcohol extractions were continued until a negative test for acetate was obtained. The material (29.3 g)was then dissolved in 100 ml of hot water, and 500 ml of hot ethyl alcohol was added slowly with swirling to precipitate the dye. The mixture was digested on a steam bath for one hour, allowed to cool slowly, and filtered while slightly warm. The precipitate was washed on the filter with another 100 ml of warm alcohol, dried in the air, and pulverized and dried further, first at 100°C., then at 135°C., and finally over phosphorous pentoxide in an Abderhalden dryer at the temperature of boiling toluene. Analysis:

Calcd: $N = 8.25\%$; $S = 12.59\%$; $Na = 9.03\%$; ml 0.1 N $TiCl_3$ per gram = 78.49.

Found: $N = 8.15\%$; $S = 12.56\%$; $Na = 8.98\%$; ml 0.1 N $TiCl_3$ per gram = 78.23.

This preparation contained 0.9% of Acid Fuchsin D as determined by the procedure given below. It appears that some hydrolysis occurred during the purification process.

From the data obtained in the previous investigation of D&C Red No. 33 (1), it is apparent that the equivalent weight and the spectrophotometric properties of a sample of Ext. D&C Red No. 11 will be almost unaffected by the presence of less than 1% of the unacetylated dye.

SPECTROPHOTOMETRIC DATA

Beer's Law was found to apply to solutions containing 5 to 12 mg of the dye per liter. The solvent was 0.04 N aqueous ammonium acetate. Spectrophotometric measurements were made with a General Electric recording spectrophotometer, using a wavelength band of 8 mμ, and with a Cary recording spectrophotometer. Data in the visible region obtained from solutions at various pH levels are shown in Figure 1.

SUBSIDIARY DYES IN EXT D&C RED NO. 11

The principal subsidiary dye expected in commercial samples of Ext. D&C Red No. 11 is Acid Fuchsin D, *Colour Index No. 30* (5), certifiable as D&C Red No. 33. This dye is formed when unacetylated H acid is coupled with diazotized aniline.

If chromotropic acid (1,8-dihydroxynaphthalene-3,6-disulfonic acid), a decomposition product of H acid, is present in the starting material, it is acetylated and then de-acetylated completely during the preparation

of the acetyl H acid. When chromotropic acid is coupled with diazotized aniline the product is Chromotrope 2R, *Colour Index No. 29*. It was expected that this dye is present in commercial samples of Ext. D&C Red No. 11.

Separation of Chromotrope 2R, Acid Fuchsin D, and Ext. D&C Red No. 11.—Mixtures of these dyes can be separated by paper chromatography when the solvent is prepared by mixing one volume of concentrated ammonium hydroxide solution and one volume of formamide with fifty volumes of 0.2 *M* boric acid solution and diluting with water to one hundred volumes. Strips of Whatman No. 1 paper are used.

Chromotrope 2R appears as the upper spot, Ext. D&C Red No. 11 as the middle spot, and Acid Fuchsin D as the lower spot.

When commercial samples of Ext. D&C Red No. 11 were chromatographed by

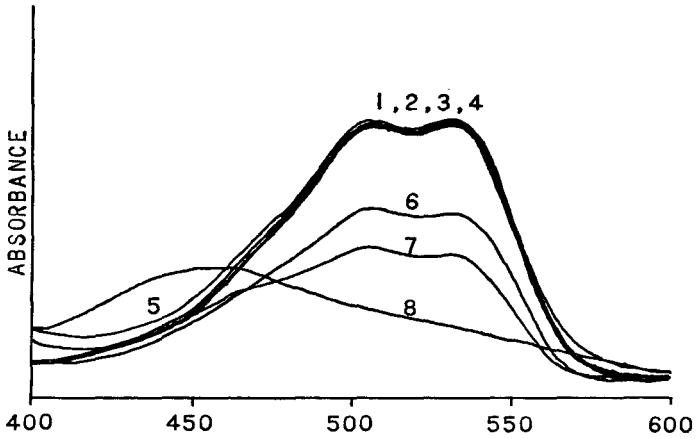

FIG. 1.—Absorption curves of Ext. D&C Red No. 11 in aqueous solutions (conc 11.04 mg/liter. Laboratory preparation containing 0.9% Acid Fuchsin D.) Curve 1.—0.1 *N* HCl. Curve 2.—*p*H 3. Curve 3.—*p*H 5. Curve 4.—*p*H 7. Curve 5.—*p*H 9. Curve 6.—*p*H 10. Curve 7.—ca *p*H 11.3. Curve 8.—0.1 *N* NaOH.

