TUESDAY—MORNING SESSION

REPORT ON ENZYMES

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

The 1950 report on enzymes summarized their use in A.O.A.C. methods (1). One more method, that of the use of a urease-bromothymol blue test paper for the detection of urea (2), has been added.

This year's report consists solely of a recommendation for future work. A partial survey of the literature has shown that there are a number of possibilities for the use of enzymes to aid our method's work. One of the most important uses may be in the detection and determination of small quantities of some of the organic insecticides, especially the organic phosphorus compounds. Giang and Hall (3) have published a method for some of the organic phosphorus insecticides based upon their inhibition of the cholinesterase enzyme system. They report that the method is highly sensitive, but that in a general sense it is nonspecific. They state, however, that one can distinguish among certain of the organic phosphorus insecticides that have large differences in inhibitory power.

It may be possible to increase the specificity of the above method. Giang and Hall (3) used the cholinesterase system from mixed human and bovine blood serum. Jansen, *et al.* (4), and Metcalf and March (5) have studied the effect of some of the same insecticides on other esterase and acetylcholinesterase systems. They found differences in the effects of some of the insecticides on different enzyme systems.

Similarly, there is evidence to show that enzymic methods might possibly be developed for benzenehexachloride (6) and 1080 (7, 8). An enzymic method for fluorine has been developed which is reported to be more sensitive than our current chemical one (9).

RECOMMENDATION

It is recommended* that an Associate Referee be appointed to develop enzymic methods of analysis for small quantities of insecticides, when it appears that such methods are more specific, more sensitive, or simpler to perform than other methods.

REFERENCES

(1) Cook, J. W., *This Journal*, **34**, 539 (1951).
(2) *Changes in Methods, ibid.*, **35**, 100 (1952).
(3) Giang, P. A., and Hall, S. A., *Anal. Chem.*, **23**, 1830 (1951).
(4) Jansen, E. F., Nutting, M. D. F., Jang, R., and Balls, A. K., *J. Biol. Chem.*, **179**, 189 (1949).
(5) Metcalf, R. L., and March, R. B., *J. Econ. Entomol.*, **43**, 670 (1950).

* For report of Subcommittee C and action of the Association, see *This Journal*, **36**, 59 (1953).

(6) LANE, R. L., and WILLIAMS, R. J., *Arch. Biochem.*, **19**, 329 (1948).
(7) BARTLETT, G. R., and BARRON, E. S. G., *J. Biol. Chem.*, **170**, 67 (1947).
(8) KALNITSKY, G., and BARRON, E. S. G., *ibid.*, **170**, 83 (1947).
(9) STETTER, H., *Chem. Ber.*, **81**, 532 (1948) through *Chem. Abstr.*, **43**, 5065i (1949).

No report was received from the Associate Referee on hydrocyanic acid glucosides.

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*

Past reports have emphasized the difficulties in finding acceptable chemical indices for decomposition in foods. Many indices have been proposed and are being studied.

A proposed method for succinic acid and other acids in tomatoes was submitted to collaborative study. The results indicated either that the method does not check well in different laboratories, or that the directions in their present form can be misinterpreted.

Histamine is another promising index of decomposition in some species of fish. The method presently used is a bio-assay for which few regulatory laboratories have proper equipment. A chemical test is now being tried.

Galacturonic acid has been studied for several years as an index of decomposition in fruits. The method first proposed has gone through several modifications. Collaborative studies are not yet good enough to recommend its acceptance. The carbazole procedure also has this status.

As the result of search for some substance which cannot be formed by degradation of naturally occurring components of fruits, as galacturonic acid may be formed from pectin, a substance believed to be gluconic acid, presumably formed by oxidation of glucose, shows promise as an index of decomposition.

It is recommended*—

(1) That studies on the naphthoresorcinol and carbazole methods for galacturonic acid be discontinued.

(2) That studies of the method for intermediate polygalacturonides as an index of decomposition in fruits be discontinued.

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

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

(5) That studies to determine the correlation of rot and succinic acid in tomatoes be expanded to include (*a*) study of bacterial rots, and (*b*) storage experiments with processed products made from sound tomatoes.

(6) That the study of histamine in fish be continued.

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

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

(8) That the study of uric acid as a possible criterion of insect filth in nuts, cereals, and eggs be continued, and that study of uric acid in fruits be discontinued.

(9) That search for other chemical indices of fecal matter in foods be continued.

REPORT ON DECOMPOSITION IN FRUIT PRODUCTS

GALACTURONIC ACID IN STRAWBERRY JUICE

By PAUL A. MILLS (Food and Drug Administration, Department of Health, Education, and Welfare, San Francisco 2, California), *Associate Referee*

Last year, the Associate Referee reported the use of small amounts of 1 per cent hydrogen peroxide to develop maximum color where laboratory synthesized naphthoresorcinol was used for the determination of galacturonic acid. Guerrero and Williams (1) have presented a theory of the reaction between naphthoresorcinol and galacturonic acid, in which the presence of oxygen would tend to produce the colored quinoid configuration in the complex. This theory seems to be in accord with observed effects of hydrosulfite and hydrogen peroxide on the colored compound, in that an oxidizing atmosphere should force the reaction toward the colored form and tend to reduce variations in the development of the colors. It was thought that this might also eliminate the variations observed with different batches of naphthoresorcinol. In order to test this hypothesis, four samples of naphthoresorcinol, differing in color and presumably in purity, were tested as follows: four tubes for each sample were prepared, containing 100 micrograms of galacturonic acid, 20 mg of naphthoresorcinol, 2 ml of 1+3 acetone solution, and 2 ml of hydrochloric acid, respectively. Quantities of 0, 0.05, 0.10, and 0.20 ml of 1 per cent hydrogen peroxide were added to the tubes which were then heated for seventy-five minutes at 65°C. for color development. Absorbancies are listed in Table 1.

TABLE 1.—*Effect of H_2O_2 on naphthoresorcinol reaction with galacturonic acid*
(Absorbancies)

SAMPLE	ML 1% H_2O_2 SOLUTION			
	0 ML	0.05 ML	0.10 ML	0.20 ML
1	0.43	0.89	0.80	0.62
2	0.34	0.86	0.89	0.74
3	0.76	0.94	0.91	0.80
4	0.73	0.88	0.88	0.75

These data show that 0.05 to 0.10 ml of 1 per cent hydrogen peroxide gives reasonably consistent results. In an earlier report (2), 0.15 ml was found to be optimum for the naphthoresorcinol used. Absorbancies were also obtained for different amounts of galacturonic acid, as shown in Table 2.

TABLE 2.—*Absorbancies obtained with different levels of galacturonic acid using 4 different samples of naphthoresorcinol (hydrogen peroxide solution included)*

SAMPLE	ML 1% H_2O_2	GALACTURONIC ACID, MMG			
		30	50	70	100
1	0.05	0.27	0.44	0.59	0.84
2	0.10	0.28	0.41	0.59	0.85
3	0.05	0.28	0.42	0.61	0.87
4	0.10	0.26	0.42	0.60	0.84

It will be observed that there is essentially no difference in the values obtained with all samples at each level of galacturonic acid.

The limited data presented indicate that the use of hydrogen peroxide in the proper amount will eliminate the uncertainties introduced in the method because of variations in purity of the naphthoresorcinol reagent.

In an earlier report (3) a temperature of 50°C. for three hours was recommended for color development. This year the effect of temperature on the color development was further studied: a number of glass-stoppered test tubes, each containing 100 micrograms of galacturonic acid in 1 ml water, 20 mg of naphthoresorcinol, 2 ml of 1:3 acetone solution and 2 ml of hydrochloric acid were placed in a controlled water bath. At intervals, one tube was removed and the developed color was measured. Trials were made at 50, 60, 65, 70, and 80°C. Absorbancies are listed in Table 3.

As might be expected, the speed of reaction is increased by elevating the temperature. Above 60°C., there was a slight decrease in total absorbance, and above 65°C., prolonged heating led to losses. At 65°C., a maximum was reached in one hour, and no loss was observed after 2.5 hrs of heating. At temperatures of 60°C. and lower, a considerably longer time was required to achieve maximum color formation, and longer heating gave variable results.

Based on these observations, it was decided that color development at 65°C. was optimum, since the reaction was complete in one-third the time required at 50°C., and the results were less variable. A reaction time of 1.25 hours at 65°C. seemed sufficient to achieve maximum color formation and yet not prolong the heating unnecessarily.

The method previously reported (3) was revised to include the use of hydrogen peroxide and the higher temperature in the color development. It was tested, under practical conditions, on samples of strawberry juice, with and without added galacturonic acid (Table 4).

TABLE 3.—*Relation of temperature of reaction to color development for 100 mmg of galacturonic acid and 20 mg of naphthoresorcinol*
(Absorbancies at 570 mμ)

REACTION TIME	TEMPERATURE, °C.									
	80	70	70	65	65	60	60	60	50	50
hours										
0.125	0.480	—	—	—	—	—	—	—	—	—
0.25	0.722	—	—	—	—	—	—	—	—	—
0.50	0.815	0.748	0.755	—	0.700	—	—	—	—	—
0.75	0.815	0.850	0.835	0.775	0.795	—	—	—	—	—
1.00	0.750	0.850	0.850	0.855	0.855	0.698	—	—	—	—
1.25	0.750	0.850	0.850	0.855	0.855	—	—	—	—	—
1.50	—	0.820	0.815	0.855	0.855	0.840	—	—	—	—
1.75	—	0.760	0.765	0.855	0.855	—	0.900	0.895	—	—
2.00	—	—	—	0.840	0.875	0.875	0.900	0.905	0.739	0.728
2.25	—	—	—	—	—	0.875	0.920	0.895	—	—
2.50	—	—	—	0.880	—	0.895	0.890	0.895	0.786	0.850
2.75	—	—	—	—	—	0.865	0.890	0.835	0.798	0.845
3.00	—	—	—	—	—	0.895	—	0.840	0.782	0.835
3.25	—	—	—	—	—	—	—	—	0.850	0.890
3.50	—	—	—	—	—	0.890	—	—	0.875	0.905
3.75	—	—	—	—	—	—	—	—	—	—
4.00	—	—	—	—	—	—	—	—	0.870	0.905

TABLE 4.—*Recovery experiments with strawberry juices*

GALACTURONIC ACID		RECOVERY	
ADDED, MMG	FOUND, MMG	MMG	PER CENT
0	23	—	—
50	75	52	104
50	68	45	90
50	71	48	96
0	82	—	—
10	93	11	110
50	129	47	94
0	72	—	—
50	124	52	104

REVISED METHOD

APPARATUS

(a) *Glass stoppered test tubes.*—Ca 23×150 mm.

(b) *Water bath.*—Controlled at 65°C. ±1°.

(c) *Photometer.*—Capable of determining absorbancies at 570 mμ.

(d) *Absorption cells with stoppers (not rubber).*—Druggist's vials, 15×45 mm with cork stoppers were used after careful optical matching.

(e) *Glass beads.*—Ca 1 mm in diameter.

(f) *Adsorption columns.*—Construct adsorption columns as in *This Journal*, **34**, 517 (1951). Place a quantity of the screened dry resin (see Reagents) in beaker and cover with H_2O for 1 hr or longer to expand (do not expand the resin in the adsorption tubes or breakage may result). Fill adsorption tube about half full of expanded wet resin (wash in with H_2O). Exhaust the cation resin (C-3) by slowly adding 4 per cent NaOH to the column from a dropping funnel until the effluent is alk. Wash with H_2O until most of the excess NaOH is removed. Back-wash the column by forcing H_2O thru delivery tube, allowing the H_2O to overflow the top of the column at such a rate that the resin is thoroly agitated but only the very fine particles are lost. Allow the tube to drain until the resin is just covered, rinse down the tube 2 or 3 times, and then wash with H_2O from the top at a rate of about 5 ml/min. until most of the NaOH is removed. Regenerate the resin by adding 1+3 HCl until the effluent is acid, and wash with H_2O from the top at a rate of 2 ml/min. until the excess acid is removed (*p*H of effluent: 5–6 with *p*H paper). Repeat this cycle twice before putting columns into use for the first time.

In the same way, exhaust the anion resin (A-4) with 1+3 HCl, backwash, wash with H_2O and then regenerate with 4 per cent NaOH. Wash until free from alk. Recycle twice before putting columns into use for the first time.

(Backwashing reclassifies the resin particles so that the larger particles are at the bottom, breaks up channels that may have formed, and removes any entrapped air bubbles which interfere with adsorption).

To prepare the columns for re-use.—After a sample has been adsorbed and the anion resin stripped, the anion column is already exhausted. Backwash, regenerate with 4 per cent NaOH, and wash free of alk. Prepare the cation resin for re-use by exhausting with 4 per cent NaOH, washing from the top to remove excess NaOH, fruit color, etc., and backwashing. Then regenerate with 1+3 HCl, and wash free of excess acid.

REAGENTS

(a) *α,D-Galacturonic acid.*—(Eastman No. 4607). Dissolve 1.000 g in H_2O and dil. to one l. Add a drop of $CHCl_3$ for preservative. 1 ml = 1 mg.

(b) *Naphthoresorcinol.*—Highest purity. Det. optimum quantity of H_2O_2 required for the reaction with galacturonic acid as follows: in each of 5 glass-stoppered test tubes, place 1 ml of galacturonic acid soln (50 mmg per ml), 20 mg of naphthoresorcinol, 2 ml of 1+3 acetone soln, 2 ml of HCl, and 0, 0.05, 0.10, 0.15, and 0.20 ml of 1 per cent H_2O_2 soln. Develop colors as directed in method. That quantity of H_2O_2 soln producing most intense color is optimum for the lot of naphthoresorcinol.

(c) *Activated carbon.*—Nuchar W or equivalent.

(d) *Acetone solution.*—1 + 3.

(e) *Anhydrous sodium sulfate.*—Powdered.

(f) *Hydrogen peroxide.*—Dilute 3 ml of 30% H_2O_2 to 100 ml. Prepare fresh daily.

(g) *Exchange resins.*—Cation resin (C-3) and anion resin (A-4); 20–60 mesh.*

PREPARATION OF STANDARD CURVE

Mount cation column so that its outlet tube delivers into the anion column, and place a dropping funnel of ca 100 ml capacity in the top of the cation column by means of a rubber stopper. Transfer 25 ml 1 per cent citric acid soln and the required quantity of standard galacturonic acid soln to the dropping funnel. Allow mixt. to drop into cation column at the rate of ca 2 ml per min. (Note 1) with the column discharging into the anion tube. After sample has passed into the resin, rinse dropping funnel 2 or 3 times, remove, attach a gravity source of distd H_2O, and wash at a rate of 2–3 ml per min. with ca 150 ml. Discard washings. Remove cation column, place a 100 ml vol. flask under the discharge from the anion column and add by means of a dropping funnel 25 ml of 1+3 HCl to the top of the anion column at the rate of 2 ml/min. Attach the H_2O supply, and wash at a rate of 2 ml/min. until 100 ml has been collected. Add ca 0.1 g of activated carbon, heat in boiling H_2O ca 10 min., cool, and filter through quantitative paper, pouring back until clear. Add a drop of $CHCl_3$ for preservative. Adsorb and elute 0, 3, 5, 7 (etc.) mg, respectively, of galacturonic acid in this manner (Note 2). Ten mg/100 ml is equivalent to 100 micrograms/ml.

Pipet accurately 1 ml of each of the standard solns into glass-stoppered test tubes and add 2.0 ml of concd HCl to each test tube from a buret. Prepare a soln of 110 mg of naphthoresorcinol in 11.0 ml of 1+3 acetone soln and add 2 ml of this soln, accurately measured, to each test tube. Add optimum quantity (see REAGENTS) of H_2O_2 soln to each tube, mix, and place in the H_2O bath at 65°C. for 1.25 hrs. Cool tubes under a H_2O tap, add 20.0 ml of ethyl ether and ca 12 glass beads to each; stopper, shake vigorously *at least* 5 min., (Note 3) and allow to settle.

Place ca 0.25 g of anhyd. Na_2SO_4 in each adsorption cell and fill one cell with the ether layer from each test tube. Stopper, shake, tap gently to settle the Na_2SO_4, and measure absorbancies at 570 mμ.

Plot absorbancies against mmg of galacturonic acid. This should yield an essentially straight line.

ISOLATION OF ACIDS FROM STRAWBERRY JUICE AND DETERMINATION OF GALACTURONIC ACID

Proceed as directed under Preparation of Standards, using 25.0 g of strawberry juice. Transfer to dropping funnel with ca 25 ml of H_2O. Proceed as directed under

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

standards, beginning "pipet accurately 1 ml each . . . " but substitute 1 ml of the decolorized soln of fruit acids for the standard solns. Include a zero standard and a galacturonic acid standard as a check.

NOTES

(1) Volumes required for adsorption and stripping of the acid from the anion column vary directly with the size of the column and the quantity of resin used. Hence, dimensions of the column have been specified. The exchange of ions in the resins is not instantaneous and the solns must be percolated through the resins slowly to allow sufficient time for reactions.

(2) The adsorption procedure should be carried through without delay. Allowing the fruit acids to remain on the anion resin overnight causes low results. The soln of acids stripped from the anion column is stable for several days if preserved with a drop or two of $CHCl_3$.

(3) The colored compound is rather slowly soluble in ether; hence the tubes must be shaken vigorously for at least 5 min. Machine shaking for 10 min. is recommended. The presence of glass beads aids in soln. The color development procedure should be carried through without delay. The ether solns of the color are stable for an hour.

The galacturonic acid content of fresh sound strawberries of commercial varieties, obtained both in the field and in placking plants, was inves-

TABLE 5.—*Galacturonic acid in fresh sound berries*

VARIETY	SEASON	MMG/G JUICE
Z2	1951	44
H1	1951	48
Sierra	1951	40
Shasta	1952	44
Shasta	1952	52
Lassen	1952	38
Lassen	1952	54
J7	1952	52
Mixed from factory	1952	36
Mixed from factory	1952	40
Mixed from factory	1952	40
Mixed from factory	1952	56

tigated. The field samples consisted of carefully selected fresh sound berries, processed by heat immediately after selection. Packing-house samples were carefully inspected to eliminate any visible rot and were immediately quick frozen. After the berries were disintegrated in a Waring blendor, juice was extracted by centrifuging and filtering through cotton. The results are listed in Table 5, and indicate reasonable uniformity in galacturonic acid content.

SUMMARY

Hydrogen peroxide was found to reduce variations in color development when used in proper amount.

The temperature of color development was increased to 65°C. This resulted in more concordant results and shortened the procedure.

The method as previously reported (2) was revised to include these improvements. It is recommended* that this method be submitted to collaborative study.

REFERENCES

(1) GUERRERO, A. H., and WILLIAMS, R. T., *Nature*, **161**, 930 (1948).
(2) MILLS, P. A., *This Journal*, **35**, 520 (1952).
(3) ———, *ibid.*, **34**, 514 (1951).

REPORT ON DECOMPOSITION IN FRUITS

GALACTURONIC ACID, GALACTURONIDES, AND GLUCONIC ACID

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

Galacturonic Acid.—Previous studies have shown that galacturonic acid is a fairly good criterion of decomposition in fruits when no agent which would cause pectin degradation has been used. Two years ago, two methods were proposed for galacturonic acid (1), both based on the naphthoresorcinol reaction; one was developed by P. A. Mills and the other by the author. Due to difficulties with the naphthoresorcinol reagent, the writer last year proposed a method based on the reaction with carbazole, a modification of the method of Dische (2). The naphthoresorcinol method is reported to determine only the monogalacturonic acid, and not to react with the higher homologs or polygalacturonides. The carbazole method, on the other hand, will determine higher units which result from the degradation of pectin if these are held on the ion exchange column and are therefore present in the final effluent.