this procedure, it was found that Chromotrope 2R was present in all samples examined but in amounts estimated to be less than 0.5%. Acid Fuchsin D was present in significant amounts. It is assumed that this procedure succeeds because the hydroxyl groups in the *peri* position form a complex with boric acid. The principle of this method is probably similar to that described by Wachtmeister (6). This author reported that organic compounds containing adjacent hydroxyl groups, or a hydroxyl group in the *ortho* position to a free carboxyl group, form complexes with boric acid which can be separated by chromatography on borate impregnated paper. Mixtures of organic solvents and water are used as the developing solvents.

Separation of Acid Fuchsin D from Ext. D&C Red No. 11.—The cellulose column chromatographic technique is used. The column is prepared and the separation carried out as described in the previous paper (1) except that the developing solvent is a 5% salt solution to which 1% by volume of concentrated NH_4OH is added. The

Ext. D&C Red No. 11 is eluted first; when all of this color has been removed from the column the Acid Fuchsin D is eluted with water.

The amount of each dye present can readily be determined spectrophotometrically.

ANALYSIS OF COMMERCIAL SAMPLES

Stock solutions of four batches of commercial dyes were prepared. One aliquot of each solution was chromatographed on cellulose as described above and fractions were examined spectrophotometrically. An additional aliquot of each solution was directly examined in the same

TABLE 1.—*Analysis of commercial Ext. D&C Red No. 11*

SAMPLE	EXT. D&C RED NO. 11	ACID FUCHSIN D	TOTAL DYE (CHROMATOGRAPHED FRACTIONS)	TOTAL DYE (BY $TiCl_3$ TITRATION)[a]
	per cent	*per cent*	*per cent*	*per cent*
1	80.2	1.3	81.5	80.8
	80.2	1.3	81.5	
2	77.2	2.0	79.2	78.8
	77.2	2.0	79.2	
3	80.0	1.0	81.0	80.1
	79.9	0.9	80.8	
4	76.5	0.9	77.4	77.5
	76.2	1.0	77.2	

[a] Sample titrated without chromatographic separation. Buffer: Sodium bitartrate.

manner. The results showed that at least 99 per cent of the material was recovered from the column.

Table 1 shows the composition of the four samples as determined by the chromatographic method. The amounts of the two dyes present were determined from spectrophotometric measurements. The Ext. D&C Red No. 11 was calculated from measurements at 505 mμ, the Acid Fuchsin D from measurements at 530 mμ. The total dye (calculated as Ext. D&C Red No. 11) in each sample, as determined by $TiCl_3$ titration, is also shown for comparison.

DISCUSSION

Visible and ultraviolet absorption spectra of Ext. D&C Red No. 11 and of Acid Fuchsin D in acid, basic, and neutral solutions are sufficiently different to permit identification of either dye when present alone. The difference in the absorption curves is not sufficient to permit detection of a small percentage of the subsidiary color in commercial samples of Ext. D&C Red No. 11. The two dyes could not be separated by extraction according to the Koch scheme (7), since they both fall into his Group 6.

Chromatographic analysis of commercial samples of Ext. D&C Red No. 11 is a simple and sensitive method for the isolation of small amounts of colored impurities. Practically quantitative results can be obtained.

REFERENCES

(1) SCLAR, RACHEL N., *This Journal*, **36**, 930 (1953).
(2) FIERZ-DAVID, H. E., and BLANGEY, L., *Fundamental Processes of Dye Chemistry*. Trans. from 5th Ed., Interscience Publishers, Inc., New York, 1949, p. 263.
(3) *Ibid.*, p. 243.
(4) HARTWELL, J. L., and FIESER, L. F., *Organic Syntheses*, John Wiley & Sons, Inc., New York, 1936, p. 12.
(5) ROWE, F. M., *Colour Index of the Soc. of Dyers and Colourists*, Yorkshire England, 1924, p. 7.
(6) WACHTMEISTER, C. A., *Acta Chemica Scandinavica*, **5**, No. 6, 976 (1951).
(7) KOCH, L., *This Journal*, **26**, 245 (1943).