In cooperation with P. A. Mills, Associate Referee, who sent out samples of strawberry juice, the carbazole method was given some collaborative study. The results were disappointing, as "off" colors developed in the determinations. The method had previously been applied chiefly to samples of apple juice or sauce, and here this difficulty was not encountered. It had been observed, however, that some batches of sulfuric acid used in the determination gave "off" colors, and the blanks contained a greenish color. There is some evidence that this effect is due to iron, and it seems likely that some preliminary treatment will have to be made on samples where such interference is likely to occur.

Intermediate Polygalacturonides.—A carbazole method was also proposed last year for intermediate degradation products of pectin. A con-

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

siderable number of these intermediate products are found in its enzymic degradation (3). Polygalacturonides containing up to six galacturonic anhydride units were separated and identified by the use of paper chromatography. These materials may also be valuable in assessing the extent of decomposition.

Other Substances.—Another substance was found in significant amount in studies on rotten appples. Preliminary tests indicate that gluconic acid is presumably formed by oxidation of glucose. The material had a positive rotation and did not reduce Benedict's solution. Only minor quantities of this substance are found in good apples. For use as a measure of decomposition, gluconic acid may not have the disadvantage of being formed by artificial degradation of known components of fruits, which may be the case with galacturonic acid formation from pectin clarification.

RECOMMENDATIONS

It is recommended*—

(1) That methods for galacturonic acid be studied further.

(2) That determination of galacturonides intermediate between pectin and galacturonic acid be studied further.

(3) That gluconic acid as a measure of decomposition in fruits be studied.

REFERENCES

(1) MILLS, P. A., *This Journal*, **34**, 506, 513 (1951).
(2) DISCHE, Z., *J. Biol. Chem.*, **167**, 189 (1947).
(3) ALTERMATT, H., and DEUEL, H., *Helv. Chim. Acta*, **35**, 1422 (1952).

REPORT ON URIC ACID IN FRUIT PRODUCTS

By DORIS H. TILDEN (Food and Drug Administration, Department of Health, Education, and Welfare, San Francisco, California), *Associate Referee*

It was recommended in the 1951 report (1) that work be continued with ion exchange resins in connection with paper partition chromatography in an attempt to (*a*) eliminate the interfering caramel from concentrated fruit syrups and (*b*) concentrate small amounts of uric acid when it is necessary to work with as much as a liter of sample. The present report describes experimental work with Duolite C-3 (cation) and A-4 (anion) resins.†

Although many limiting factors have developed, the use of ion exchange resins appears to offer considerable promise for this work. The degree of discoloration due to caramelization of sugars in the sample solution should not be much deeper than Lovibond 7 or 8 (Series 52), the

* For report of Subcommittee C and action of the Association see *This Journal*, **36**, 55 (1953).
† Procurable at the Chemical Process Company, 901 Spring St., Redwood City, Cal.

total acid content of the soln should not exceed the capacity of the A-4 column, and the sugar content of a syrup should probably be limited. Allowing for these conditions, experiments were conducted with 1 liter of diluted fruit juice concentrate to which was added 2 mmg/ml of uric acid. Recoveries have been about 80 per cent when uric acid was present in amounts of 10–40 mmg in the aliquot taken for chromatographing. Experiments have also been conducted with 1 liter of a diluted pear juice concentrate containing the water extract from 100 mg of weevil excreta pellets. The 0.01 ml portion chromatographed on paper developed a heavy spot of mercury-uric acid complex.

EXPERIMENTAL

A series of columns, or one relatively large column, of C-3 resin was employed. A small column of A-4 resin was used in order to reduce the quantity of acid necessary for stripping the uric acid. However the small A-4 column limits the amount of total acid which can be present in the portion of fruit syrup to be examined. In an attempt to reduce the quantity of organic acids in a sample, lead acetate was used as a precipitant, and the filtrate was de-leaded. Acetic acid, formed in the passage of this solution through the C-3 column, was as objectionable as the acids originally present, and the columns were soon "poisoned" beyond cleaning.

In order to concentrate small amounts of uric acid present in the eluate from the A-4 column, the solution must be evaporated, preferably to dryness. For the determination to be in some degree quantitative, it should be possible to dissolve this residue in a known small volume (1 ml) of a saturated Li_2CO_3 solution. Aliquots of 0.01 ml may then be spotted on the chromatographic strip. If large quantities of salts are formed during neutralization of the stripping acid, solutions in 1 ml of Li_2CO_3 become slurries which are difficult to filter, and loss of uric acid results.

Apparently sugars are also retained on the A-4 column, and strip with acid to caramelize or even char when the strength of acid increases as the eluate is evaporated. Spotting such a solution produces streaks of brown the entire length of the chromatogram, with little or no separation of uric acid.

It was found that eluting the A-4 column with glacial acetic acid instead of with HCl as used previously (2) was quite satisfactory. A large volume of eluate is necessary since the acetic acid apparently removes uric acid from the anion resin much more slowly than does a stronger acid. The acetic acid eluate can be concentrated by boiling, and repeated addition of water during evaporation reduces the acid content so that very little caramelization occurs. When the residue is finally brought to dryness, it may be taken up in 1 ml of saturated Li_2CO_3 solution and spotted on paper in increments of 0.01 ml. Upon development, good resolution of uric acid is obtained.

SUMMARY

Experimental work has been continued on the use of ion exchange resins in connection with paper partition chromatography for concentrating and isolating small amounts of uric acid in fruit syrup. It was found that for best results, the amount of caramel and total acid present in a sample solution must be limited. Glacial acetic acid proved to be a better eluant than hydrochloric acid for the A-4 column, which retains uric acid.

REFERENCES

(1) TILDEN, D. H., *This Journal*, **34**, 498 (1951).
(2) ———, *ibid.*, **35**, 526 (1952).

REPORT ON DECOMPOSITION IN TOMATO PRODUCTS

DETERMINATION OF ACETIC, FORMIC, LACTIC, SUCCINIC, MALIC, AND CITRIC ACIDS

By HALVER C. VAN DAME (Food and Drug Administration, Department of Health, Education, and Welfare, Cincinnati, Ohio), *Associate Referee*

The decomposition of tomatoes by molds and bacteria is a complex process and causes changes in the composition of the tomato which depend on the particular organism involved. In last year's report it was shown that various molds produce succinic acid in tomatoes. Hillig (1) has shown that acetic, formic, and lactic acids are produced in tomatoes by molds and bacteria. Bacon and Dunbar (2) have shown that lactic acid is formed, and that citric acid disappears with decomposition due to molds and bacteria.

In order to obtain as much information as possible about the change in composition in tomatoes during decomposition, the Associate Referee attempted to formulate a method which would determine acetic, formic, lactic, succinic, malic, and citric acids with a minimum number of operations. A combination of existing methods was used. The sample was prepared and extracted by a method similar to the method given in the report on succinic acid (3). The separation of acetic, formic, succinic, and lactic acids from the ether extract was accomplished by the chromatographic method developed by Bulen, Varner, and Burrell (4). Citric and malic acids are separated from a separate portion of the filtered juice by a similar chromatographic procedure. The method follows:

PROCEDURE FOR ORGANIC ACIDS IN TOMATOES

Preparation of sample.—To 100 g of puree, tomato juice, or other tomato products, add 25 ml of 1 *N* H_2SO_4. Make to 200 g with H_2O in a 250 ml centrifuge bottle. Shake well for 1 min. and centrifuge for 10 min. Filter thru large coarse fluted paper. To 100 ml of filtrate, add 50 g of $(NH_4)_2SO_4$ and 0.5 ml of 1 + 1 H_2SO_4.

Transfer to a suitable continuous ether extractor and extract for 5–6 hours or until completely extracted. Save the remainder of the filtrate for citric and malic acid detns.

Shake ether extract with 5 ml of 0.2 *N* NaOH to which several drops of phenolphthalein indicator have been added. Add more alkali, if necessary, to convert acids to sodium salts, and 0.5 ml in excess. Shake well and pour entire mixt. into large beaker. Pour aq. soln and ether remaining in extractor into a 500 ml separatory funnel and shake well. If only a small amount of ether remains, add sufficient to make a total of 200 ml. Discard aq. layer and filter ether into a second separatory funnel. Shake well with 5 ml of 0.2 *N* NaOH as above and add mixt. to beaker contg original ether extract. Evap. ether on steam bath and transfer aq. soln to 100 ml beaker. Continue to evap. to 1–2 ml on steam bath. Transfer to vacuum desiccator and evap. to dryness.

Preparation of standard chromatographic column.—Add 5 ml of 0.5 *N* H_2SO_4 to 10 g silicic acid in a mortar and mix well. Add 40–50 ml $CHCl_3$ gradually and make into paste and then to pourable slurry. Pour mixt. into a chromatographic column 12 mm (i. d.) by 40–50 cm long, containing a small piece of cotton in constricted end. Force excess solvent from column dropwise by applying pressure to top of column. Do not allow column to dry below surface of gel, as drying or "cracking" renders column useless.

SEPARATION OF ACETIC AND FORMIC ACIDS AND PRELIMINARY SEPARATION OF SUCCINIC AND LACTIC ACIDS

Add 2 drops phenolphthalein indicator and 3 or 4 drops H_2O to beaker contg acid salts. Mix with glass rod and add sufficient 1:4 H_2SO_4 to change indicator to colorless. Add 2 drops excess. Total vol. of liquid should be kept to about 1.0 ml. Add 2 g silicic acid and mix well. Transfer powder quantitatively to column after adding small piece of cotton on top of gel. Rinse beaker with 5 ml of $CHCl_3$ and add to top of column. Make powder into slurry with $CHCl_3$, using a long thin glass rod. Rinse beaker, rod, funnel, and sides of tube with an addnl 5 ml of $CHCl_3$. Force $CHCl_3$ into column by means of pressure. When $CHCl_3$ level reaches level of gel, add another small piece of cotton to top of column. Develop column by adding 100 ml of 5% butanol-95% $CHCl_3$ mixt. and then 125 ml of 15% butanol-85% $CHCl_3$ mixt.

Collect eluate in 5 ml fractions, and titrate each fraction with 0.01 *N* NaOH to a blue color, using thymol blue indicator. The graduated cylinder used for collection of the eluate is washed into a small glass-stoppered flask with 5 ml of recently boiled distd H_2O. Acids will elute in following order:

(1) Acetic—about 40–60 ml.
(2) Formic—about 100–135 ml.
(3) Succinic and lactic—about 140–175 ml.

The exact position is detd by the titration values found while the acids are eluting. Combine fractions containing lactic and succinic acids for subsequent separation.

SEPARATION OF SUCCINIC AND LACTIC ACIDS

Make combined fractions contg lactic and succinic acids distinctly alk. by adding 1 ml of 0.5 *N* NaOH. Evap. to about 5 or 10 ml and transfer to a 50 ml beaker. Continue to evap. to about 1 ml. Evap. to dryness in a vacuum desiccator. Moisten with 2 drops phenolphthalein indicator and 2 drops H_2O and make acid with 1:4 H_2SO_4 as before but keep final vol. to about 0.5 ml. Add 1 g silicic acid, mix well, and transfer to prepared standard chromatographic column. Develop column with 25% butanol-75% benzene mixt. Acids will elute in the following order:

(1) Succinic—about 30–55 ml.
(2) Lactic—about 60–90 ml.

Titrate each 5 ml fraction as before.

TABLE 1.—*Determination of acids in tomato puree*

COLLABORATOR	MG ACID/100 GRAMS OF SAMPLE	MG ACID ADDED/100 GRAMS SAMPLE	MG ACID/100 GRAMS FOUND	RECOVERY, PER CENT
		Acetic Acid		
1	23.1	10.1	32.7	95.3
2	21.1	10.0	31.1	100.1
3	20.8	10.0	30.9	100.8
4	22.4	9.9	30.0	76.9
5	26.1	10.5	33.3	68.8
6	24.4	—	—	—
7	26.2	8.5	33.7	88.1
8	22.6	10.0	32.3	96.6
Av.	23.3	—	—	89.5
		Formic Acid		
1	5.8	10.4	15.7	95.2
2	7.2	10.7	17.3	94.2
3	6.3	10.7	16.5	95.1
4	13.9	10.4	18.3	43.8
5	9.2	9.4	15.4	66.1
6	6.9	—	—	—
7	7.8	9.5	17.6	103.7
8	5.4	10.1	11.2	57.8
Av.	7.8	—	—	78.2
		Lactic Acid		
1	11.9	8.7	20.1	94.3
2	11.9	8.6	20.7	101.7
3	12.6	8.6	21.8	106.0
4	9.2	8.5	17.8	98.1
5	12.6	—	—	—
8	12.8	10.3	22.1	90.2
Av.	11.8	—	—	98.1
		Succinic Acid		
1	11.1	10.0	21.0	99.1
2	12.0	9.9	22.0	100.4
3	11.6	9.8	21.1	97.2
4	10.8	10.3	16.3	49.0
5	8.6	—	—	—
8	11.6	10.0	22.2	105.7
Av.	10.9	—	—	90.3

TABLE 1—(*continued*)

COLLABORATOR	MG ACID/100 GRAMS OF SAMPLE	MG ACID ADDED/100 GRAMS SAMPLE	MG ACID/100 GRAMS FOUND	RECOVERY, PER CENT
		Malic Acid		
1	122	408	545	103.8
2	107	388	485	97.2
3	128	392	542	105.6
4	127	396	494	92.7
5	136	398	500	91.6
7	139	405	516	93.2
8	101	406	512	101.2
Av.	123	—	—	97.9
		Citric Acid		
1	692	408	1087	96.9
2	668	357	1030	101.5
3	677	369	1042	101.2
4	658	396	1020	91.5
5	702	400	1112	102.5
6	672	—	—	—
7	656	389	1095	112.5
8	703	399	1104	100.7
Av.	678	—	—	101.0

SEPARATION OF CITRIC AND MALIC ACIDS

Neutralize 5 ml original filtrate with 2*N* NaOH (phenolphthalein indicator) and add 5 drops in excess. Evap. to 1 or 2 ml on steam bath and finish evapn to dryness in vacuum desiccator. Add a few drops of H_2O and 2 drops of indicator and make distinctly acid with 1:4 H_2SO_4. The final vol. should be kept to about 1.0 ml. Add 2 g of silicic acid, mix well, and add powder to prepared standard column. Develop column first with 100 ml of 25% butanol-75% $CHCl_3$ mixt. and then with 200–300 ml of a 35% butanol-65% $CHCl_3$ mixt. Begin titration of 10 ml fractions after the first 100 ml has eluted. The acids elute in the following order:

(1) Malic—about 160–200 ml.
(2) Citric—about 250–350 ml.

COLLABORATIVE RESULTS

A sample of tomato puree was sent out for collaborative study to test the above method. The sample was prepared from a batch of tomato puree known to contain significant amounts of the 6 acids mentioned above. The puree had a mold count of 66 per cent. The puree was thoroly mixed, placed in sterile jars, and reprocessed in a boiling water bath for 1 hour. The collaborators were instructed to make a determination of the 6 acids on one portion of the sample and on another portion to which known amounts of the 6 acids had been added. Eight collaborators reported results, three from the Associate Referee's laboratory and five from other

laboratories of the Food and Drug Administration. The results are given in Table 1.

The Associate Referee analyzed two samples of pulped fresh sound tomatoes by this method. The tomatoes were hot-house tomatoes; one sample was rather green and the other normally ripe. The results are as follows (Table 2):

TABLE 2.—*Analysis of pulped fresh tomatoes*

ACID	RIPE TOMATOES, MG ACID/100 GRAMS	GREEN TOMATOES, MG ACID/100 GRAMS
Acetic	2.32	2.62
Formic	1.22	1.52
Lactic	1.85	3.19
Succinic	0.86	1.41
Malic	97.0	91.6
Citric	621.6	634.8

COMMENTS

The results as a whole are in fairly good agreement with the exception of formic acid, although a few results are out of line. The results of collaborators No. 5 and No. 7 are not recorded for succinic and lactic acid because of an incomplete separation of the two acids on the second column. Collaborator No. 6 did not submit results on the sample to which known acids were added; thus no results are recorded for that collaborator. Collaborator No. 4 had low recoveries on acetic, formic, and succinic acids. This appears to be due to an incomplete extraction of the sample to which the known acids were added.

The results for formic acid appear to be rather erratic, although several of the collaborators were able to get good recoveries. It is felt that the formic acid may be held by the silicic acid to a greater extent than the other acids and the type of silicic acid used may be the reason for the variance among collaborators. The value for succinic acid obtained by collaborator No. 5 is low on the sample to which no acids were added, yet the total value for succinic and lactic acids obtained on the first separation was correct. This suggests that part of the succinic acid was lost at this point.

CONCLUSIONS

(1) The results on the collaborative sample are in fair agreement for a chromatographic procedure.

(2) The results show that good recoveries of the six acids can be obtained by this method.

(3) The method is sound but requires some practice in technique.

(4) There are relatively small amounts of acetic, formic, lactic, and succinic acids in samples of fresh tomatoes.

ACKNOWLEDGMENTS

The Associate Referee wishes to thank Theodore E. Byers, Luther G. Ensminger, Mary McEniry, D. W. Johnson, F. Y. Mendelsohn, J. T. Welch, and L. W. Ferris, all of the Food and Drug Administration, for their help in this work.

RECOMMENDATIONS*

(1) It is recommended that the method for malic and citric acids be adopted, First Action.

(2) It is recommended that the method for acetic, formic, succinic, and lactic acids be given further collaborative study.

(3) It is recommended that these acids be determined on good tomatoes and on samples of tomatoes which have become decomposed by the action of molds, yeasts, or bacteria.

REFERENCES

(1) HILLIG, F. *This Journal*, **28**, 525 (1945).
(2) BACON, R,. and DUNBAR, P., Bureau of Chemistry Circular No. 78 (1911).
(3) VAN DAME, H. C., *This Journal*, **34**, 522 (1951).
(4) BULEN, W. A., VARNER, G. E. and BURRELL, R. C., *Anal. Chem.*, **24**, 188 (1952).

No reports were received on: fish products, shellfish, animal fecal matter, pineapple decomposition, pigments in strawberries, uric acid in cereal products and in nuts, histamine in fish, or galacturonic and succinic acids in spinach.

REPORT ON METALS, OTHER ELEMENTS, AND RESIDUES IN FOODS

By P. A. CLIFFORD (Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C.), *Referee*

Ten topics are listed under this chapter and an eleventh, "Sodium in Foods" was added during the year. No reports were received on copper and zinc, and because of the press of other work, Mr. Brandon has asked to be relieved as Associate Referee on copper.

For the third successive year there has been no report of further work on the fate of insecticides, existing as residues, in the canning process. A good start has been made by Tressler†, and by Britten and Fairing†. Where sensitive and specific methods are still lacking, perhaps a series of relatively simple biological tests on the newer organic pesticides, as for example, with flies, could indicate if their toxicity is altered during processing. We might find that most of them are destroyed or changed

* For report of Subcommittee C and action of the Association, see *This Journal*, **36**, 56 (1953).
† *This Journal*, **30**, 140 (1947); *ibid.*, **33**, 599 (1950).

to nontoxic forms during the canning process. Such knowledge would be of reassurance to food chemists.

The Referee did not find time to do further work on parathion. The difficulty here has been a small and variable natural "blank" which interferes in the determination of trace amounts. Before progress towards adoption of a method can be made, means must be found to eliminate or minimize this blank.

The Associate Referee on 1080 received some poor collaborative results in spite of the fact that results reported in 1949 were quite good. There appears to be no alternative except to continue work upon both the quantitative and qualitative tests for 1080.

No report on methoxychlor was given, but the Associate Referee intends shortly to resume work on the pharmacology of this insecticide. Further work and a collaborative study should result in the adoption of a method for methoxychlor.

Methods for BHC have progressed to the point where collaborative study seems in order. The Associate Referee expects to submit his selected method for total BHC and his differential hydrolysis method for the *beta* isomer to collaborative test during the year.

The problem of the determination of fluorine in high-silica plant materials was revived last year. A procedure by L. F. Remmert and T. D. Parks, designed to eliminate the silica interference, has appeared in *Analytical Chemistry*.* It will be made the basis of a collaborative study of the determination of fluorine in refractory materials.

Work upon the determination of sodium in foods is progressing well and the Referee should be able to recommend methods next year.

RECOMMENDATIONS

It is recommended†—

(1) That study of methods for copper and zinc in foods be continued.

(2) That the First Action method for mercury, **35,** 80 (1952), be adopted as official.

(3) That in view of possible future refinements, the colorimetric and organic chloride methods for DDT be maintained in First Action status for another year and that the Stiff-Castillo colorimetric method be developed as an alternate procedure for the determination of DDT as spray residue.

(4) That studies of the effect of canning and other processes upon the newer pesticides be continued.

(5) That the quantitative and qualitative methods for sodium fluoroacetate (1080) be further studied.

(6) That methods for the determination of methoxychlor and its differentiation from DDT in plant and animal products be continued.

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* *Anal. Chem.*, **25,** 450 (1953).

† For report of Subcommittee C and action of the Association, see *This Journal*, **36,** 56 (1953).

(7) That methods for the determination of BHC and its *beta* isomer be studied collaboratively.

(8) That collaborative study be made of modifications designed to eliminate the effect of silica in the determination of fluorine.

(9) That methods for the determination of trace amounts of phosphorus-containing insecticides, including parathion, be studied.

(10) That methods for the determination of trace amounts of chlordane, heptachlor, aldrin, dieldrin, toxaphene, and other chlorinated insecticides be investigated.

(11) That flame photometric and chemical methods for the determination of sodium in foods be developed.

REPORT ON THE DETERMINATION OF DDT AS SPRAY RESIDUE ON FOODS

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

The Seventh Edition of *Methods of Analysis* of the A.O.A.C. in the chapter on "Metals, Other Elements, and Residues in Foods," describes methods for the determination of DDT residues in fruits, vegetables, and cereal, dairy, and meat products. These methods are based on the determination of the organic-chlorine content of the materials or on a colorimetric determination specific for DDT. The procedures are classified as First Action.

At the 1951 meeting of the Association, it was recommended that the study of methods of analysis be continued. Two units of the Food and Drug Administration, one State Chemist, and the Bureau of Entomology and Plant Quarantine expressed interest and willingness to cooperate in collaborative studies.

The Associate Referee corresponded with the three collaborators and several other people, who stated that the methods appeared satisfactory and did not suggest any particular points to be investigated. Accordingly no samples were sent out to collaborators.

Since the publication of the Seventh Edition, some modifications of the colorimetric and chlorine methods have been proposed.

Downing and Norton (1) have proposed a modification of the Schechter-Haller method which shortens the time for routine determinations without reducing their accuracy or precision.

Mann and Carter (2) have recommended a shorter and improved procedure for extraction of DDT from milk.

Fahey and Rusk (3) have made a study of three analytical methods—the total organic chlorine method, the Schechter-Haller colorimetric

method, and the Stiff-Castillo colorimetric method. They concluded that the last method was applicable and possessed certain advantages over the other two.

Parks and Lykken (4) have given details of the procedure and apparatus for the amperometric titration of halogens. This procedure is more sensitive than the Volhard or the electrometric-titration procedure.

The Shell Development Company in collaboration with the Julius Hyman Company has developed procedures for the combustion of plant extracts and amperometric titration of the chlorine residues resulting from insecticide applications. By these procedures it is possible to determine much smaller amounts of organic chlorine than can be found by the sodium reduction method. A paper on these combustion procedures (5) was presented at the Milwaukee meeting of the American Chemical Society, March 30 to April 3, 1952.

Another paper (6), on the application of these procedures to the determination of dieldrin residues in citrus fruit, was presented at the same meeting.

The combustion and amperometric titration procedures have been studied in the laboratory of the Associate Referee and appear to be applicable to the determination of very small quantities of organic-chlorine residues. They are especially useful in the determinations of residues of materials for which specific methods have not been developed.

Gordon (7) has recently proposed a microdiffusion procedure for the determination of chlorides and has suggested that the procedure be used for the determination of residues of chlorinated organic insecticides in biological materials.

Activation analysis (8) has also been investigated for the determination of trace elements in biological materials. It is being investigated for the determination of chlorinated insecticides. The test sample is exposed to neutron bombardment, rendering the chlorine artificially radioactive. By this procedure, very small amounts of chlorine can be detected.

The colorimetric-microdiffusion procedure and activation analysis appear to be promising leads.

D. E. H. Frear, chairman of the Subcommittee on Chemistry, Food Protection Committee, National Research Council, is preparing a bibliography on analytical methods for DDT with special emphasis on the determination of residues.

RECOMMENDATION

It is recommended* that the Stiff-Castillo colorimetric method and the combustion procedure followed by amperometric titration be studied further.

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* For report of Subcommittee C and action of the Association, see *This Journal*, 36, 56 (1953).

REFERENCES

(1) DOWNING, G., and NORTON, L. B., *Anal. Chem.*, **23**, 1870–1871 (1951).
(2) MANN, H. D., and CARTER, R. H., *ibid.*, **23**, 929–930 (1951).
(3) FAHEY, J. E., and RUSK, H. W., *ibid.*, **23**, 1826–1829 (1951).
(4) PARKS, T. D., and LYKKEN, L., *ibid.*, **22**, 1444–1446 (1950).
(5) AGAZZI, E. L., PETERS, E., and BROOKS, F. R., Paper presented at the Milwaukee meeting of the American Chemical Society, March 30 to April 3, 1952.
(6) GUNTHER, F. A., EWART, W. H., and BARKLEY, J. H., Paper presented at the Milwaukee meeting of the American Chemical Society, March 30 to April 3, 1952.
(7) GORDON, H. T., *Anal. Chem.*, **24**, 857 (1952).
(8) ANON., *Chem. Eng. News*, **30**, 3306 (1952).

REPORT ON BENZENE HEXACHLORIDE IN FOODS

DETERMINATION OF TOTAL BHC AND *BETA* ISOMER

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

DETERMINATION OF TOTAL BHC

Four analytical methods, each based on a different chemical principle, have been proposed for determining residual amounts of BHC in foods. The first depended on chlorine analyses. Although chlorine methods have recently been refined (1, 2) so that micro quantities of the element may be determined accurately, this method is nonspecific for the analysis of products whose spray history is unknown. The second procedure involved estimating the amount of 1,2,4-trichlorobenzene formed by refluxing BHC with alcoholic NaOH (3). All of the four commonly known isomers present in commercial BHC yield three isomeric trichlorobenzenes on warm alkaline hydrolysis, but in each instance the proportion of 1,2,4-trichlorobenzene amounts to approximately 80 per cent. This compound is evaluated spectrophotometrically at one of its peak points of absorbancy, 286 mμ in the ultraviolet region. To circumvent background interference contributed by the sample, base line densities at 286 mμ are used instead of the total optical density at this wavelength. A linear relationship exists between amounts of BHC employed and the baseline density of the hydrolysate, measured at 286 mμ. However, the molar absorptivity of 1,2,4-trichlorobenzene at 286 mμ is only about 600 and, because the trichlorobenzene is somewhat volatile, the final solution to be evaluated should not be concentrated to a volume of less than 5 ml (3). The lower limit at which this method will accurately measure BHC, therefore, is about 100 micrograms.

The third method (4), a strictly empirical one, depends upon the formation of dichlorodiphenylamine (among other reaction products) by re-

fluxing BHC with specially purified aniline. After removal of the dichlorodiphenylamine by solvent extraction, it is treated with a sulfuric acid solution of vanadic acid, a reaction which produces a deep magenta-colored solution with an absorption maxima at about 560 mμ. Since other products are formed during the reaction of BHC and aniline, however, the final colored solution is, in practice, evaluated at 510 mμ. Although the method is empirical, linearity between optical density of the reaction products and starting amounts of BHC is obtained if the range is restricted to 0–150 micrograms. One disadvantage of the method is that not all of the isomers of technical BHC measure the same as *gamma*, the isomer of highest acute toxicity. Thus, compared to *gamma*, the others measure as follows: *alpha*, 90%; *delta*, 40%; and *beta*, 5%. For that reason, technical BHC is used in preparing the standard curve, and the assumption is made that the technical product is of uniform composition. Another disadvantage of the method is that almost all fat and other glycerides must be removed from the sample extract before refluxing with aniline. This removal, a somewhat long and undesirable step, is effected by using a modification of the procedure employed by Davidow (5) where the fat, dissolved in petroleum ether, is absorbed on Celite previously mixed with fuming sulfuric acid.

The fourth procedure (6), described by Schechter and Hornstein, is, in the Associate Referee's opinion, the best. Fats and other glycerides, as well as fruit and vegetables, do not interfere. The method depends upon the formation of benzene by refluxing an acetic acid solution of BHC with powdered zinc for 2.5 hrs. The benzene is then quantitatively nitrated as liberated to three isomeric dinitrobenzenes of which the *meta*-isomer comprises about 85 per cent. Both dehydrohalogenation and nitration are conducted in the same apparatus. *Meta*-dinitrobenzene forms an intense magenta colored solution when treated with butanone-2 and strong alkali. The other two isomeric dinitrobenzenes remain colorless. Although the conversion of benzene to *meta*-dinitrobenzene is not quantitative, the 85 per cent conversion is remarkably consistent, and a standard working curve, obtained by carrying known amounts of *gamma* through the procedure, follows Beer's law very closely. Unfortunately, not all of the isomers measure the same. Compared to *gamma*, the others measure as follows: *alpha*, 100 per cent; *beta*, 80–90 per cent (the Associate Referee obtained 90 per cent); and *delta*, about 30 per cent. However, *delta* accounts for only about 5–10 per cent of technical BHC.

In applying the Schechter-Hornstein procedure to various food products, the Associate Referee encountered no difficulty except with peanut butter. No matter which solvent was used to prepare the sample solution of this product (carbon tetrachloride, methylene chloride, or refined petroleum ether) sufficient amounts of aromatic materials, presumably amino acids, were extracted to cause a seriously high and variable false

blank. In order to remove the interference, the procedure (5) of removing fats by adsorption on Celite-fuming sulfuric acid was used. The extracted protein matter is also adsorbed and the false blank is reduced to less than 0.1 p.p.m. when a sample aliquot representing 40 g of sample is used. To check the efficiency of this method of sample preparation, technical BHC was added in the amount of 1.88 p.p.m. to 100 g of peanut butter known to be free of BHC. Recovery values of 1.80, 2.0, and 1.75 p.p.m., averaging 1.85 p.p.m., were obtained on separate petroleum ether extracts after the acid-Celite treatment.

DETERMINATION OF BETA ISOMER

None of the proposed chemical methods is specific for determining any one of the various isomers of BHC. It is desirable to devise a method for selectively estimating the *beta*-isomer since long-term feeding tests showed that the *alpha-*, *gamma-*, and *delta*-isomers are stored in the animal body fat in about the same amounts in which they are present in the diet, expressed in p.p.m. (7). In the case of the *beta*-isomer, however, the storage is about ten-fold (7). The same investigators also observed that when the toxicants are removed from the diet of the experimental animals, the *alpha-*, *gamma-*, and *delta*-isomers are rapidly eliminated, but the *beta*-persists for a comparatively long time. If the *beta*-isomer accumulates in plants in a similar manner, this accumulation might present a serious health problem because this isomer is known to persist in soils even after extended weathering periods (8).

EXPERIMENTAL

To evaluate the *beta*-isomer in a mixture of the isomers, use was made of the observations of Cristol (9) that it is somewhat resistant to alkaline hydrolysis. He observed the following order of reactivity, measured at 20°C., arranged in order of increasing rate: *beta-* (relatively inert), *gamma-*, *alpha-*, and *delta-* (very reactive). The products of hydrolysis, the three trichlorobenzenes, do not interfere in the determination of BHC by the Schechter-Hornstein procedure. A rather extended series of experiments was conducted to determine the conditions under which the *alpha-*, *gamma-*, and *delta*-isomers are completely hydrolyzed with no attendant decomposition of the *beta*. This would not have been difficult with solutions containing all of the four isomers in equal amounts, but in synthetic solutions containing the isomers in ratios approximating that present in commercial preparations, when the temperature and time of hydrolysis were so adjusted that complete hydrolysis of *alpha-*, *gamma-*, and *delta-* was effected, too much *beta-* was destroyed. Conversely, when conditions were used by which no *beta-* was hydrolyzed, too much of the other isomers remained. Consequently, a set of conditions was employed in which a consistent compromise result was obtained, one in which the amount of the three isomers remaining after hydrolysis compensated in

large measure for the amount of *beta-* destroyed. Hydrolysis at 20°C. for 3 hours of alcoholic solutions of the isomers in an alkaline medium corresponding to 0.01 *N* effected this compensation. Results are shown in Table 1.

The conditions in all the following experiments were as follows: volume, 50 ml; alkalinity, 0.01 *N* NaOH; temperature 20°C.; time, 3 hours. The residual isomers were finally referred to a standard curve developed by the Schechter-Hornstein procedure with pure *gamma*. An example follows:

A synthetic solution contained 0.560 mg of *alpha-*, 0.120 mg of *gamma-*, and 0.080 mg of *delta-*, totaling 0.760 mg. After hydrolysis, 0.008 mg was found. Under like conditions, 0.025 mg of *beta-* yielded 0.022 mg (0.008 +0.022 = 0.030 mg), an over-compensation of 0.005 mg. The above experiment was repeated and the mixture of 0.760 mg yielded 0.0045 mg

TABLE 1.—*Hydrolysis of isomers of benzene hexachloride*

BEFORE HYDROLYSIS SYNTHETIC SOLN, MG	AFTER HYDROLYSIS YIELD, MG
Experiment 1	
0.560 *alpha*	
0.120 *gamma*	0.008
0.080 *delta*	
0.025 *beta*	0.022
	0.008 +0.022 =0.030 (over-compensation)
0.760 mixt.	0.0045
0.025 *beta*	0.0195
	0.0045 +0.0195 =0.024 (under-compensation)
Experiment 2	
0.0988 mixt.	0.0039
0.010 *beta*	0.0085
	0.0039 +0.0085 =0.0124 (over-compensation)
0.0988 mixt.	0.0024
0.010 *beta*	0.009
	0.0024 +0.009 =0.0014 (over-compensation)
Experiment 3	
0.066 *alpha*	0.0078
0.006 *beta*	0.0050
0.012 *gamma*	0.0057
0.010 *delta*	
	Av.: 0.0062
0.009 *beta*	0.0076 0.0014 (under-compensation

after hydrolysis; 0.025 mg of *beta-* yielded 0.0195 mg (0.0045+0.0195 =0.024 mg), a slight under-compensation.

In order to determine if the *beta*-isomer is selectively absorbed or stored in food products, peanuts, a high fat product (approximately 50 per cent), were chosen for study. Samples of peanut butters, processed from experimental peanuts grown by the Bureau of Plant Industry, Soils, and Agricultural Engineering (BPISAE), were used.

One sample was part of a lot which had been planted on May 20, 1949, on soil which was treated on June 6, 1949, with commercial BHC at a dosage rate to give 1 pound of *gamma*-isomer per acre (8 pounds of technical BHC per acre). This corresponds to an application of 8 p.p.m., assuming uniform distribution both at the surface and to a depth of 3 inches. Hornstein found 8–9 p.p.m. of total BHC; the Associate Referee obtained a value of 7.1 p.p.m. This sample should be ideal for the purpose since the planting was done almost simultaneously with spray application so that no immediate "weathering" of the isomers should take place. Moreover, sufficient *beta-* should be present to eliminate the possibility of gross analytical errors. A value of 0.37 p.p.m. of *beta-* was obtained, corresponding to about 5 per cent of the total BHC. A check analysis yielded 0.50 p.p.m. of *beta-* or about 7 per cent of the total BHC. Commercial BHC contains about 10 per cent *beta-*.

Seven additional experimental samples of peanut butter were submitted by the BPISAE. The description of the original planting is as follows: the first three were "grown on plots planted in cotton the previous year. Cotton dusted with seven applications of BHC. Total *gamma*-isomer per acre, 3.80 lb." This would constitute an application of 30 pounds per acre of the technical product. Hornstein found an average of 0.37 p.p.m. of BHC in these samples. The remaining four were processed from peanuts which had been "grown on plots planted in cotton the previous year, dusted with 10 applications of BHC. Total *gamma*-isomer per acre, 5.14 lb." This amounts to an application of 41 pounds per acre of commercial BHC. Both of the spray schedules are very heavy ones. Hornstein obtained an average of 0.88 p.p.m. total BHC in the last four samples. The Associate Referee obtained 0.84 p.p.m. in one, and 0.60 and 0.73 p.p.m. in another. In the latter, 0.16 and 0.20 p.p.m. of *beta-* were found, the average of which is 27 per cent of the average total BHC.

These experiments demonstrate that peanuts planted in soil almost simultaneously with BHC application absorb a large amount of the insecticide but the ratio of the *beta*-isomer is a normal one. When the peanuts were grown one year later on soils treated originally at very high dosage levels, the absorption of BHC was less than 1.0 p.p.m., a fact which is in accord with Hornstein's experience. As a result of many analyses, he concluded that peanuts thus grown always contain less than 1.0 p.p.m., and average about 0.4 p.p.m. Since the *beta*-isomer has a

tendency to persist in soil, data show that peanuts grown a year after spraying contain an increased ratio of absorbed *beta-*, but the actual amount present is less than 0.3 p.p.m.

RECOMMENDATIONS

It is recommended* that the Schechter-Hornstein method for the determination of benzene hexachloride be submitted to collaborative study.

REFERENCES

(1) AGAZZI, E. J., PETERS, E. D., and BROOKS, F. R., *Anal. Chem.*, **25**, 237 (1953).
(2) PARKS, T. D., and LYKKEN, L., *Anal. Chem.*, **22**, 1444 (1950).
(3) DAVIDOW, B., and WOODARD, G., *This Journal*, **32**, 751 (1949).
(4) PHILLIPS, W. F., *Anal. Chem.*, **24**, 1976 (1952).
(5) DAVIDOW, B., *This Journal*, **33**, 130 (1950).
(6) SCHECHTER, M. S., and HORNSTEIN, I., *Anal. Chem.*, **24**, 544 (1952).
(7) DAVIDOW, B., and FRAWLEY, J. P., *Proc. Soc. Exptl. Biol. Med.*, **76**, 780 (1951).
(8) FOSTER, A. C., Beltsville, Md., Experimental Station, BPISAE (private communications).
(9) CRISTOL, S. J., *J. Am. Chem. Soc.*, **69**, 333 (1947).