A SEPARATION OF THE TRIPHENYLMETHANE FOOD COLORS BY COLUMN CHROMATOGRAPHY

By G. G. MCKEOWN and J. L. THOMSON (Food and Drug Laboratory, Department of National Health and Welfare, Ottawa, Canada)

Five of the colors permitted for food use in Canada and the U.S.A. belong to the triphenylmethane class. These are Brilliant Blue FCF (FD&C Blue No. 1), Light Green SF Yellowish (FD&C Green No. 2), Fast Green FCF (FD&C Green No. 3), Guinea Green B (FD&C Green No. 1), and Benzyl Violet 4B (FD&C Violet No. 1). This paper describes the behavior of these colors on columns of alumina when a special method of adsorption and development is used.

Several investigators (1–3) have applied column chromatography to the separation of water-soluble food colors. These workers effected physical adsorption of the colors on columns of alumina from small volumes of neutral aqueous or alcoholic solutions. Water or alcohol was also used for the development. The colors studied included two of the above, viz., Light Green SF Yellowish and Guinea Green B. In the present work, the colors are adsorbed from acidic solutions, which causes a chemical fixation of the colors as alumina lakes. The procedure has an advantage in that, even with the rapid passage of a large volume of color solution, the colors are quantitatively fixed in a narrow band at the top of the column. Development and elution is accomplished by washing with weakly basic solvents. A similar study involving amaranth and tartrazine has been carried out by one of the authors (4).

EXPERIMENTAL

A column was prepared using a chromatographic tube of length 150 mm and I.D. 15 mm. A glass wool plug was covered with a 5 mm layer of Ottawa sand to

provide a regular foundation for the adsorbent. A slurry of alumina in water was slowly run into the tube to a height of 100 mm. The column was washed with distilled water until a clear percolate was obtained. (Adsorption alumina (80–200 mesh), supplied by the Fisher Scientific Company, was used.)

The colors used were commercial samples supplied by the Dye and Chemical Company of Canada, Ltd., Kingston, Ontario. They were not purified before use. Aqueous solutions were prepared from which aliquots for the separations were drawn.

The colors were fixed on the column as follows: An aqueous solution of color was adjusted to about 0.1 *N* with dilute acetic acid and passed through the column. This was followed by about 50 ml of distilled water to free the column of excess acid. The colors were found to be fixed on the upper 2–3 mm of the column.

Development was accomplished by washing the column with a dilute solution of pyridine in water at a flow rate of 2–3 ml per minute. The concentration of pyridine in the developer solution was found markedly to affect the rate of development and degree of separation. While a 5 per cent solution provided rapid development but poor separation, a 0.5 per cent solution gave excellent separation but impractically slow development. A compromise, using solutions containing from 1 to 3 per cent pyridine, was adopted.

During development, the influence of the basic solvent caused some alteration in the color of the dyes. Guinea Green B and Light Green SF Yellowish were decolorized, Fast Green FCF became a dark blue, and Brilliant Blue FCF and Benzyl Violet 4B remained unchanged. To observe the position of the colors after development, it was necessary to pass 100 ml of water through the column to wash out the remaining developer solution, followed by about 100 ml of 1+100 acetic acid solution. The dyes then regained their original color.

RESULTS

Chromatograms of single colors were first run to observe individual behavior and chromatographic purity. Brilliant Blue FCF passed rapidly down the column in a discrete band. Guinea Green B and Benzyl Violet 4B travelled less rapidly in somewhat diffuse bands. Light Green SF Yellowish and Fast Green FCF traveled most slowly in fairly compact bands. Fast Green FCF separated into two colors, a main blue band and a green band which remained at the top of the column. The latter was not removed by washing with ammonia solution.