REPORT ON SODIUM IN FOODS

By PAUL A. CLIFFORD (Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C.), *Referee*

Several years ago, probably no one would have predicted that the food chemist would become concerned about sodium in foods; it was known that all foods contain more or less sodium and little was thought about the matter. The reason for the present interest in the topic is, of course, the discovery that a lowered dietary intake of sodium may alleviate such conditions as high blood pressure and congestive heart failure. The severely restricted diet may contain only 250 mg of sodium or less. Since the average person, consuming an ordinary salt-seasoned mixed diet, ingests from 6 to 10 g of sodium chloride daily, it is seen how drastically sodium intake may be reduced.

In the first place, doctors and dietitians want to know the natural sodium content of many foods so that they may plan proper diets, and it is the function of the food chemist to supply such data. Various tabulations already exist; the most modern and extensive is that of Bills and coworkers,† who list the sodium and potassium contents of some 500 foods. These authors did not analyze a large number of samples of each food; they point out that even if significant average values for a particular food item had been derived, the user of the tables would have no assurance that his sample was of normal sodium content. Consequently, the nat-

* For report of Subcommittee C and action of the Association, see *This Journal*, **36**, 57 (1953).
† Bills, C. E., McDonald, F. G., Niedermeier, W., and Schwartz, M. C., *J. Am. Dietet. Assoc.*, **25**, 304 (1949).

ural variability in sodium content of many foods has yet to be appraised. Bills' results do show, however, that the sodium content of foods follows broad generic classifications; fruits and cereals are low in sodium, vegetables contain more, and animal products such as meats, milk, and eggs contain still more. The tabulation of Bills and his colleagues is the one most used by physicians and dietitians. Work by other investigators indicates that the variability of sodium content for a particular food may be very great. At present, the Referee is engaged in what might be considered a spot check of Bills' figures. In the analysis of some 40 samples, quite good agreement for sodium and potassium content was usually found. Exceptions are as follows: 1) *fresh beets,* where Bills reports 110 mg Na/100 g and the Referee could find only 30 mg; one sample. It is noted that Bills reports 36 mg on a sample of canned beets; 2) *dried peas,* where Bills reports 42 mg/100 g and the Referee found 12 mg; and especially, 3) *fresh spinach* where the referee (one sample) found only 3 mg/100 g as against Bills' figure of 82 mg.

It is tentatively noted that animal food products—meats, milk, etc., are quite uniform in sodium content and this uniformity could conceivably be tied in with bodily metabolic processes. Greater variation is noted with foods of plant origin. It is seen that much more work needs to be done before normal, practical values may be assigned to each particular food.

In the second place, the labeling of special low-sodium dietary foods is sure to pose regulatory problems. The Food and Drug Administration has recently reviewed the labeling of 128 various products represented as "salt-free," "low-sodium," "for use in low-sodium diets," etc. Only about 25 per cent of the labels declared the actual amount of sodium present, and it is suspected that in many cases these figures were not the result of actual analysis, but were taken from values reported in the literature. We have done enough work to know that the natural sodium content of foods may vary highly. Adequate control by chemical analysis is essential, if the label statement is to be "true to fact," according to the words of the Commissioner of the Food and Drug Administration, who mentioned this subject in his address of last year.

And in the third place, and as far as we are concerned the most important one, there is the consideration of methods. It is an interesting coincidence that flame photometric methods for sodium were being perfected at about the time of the new demand for more complete and reliable figures for the sodium content of foods. Bills' figures were obtained by the flame photometer and he and his co-workers helped to develop the flame technique. It has been noted that their figures for sodium, as a rule, are lower than previously-reported values. The Referee considers these lower figures as more nearly correct. In his experience, certain products such as corn and soybeans contain so little sodium (<5 p.p.m.) that its detection may

tax the sensitivity of even flame photometric methods. In such cases sodium could truly be classed as a trace element.

Flame photometric methods are attractive because of their speed and inherent specificity. In this respect they are akin to arc spectrographic methods but have a great advantage in that it is easier to control the temperature, shape, and position of a flame than of an arc. It is predicted that they will be made more and more accurate. At present the Referee is working with two flame instruments which may be considered of different type and method of operation: the Perkin-Elmer Model 52C with a relatively low-temperature air-propane flame and lithium internal standard, and the Beckman DU spectrophotometer, with the oxygen-acetylene flame accessory, which operates on the direct measurement principle. A tentative procedure has been devised which, in the sodium determination, eliminates the interference of all common ions except that of potassium. It is necessary to estimate potassium and to correct for it. Sodium results by both instruments agree quite well.

It is noted that the magnesium uranyl acetate method, official for the determination of sodium in plants, fails in the determination of very low amounts of sodium, especially when a large proportion of potassium is present. Modifications designed to eliminate a potassium interference, such as that of Williams and Haines*, will be investigated.

Some collaborative work on sodium has been started. The Referee would welcome collaborative aid from those laboratories which are equipped with flame photometers. It is hoped that procedures for the determination of sodium in foods can be recommended next year.

REPORT ON MERCURY

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

As a result of last year's collaborative results, the mercury method, designed for evaluating micro quantities of the element, was given First Action status (*This Journal*, **35**, 80 (1952)). During the spring and summer a number of micro mercury determinations were conducted on wheat in which neither the Associate Referee nor other chemists experienced any difficulties in the application of the method. The Associate Referee therefore feels justified in recommending† that the method be adopted as official, Final Action.

* *Anal. Ed.*, 16, 157 (1944).
† For report of Subcommittee C and action of the Association, see *This Journal*, 36, 56 (1953).

REPORT ON SODIUM FLUOROACETATE (1080)

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

In accordance with the recommendations of Subcommittee C [*This Journal*, **35**, 51 (1952)], the quantitative method of Ramsey and Clifford [*ibid.*, **32**, 788 (1949)] and the qualitative method of Ramsey and Patterson [*ibid.*, **34**, 828 (1951)] were subjected to collaborative study. Four food samples were prepared; the collaborators were instructed to make a single qualitative determination on each sample and to analyze quantitatively, in duplicate, three of the four samples (not all collaborators were assigned the same three samples). Only three out of the five collaborators had time to do the work, and the results in general were not satisfactory. With the quantitative method only one collaborator* obtained good results on the samples. His recoveries on the flour were 97 and 98 per cent; on the sugar, 90 and 92 per cent; and on the hamburger 93 and 96 per cent. The other two collaborators obtained widely varying results and it is not clear why they failed to obtain quantitative recoveries.

With the qualitative method, two analysts obtained perfect results, but the third analyst failed to obtain any positive test, although three of the four samples actually contained 1080. This analyst attributed his failure to obtain satisfactory results on the flour to a dark brown discoloration, which occurred during the concentration of the filtrate prior to extraction, and on the sugar to a color which was extracted by the ether; these brown colors persisted in the determination and masked the thioindigo color.

In order to get satisfactory results with these methods, it may be necessary to instruct the collaborators to familiarize themselves adequately with the procedures before doing the collaborative work, by adding a small amount of 1080 (0.5 to 1.0 mg) to a food and running thru the determinations, particularly the quantitative analysis. Possibly the alternative to a large extractor, concentrating the filtrate prior to extraction in order to use a small extractor, should be eliminated. In the qualitative method, it may be necessary to substitute another agent for the carbon in the purification step. Chromatography, although it is best for the purpose, is quite time-consuming. It also appears that certain precautions and clarifying instructions should be inserted in both methods.

It should be noted that in the first collaborative study of the quantitative method [*This Journal*, **33**, 608 (1950)], excellent results were obtained by all of the collaborators.

* Harry W. Conroy, Kansas City District, Food and Drug Administration.

RECOMMENDATIONS

It is recommended* that the qualitative and quantitative methods for 1080, after appropriate changes are made as indicated, be again studied collaboratively.

No reports were received on: copper and zinc, insecticides in canned foods, parathion, methoxychlor, and fluorine.

REPORT ON GUMS IN FOOD

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

The Associate Referee on gums in cacao products has submitted a report on gums in chocolate milk, and recommends that the method submitted, which is supported by collaborative results, be adopted, First Action. The Referee concurs in the Associate Referee's recommendation.

No reports were received from the Associate Referee on gums in catsup and related tomato products and from the Associate Referee on gums in frozen desserts. This undoubtedly was caused primarily by the failure of the Referee to cooperate with them due to circumstances beyond his control. Both have done considerable work on their subjects and the Referee looks forward to reports from them next year.

No report was submitted by the Associate Referee upon alginates in cheese and by the Associate Referee upon alginates in salad dressings, although some work was done in the case of salad dressings.

RECOMMENDATIONS

It is recommended†—

(1) That the method for the detection of gums in chocolate milk be adopted, First Action.

(2) That work be continued on detection of alginates in chocolate milk.

(3) That the method for the detection of gums in catsup and related products be submitted to collaborative study.

(4) That work be done by the Associate Referee on gums in cheese on the detection of alginates.

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

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

(7) That work be continued on detection of the other emulsifying agents which are permitted by the Federal standard for salad dressing.

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

REPORT ON THE DETECTION OF ALGIN AND GUMS IN CACAO PRODUCTS

By FLORA Y. MENDELSOHN (Food and Drug Administration, Department of Health, Education, and Welfare, Los Angeles, California), *Associate Referee*

The previous report indicated that an attempt was being made to work out methods for the detection of both gums and algin in chocolate flavored milk. The work on gums has been successful, but methods for algin are still unsatisfactory. A report on the detection of gums is submitted at this time. Since algin was present in some of the collaborative samples it is clear that it does not interfere with the test for gums.

Samples for laboratory analysis and collaborative work were prepared, using the following formula:

100.0 ml reconstituted skim milk*
5.0 g sugar
1.5 g cocoa
0.2 g gum

The reconstituted skim milk was heated to 74°C. and the cocoa was added with stirring, followed by a dry mixture of the sugar and gum. The total mixture was held at 74°C. for 30 minutes, and then cooled and bottled.

The following method was used:

GUMS IN CHOCOLATE FLAVORED MILK

REAGENTS

(a) *Trichloroacetic acid soln*, 20%.—Use fresh soln made up just before use from non-hydrolyzed reagent.

(b) *Alcohol.*—95% and 70% v/v.

(c) *Acetic acid.*—Reagent grade, glacial, 99.5%.

(d) *NaOH.*—10% w/v.

(e) *Benedict's soln (qualitative).*—Dissolve 17.3 g Na citrate and 10 g anhydrous Na_2CO_3 in ca 80 ml of hot H_2O; dissolve 1.73 g of $CuSO_4 \cdot 5H_2O$ in 10 ml H_2O. Filter the alk. citrate soln, add the $CuSO_4$ soln slowly, with constant stirring, and dilute with H_2O to 100 ml.

SEPARATION OF GUM

Thoroly shake entire sample to insure uniform composition. Transfer 50 ml of sample to a 250 ml Pyrex centrifuge bottle. Add 50 ml of 20% trichloroacetic acid soln (reagent a), stopper bottle, and shake thoroly (15 min., in mechanical shaker). Allow mixt. to stand at least 2 hrs and then centrifuge 15 min. at 1500 r.p.m. Decant the clear liquid into a 600 ml beaker, add four volumes of 95% alcohol, and allow to stand overnight or until the ppt. has settled. Decant off the clear liquid, reserving only an amount sufficient to transfer the ppt to a 250 ml Pyrex centrifuge bottle. Centrifuge for 20 min. at 1500 r.p.m. Decant off the clear liquid and add 20 ml of

* 100 g of non-fat dry milk solids was diluted with H_2O to make 1000 g of reconstituted skim milk. As a preservative, 0.1% sodium benzoate was added.

70% alcohol to the residue in the bottle. Stopper bottle and shake to break up the residue thoroly. Centrifuge, decant, and discard alcohol. Wash again with a second 20 ml portion of 70% alcohol. Dissolve the residue in ca 40 ml of hot H_2O. Centrifuge to remove any undissolved material. Decant off the clear liquid into a 250 ml Pyrex centrifuge bottle, add four portions of 95% alcohol, and three drops of acetic acid. Allow to stand until the ppt has settled. Centrifuge and decant off the clear liquid. Wash once with 10 ml of 70% alcohol. Centrifuge and again decant.

TEST FOR GUM

Add 20 ml of hot H_2O to residue and transfer to 50 ml beaker. Warm to dissolve gum and evap. to 10 ml. Add 2 ml of concd HCl. Boil one min. Transfer 1 ml of this soln to a test tube and neutralize with NaOH soln, using litmus paper as an indicator. Remove litmus paper, add 5 ml of Benedict's soln, and boil 2 min. Allow to cool spontaneously. A ppt which may be yellow, orange, or red indicates reducing sugars (hydrolyzed gums).

EXPERIMENTAL RESULTS

Samples of chocolate flavored milk prepared as follows were submitted for collaborative work:

(1) No gum or algin added.
(2) 0.2 per cent locust bean gum added.
(3) 0.1 per cent locust bean gum and 0.1 per cent algin added.

Positive results for the presence of gum in samples 2 and 3 were reported by:

(1) T. E. Strange—Food and Drug Administration, Portland, Oregon
(2) E. W. Coulter—Food and Drug Administration, Chicago, Illinois
(3) M. J. Gnagy—Food and Drug Administration, Los Angeles, California

Additional work was done in the Los Angeles District laboratory on samples containing 0.2 per cent Irish Moss and samples containing 0.2 per cent gum arabic. Both samples gave a positive test for gum with Benedict's solution.

DISCUSSION

Although only three gums were used in this work, the literature indicates that this method would be suitable for the separation and identification of the many other gums used in food products.

In samples containing a mixture of algin and gum, one portion was treated with trichloroacetic acid solution and a second portion was treated with tannic acid solution for the separation of sodium alginate. Positive tests for gums, but negative tests for algin, were obtained.

Recent information indicates that sodium alginate tends to polymerize on standing at room temperature and its emulsifying property is thereby weakened. As the use of algin is continually increasing, further work is planned.

RECOMMENDATIONS

It is recommended*—

(1) That further work be done on the separation and detection of algin, using freshly manufactured products.

(2) That the method for the detection of gum in chocolate flavored milk be adopted as First Action.

No reports were received on alginates in cheese, and gums in desserts, catsup and related tomato products, and in salad dressings.

REPORT ON GELATIN, DESSERT PREPARATIONS, AND MIXES

By S. C. Rowe (Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C.), *Referee*

The Associate Referee on Gelatin Dessert Constituents, Dr. Joseph H. Cohen, has submitted a detailed report on a further study of methods† for determining sucrose and dextrose in gelatin dessert powders. Collaborative work in 1949 showed that the reproducibility of the results obtained by the methods was good but no attempt was made to test the accuracy of the methods with samples of known composition.

This year, four samples of known composition were sent to 6 collaborators. The samples contained different amounts of acid or acid-buffer, and different amounts of sucrose and dextrose. Each collaborator was instructed to give his results in duplicate. The Associate Referee has reported all of the results obtained and has accompanied them with a statistical interpretation. He concludes that the reproducibility of the dextrose results is not good, although the precision in the case of sucrose is fairly good, and recommends that Final Action on the methods be deferred until further work is done in an effort to improve their precision.

A study of the results obtained by the Associate Referee would indicate that, with the exception of the dextrose figures reported by one collaborator, (F), the results are quite good for determinations of this type. The largest variations appear to be due to factors other than the lack of precision of the methods. However, the Referee concurs in the recommendation of the Associate Referee that Final Action on the methods for sucrose and dextrose be deferred and recommends that no action be taken for at least another year.

During the past two years, the methods for jelly strength of gelatin and gelatin dessert preparations which appear under sections **21.6** and

* For report of Subcommittee C and action of the Association, see *This Journal*, **36**, 57 (1953).
† *Official Methods of Analysis*. 7th Ed., Sections, **21.13, 21.14, and 21.15.**

21.12 as Official, First Action, in the Seventh Edition of *Methods of Analysis*, have been given further study. These methods have been adopted by the Chicago Quartermaster Depot of the U. S. Army and other Federal agencies in specifications for purchase of food supplies. They appear in the new Federal Specification entitled Dessert Powders, and Plain Gelatin (C-D-221).

In view of the fact that the methods for jelly strength have been given further study by Government and industry and found to be acceptable, it is recommended* that they be made official.

REPORT ON GELATIN DESSERT CONSTITUENTS

By JOSEPH H. COHEN (Atlantic Gelatin Division, General Foods Corporation, Woburn, Mass.), *Associate Referee*

Further study of methods for determining sucrose and dextrose in gelatin dessert powder was recommended at the 1949 meeting of the Association of Official Agricultural Chemists. The methods have been adopted, first action, and appear in the Seventh Edition of the *Official Methods of Analysis*, sections **21.13, 21.14,** and **21.15.**

SAMPLING SCHEME

A sampling scheme was devised to test the interaction effect of the acid or acid-buffer system as well as the variation of sucrose and dextrose content of the samples. The samples were prepared, as shown in Table 1, with varying amounts of sucrose and dextrose and various acid or acid-buffer combinations.

TABLE 1.—*Composition of samples*

SAMPLE NO.	1	2	3	4	5	6	7	8	9	10	11	12
Buffer Acid[a]	I	II	III	IV	I	II	III	IV	I	II	III	IV
Sucrose, %	85	85	85	85	78	78	78	78	70	70	70	70
Dextrose, %	0	0	0	0	7	7	7	7	15	15	15	15

[a] I = Citric Acid—Sodium Citrate; II = Tartaric Acid—Sodium Bitartrate; III = Citric Acid—Mixed Phosphates; IV = Citric Acid.

Four samples were sent to each collaborator as shown in Table 2. This set-up ensured that:

(1) Each sample was received by two different collaborators.
(2) Each collaborator received samples representing all levels of sucrose and dextrose and all types of acid or acid-buffer systems.
(3) Each level of dextrose was represented 8 times.
(4) Each acid or acid-buffer combination was represented 12 times.

Each collaborator was instructed to make duplicate determinations on each of his samples.

* For report of Subcommittee C and action of the Association, see *This Journal*, 36, 56 (1953).

TABLE 2.—*Distribution of samples*

COLLABORATOR	SAMPLES			
A	4	1	6	11
B	3	8	5	10
C	2	7	12	9
D	1	10	7	4
E	5	2	11	8
F	9	6	3	12

RESULTS AND DISCUSSION

The results of the collaborative work are shown in Table 3. Statistical interpretation of these data indicates the following:

(1) Differences have been found between collaborator's results on sucrose and dextrose. These are significant at odds of 20 to 1.

(2) In the determination of sucrose, systematic differences with odds of 20 to 1 or greater have been found only between the results reported by collaborators F and those reported by collaborators A and B.

(3) In the determination of dextrose, only the results of collaborator F were found to be systematically different by odds of 20 to 1 or greater from the results of the other collaborators.

(4) The sucrose content of samples as determined in duplicate by different collaborators must differ from one another by more than 5.5 per cent in order for that difference to be judged significant at 20 to 1 odds. Lesser differences can be attributed to the lack of reproducibility of the method and to personal factors.

(5) The dextrose content of samples as determined in duplicate by different collaborators must differ from one another by more than 7.6 per cent in order for that difference to be judged significant at 20 to 1 odds. Lesser differences can be attributed to the lack of reproducibility of the method and to personal factors.