A mixture containing 0.5 mg of each color was developed with 350 ml of 1.5 per cent pyridine. The colors were found to be positioned on the column as follows: Fast Green FCF, 20–28 mm; Light Green SF Yellowish, 22–34 mm; Benzyl Violet 4B, 36–50 mm; Guinea Green B, 55–75 mm; and Brilliant Blue FCF, 56–72 mm. Binary mixtures of the various combinations were run, using 1 per cent pyridine. Good separations were obtained except in the case of Light Green SF Yellowish and Fast Green FCF, and Guinea Green B and Brilliant Blue FCF. These pairs could not be resolved.

The possibility of quantitatively separating a mixture of Brilliant Blue FCF and Light Green SF Yellowish was investigated. A mixture containing 0.5 mg of each color was fixed on the column and developed with a 1

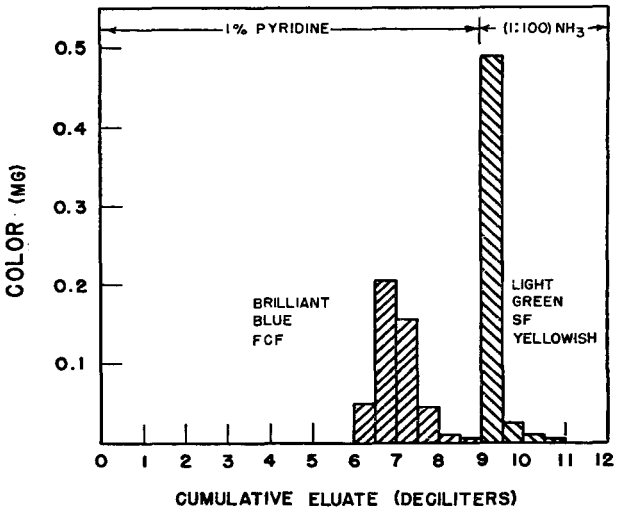

FIG. 1.—Separation of brilliant blue FCF and light green SF yellowish on a column of alumina.

per cent pyridine solution. The eluate was collected in 50 ml fractions. After the passage of 900 ml of developer solution, almost all of the Brilliant Blue FCF had appeared in the eluate. The column was then washed with 300 ml of 1+100 ammonia solution to recover the Light

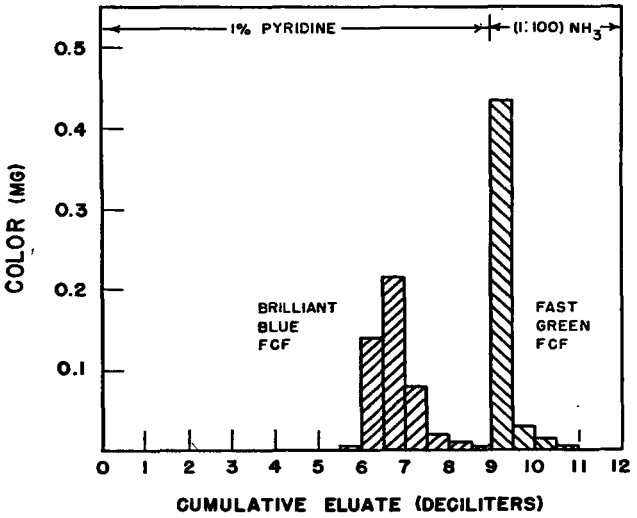

FIG. 2.—Separation of brilliant blue and fast green FCF on a column of alumina.

Green SF Yellowish. Each fraction was acidified by adding 1 ml of concentrated acetic acid, and examined spectrophotometrically. A similar experiment was carried out with a mixture of Brilliant Blue FCF and Fast Green FCF. The results are shown in Figures 1 and 2.

It will be seen that separations of well over 90 per cent are obtainable. In the first separation, the pyridine fractions contain 94 per cent of the Brilliant Blue FCF while the ammonia fractions show an apparent 106 per cent recovery of Light Green SF Yellowish. The latter undoubtedly contains the remaining Brilliant Blue FCF. The second experiment shows recoveries of 95 per cent Brilliant Blue FCF and 97 per cent Fast Green FCF. Similarly, the 97 per cent figure probably includes about 5 per cent Brilliant Blue FCF. The resulting low recovery for Fast Green FCF is to be expected, as it has been shown previously to contain an impurity not eluted with pyridine or ammonia solutions.