(6) All of the collaborators, excluding F, give consistently low values when compared with the known content of sucrose of the samples. Laboratory biases associated with the different levels of sucrose are as follows:

Sucrose, %	*Bias (% Sucrose)*
85	-2.1 ± 0.9
78	-1.6 ± 0.9
70	-1.1 ± 0.9

(7) The average bias in determining dextrose over all the laboratories is as follows:

Dextrose, %	*Bias (% Dextrose)*
0	$+1.34 \pm 0.5$
7	$+0.83 \pm 0.5$
15	-0.36 ± 0.5

TABLE 3.—*Results of sucrose and dextrose determinations by collaborators*

COLLABORATOR	SAMPLE NO.	SUCROSE	DEXTROSE
		per cent	*per cent*
A	1	80.00	2.60
		81.11	−3.36
	4	82.08	2.14
		82.08	2.14
	6	75.25	7.85
		75.08	8.06
	11	66.85	15.55
		65.62	15.95
B	3	82.42	0.98
		82.42	0.98
	5	78.18	5.35
		78.18	5.35
	8	77.27	7.00
		77.27	7.00
	10	69.39	12.92
		69.39	12.92
C	2	82.39	0.00
		81.89	0.00
	7	76.37	6.37
		76.40	6.07
	9	72.12	11.50
		72.12	11.50
	12	69.29	13.57
		69.29	13.57
D	1	81.7	−0.2
		82.2	−0.2
	4	83.2	−0.6
		82.9	−1.1
	7	74.5	6.9
		74.7	7.2
	10	66.6	13.9
		67.3	13.9

TABLE 3—(*continued*)

COLLABORATOR	SAMPLE NO.	SUCROSE	DEXTROSE
		per cent	*per cent*
E	2	83.33	0.00
		82.42	0.00
	5	78.18	5.35
		78.48	4.96
	8	76.67	5.73
		76.97	5.87
	11	68.79	13.69
		69.09	13.31
F	3	85.60	4.36
		87.31	1.18
	6	76.58	7.68
		76.01	13.98
	9	75.16	20.53
		76.01	11.75
	12	69.21	23.91
		67.90	16.04

CONCLUSIONS AND RECOMMENDATIONS

In the previous report it was concluded that the reproducibility of the results by these methods was good. This conclusion does not hold now in the case of dextrose, although the precision in the case of sucrose is fairly good.

It is recommended* therefore that Final Action on methods **21.13, 21.14,** and **21.15** not be taken until further work is done in an effort to improve their precision.

ACKNOWLEDGMENT

The Associate Referee wishes to acknowledge the assistance of the following collaborators:

(1) Mr. A. E. Anderson, Quaker Maid Co., Inc., New York, New York.
(2) Mr. Wm. H. Case, Food and Container Institute, Chicago, Illinois.
(3) Mr. N. H. Ishler, General Foods Corporation, Central Laboratories, Hoboken, New Jersey.
(4) Mrs. D. Montgomery, Food and Drug Administration, Washington, D. C.
(5) Mr. E. J. Pelaccio, Standard Brands, Inc., Peekskill, New York.
(6) Mr. C. B. Streightif, U-COP-CO., Gelatine Corp., Calumet City, Illinois.

* For report of Subcommittee C and action of the Association, see *This Journal*, 36, 56 (1953).

REPORT ON FISH AND FISH PRODUCTS

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

The Associate Referee on total solids in oysters has completed an investigation of the drying time required for total solids determinations and the use of the Waring blendor in preparing oyster samples. Authentic samples were subjected to collaborative study.

Due to a change in duties, this Referee has done no further work on a method for determining total solids in fish and other marine products. An Associate Referee has been appointed to complete this work and to investigate a new rapid method for the determination of ether extract in fish. A preliminary report on this method has been submitted.

RECOMMENDATIONS

It is recommended*—

(1) That the method described by the Referee (*This Journal*, **35**, 216 (1952)) for the determination of total solids in fish and other marine products, except oysters, be submitted to collaborative study.

(2) That the use of the forced draft oven in connection with the determination of total solids in fish and other marine products, including oysters, be investigated.

(3) That the rapid method for ether extract in fish described by the Associate Referee be subjected to collaborative study.

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

The Associate Referee on total solids in oysters has recommended the following changes in official methods for oyster work. It is recommended that these changes be accepted by the Association. It is therefore further recommended—

(5) That the drying time in the first action method for oysters (*Official Methods of Analysis*, 7th Ed., **18.4**) be reduced to "3 hrs. in oven at 98–100°."

(6) That the Waring blendor be specified for preparation of samples of shucked oysters by deleting the entire paragraph (Sec. **18.1** (f) second par.) as applied to oysters, and substituting "Grind meats, including liquid, 1–2 min. in Waring blendor."

(7) That the requirement for separate analysis of meats and liquid be deleted (Sec. **18.1** (f) first par., last sentence.)

* For report of Subcommittee C and action of the Association, see *This Journal*, **36**, 56 (1953).

REPORT ON TOTAL SOLIDS AND ETHER EXTRACT IN FISH

By H. M. RISLEY (Food and Drug Administration, Department of Health, Education, and Welfare, Seattle, Washington), *Associate Referee*

Last year, the Referee submitted a method for total solids in fish and other marine products (except oysters) with the recommendation that it be subjected to collaborative study. He also mentioned that use of a forced draft oven, if available, might reduce the drying time required. Next year the forced draft oven will be tried and the method will be given collaborative study if time permits.

A rapid method for the determination of ether extract in canned salmon has been developed and used at the Seattle District of the Food and Drug Administration during the past two years. Preliminary results indicate that much time can be saved over the present official method and that the accuracy is good.

RAPID METHOD—ETHER EXTRACT IN 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 and fasten stopper in place with wide rubber band. Shake vigorously by hand to break up and disperse fish solids. Shake 30 min. vigorously enough to keep solids in suspension. Centrifuge to form tight cake under ether (ca 10 min. at 1200 r.p.m.). Carefully pipet 20 ml of the clear ether layer into a 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 an air oven at 100°C. Cool in air and weigh. Calc. % fat according to the following formula, which corrects for the vol. of fat contained in the aliquot:

$$\text{Per cent fat} = \frac{W\,(50)(100)}{(20 - W/0.92)(S)}$$

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

NOTES

(1) It has been found that the ratios of solvent volume to sample size and to size of centrifuge bottle are somewhat critical and should not be varied too greatly from those given in the method. In practice, it has been found satisfactory to weigh exactly 5 g of sample in a tared scoop and transfer into the centrifuge bottle by scraping with a spatula.

(2) Aliquots of other than 20 ml can be used, although this was approximately the largest that could be taken without danger of disturbing the "cake" on the bottom of the bottle.

(3) The Ottawa sand seems to act as a dispersing and grinding agent in the mixture. If the sodium sulfate is omitted, a cloudy ether layer occasionally results. These reagents are measured by volume, rather than by weight, simply as a matter of convenience. Their proportion may be critical, but this factor was not investigated.

RECOMMENDATION

It is recommended* that the method be subjected to collaborative study and further comparison with the official method.

REPORT ON SOLIDS IN RAW OYSTERS

By DOUGLAS C. PRICE†, *Former Associate Referee*, and JOHN P. TRAYNOR, *Associate Referee* (Food and Drug Administration, Department of Health, Education, and Welfare, Baltimore, Maryland)

The A.O.A.C. method (1) for preparation of samples of raw oysters for total solids, ash, and salt determinations specifies the use of the meat chopper and, indirectly, the use of the Waring blendor.

During the past several years, considerable work has been carried out comparing the performance of the meat or food chopper with the Waring blendor. In general, the results favor its use. This comparison is included in this report.

The official method (1) specifies that when the drained liquid in oysters is more than 10%, the meats will be analyzed separately from the liquid. It has been the experience of analysts who have used the Waring blendor that such separate analysis is not necessary and that a considerable saving of time and effort will result from analysis of the well-mixed whole sample. This comparison is also a part of this report.

Official Methods of Analysis (1) specifies a 4-hour drying time for total solids. Preliminary work has indicated that when samples are prepared with a Waring blendor, considerably less time is required. Evaluations of 2, 3, and 4-hour drying periods have been included in this report.

PREPARATION OF COLLABORATIVE SAMPLES

A supply of fresh raw oysters of variable drained liquid contents was obtained. In some cases, the samples were further diluted with city water so that a 16–34 per cent range in drained liquid was achieved (samples 1–6). Authentic samples of raw oysters having 0.7 to 3.5 per cent drained liquid were included as collaborative samples for control purposes (samples 7–12). The range of the total solids content for the 12-sample series was approximately 10–16 per cent. In all cases, samples from time of initial packing by the plant were kept well-iced and were promptly shipped to collaborators in order to avoid decomposition and its effect on the analysis.

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* For report of Subcommittee C and action of the Association, see *This Journal*, **36**, 56 (1953).
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INSTRUCTIONS TO COLLABORATORS

The following instructions were furnished collaborators well in advance of the samples:

(1) Determine drained liquid by **18.2** and **18.3** of *Methods of Analysis*, 7th Ed. Retain all the drained liquid.

(2) If the drained liquid is less than 10%, reserve two representative samples of about 1 pt. each and keep them in closed pint jars to eliminate evaporation. Use 1 pt. for preparing by **18.1 (a)**, *Methods of Analysis*, 7th Ed., using the meat grinder. The other pint is blended as follows: A representative sample is best obtained by mixing the meats and drained liquid together again, vigorously stirring in a dish pan, then immersing a ladle below the surface (allowing a few seconds for the liquid to fill the oyster interstices), transferring the full ladle to the sample jar, and taking the number of whole ladlefuls which will fill the pint jar nearly full without overflowing. It has been found unsatisfactory to handle part ladlefuls. The sample is then prepared by using the Waring blendor.

(3) If the drained liquid is more than 10%, a portion of the skimmed meats and of the liquid must be set aside for separate examination by **18.1 (a)** and **(f)**, *Methods of Analysis*, 7th Ed., using the meat grinder for the meat portion. In addition, weigh a portion of skimmed meats, and reconstitute drained liquid to these meats equal to the percentage of liquid in the original sample. This representative sample of about 1 pt. will be prepared by the Waring blendor.

Using this sample, blend for 3–4 min. in a quart size Waring blendor jar. Discard any part of this emulsion which fills the pint jar more than ¾ full, (to allow for vigorous stirring in this jar when taking sample). Use a long-handled spoon for stirring and transfer of sample and weigh ca 10 g as quickly as possible to prevent evapn.

(4) Dry both samples as described in *Methods of Analysis*, **18.4**, 7th Ed., except for the drying time. Make duplicate determinations, since the important comparison will be the variations within the two sets of duplicates. In case liquids and solids had to be prepared separately (i.e., drained liquid more than 10% by weight, calc. the solids in the original sample by multiplying the % liquid × % solids in the liquid and the % solids (100 − % liquid) by the % solids in the solids and adding the two figures.

Dry the 4 (or 6 if the liquid is determined separately) dishes for 2 hrs at 98–100°C., remove from oven, cool in desiccator, and weigh. Dry the same dishes another hour, cool, and weigh. Finally, repeat at 4 hours total drying time. Report % total solids by the two methods at the three different drying times. A full report on one sample, excluding averages, can then be made by recording 12 or 18 values.

RESULTS

Table 1 lists the total solids obtained in 4 hours drying by 9 collaborators on the 12 samples of raw oysters, when the Waring blendor and the food chopper method for preparing the sample were used.

Table 2 lists the total solids obtained by four hours drying on raw oysters which have a drained liquid content greater than 10 per cent. The mixed meats and liquid were prepared by using the Waring blendor, and the food chopper was used when the meats and liquid were analyzed separately.

Table 3 is a summary of the total solids obtained by 2, 3, and 4 hours drying on samples prepared by the Waring blendor. Results are averages of duplicate determinations.

TABLE 1.—*Total solids on samples prepared by Waring blendor and food chopper*

ANALYST	SAMPLE	DRAINED LIQUID	WARING BLENDOR METHOD		FOOD CHOPPER METHOD	
			AV. SOLIDS 4 HOURS DRYING	DIFFERENCE BETWEEN DUPLICATE DETERMINATIONS	AV. SOLIDS 4 HOURS DRYING	DIFFERENCE BETWEEN DUPLICATE DETERMINATIONS
			per cent	*per cent*	*per cent*	*per cent*
1	1	24.0	11.88	0.08	11.82	0.24
3	1	31.0	11.63	0.03	12.07	0.10
4	1	32.9	12.02	0.02	12.38	0.08
5	1	31.0	11.61	0.06	12.28	0.23
6	1	33.7	12.03	0.04	11.88	0.11
7	1	24.0	12.15	0.01	12.03	0.28
1	2	18.9	12.28	0.09	12.31	0.29
3	2	28.1	11.57	0.01	12.85	0.02
4	2	27.9	12.70	0.01	12.91	0.02
5	2	28.1	11.76	0.00	13.14	0.06
6	2	29.0	12.89	0.03	13.04	0.12
7	2	18.9	12.42	0.05	12.32	0.24
7	3	20.6	10.98	0.03	10.99	0.02
7	4	18.5	11.65	0.03	11.70	0.35
2	5	22.8	10.01	0.01	9.58	0.05
3	5	21.3	10.02	0.03	9.63	0.15
8	5	21.3	10.10	0.00	9.69	0.02
9	5	24.1	9.82	0.02	9.67	0.06
2	6	19.8	10.35	0.02	10.59	0.13
3	6	16.5	10.81	0.03	10.54	0.02
8	6	16.5	10.88	0.00	10.59	0.01
9	6	20.5	10.69	0.01	10.14	0.03
2	7	0.7	15.48	0.00	15.33	0.11
3	7	1.5	15.13	0.03	15.14	0.01
5	7	1.5	15.19	0.05	15.16	0.02
7	7	1.1	15.27	0.02	15.27	0.13
2	8	0.7	14.96	0.01	15.09	0.19
3	8	0.8	14.57	0.06	14.46	0.00
5	8	0.8	14.56	0.02	14.46	0.06
7	8	1.1	14.67	0.02	14.70	0.11
6	9	3.5	13.89	0.04	—	—
7	9	2.8	13.84	0.31[a]	14.21	0.40
6	10	1.8	14.36	0.03	—	—
7	10	1.1	14.88	0.08	14.84	0.25
4	11	0.8	15.46	0.00	15.02	0.01
6	11	1.5	15.22	0.02	15.69	0.06
7	11	1.2	15.48	0.02	15.99	0.28
4	12	0.8	14.81	0.03	14.25	0.01
6	12	1.2	14.78	0.01	15.56	0.02
7	12	1.1	15.04	0.08	14.79	0.11
	Av.	—	12.95	0.04	12.92	0.12

[a] Probably an error; included in average, however.

TABLE 2.—*Total solids on whole sample and meat and liquid separately*

ANALYST	SAMPLE	DRAINED LIQUID	TOTAL SOLIDS 4 HOURS DRYING			
			WARING BLENDOR	WHOLE SAMPLE	FOOD CHOPPER MEAT AND LIQUID SEPARATELY	
			per cent	*average*	*per cent*	*average*
			11.84		11.94	
1	1	24.0	11.92	11.88	11.70	11.82
			11.61		12.02	
3	1	31.0	11.64	11.63	12.12	12.07
			12.03		12.42	
4	1	32.9	12.01	12.02	12.34	12.38
			11.58		12.39	
5	1	31.0	11.64	11.61	12.16	12.28
			12.01		11.82	
6	1	33.7	12.05	12.03	11.93	11.88
			12.14		12.17	
7	1	24.0	12.15	12.15	11.89	12.03
			12.23		12.45	
1	2	18.9	12.32	12.28	12.16	12.31
			11.56		12.86	
3	2	28.1	11.57	11.57	12.84	12.85
			12.70		12.92	
4	2	27.9	12.69	12.70	12.90	12.91
			11.76		13.17	
5	2	28.1	11.76	11.76	13.11	13.14
			12.90		13.10	
6	2	29.0	12.87	12.89	12.98	13.04
			12.39		12.44	
7	2	18.9	12.44	12.42	12.20	12.32
			10.96		11.00	
7	3	20.6	10.99	10.98	10.98	10.99
			11.63		11.52	
7	4	18.5	11.66	11.65	11.87	11.70
			10.01		9.55	
2	5	22.8	10.00	10.01	9.60	9.58
			10.02		9.70	
3	5	21.3	10.02	10.02	9.55	9.63
			10.10		9.76	
8	5	21.3	10.10	10.10	9.61	9.69
			9.82		9.70	
9	5	24.1	9.81	9.82	9.64	9.67
			10.34		10.65	
2	6	19.8	10.36	10.35	10.52	10.59
			10.82		10.55	
3	6	16.5	10.79	10.81	10.53	10.54
			10.88		10.59	
8	6	16.5	10.88	10.88	10.58	10.59
			10.69		10.15	
9	6	20.5	10.68	10.69	10.12	10.14
			Av.	11.38		11.46

TABLE 3.—*Total solids at 2, 3, and 4 hours—Samples prepared by Waring blendor*

ANALYST	SAMPLE	DRAINED LIQUID	PER CENT TOTAL SOLIDS			PER CENT DIFFERENCE BETWEEN HOURS	
			HOURS DRIED				
			2	3	4	2 AND 4	3 AND 4
1	1	24.0	12.02	11.90	11.88	0.14	0.02
3	1	31.0	11.73	11.67	11.63	0.10	0.04
4	1	32.9	12.14	12.07	12.02	0.12	0.05
5	1	31.0	11.81	11.68	11.61	0.20	0.07
6	1	33.7	12.18	12.07	12.03	0.15	0.04
7	1	24.0	—	12.19	12.15	—	0.04
1	2	18.9	12.44	12.33	12.28	0.16	0.05
3	2	28.1	11.66	11.61	11.57	0.09	0.04
4	2	27.9	12.85	12.77	12.70	0.15	0.07
5	2	28.1	11.90	11.80	11.76	0.14	0.04
6	2	29.0	13.05	12.93	12.89	0.16	0.04
7	2	18.9	—	12.49	12.42	—	0.07
7	3	20.6	11.06	11.01	10.98	0.08	0.03
7	4	18.5	11.71	11.68	11.65	0.06	0.03
2	5	22.8	10.02	10.01	10.01	0.01	0.00
3	5	21.3	10.05	10.04	10.02	0.03	0.02
8	5	21.3	10.17	10.11	10.10	0.07	0.01
9	5	24.1	9.85	9.83	9.82	0.03	0.01
2	6	19.8	10.38	10.34	10.35	0.03	0.01
3	6	16.5	10.89	10.81	10.81	0.08	0.00
8	6	16.5	10.96	10.90	10.88	0.08	0.02
9	6	20.5	10.72	10.70	10.69	0.03	0.01
2	7	0.7	15.07	15.03	15.00	0.07	0.03
3	7	1.5	15.23	15.14	15.13	0.10	0.01
5	7	1.5	15.30	15.26	15.19	0.11	0.07
7	7	1.1	15.34	15.29	15.27	0.07	0.02
2	8	0.7	15.05	14.99	14.96	0.09	0.03
3	8	0.8	14.74	14.59	14.57	0.17	0.02
5	8	0.8	14.90	14.65	14.56	0.34	0.09
7	8	1.1	14.76	14.70	14.67	0.09	0.03
6	9	3.5	13.96	13.91	13.89	0.07	0.02
7	9	2.8	13.91	13.87	13.84	0.07	0.03
6	10	1.8	14.40	14.38	14.36	0.04	0.02
7	10	1.1	14.94	14.93	14.88	0.06	0.05
4	11	0.8	15.55	15.49	15.46	0.09	0.03
6	11	1.5	15.31	15.26	15.22	0.09	0.04
7	11	1.2	15.58	15.56	15.48	0.10	0.08
4	12	0.8	14.86	14.82	14.81	0.05	0.01
6	12	1.2	14.84	14.81	14.78	0.06	0.03
7	12	1.1	15.12	15.05	15.04	0.08	0.01
				Av.		0.09	0.03

TABLE 4.—*Total solids at 2, 3, and 4 hours—Samples prepared by food chopper method*