REFERENCES

(1) RUGGLI, P., and JENSEN, P., *Helv. Chim. Acta.*, **18**, 624 (1935); **19**, 64 (1936).
(2) RUIZ, SAENZ LASCANO, I., *Ind. Parfum.*, **1**, 187 (1946).
(3) ———, *Ann. fals. et fraudes*, **41**, 211 (1948).
(4) MCKEOWN, G. G., *This Journal*, **37**, 527 (1954).

NOTE

ESTER DETERMINATIONS IN DISTILLED LIQUORS WITH INCREASING ALKALI CONCENTRATIONS*

By ROBERT L. SCHOENEMAN (Alcohol and Tobacco Tax Division Laboratory, Internal Revenue Service, Washington 25, D. C.)

The present method for the determination of esters in distilled liquors (*Official Methods of Analysis*, A.O.A.C., 1950, 9.17) calls for an excess of alkali in the hydrolysis but does not specify a method for determining the excess. This must be estimated by the analysts' fore-knowledge of the type of product or must be determined by trial and error. Any added alkali will bring about a degree of saponification, and

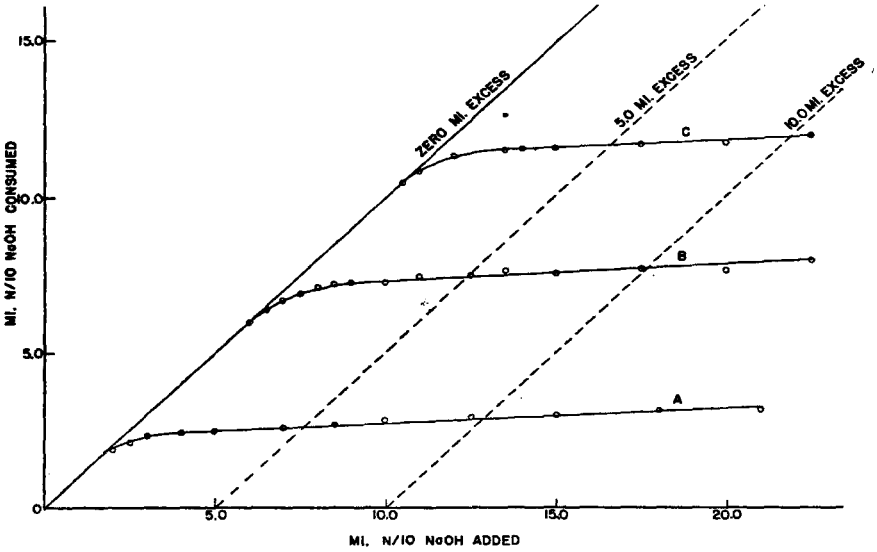

FIG. 1.—Ester hydrolysis with increasing alkali concentrations.

as an equivalence is approached, the phenolphthalein endpoint indicates an excess of alkali before the hydrolysis curve flattens. (Since the saponification of esters is an equilibrium type reaction, absolute ester content cannot be determined without an infinite excess of alkali.)

For this reason the effect of increasing alkali concentration in the determination of esters in distilled liquors was studied. Three samples covering the range of ester content normally encountered were used: A, Light-bodied whisky, B, Heavy-bodied whisky, and C, Apple brandy.

In Fig. 1, ml of 0.1 *N* NaOH added in each ester determination are plotted against ml of 0.1 *N* NaOH consumed in the hydrolysis. The "zero ml excess" line

* Presented at the Sixty-seventh Annual Meeting of the Association of Official Agricultural Chemists, held Oct. 12, 13, and 14, 1953, at Washington, D. C.

marks the area beyond which phenolphthalein indicates an excess of alkali. The dotted lines intersect the curves at points where the alkali is in excess by 5.0 and by 10.0 ml.

Table 1 shows the ester values calculated as grams of ethyl acetate per 100 liters (1 ml 0.1 *N* NaOH = 0.0088 g ethyl acetate).

TABLE 1.—*Ester values with increasing alkali concentrations*

SAMPLE	0.1 *N* NaOH IN EXCESS OF PHENOLPHTHALEIN ENDPOINT				
	0.0	0.5	2.0	5.0	10.0
A	16.3	20.2	22.0	23.3	25.5
B	55.9	60.3	64.2	65.6	68.2
C	92.0	98.1	101.6	103.0	105.6

These data indicate that an excess of 5 to 10 ml of alkali will result in variations of ester content no greater than 2.6 per 100 liters. Less than 5.0 ml excess of 0.1 *N* NaOH results in variations as high as 11.0 g per 100 liters. The range of this variation can be limited by specifying the amount of excess alkali to be used in the method.