ANALYST	SAMPLE	DRAINED LIQUID	PER CENT TOTAL SOLIDS			PER CENT DIFFERENCE BETWEEN HOURS	
			HOURS DRIED				
			2	3	4	2 AND 4	3 AND 4
1	1	24.0	12.04	11.85	11.82	0.22	0.03
3	1	31.0	12.17	12.12	12.07	0.10	0.05
4	1	32.9	12.56	12.46	12.38	0.18	0.08
5	1	31.0	12.46	12.32	12.28	0.18	0.04
6	1	33.7	12.00	11.91	11.88	0.12	0.03
7	1	24.0	12.15	12.04	12.03	0.12	0.01
1	2	18.9	12.42	12.36	12.31	0.11	0.05
3	2	28.1	12.96	12.91	12.85	0.11	0.06
4	2	27.9	13.08	12.98	12.91	0.17	0.07
5	2	28.1	13.29	13.19	13.14	0.15	0.05
6	2	29.0	13.23	13.10	13.04	0.19	0.06
7	2	18.9	12.44	12.36	12.32	0.12	0.04
7	3	20.6	11.11	11.04	10.99	0.12	0.05
7	4	18.5	11.82	11.73	11.70	0.12	0.03
2	5	22.8	9.60	9.59	9.58	0.02	0.01
3	5	21.3	9.63	9.63	9.63	0.00	0.00
8	5	21.3	9.76	9.70	9.69	0.07	0.01
9	5	24.1	9.72	9.69	9.67	0.05	0.02
2	6	19.8	10.64	10.59	10.59	0.05	0.00
3	6	16.5	10.61	10.54	10.54	0.07	0.00
8	6	16.5	10.67	10.59	10.59	0.08	0.00
9	6	20.5	10.18	10.15	10.14	0.04	0.01
2	7	0.7	15.42	15.36	15.33	0.09	0.03
3	7	1.5	15.35	15.15	15.14	0.21	0.01
5	7	1.5	15.36	15.25	15.16	0.20	0.09
7	7	1.1	15.34	15.29	15.27	0.07	0.02
2	8	0.7	15.15	15.12	15.09	0.06	0.03
3	8	0.8	14.64	14.49	14.46	0.18	0.03
5	8	0.8	14.64	14.53	14.46	0.18	0.07
7	8	1.1	14.75	14.71	14.70	0.05	0.01
6	9	3.5	—	—	—	—	—
7	9	2.8	14.41	14.32	14.21	0.20	0.11
6	10	1.8	—	—	—	—	—
7	10	1.1	15.04	14.94	14.84	0.20	0.10
4	11	0.8	15.07	15.03	15.02	0.05	0.01
6	11	1.5	15.79	15.71	15.69	0.10	0.02
7	11	1.2	16.25	16.11	15.99	0.26	0.12
4	12	0.8	14.30	14.26	14.25	0.05	0.01
6	12	1.2	15.64	15.57	15.56	0.08	0.01
7	12	1.1	15.04	14.82	14.79	0.25	0.03
				Av.		0.12	0.04

Table 4 is a summary of the total solids obtained by 2, 3, and 4 hours drying on samples prepared by the food chopper method. Results are averages of duplicate determinations.

COMMENT OF COLLABORATORS

(1) *Rapidity of method*
   (a) *William C. Woodfin:* There is a distinct saving in time in preparation of the sample by the blendor method; it takes only about 5 min. (or 10 min. if a quart of sample is prepared in 2 portions) compared with 20 to 30 min. with the grinder and stirrer.

(2) *Reliability of method*
   (a) *Frank H. Collins:* The Waring blendor is so superior that the food chopper method should be eliminated from the official category as soon as possible.
   (b) *D. J. Miller and W. C. Woodfin:* The subdivisions prepared by the Waring blendor give better reproducibility in nearly all cases.

(3) *Loss of weight during blending*
   (a) *Frank H. Collins:* The Waring blendor preparation is a homogeneous mixture, prepared in a closed vessel without much chance of moisture loss. There is more danger of moisture loss during the prolonged chopping and mixing with an electric mixer.
   (b) *Theodore E. Byers and Arthur C. Thomson:* Loss of moisture in mixing in the Waring blendor was determined as directed and found to be insignificant (less than 0.01 oz.).
   (c) *W. C. Woodfin and D. J. Miller:* On a theoretical basis, there would be much less risk of loss in this closed container than there would be in the preparation of samples by the food chopper method with the oysters exposed to the atmosphere over a much longer period of time and particularly where the muscles have to be removed from the grinder and chopped up with a knife and fork, which has always seemed a crude and messy method of preparing a sample for analysis.

(4) *General comment on 1, 2, and 3 (above)*
   From reports received, the views set forth in 1, 2, and 3 are also shared by collaborators H. E. Gakenheimer, D. D. Price, and M. A. McEniry.

DISCUSSION OF RESULTS

TABLE 1.—Although some of the samples examined by the same analysts using both methods showed appreciable deviations in solids, the average solids figures by the two methods for the 40 samples agree quite closely (12.92% *vs.* 12.95%).

The deviation in individual results is probably explainable on the basis of:

(1) *Imperfect sampling:* It is rather difficult to select a representative sample from a gallon of highly watered oysters. Instructions providing for a quart sample and the use of full ladles (see instructions to collaborators) should eliminate this difficulty.

(2) *Moisture loss:* The excess time required for the food chopper method results in evaporation and gives a slightly higher percentage of solids. Experiments by the Associate Referee and several collaborators showed the loss due to evaporation, using the blendor, to

be a few hundredths of 1 per cent as a maximum, even when samples at room temperature were blended for 5 minutes. On the other hand, when the grinder is used, there is considerably greater chance of evaporation and higher solids.

(3) *Blending:* Samples of low free liquid content apparently yield a slightly less homogeneous mass on blending than do samples with more liquid. For this reason, adequate blending for 3 full minutes should be specified.

(4) *Differences:* Table 1, under the columns headed "Difference Between Duplicate Determinations," under "Waring blendor Method" and "Food Chopper Method," demonstrates that the duplicates in the blendor method check more closely than the chopper method. The average difference for the former is 0.04 per cent and for the latter 0.12 per cent, indicating greater precision when the blendor method is used.

TABLE 2.—A careful study of the data in this table shows that it is not necessary to determine meats and liquid separately in a sample containing more than 10 per cent free liquid if the Waring blendor method is used. Some of these samples contain as much as 33 per cent added water; yet, by using the blendor method, results were obtained comparable to the chopper method by which meat and liquid were determined separately. A survey of the duplicate determinations shows that the blendor method is more satisfactory in regard to precision. Note that individual variations in the duplicate determinations in the food chopper method are as high as 0.40 per cent while by the blendor method, the differences are less than 0.1 per cent in all the samples examined.

The somewhat higher solids obtained by the food chopper method are probably attributable to the moisture loss experienced in the grinding procedure.

TABLE 3.—This table shows the per cent of solids of 40 samples using the Waring blendor method at 2, 3, and 4 hours drying and tabulates the per cent difference in weight between the 2nd and 4th hours of drying and the 3rd and 4th hours of drying.

The average difference in weight between the 2nd and 4th hours of drying is 0.09 per cent, indicating that it is necessary to dry for more than 2 hours. The difference in weight between the 3rd and 4th hours, however, is 0.03 per cent, indicating that drying beyond the 3rd hour is unnecessary.

TABLE 4.—This table shows the per cent solids of 40 samples using the food chopper method at 2, 3, and 4 hours drying time, and tabulates the per cent difference in weight between the 2nd and 4th hours of drying and the 3rd and 4th hour of drying.

The average difference in weight between the 2nd and 4th hours of drying is 0.12 per cent, indicating that it is necessary to dry for more than 2 hours. The difference in weight between the 3rd and 4th hours, how-

ever, is 0.04 per cent, indicating that drying beyond the 3rd hour is unnecessary.

SUMMARY

(1) The Waring blendor method for preparation of samples is more efficient and the results are more accurate than those obtained by the food chopper method.

(2) When the Waring blendor is used, it is unnecessary to analyze meats and liquids separately.

(3) Three hours is sufficient drying time for total solids, using an oven at 98–100°C.

RECOMMENDATION

It is recommended that*—

(1) The drying time be reduced to 3 hours in an oven at 98–100°.

(2) The Waring blendor be specified for preparation of sample of shucked oysters.

(3) The requirement for separate analysis of meats and liquid be deleted.

It is further recommended that additional study be undertaken to—

(1) Evaluate the drying time required when using a forced draft oven, with and without the addition of asbestos or sand (suggestion by M. D. Voth).

(2) Consider the chemical determination of water by the titrimetric procedure of McComb and McCready (2).

(3) Consider viscosimetric methods to determine the solids content (suggested by E. M. Hoshall).

ACKNOWLEDGMENT

Appreciation is extended to the following collaborators of the Food and Drug Administration, without whose generous efforts this work would not have been possible:

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(5) D. J. Miller, Pittsburgh Sub-District
(6) D. D. Price, Baltimore District
(7) H. E. Theper, St. Louis District
(8) A. C. Thompson, Cincinnati District
(9) W. C. Woodfin, Pittsburgh Sub-District

REFERENCES

(1) *Methods of Analysis*, 7th Ed., *A.O.A.C.*, 18.1 thru 18.7.
(2) McComb, E. A., and McCready, R. M., *This Journal*, 35, 437 (1952).

* For report of Subcommittee C and action of the Association, see *This Journal*, 36, 56 (1953).

REPORT ON FERTILIZER

By F. W. QUACKENBUSH (Department of Agricultural Chemistry, Purdue University, Lafayette, Indiana), *Referee*

Reports were received from Referees on nitrogen, potash, inert materials, boron, manganese, phosphoric acid, and sampling. The recommendations are approved. No reports were received from Referees on free water, acid-base balance, sulfur, copper, and zinc.

RECOMMENDATIONS

It is recommended*—

(1) That all Referees continue their work during the coming year.

(2) That an additional Associate Referee be appointed to study sampling and sample preparation of ammoniacal solutions.

(3) That the Referees on various "trace" elements each give particular attention to analysis of products of low solubility which contain these elements.

The Referee concurs with the recommendations of the Associate Referees.

REPORT ON SAMPLING AND PREPARATION OF FERTILIZER SAMPLE

By STACY B. RANDLE (New Jersey Agricultural Experiment Station, New Brunswick, New Jersey), *Associate Referee*

In a report to this Association in 1940, Ross, Rader, and Hardesty (1) pointed out that there had been little investigation on sampling and the preparation of fertilizer samples for many years. These authors report the results of a collaborative study of the fineness to which fertilizers should be ground in the preparation of samples for analysis. A further report on the sampling of fertilizers was presented the same year by these authors (2). The latter paper presents the results of a questionnaire obtained from 32 state control officials. Apparently, however, there was no further investigation or report on this subject until the 1946 Association meeting when the Referee on fertilizers recommended "that methods of sampling be studied."

During the 1947 meeting Allen (3) reported results of sampling with two types of triers and a comparison of the moisture content of samples sealed in glass and in paper envelopes. In 1948, Allen (4) reported the results of (*a*) a questionnaire on sampling fertilizers and preparation of samples for analysis; (*b*) a collaborative study of the effectiveness of three kinds of sample containers in preventing change of moisture; (*c*) a comparison of the slotted single-tube and the slotted double-tube sampler

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

in taking samples; (*d*) a comparison of preparation of samples for analysis with and without screening the sample through 10 mesh. The 1949 report on sampling by Allen (5) "consisted chiefly of a preliminary investigation on reducing the inspection sample to the desired size and on preparation of sample for analysis." "Two grades of mixed fertilizer, 5-10-10 and 4-12-8, were used in this study." Approximately 20 pounds of each mixture was mixed on an oilcloth, and portions of varying size were taken for grinding and analysis. The Associate Referee summarized the results as follows: "This is a preliminary study of reducing the inspection sample to the desired size and of preparation of sample for analysis. Results from different quart portions of two grades of mixed fertilizer indicate it is not difficult to obtain a quart portion from 15 or 20 pounds of fertilizer that is representative of that amount. Results also show that analyses obtained from grinding $\frac{1}{2}$ to 1 pound of sample without previous sieving are at least as accurate as analyses obtained by first sieving the whole sample through 10 mesh, the A.O.A.C. procedure." There was also a study made of single-tube and double-tube samplers in sampling a 27-0-0 grade composed of urea and peanut hulls. No significant differences were found in the use of the two type samplers. The 1950 report on sampling as reported by Allen (6) consisted of a comparison of fineness of grind by several methods. The Associate Referee submitted a one-half pound sample to fertilizer control officials for them to grind in their own laboratory. The ground sample was returned to the Associate Referee for a sieve test in order to obtain an indication of the particle size that would be obtained in each laboratory. The results of these findings were summarized by the Associate Referee.

During 1950, Miles and Quackenbush (7) initiated a study of "Precision of Samples and Analyses of Fertilizers and Feeds." The conclusions from this study resulted in a formula for intensiveness of sampling fertilizers and feeds which was adopted by the Association.

The General Referee on Fertilizers, Dr. Quackenbush, has appointed a fertilizer sampling committee to study the problems associated with the sampling of fertilizers. This committee is composed of representatives from the fertilizer industry, the U. S. Department of Agriculture, and state control officials. The committee met during the 1951 A.O.A.C. convention and discussed several sampling problems which should be investigated. It was agreed there are a number of problems which should be studied and it probably will require several years to resolve all of them. The General Referee and Associate Referee studied the suggestions of the Committee and found it advisable to begin with a study of the effects of quartering the sample in the field *vs.* in the laboratory, upon the reliability of the sample, and also that the study should be performed with several grades of fertilizer particularly those of high analysis which are frequently a source of trouble.

A questionnaire on sampling and preparation of the sample was sent to all state fertilizer control officials and a number of industry representatives. A fair number of returns was obtained, although only 13 collaborators participated in the study. One of the group was an industry representative.

PROCEDURE

The instructions for this study were as follows: sample shipment of high analysis fertilizer according to A.O.A.C. sampling procedure (*Methods of Analysis*, 7th Ed.). Quarter sample in field. Transfer quarter sample into container used in your state. Save remaining three-quarters and place in the same type container as the regular sample. In those states where sample is put into small glass jars, it will be necessary to place the remaining three-quarters in a larger glass jar. Be sure that both samples are sealed. (In those states using paper bags, the bag probably will be large enough to hold the three-quarters portion.)

Transmit both samples to the laboratory and grind each portion for analysis. Make duplicate determinations on each sample and report average analysis on the enclosed form.

If it is found that the two portions are too large for laboratory samples, they may both be further quartered after grinding and thoroly mixing.

DISCUSSION

This report represents an initial study of fertilizer sampling and preparation of the sample for analysis by quartering in the field.

The methods of inspection and sample preparation are outlined in Table 1. The chemical analysis of the one-quarter and three-quarter portions of 69 fertilizer samples representing 47 grades studied by 13 collaborators are presented in Table 2. The deviation for each constituent is shown, using the one-quarter as the base. In some instances there is close agreement of the analysis of each constituent in the two samples, while in others there is a wide variation which appears to be greater with the higher

TABLE 1.—*Collaborator's usual method of inspection and sample preparation*

COLLABORATOR	QUARTER IN FIELD	TYPE GRIND	PART TIME INSPECTOR
1	No	Mikromill	Yes
2	Yes	Mikromill	No
3	Yes	Mikromill	No
4	Not stated	Mikromill	No
5	Yes	Mikromill	Not stated
6	Not stated	Mikromill	Not stated
7	No	Buck board and mortar	No
8	No	Mikromill	No
9	Yes	Mikro and others	No
10	No	Labconco mill Braun pulverizer	No
11	No	Mikromill	Yes
12	No	Mikromill	No
13	Yes	Mikromill	No

TABLE 2.—*Analyses of one-quarter and three-quarter fertilizer samples*

COLLABORATOR	GRADE	ONE-QUARTER SAMPLE				THREE-QUARTER SAMPLE				DEVIATION×100			
		N	TPA[a]	APA[a]	K_2O	N	TPA[a]	APA[a]	K_2O	N	TPA[a]	APA[a]	K_2O
1	10-20-10	9.94	20.17	19.60	10.27	9.97	19.94	19.26	10.22	+03	−23	−34	−05
	10-20-10	9.96	20.37	19.86	10.23	10.18	20.17	19.53	10.23	+22	−20	−33	00
	10-20-10	10.06	21.31	20.25	10.82	9.98	21.64	20.40	10.52	−08	+33	+15	−30
	12-12-12	12.02	12.51	12.10	12.56	12.08	12.25	11.74	12.30	+06	−26	−36	−26
	12-12-12	11.64	13.43	13.04	12.79	11.70	13.12	12.69	12.96	+06	−31	−35	+17
2	8-10-12	8.27	11.30	10.95	13.01	8.27	11.15	10.70	13.42	00	−15	−25	+41
	8-10-12	8.42	10.40	10.18	12.12	8.43	10.20	10.00	12.58	+01	−20	−18	+46
	15-10-10	15.23	11.20	10.85	10.30	14.96	11.10	10.70	10.18	−27	−10	−15	−12
	12-8-12	12.37	8.20	8.10	13.20	11.86	8.20	8.05	13.78	−51	00	−05	+58
	4-18-18	4.52	19.90	19.40	16.89	4.63	20.35	19.90	16.92	+11	+45	+50	+03
3	9-5-7	7.85	6.35	—	6.75	7.85	6.30	—	6.90	00	−05	—	+15
	5-10-13	5.15	11.00	—	13.50	5.18	10.86	—	13.75	+03	−14	—	+25
	3-18-9	3.41	17.85	—	9.45	3.38	17.77	—	9.60	−03	−08	—	+15
	4-24-12	4.81	26.65	—	11.10	4.81	26.75	—	11.15	00	+10	—	+05
	8-8-8	7.34	9.24	—	7.80	7.39	9.25	—	7.90	+05	+01	—	+10
4	0-46-0	—	48.06	46.67	—	—	49.22	47.91	—	00	+116	+124	00
	33-0-0	33.93	—	—	—	33.17	—	—	—	−76	00	00	00
	0-19-0	—	22.23	21.57	—	—	22.08	21.41	—	00	−15	−16	00
	4-24-12	3.45	25.03	23.74	12.15	3.21	24.84	23.67	11.96	−24	−19	−07	−19
	8-32-0	7.65	36.99	34.66	—	7.47	36.96	34.65	—	−18	−03	−01	00
5	10-10-10	9.82	10.46	—	11.29	9.83	10.47	—	11.36	+01	+01	—	+07
	8-16-16	7.85	16.09	—	17.01	7.82	16.17	—	17.18	−03	+08	—	+17
	8-12-12	8.28	12.84	—	12.05	8.26	12.54	—	12.22	−02	−30	—	+17
	5-20-10	4.81	22.46	—	9.15	5.05	21.36	—	10.08	+24	−110	—	+93
	5-20-10	5.36	19.68	—	10.52	5.40	19.70	—	10.25	+04	+02	—	−27
	0-20-20	—	20.54	—	19.99	—	20.74	—	20.00	00	+20	—	+01
	5-10-10	5.10	10.76	—	10.58	5.13	10.61	—	10.70	+03	−15	—	+12
6	6-9-12	6.10	10.25	9.57	12.91	6.18	10.25	9.54	13.01	+08	00	−03	+10
	3-12-6	3.31	12.56	11.67	6.86	3.25	12.37	11.45	6.73	−06	−19	−22	−13
	9-14-18	8.72	15.05	14.75	20.24	9.30	15.08	14.62	19.26	+58	+03	−13	−98
	10-12-20	10.05	12.66	12.31	19.97	10.17	12.22	11.87	20.34	+12	−44	−44	+37
	7-7-7	6.94	8.23	7.82	7.66	7.04	8.13	7.60	7.91	+10	−10	−22	+25
7	8-32-0	8.74	32.65	30.29	—	8.76	32.45	30.34	—	+02	−20	+05	00
	8-24-8	8.12	25.33	24.13	8.48	8.10	25.53	24.34	8.30	−02	+20	+21	−18
	8-24-8	7.92	25.43	23.51	8.96	7.96	25.25	23.50	8.85	+04	−18	−01	−11
	8-24-8	7.02	24.75	23.08	10.29	6.98	24.88	23.28	9.94	−04	+13	+20	−35
	10-30-10	10.81	30.93	29.39	10.87	10.54	31.10	29.54	10.41	−27	+17	+15	−46