Organic Syntheses, Volume 33. By CHARLES C. PRICE, Editor-in-Chief. John Wiley & Sons, Inc., New York, 1953. vi+115 pp. Price $3.50.

Since each volume of this series of annual publications is essentially a continuation of the preceding one, the reviewer can add but little to that which has been written about previous volumes. The high standards attained in these books are well known to chemists everywhere, and Volume Thirty-three continues those standards. In this reviewer's opinion, the book is a valuable addition to any chemical library.

Directions are given for the preparation of the following materials:

3-Acetamido-2-butanone; alloxantin dihydrate; atrolactic acid; benzhydryl β-chloroethyl ether; benzoguanamine; butyrchloral; cresol; di-*tert*-butyl malonate; diethyl 1,1-cyclobutanedicarboxylate; diethyl-oxopimelate; *p*-dimethyl-aminobenzaldehyde; dimethylketene; 2,2-dimethylpyrrolidine; ethyl α-(1-pyrrolidyl)-propionate; 9-fluorenecarboxylic acid; furfural diacetate; itaconyl chloride; 2-methylcoumarone; 1,5-naphthalenedithiol; 1,4-naphthoquinone; nicotinonitrile; *m*-nitrobenzazide; *m*-nitrobiphenyl; *o*-nitrocinnamaldehyde; *m*-nitrostyrene; 6-nitroveratraldehyde; 4-pentyn-1-ol; α-phenylcinnamic acid; 4-phenyl-*m*-dioxane; *o*-phenylene carbonate; 3-phenyl-1-propanol; pyridine-N-oxide; 2-(1-pyrrolidyl)-propanol; stearone; *cis*-stilbene; α-tetralone; 3-thenaldehyde; 3-thenoic acid; 3-thenyl bromide; α,β,β-triphenylpropionic acid.

KENNETH A. FREEMAN

Standard Methods of Clinical Chemistry, Vol. I. By the American Association of Clinical Chemists. Miriam Reiner, Editor-in-Chief. Academic Press, Inc., New York, 1953. x+142 pages. Illus., index. Price $4.50.

This manual, the first of a series, is the outgrowth of a long-felt need to evaluate, to standardize, and to adopt reliable methods of analysis in this all-important field. The foreword recalls the names of Thudichum, Bang, Folin, Benedict, van Slyke, and others—all dedicated investigators who (with many others) created the structure of clinical chemistry. The Editorial Committee of the American Society of Clinical Chemists hopes that the book "will raise the standards of clinical chemistry and will ultimately improve the medical care of the population."

The title should not give an impression of completeness; only 19 tested methods are presented in this first volume, and these deal mainly with the constituents of blood. The Editor states, "the exact order of importance of those [the methods] chosen here may be debatable, but almost all laboratories include the analysis of these constituents in their daily repertoire." It is expected that many more tested methods will appear in succeeding volumes.

It is gratifying to note that in the testing of methods, the American Association of Clinical Chemists has adopted a procedure similar to that of the A.O.A.C. The method is studied by the "Submitter"; then it is sent to the "Checker" or "Checkers" who re-test it in their own laboratories for its validity and practicability. The original test selected by the Submitter is usually one enjoying long usage in the field of clinical chemistry and is selected for speed and simplicity, as well as precision, so as to be consistent with the vigorous regime of a clinical laboratory conducting many routine tests.

Although the method is checked by analysis of a number of samples from control and pathological cases, in the opinion of this reviewer, the checking procedure is not quite as rigorous as the collaborative work which is required for the Official Methods

of Analysis of the A.O.A.C. In the present instance the checking may be, and often is, done by one person.

The excellent introduction to this book may well be considered an integral part of each procedure. It discusses the methods to follow for specimen collection, preparation and preservation, test performance, reporting of results, limitations of methods, pitfalls, interpretation of results, and use and care of equipment.

Each analysis is contained in a separate chapter headed by the name of the Submitter, the Checker, and the reference to the author's original work. The format of individual chapters is good. Included are the reagents and procedure, and in addition, a general introduction and discussion of underlying principles, historical and chemical background, and methods of calculation. Some chapters include, too, the range of normal and abnormal values, interpretations of results, comments, precautions, and notes. An excellent bibliography is appended to each chapter.

The chapter "Sodium and Potassium by Flame Photometry" includes a good general introduction describing the elementary principles of flame photometry, the equipment, and its use.

The book is well written in sufficient detail to warrant inclusion in the clinical library and at the workbench of the clinical chemist and technician.

BENJAMIN M. GUTTERMAN

Toxicity of Industrial Solvents. By E. BROWNING. The Chemical Publishing Co., Inc., New York, 1954. viii+411 pp. Price $8.00.

To help prevent poisoning from the handling of solvents is the declared objective of this book. To accomplish this aim the author has prepared a comprehensive review of the effects on man and animals of a great variety of solvents used in the laboratory and in industrial plants. The subject matter is organized so that each chapter deals with a group of solvents classified as hydrocarbons, chlorinated hydrocarbons, alcohols, ethers, esters, ketones, glycols and their derivatives, amines and coal-tar bases, nitro compounds, and a number of miscellaneous compounds, which include carbon disulfide, silicones, and silane intermediates.

There is a discussion of the properties, uses, and toxic effects of each solvent. The toxic effects in man and animals are treated separately, which in many instances makes for duplication. The toxicity discussion includes the symptoms of acute and chronic poisoning; the absorption, metabolism, and excretion of the solvent; and its effects upon tissues such as blood, liver, kidney, etc. Of special interest are the air concentration of solvents found under actual working conditions, and the tables which relate air concentration to toxicity.

The text is clearly written with hundreds of references, some as recent as 1950, and should gain wide popularity as a reference book for industrial toxicologists and pharmacologists.

BERNARD DAVIDOW

Proceedings of the International Congress on Analytical Chemistry. Published under the patronage of the International Union of Pure and Applied Chemistry, Oxford, England, September 4–9, 1952. W. Heffer and Sons, Ltd., Cambridge, England, 1953. xii+493 pp. Price 42 *s.*

The Analytical Section (Section No. V of the International Union of Pure and Applied Chemistry) held a full international congress at Oxford in September of 1952, with nearly 700 delegates from 26 countries in attendance. It was decided to publish all lectures and papers in a scientific journal, and accordingly they appeared in the *Analyst*, **77**, 557–1024 (Nov.–Dec. 1952). The present book is a reprint of the

proceedings as they appeared in the *Analyst;* it contains, in addition, a brief foreword by President Sir Robert Robinson, and various committee and subcommittee listings. Three stimulating Congress lectures, by R. H. Müller, L. H. Lampitt, and C. J. van Nieuwenburg, are reprinted. There follows a total of 43 program papers, with summaries in English, French, and German, along with the usual discussions. The papers are grouped under 9 sub-heads: microchemical, biological, electrical, optical, radiochemical, organic complexes, presentation of data, adsorption and partition methods, and general. The papers are of the high caliber a reader would expect of internationally known authorities.

However, one opens the book, for example, to page 311, "The Fractionation of Ozokerites . . . " and, again for example to page 359, "Gas-Liquid Partition Chromatography." It is seen that the book is a grab-bag of brilliant miscellaneous information and the reader is almost sure to forget what particular topics are dealt with therein. If an investigator is interested in a particular phase of analytical chemistry, he would normally consult the abstracts, which would lead him to the corresponding pages of the *Analyst.* However, the book remains an interesting souvenir volume marking the status of analytical chemistry to date.

P. A. CLIFFORD

Instrumental Analysis. By JOHN H. HARLEY and STEPHEN E. WIBERLEY. John Wiley & Sons, Inc., New York, 1954. vii+440 pp. Price $6.50.

The preface states that this book "has been written as a text for a course in instrumental analysis given to senior and graduate chemistry students," and the volume appears to be an excellent text for this purpose. Obviously in a book of this size (440 pages) covering so many topics, only a limited amount of material can be included on each topic. The material given, however, does provide a sound introduction to the subjects covered. The discussions of theory, instrumentation, and applications are clear and concise, and in most instances, the discussion of the limitations of different methods as well as their advantages is good.