TABLE 2.—(*continued*)

COLLABORATOR	GRADE	ONE-QUARTER SAMPLE				THREE-QUARTER SAMPLE				DEVIATION ×100			
		N	TPA	APA	K_2O	N	TPA	APA	K_2O	N	TPA	APA	K_2O
8	5-10-16	4.85	11.05	10.49	16.22	4.86	11.02	10.49	16.09	+01	−03	00	−13
	4-8-12	4.07	9.00	8.54	12.38	4.08	8.95	8.53	12.21	+01	−05	−01	−17
	6-8-8	5.98	8.72	8.47	6.99	5.95	8.75	8.49	7.03	−03	+03	+02	+04
	3-12-12	3.11	12.67	12.05	12.86	3.15	12.67	12.06	13.21	+04	00	+01	+35
	4-8-16	3.97	10.27	9.61	14.80	3.98	10.27	9.50	14.56	+01	00	−11	−24
9	5-10-15	5.05	11.69	10.95	15.06	5.11	11.76	11.03	14.72	+06	+07	+08	−34
	10-10-10	10.08	11.56	11.25	9.28	10.04	11.47	11.14	9.34	−04	−09	−11	+06
	5-10-10	5.02	10.85	10.19	9.46	4.84	10.94	10.30	9.28	−18	+09	+11	−18
	6-12-12	5.99	13.69	13.16	11.11	5.94	13.03	12.49	11.27	−05	−66	−67	+16
10	10-20-10	10.06	20.10	19.61	10.10	10.05	20.65	20.22	10.59	−01	+55	+61	+49
	5-10-10	5.25	10.70	9.82	9.96	5.24	10.30	9.42	10.23	−01	−40	−40	+27
	0-14-7	—	14.55	14.12	7.30	—	15.03	14.58	7.84	00	+48	+46	+54
	12-24-12	12.04	23.50	23.39	11.15	12.03	25.38	25.33	11.62	−01	+188	+194	+47
	16-20-0	13.50	20.65	20.44	—	13.32	22.30	22.12	—	−18	+165	+168	00
	8-8-8	5.88	11.44	10.35	7.30	5.89	11.57	10.38	7.96	+01	+13	+03	+66
	0-60-0	—	—	—	49.01	—	—	—	48.84	00	00	00	−17
11	5-10-5	4.60	12.10	11.35	9.90	4.60	12.20	11.50	9.95	00	+10	+15	+05
	4-16-16	4.65	16.60	16.20	15.32	4.62	16.50	16.05	15.25	−03	−10	−15	−07
	8-8-8	7.66	9.30	8.30	8.52	7.63	9.40	8.20	8.60	−03	+10	−10	+08
	3-12-12	3.01	13.70	13.30	12.34	3.08	13.80	13.35	12.46	+07	+10	+05	+12
	0-9-27	—	10.50	9.50	24.32	—	10.55	9.65	24.92	00	+05	+15	+60
12	0-9-27	—	10.40	9.39	24.15	—	10.30	9.15	24.18	00	−10	−24	+03
	4-16-8	3.98	16.90	15.61	8.45	3.98	17.10	15.73	7.78	00	+20	+12	−67
	10-6-4	9.14	6.80	6.21	4.28	9.24	6.20	5.61	4.05	+10	−60	−60	−23
13	7-7-7	6.80	8.16	8.06	7.20	6.76	8.19	7.78	7.16	−04	+03	−28	−04
	5-10-10	5.02	10.55	9.95	10.38	5.06	10.50	9.98	10.51	+04	−05	+03	+13
	5-15-10	5.43	13.99	13.79	10.25	5.48	14.15	13.48	10.24	+05	+16	−31	−01
	8-16-16	7.82	14.56	14.26	16.12	7.79	15.29	14.68	16.68	−03	+73	+42	+56
	10-10-10	10.15	10.14	9.84	10.28	10.13	10.41	10.09	10.49	−02	+27	+25	+21
	4-12-16	3.95	12.60	12.40	13.70	3.93	12.49	12.11	13.68	−02	−11	−29	−02
	15-30-14	13.93	30.70	30.70	16.56	13.70	31.66	31.66	16.08	−23	+96	+96	−48
	8-16-16	8.11	15.93	15.63	15.64	7.88	15.85	15.35	15.79	−23	−08	−28	+15

TPA: Total Phosphoric Acid. APA: Available Phosphoric Acid.

analysis fertilizers. It should be observed that some of these samples represent very high analysis products.

The purpose of this investigation was to determine if there is a variation in analysis of samples quartered in the field, and the results show there are variations. The study was not designed to show the causes. It is evident there should be further study of sampling and sample preparation.

One collaborator used a riffle sampler, in addition to the outlined quartering study. The variations with the riffle were about of the same order as the one-quarter and three-quarter samples. Further study should be made of the use of the riffle as a quartering device.

RECOMMENDATIONS

It is recommended* that there be further study of sampling and sample preparations.

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REFERENCES

(1) Ross, W. H., Rader, L. F., Jr., and Hardesty, J. O., *This Journal*, **24**, 253 (1941).
(2) ———, *ibid.*, **24**, 499 (1941).
(3) Allen, H. R., *ibid.*, **31**, 205 (1948).
(4) ———, *ibid.*, **32**, 193 (1949).
(5) ———, *ibid.*, **33**, 229 (1950).
(6) ———, *ibid.*, **34**, 620 (1951).
(7) Miles, S. R., and Quackenbush, F. W., *ibid.*, **33**, 424 (1950).

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

REPORT ON BORON IN MIXED FERTILIZERS

By RODNEY C. BERRY (Virginia Department of Agriculture, Richmond Virginia), *Associate Referee*

Recent dissatisfaction with the Official Method for determining water-soluble boron in fertilizers led to efforts to develop a more satisfactory procedure. Using the samples employed by D. S. Taylor in his work on the "identical *p*H method," a modified method was developed but was not completed in time for presentation to the A.O.A.C. at its annual meeting. However, results obtained in this laboratory and in collaborating laboratories showed that the modified method gave results as precise as the identical *p*H method with less difficulty.

COMPARISON

The two methods may be compared as follows:

Sample.—For the "Virginia modified method," 2.5 g; for the identical *p*H method, 1 g. The larger amount seems to give a more representative sample, especially where segregation occurs.

Precipitant.—In the Virginia method, $BaCl_2$; in the *p*H method, $Pb(NO_3)_2$. The absence of nitrate ion in the Virginia method reduces the possibility of bleaching the indicator.

Neutralization Prior to Precipitation.—Obtained by $Ba(OH)_2$ in the Virginia method; by $NaHCO_3$ in the *p*H method. The use of $Ba(OH)_2$ is less time-consuming and less dangerous.

Neutralization with $Ba(OH)_2$ and precipitation with $BaCl_2$ provides an excess of precipitating ions, and completely removes phosphates, sulfates, and carbonates.

Number of Filtrations.—One for the *p*H method; two for the Virginia method to eliminate all organic matter before precipitation of the phosphates, sulfates, and carbonates.

No preliminary acidification of sample is used in the Virginia method, and no instruments are required in the titration.

VIRGINIA MODIFIED METHOD

Weigh 2.5 g sample into a 250 ml beaker. Add 125 ml H_2O and boil gently for ca 10 min, filter hot thru #40 Whatman paper into 400 ml beaker. Wash solids well with hot H_2O (6 washings) and make vol. to at least 200 ml with distd H_2O. Heat filtrate just to boiling. Add 15 ml 10% $BaCl_2$ to ppt sulfates and phosphates and add powd $Ba(OH)_2$ cautiously with stirring, until just alk. to phenolphthalein, avoiding large excess. Boil in open beaker at least 60 min. to expel NH_3. Longer boiling for samples colored by organic matter is desirable. If necessary, add H_2O to keep vol. to at least 150 ml. Add and stir 1–2 tsp. of Filter-cel or other inert filtering aid, and filter with suction thru packed paper pads into 500 ml Pyrex Erlenmeyer flask. Wash ppt 6 times with hot boiled distd H_2O. (Too large wash volumes are to be avoided; such washings increase the vol. in the flask to the point of dangerous bumping in the next step.) Make filtrate just colorless to phenolphthalein with 6% HCl, add methyl red indicator and make just pink with the acid. Add 5 or 6 boiling stones

TABLE 1.—*Composition of collaborative samples*

	SAMPLE 1[a]	SAMPLE 2[b]	SAMPLE 3[c]
	(LBS)	(LBS)	(LBS)
Ground tobacco stems	50	75	—
Muriate of potash	473	422	110
Superphosphate (19%)	1,435	1,285	960
Superphosphate (45%)	25	—	—
Urea ammonia soln (45.5%)	—	88	—
Limestone, dry	—	60	210
Cal. mag.	15	—	—
Nitrogen soln 2A (40.6%)	—	—	120
Process tankage	—	—	145
Sulfate of potash	—	—	121
Inert filler	—	60	379
High grade fertilizer borate, anhydrous, (121% borax)	52 (actual borax = 62.92)	52 (actual borax = 62.92)	5 (actual borax = 6.05)
Total	2,050	2,042	2,050
Allowed for shrinkage	50	42	50
Net	2,000	2,000	2,000

[a] 0-14-14 fertilizer. Formulated to contain 60 lbs. borax/ton.
[b] 2-12-12 fertilizer. Formulated to contain 60 lbs. borax/ton.
[c] 3-9-6 fertilizer. Formulated to contain 5 lbs. borax/ton.

and a stirring rod, cover with watch glass and boil for 5 min. to remove CO_2. Cool in cold water, covered. Wash cover glass, stirrer and sides of flask. Titrate to the yellow of methyl red with standard 0.05 *N* NaOH. Add 20 g D-mannitol and 1 ml or more of 1% phenolphthalein soln, shake and wash down sides. Titrate to pink end point. Run blank in exactly same manner as sample. 1 ml 0.05 *N* NaOH = 0.000540 g B or 0.00477 g borax. Or (Titer − blank) × factor = lb borax per ton (factor = 3.807 for 0.05 *N* NaOH).

NOTES

(1) Calcium borate and other borax concentrates may not be dissolved in water unless residue is thoroly washed and sample dissolved by boiling in over 100 ml vol.

(2) A large excess of Ba ions is necessary for complete removal of phosphates.

(3) Less than 20 g of mannitol gives an indecisive end-point. Also, ca 1 ml or more of 1% phenolphthalein is necessary for a strong end-point.

RESULTS

Three samples of mixed fertilizers (Table 1) were formulated and sent to collaborators, all of whom were asked to make determinations of boron by the A.O.A.C. Method I, **2.45** (identical *p*H method) and the Virginia Modified Method. Three collaborators also reported results using A.O.A.C Method II, **2.48** (Distillation Method). One collaborator used a spectrographic method (Table 2):

TABLE 2.—*Boron in mixed fertilizer. Collaborative results*[a]

COLLABORATOR		SAMPLE 1				SAMPLE 2				SAMPLE 3			
		A.O.A.C. 2.45 (IDEN. *p*H)	VIRGINIA METHOD	A.O.A.C. 2.48 (DISTILLATION)	SPECTROGRAPHIC	A.O.A.C. 2.45 (IDEN. *p*H)	VIRGINIA METHOD	A.O.A.C. 2.48 (DISTILLATION)	SPECTROGRAPHIC	A.O.A.C. 2.45 (IDEN. *p*H)	VIRGINIA METHOD	A.O.A.C. 2.48 (DISTILLATION)	SPECTROGRAPHIC
2	No. of Detns	2	3	—	—	3	3	—	—	3	—	—	—
	Max.	74.38	64.56			70.50	66.47			5.29			
	Min.	66.80	62.67			68.74	63.62			4.76			
	Av.	70.59	63.30			71.90	64.55			4.94			
	Dev.	7.58	1.89			1.76	2.75			0.53			
4	No. of Detns	3	4	—	—	5	4	—	—	4	—	—	—
	Max.	66.9	64.6			72.2	73.80			5.9			
	Min.	63.4	61.5			65.2	71.70			2.9			
	Av.	65.17	62.4			68.3	72.60			3.9			
	Dev.	3.5	3.1			7.0	2.10			3.0			
5	No. of Detns	—	—	—	3	—	—	—	3	—	—	—	3
	Max.				61.6				61.6				5.3
	Min.				53.6				54.6				4.9
	Av.				56.6				57.5				5.2
	Dev.				8.0				7.0				0.4
6	No. of Detns	3	3	—	—	3	3	—	—	2	3	—	—
	Max.	71.0	63.03			64.8	71.73			4.22	10.43		
	Min.	66.2	61.00			63.6	69.56			3.79	8.70		
	Av.	68.8	62.35			64.4	70.64			4.01	9.56		
	Dev.	4.8	2.03			1.2	2.17			0.43	1.73		
7	No. of Detns	3	3	—	—	3	3	—	—	2	3	—	—
	Max.	62.5	63.5			58.5	65.6			3.3	5.6		
	Min.	60.4	62.7			57.9	64.3			3.2	5.2		
	Av.	61.6	63.0			58.3	65.0			3.3	5.4		
	Dev.	2.1	0.8			0.6	1.3			0.1	0.4		

TABLE 2.—(*continued*)

COLLABORATOR		SAMPLE 1				SAMPLE 2				SAMPLE 3			
		A.O.A.C. 2.45 (IDEN. *p*H)	VIRGINIA METHOD	A.O.A.C. 2.48 (DISTILLATION)	SPECTROGRAPHIC	A.O.A.C. 2.45 (IDEN. *p*H)	VIRGINIA METHOD	A.O.A.C. 2.48 (DISTILLATION)	SPECTROGRAPHIC	A.O.A.C. 2.45 (IDEN. *p*H)	VIRGINIA METHOD	A.O.A.C. 2.48 (DISTILLATION)	SPECTROGRAPHIC
	No. of Detns	3	3	—	—	3	3	—	—	3	3	—	—
	Max.	72.0	69.2			73.6	72.6			4.4	8.8		
8	Min.	69.4	65.8			69.4	70.1			4.0	8.0		
	Av.	70.7	67.0			71.9	71.1			4.3	8.4		
	Dev.	2.6	3.4			4.2	2.5			0.4	0.8		
	No. of Detns	3	2	—	—	3	3	—	—	3	3	—	—
	Max.	67.63	59.92			70.98	67.46			7.41	8.67		
9	Min.	67.63	56.80			70.03	66.69			6.45	6.41		
	Av.	67.63	58.45			70.30	67.14			7.07	7.90		
	Dev.	0.00	5.02			0.95	0.77			0.96	2.26		
	No. of Detns	—	3	3	—	—	3	3	—	—	3	3	—
	Max.		69.4	68.6			72.4	64.0			8.0	8.0	
10	Min.		67.8	62.6			70.8	60.2			6.8	5.3	
	Av.		68.6	66.3			71.3	62.0			7.6	6.2	
	Dev.		1.6	6.0			1.6	3.8			1.2	2.7	
	No. of Detns	—	3	3	—	—	3	3	—	—	3	3	—
	Max.		63.9	65.4			70.0	67.0			6.9	9.5	
11	Min.		53.9	61.4			68.5	60.8			6.5	7.2	
	Av.		63.4	63.3			69.0	64.7			6.6	8.1	
	Dev.		1.5	3.0			1.5	6.2			0.4	2.3	
	No. of Detns	5	6	—	—	5	5	—	—	3	3	—	—
	Max.	71.4	70.8			67.2	70.4			5.2	8.6		
14	Min.	68.4	66.6			66.6	68.8			4.6	7.2		
	Av.	69.9	69.0			67.0	70.0			5.0	7.8		
	Dev.	3.0	4.2			0.6	1.6			0.6	1.4		

TABLE 2.—(*continued*)

COLLABORATOR		SAMPLE 1				SAMPLE 2				SAMPLE 3			
		A.O.A.C. 2.45 (IDEN. *p*H)	VIRGINIA METHOD	A.O.A.C. 2.48 (DISTILLATION)	SPECTROGRAPHIC	A.O.A.C. 2.45 (IDEN. *p*H)	VIRGINIA METHOD	A.O.A.C. 2.48 (DISTILLATION)	SPECTROGRAPHIC	A.O.A.C. 2.45 (IDEN. *p*H)	VIRGINIA METHOD	A.O.A.C. 2.48 (DISTILLATION)	SPECTROGRAPHIC
15	No. of Detns	—	3	—	—	—	3	—	—	—	3	—	—
	Max.		68.6				69.9				8.06		
	Min.		66.2				68.6				7.39		
	Av.		67.47				69.2				7.61		
	Dev.		2.40				1.3				0.67		
16	No. of Detns	—	3	3	—	—	3	—	—	—	3	—	—
	Max.		71.50				69.0				7.0		
	Min.		71.15				68.5				6.0		
	Av.		71.33	60.0			68.8				6.7		
	Dev.		0.35				0.5				1.0		
17	No. of Detns	11	7	—	—	15	12	—	—	12	6	—	—
	Max.	70.62	63.89			71.83	69.78			3.90	6.97		
	Min.	67.79	62.54			68.13	67.97			3.51	6.06		
	Av.	69 57	63.44			69.13	68.68			3.69	6.44		
	Dev.	2.83	1.35			3.70	1.81			0.39	0.91		
Total	No. of Detns	34	44	9	3	40	48	6	3	34	33	6	3
	Max.	74.38	71.50	68.6	61.6	73.6	73.80	67.0	61.6	7.41	10.43	9.5	5.3
	Min.	60.4	56.89	60.0	53.6	57.9	63.62	60.2	54.6	2.90	5.2	5.3	4.9
	Av.	68.45	65.15	63.2	56.6	67.45	69.06	63.3	57.5	4.28	7.29	7.2	5.2
	Dev.	13.98	14.61	8.6	8.0	15.7	10.18	66.8	7.0	4.51	5.23	4.2	0.4

[a] All results in lbs borax/ton.

COLLABORATORS

W. S. Thompson, Columbus, Ohio*.
M. P. Etheredge, State College, Mississippi.
J. W. Kuzmeski, Amherst, Massachusetts*.
H. J. Fisher and S. R. Squires, New Haven, Connecticut.
W. T. Mathis and R. A. Botsford, New Haven, Connecticut.
C. V. Marshall and W. H. Hollington, Ottawa, Ontario, Canada.
W. R. Austin, and T. G. Brandon, Nashville, Tennessee.
W. McAllister, Baltimore, Maryland.
F. W. Quackenbush and O. W. Ford, Lafayette, Indiana.
D. Long, Tallahassee, Florida.
Cordelia B. Hoffman, Tallahassee, Florida.
J. E. Moore, Atlanta, Georgia*.
L. E. Bopst, College Park, Maryland.
H. R. Allen, Lexington, Kentucky.
H. L. Moxon and R. O. Powell, Richmond, Virginia.
R. L. Jones, and Paul Lineberry, Wilmington, North Carolina.
R. C. Berry, J. H. Elder, and J. A. Dobyns, Jr., Richmond, Virginia.