The major problem in preparing a book of the survey type is what material to include and what to omit. On the whole the authors have done an excellent job in the selection of material to be presented. It is the reviewer's opinion, however, that a more complete discussion of spectrophotometric methods, particularly ultraviolet procedures, would be desirable.

The reviewer noted no errors of fact, but did observe some statements that might be easily misinterpreted. For example, the discussion of solvents on pp. 55–57 leaves the impression that water is not a useful solvent for ultraviolet absorption measurements. Also, it might be inferred from the discussion on pp. 59–60 that ultraviolet spectra are of little use in qualitative identification of organic compounds. The reviewer was surprised to find only one obscure reference to "Analytical Absorption Spectroscopy" by Mellon, *et al.*

The proposed laboratory experiments seem inconsistent in that detailed, very specific directions are given in some cases, yet only general directions in other instances. It might have been preferable to omit the laboratory experiments and instrument operating instructions, since each college using the book as a text will have its own preferred list of experiments adaptable to the instruments available.

Although this volume is intended primarily to be a text for college students it should be useful as a reference book for chemists who are not experts in the field but who wish to determine possible applications of instrumental methods to their particular problem.

JOHN H. JONES

Fertilizers. Recent publications of the Food and Agriculture Organization of the United Nations. Available in the United States from Columbia University Press, International Documents Service, 2960 Broadway, New York 27, N. Y.

Report of the Meeting on Fertilizer Production, Distribution and Utilization in Latin America. Edited by H. G. DION and G. J. CALLISTER. FAO Development Paper No. 36, Agriculture. Rome, 1953. 52 pp. Price $0.50.

This publication relates to fertilizer resources, processing facilities, production, consumption, trade, distribution, requirements, and economics in Latin American countries. Included are the recommendations to the country Governments and to the FAO made at the meeting convened at Rio de Janeiro, December 4–12, 1951. As a source of authoritative information presented in concise, readable form, it will be welcomed by all persons interested in the fertilizer problems of Latin America.

Report of the Third Meeting of the International Rice Commission's Working Party on Fertilizers. Edited by H. G. DION. FAO Development Paper No. 39, Agriculture. Rome, 1953. 46 pp. Price $0.50.

This report of the meeting held at Bangkok in September, 1953 summarizes the discussions on fertilizer problems; physiological diseases of rice; experimental designs for variety testing and fertility surveys; soil, water, and plant relationships; and productivity of paddy soils in Cambodia, India, Japan, Korea, Taiwan, and Viet-Nam. The recommendations of the Working Party are set forth.

An Annual Review of World Production and Consumption of Fertilizers, 1953. FAO, Rome, 1953. 58 pp. Price $0.25.

In 1949 the FAO commenced the publication of a series of annual reports on the production and consumption of commercial fertilizers in the individual countries of the world, based chiefly on information supplied by the respective Governments. The present report, the fifth in the series, gives the data for 1951–1952, together with preliminary figures for 1952–1953 and forecasts for 1953–1954, in terms of nitrogen (N), phosphoric oxide (P_2O_5), and potash (K_2O). Unofficial estimates for the countries of Eastern Europe are included, but all data are exclusive of the USSR and the Chinese Peoples Republic. Recent developments in fertilizer resources, processes, manufacturing facilities, and use are summarized.

Previous FAO publications of interest to agricultural chemists include: *Preservation of Grains in Storage* ($1.50); *Using Salty Land* ($0.50); *Nutritional Deficiencies in Livestock* ($1.00); *Storing and Drying Grain in Canada, in the United States, in the United Kingdom* ($0.50); *Better Utilization of Milk* ($0.75); *Efficient Use of Fertilizers* ($2.00); *Some Aspects of Food Refrigeration and Freezing* ($2.00); *Weed Control by Growth-Regulating Substances* ($0.50); *Soil Surveys for Land Development* ($1.00); *Report of the First Meeting of the International Rice Commission's Working Party on Fertilizers* ($0.25); *Report of the Second Meeting of the International Rice Commission's Working Party on Fertilizers* ($0.50).

K. D. JACOB