RECOMMENDATIONS

Although some difficulty was experienced in both methods, less trouble was reported with the Virginia modified method. Fairly good results were obtained by both methods on samples 1 and 2 but erratic values resulted from both methods on sample 3. Only one analyst reported the correct amount of borax in sample 3 by A.O.A.C. Method I; with the Virginia method, most analysts obtained satisfactory values.

On the basis of the reports of collaborators, the Virginia method seems to be equally satisfactory as, and less difficult than, A.O.A.C. Method I.

The Associate Referee recommends† that the Virginia modified method be adopted, First Action, and that referee work be continued.

REPORT ON MAGNESIUM IN FERTILIZERS‡

By JOHN B. SMITH, *Associate Referee*, and CHARLES E. OLNEY
(Agricultural Experiment Station, Kingston, R. I.)

The feasibility and desirability of measuring magnesium in the coarser particles of a mixed fertilizer was discussed in a previous report (*This Journal*, **35**, 663 (1952)). If limited to water-insoluble substances, this fraction contains the particles of magnesic limestone that are slow to decompose in soil. There is no exact dividing line between large and small particles, but from published rates of limestone decomposition, the 40 mesh size (420 microns) was chosen as the most suitable. This choice is confirmed by the recent work of Meyer and Volk§. A procedure was

* No report.
† For report of Subcommittee A and action of the Association, see *This Journal*, **36**, 48 (1953).
‡ Contribution No. 809 of this Station.
§ Meyer, T. A., and Volk, G. W., *Soil Sci.*, **73**, 37–52 (1952).

devised that gave satisfactory results in this laboratory and the method has been studied collaboratively this year.

SAMPLES AND METHODS

Three large samples of representative brands of commercial fertilizers were donated by three manufacturers from different sections of the country. These unground samples were mixed and quartered; each quarter was subdivided through a riffle to make portions weighing approx. 2 lb. which were sent to 13 collaborators, with the following instructions:

Weigh a 15 g sample of unground fertilizer, using a spoon to convey the material. Transfer to 250 ml beaker, add 100 ml H_2O, cover with watch glass, and boil for 30 min. Disintegrate lumps by rubbing with rubber bulb of a medicine dropper. Pour through 40 mesh sieve, washing beaker and sieve with a stream of tap H_2O through rubber tubing attached to a faucet (a 3 in. sieve is most convenient). Transfer residue on sieve to porcelain evaporating dish, disintegrate lumps with the rubber bulb, and again wash on sieve. Repeat process until separation is complete; 3 repetitions are usually sufficient. Do not force particles through by rubbing on the screen. Wash final residue into 250 ml volumetric flask with H_2O, let stand until clear, and decant as much H_2O as possible, retaining all mineral particles in flask. Det. Mg as directed in **2.55** or **2.56** beginning with, "Add 30 ml of HNO_3 and 10 ml of HCl, and boil 30 min." in **2.55**, and report as % Mg in original sample.

After completing the analyses for Mg in the coarse particles, grind a portion of the sample, and det. acid-soluble Mg by Methods **2.55** or **2.56.**

TABLE 1.—*Collaborators' results*[a] *for Mg (per cent)*

COLLABORATOR NO.	SAMPLES						SIEVE
	1	2	3	1	2	3	
	ACID-SOLUBLE Mg			WATER-INSOLUBLE Mg IN COARSE PARTICLES			
1	2.23	2.33	1.33	0.15	1.31	0.96	
2	2.17	2.35	1.45	0.19	1.33	1.14	40 mesh, 8″
3	2.15	2.47	1.42	0.16	1.30	1.00	40 mesh, 5″
4	2.16	2.48	1.39	0.15	1.37	1.00	40 mesh, 3″
5	2.27	2.54	1.45	0.15	1.50	1.05	Tyler No. 35, 8″
6	2.30	2.55	1.41	0.19	1.26	1.12	———
7	2.24	2.58	1.44	0.29	1.55	1.14	Tyler No. 40, 6″
8	2.17	2.60	1.42	0.20	1.28	1.10	Tyler No. 35
9	2.44	2.72	1.73	0.17	1.53	1.04	Tyler No. 35, 3″
10	2.35	2.77	1.54	0.17	1.42	1.04	Bur. of Standards No. 40, 8″
11	2.43	2.77	1.55	0.15	1.04	0.94	40 mesh, 5″
12	2.50	2.97	1.78	0.21	1.49	1.21	Tyler No. 40, 8″
13	2.27	3.10	2.09	0.10	1.32	1.18	Bur. of Standards No. 40, 5″
Av.	2.28	2.63	1.54	0.18	1.36	1.07	———
Av. dev.	0.09	0.18	0.15	0.03	0.11	0.07	———

[a] Each result is the average of 3 determinations.

RESULTS

The results submitted by the collaborators appear in Table 1, arranged in the ascending order of percentages of acid-soluble Mg for Sample 2. The collaborator's number corresponds with the list published below. Obviously, there is an undesirable degree of variability for acid-soluble Mg among the different laboratories. The results for the screened fraction are more consistent. There are a number of possible variables: selection of 15 g portions from the heterogeneous unground sample, possible grinding of coarse particles by rubber bulbs of different types, variability of sieve size and screen openings, and errors in the determination of Mg in the residue. The physical variables appear to be well controlled, since the results are as uniform as could be anticipated for the determination of Mg in unmanipulated samples.

The designation of 40 mesh is an inexact description of a sieve. The U. S. Standard Sieve No. 40 has openings of 420 μ; Tyler No. 35, 420 μ; Tyler No. 42, 350 μ; and Newark Market Grade 40 mesh, 380 μ. The method should specify the standard 420 μ openings, but the results indicate that this is a minor source of variability. Rubbing the residues directly on the screen is unpermissible, and transfer from the sieve to an evaporating dish or similar utensil is necessary. Sieves of 3 inch diameter are most convenient for this transfer but larger sizes appear to be equally exact.

COLLABORATORS' COMMENTS

Collaborators were asked to comment on the method and on its applicability to the evaluation of fertilizers. Collaborator 3 ascribed inconsistency to dissimilar individual sampling and to the extent, limit, and care in sieving. Collaborator 5 found the procedure relatively simple but would like more exact control of *p*H at the initial precipitation of magnesium ammonium phosphate. Collaborator 7 liked the precision of results for Mg in coarse particles and considered the method satisfactory if results from different laboratories are consistent. Collaborator 8 thought the method as fair as anything offered so far. Collaborator 9 found 30 minutes too short a time to dissolve the screened residue completely, and also suggested that the size and type of rubber bulb be specified. Collaborator 11 made the interesting observation that greater precision was obtained by rubbing the residue with his finger than with a rubber bulb.

The Associate Referee has observed that residues from wet sieving of mineral materials often contain particles that pass the sieve after being dried. This may be caused by adhesion of particles because of the surface tension of water films. Residues from samples 1 and 2 included particles of organic matter from conditioning ingredients: an approximate separation showed 42% in the residue from Sample 1, 9% in Sample 2, and none in Sample 3. This probably does not cause much error because such materials have little Mg in the natural state and less after extraction with the hot salt solution during the boiling period required by the method, but the magnitude of the error should be determined.

INTERPRETATION OF RESULTS

More important than the technique of the method is its applicability as a quality measurement for fertilizers. The results for Sample 1 are con-

sistent with the formula for the fertilizer and the chemical and mechanical analysis of the limestone used. This fertilizer had 2.28 per cent acid-soluble magnesium, 0.52 per cent water-soluble magnesium, and only 0.17 per cent of magnesium in the screened particles. The method would be unnecessary for such fertilizers because of the small quantity of coarse dolomite. The analysis is also consistent with information available for the formula of Sample 3. This fertilizer apparently had dolomite added as make-weight after the curing period, for it contained 1.54 per cent acid-soluble magnesium but very little of this was in water-soluble forms. Because of coarse dolomite, aspersions would be cast on more than two-thirds of the magnesium in this brand. Not enough information is available for Sample 2 to permit calculation of the portion of magnesium that should be found on the 40 mesh sieve. This sample has 2.63 per cent acid-soluble magnesium, 0.2 per cent water-soluble magnesium, and 1.33 per cent in the screened particles. Thus, the method would condemn one-half of the magnesium present. These samples were chosen for testing the method, but analysis of a number of fertilizers taken at random from brands on the Rhode Island market showed that approximately one-half had very little coarse dolomite, while the others had from 20 to 33 per cent of their magnesium in that form.

Fertilizers are bought primarily for their immediate effects. Particles of magnesic limestone coarser than 40 mesh vary in their usefulness depending on soil acidity, soil texture, climate, and crop, but in the average soil, particles larger than 40 mesh are of little immediate value. In fact, 40 mesh is a very conservative limit. Therefore, the proposed method should be a useful measure of quality, and it avoids the questionable designations of "available" and "unavailable."

The method appears to be feasible technically, but the idea has had too little consideration by control officials or by members of the fertilizer trade to justify immediate recommendation for adoption.

If the procedure proves acceptable, a name is needed. "Water-insoluble magnesium in coarse particles" is descriptive but too long. Collaborator Halbrook suggests, "Water-insoluble screened magnesium." Other suggestions are solicited.

COLLABORATORS

The Associate Referee is indebted to the following collaborators for their help with analyses and for their criticisms:

(1) A. T. Blackwell, Davison Chemical Corporation, Baltimore, Maryland.
(2) Allen B. Lemon, California Department of Agriculture, Sacramento, California.
(3) L. E. Bopst, Maryland Inspection and Regulatory Service, College Park, Maryland.
(4) Charles E. Olney, Rhode Island Agricultural Experiment Station, Kingston, Rhode Island.
(5) Gordon J. Turner, Eastern States Farmers' Exchange, York, Pennsylvania.

(6) M. P. Etheredge, Mississippi State Chemical Laboratory, Jackson, Mississippi.
(7) Noah J. Halbrook, Florida Department of Agriculture, Tallahassee, Florida.
(8) W. R. Austin and T. G. Brandon, Armour Fertilizer Works, Nashville, Tennessee.
(9) Frank O. Lundstrom, U. S. Bureau of Plant Industry, Soils, and Agricultural Engineering, Beltsville, Maryland.
(10) C. R. Byers, Armour Fertilizer Works, Carteret, New Jersey.
(11) Harry A. Miller, North Carolina Department of Agriculture, Raleigh, North Carolina.
(12) R. L. Jones, Armour Fertilizer Works, Wilmington, North Carolina.
(13) Helen Kocaba, Connecticut Agricultural Experiment Station, New Haven, Connecticut.

RECOMMENDATIONS

It is recommended* that further collaborative study of the method for magnesium in coarse particles be conducted.

REPORT ON PHOSPHORIC ACID IN FERTILIZERS: DIRECT DETERMINATION OF AVAILABLE PHOSPHORIC ACID BY VOLUMETRIC AND PHOTOMETRIC PROCEDURES

By K. D. JACOB, *Associate Referee*, W. M. HOFFMAN, and F. C. SCHRAMM (Division of Fertilizer and Agricultural Lime, Bureau of Plant Industry, Soils, and Agricultural Engineering, Agricultural Research Administration, U. S. Department of Agriculture, Beltsville, Maryland)

Laboratory evaluation of phosphatic fertilizers with the aid of neutral ammonium citrate was first suggested by Fresenius, Neubauer, and Luck in Germany in 1871 (1). These workers determined the citrate-soluble P_2O_5 either directly by analysis of the citrate extract or indirectly by subtracting the sum of the water-soluble and citrate-insoluble P_2O_5 from the total P_2O_5. The neutral ammonium citrate procedure, essentially as proposed by Fresenius, *et al.*, was adopted by the Association of Official Agricultural Chemists at its first annual meeting (2) held at Philadelphia, September 8–9, 1884, with the specification that the citrate-soluble P_2O_5 be determined by the indirect procedure. Subsequently it became the general practice in the United States to express the solubility of phosphate fertilizers in terms of available P_2O_5. The available P_2O_5 is the sum of the water-soluble P_2O_5 and the water-insoluble but citrate-soluble P_2O_5, the value for which is obtained by subtracting the citrate-insoluble P_2O_5 from the total P_2O_5. It was not until 1940, however, that the expression "available P_2O_5" appeared in *Official Methods of Analysis* (3).

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

Direct determination of the soluble P_2O_5 in the citrate extract, prepared with either neutral or alkaline ammonium citrate, or in the combined water and citrate extracts is generally practiced in Europe (15), but for one reason or another has received little attention in the United States. In European practice, the P_2O_5 is determined by volumetric or gravimetric methods (usually the latter).

At the 1951 meeting of the Association, Allen, *et al.* (4), reported good agreement between the results for available P_2O_5 in samples of superphosphates and mixed fertilizers, as determined by the indirect (official) procedure (5) and by a direct procedure that involved precipitation of ammonium phosphomolybdate from an aliquot of the combined water and citrate extracts with subsequent titration of the molybdate according to the official volumetric method.

Photometric determination of available P_2O_5 in the combined water and citrate extracts, with the aid of a vanadomolybdate reagent as recommended by Barton (6), has been studied by Epps (7), who concluded that the method is sufficiently accurate for routine purposes. Allen, *et al.* (4), state that good agreement in results by the Epps and official procedures was obtained with some samples but not with others.

Since the indirect procedure requires the determination of both total and citrate-insoluble P_2O_5, application of direct procedures to the determination of available P_2O_5 could well result in considerable saving of time, especially in mass analyses where the interest is solely in available P_2O_5. The present study was undertaken for the purpose of obtaining additional data on the procedures of Epps, and of Allen, *et al.*

SAMPLES

The samples used in this study comprised 4 normal superphosphates, 3 triple superphosphates, 1 calcium metaphosphate manufactured by the Tennessee Valley Authority, 1 ammoniated superphosphate (No. 26) experimentally prepared with 5% nitrogen from anhydrous NH_3, 25 commercial mixed fertilizers, and 2 mixed fertilizers (Nos. 34 and 35) experimentally formulated with all (No. 34) or a portion (50%) of the P_2O_5 in the form of Ca metaphosphate. Ca metaphosphate was not known to be present in any of the commercial mixtures.

The samples of normal superphosphate and commercial mixtures, taken from materials marketed in the 1949–1950 fertilizer season, were kindly supplied by State fertilizer control officials. They were selected from a nation-wide series of samples collected for a survey of the acid-insoluble ash, carbonate, and forms and solubility of P_2O_5 in mixed fertilizers (8, 9). The mixtures, ranging in grade from 6-3-6 to 6-24-12, were manufactured by 23 companies—small, medium, and large—and were marketed in 15 States from Maine to California. Organic materials of one kind or another were known to be present in nine of the mixtures (Nos. 2, 3, 4, 9, 11, 15, 17, 20, and 22).

For analysis, the samples of superphosphate, ammoniated superphosphate, and commercial mixtures were ground to pass either the 20- or 35 mesh sieve. The calcium metaphosphate and the experimental mixture containing this material were ground to pass the 80- and 35 mesh sieves, respectively.

METHODS OF ANALYSIS

All of the samples were analyzed for available P_2O_5 by the official procedure (5) (Method I), the direct volumetric procedure of Allen, *et al.* (4) (Method II), and the direct photometric procedure of Epps (7) (Method III). The H_2O extraction was omitted in the case of the straight Ca metaphosphate, and a 1 g sample—placed on a dry 9 cm filter paper—was extracted directly with citrate soln. Analyses of the superphosphates, ammoniated superphosphate, and 8 of the commercial mixed fertilizers were also made by the direct volumetric procedure on citrate extracts prepared by direct digestion of the samples without prior washing with H_2O (Method IV). The several methods as used in this study are outlined as follows:

Method I.—Prepare the H_2O and citrate extracts according to the official procedure with gravity filtration during the H_2O extraction and continuous agitation during the citrate digestion. As in the detn of total P_2O_5, digest the citrate-insoluble residue with a mixt. of concd HNO_3 (25 ml) and HCl (10 ml), and analyze the soln for P_2O_5 by the official volumetric method with pptn of the phosphomolybdate at 25–30°C. and continuous agitation at room temp.

Method II.—The procedure, essentially as outlined by Allen, *et al.*, was as follows:

Add 50 ml of H_2O to a 1 g sample in a 300 ml Erlenmeyer flask and agitate continuously for 10 min. Filter the mixt. with the aid of suction through a 9 cm paper (Whatman No. 40 or equivalent) into a one l filter flask contg 5 ml of concd HNO_3, and wash the residue with small portions of cold H_2O to a vol. of 125 ml. Within 1 hr, treat the water-insoluble residue with citrate soln according to the official procedure with continuous agitation during the citrate digestion. Filter the soln through paper (Whatman No. 5 or equivalent) into the flask contg the H_2O extract, and wash the residue with H_2O at 65°C. to a total vol. of 450 ml. Transfer the contents of the filter flask to a 500 ml volumetric flask, cool to room temp., adjust the vol. to 500 ml., and mix thoroly.

For the detn of P_2O_5, use aliquots of the soln corresponding to 0.10, 0.05, and 0.025 g of sample for materials contg less than 25, 25 to 50, and more than 50 per cent of available P_2O_5, respectively. Transfer the appropriate aliquot to a 300 ml Erlenmeyer flask or other suitable vessel, adjust the vol. to 120 ml with H_2O, and heat to 50°C. Add 60 ml of molybdate soln (omitting the extra HNO_3, 5 ml/100 ml of molybdate) and 15 ml of NH_4NO_3 (10 g of NH_4NO_3), previously heated to 50°C. Agitate continuously for 30 min. at 50°C., filter, and complete the detn according to the official volumetric procedure.

With Ca metaphosphate and its mixtures, add 5 ml of concd HNO_3 to the aliquot of the extract and digest overnight on the steam bath before precipitating the phosphomolybdate.

Method III.—The procedure, essentially as outlined by Epps, was as follows:

Prepare the H_2O and citrate extracts as in Method II.

Prepare the vanadomolybdate soln according to the directions of Barton (6) for the mixed reagent. Dissolve 40 g of NH_4 molybdate in 400 ml of H_2O. Dissolve 1 g of NH_4 vanadate in 300 ml of H_2O and add 200 ml of concd HNO_3. Allow the two solns to cool, and mix by pouring the molybdate soln into the vanadate soln. Dilute to one l.

Prepare a standard phosphate soln by dissolving 0.4792 g of monopotassium phosphate in one l of H_2O. This soln contains 0.25 mg of P_2O_5 per ml.

Transfer an aliquot (0.1 to 0.4 mg of P_2O_5) of the extract of the sample to a 50 ml volumetric flask, add 10 ml of vanadomolybdate soln, bring vol. to 50 ml with H_2O, mix thoroly, and let stand for 15 min. for development of the color. Det. the light transmission of the colored soln by means of an Evelyn photoelectric colorimeter (420–515 mμ filter), with prior adjustment of the instrument against a blank soln

contg NH_4 citrate and HNO_3 in the same concns as in the soln being analyzed. Estimate the P_2O_5 by interpolation of the colorimeter reading on a curve prepd from analyses of aliquots of the standard monopotassium phosphate soln under similar conditions of temperature and of concn of NH_4 citrate and HNO_3.

With Ca metaphosphate and its mixtures, add 2 ml of concd HNO_3 to the aliquot of the extract and digest overnight on the steam bath before proceeding with the color development.

Method IV.—Omit the H_2O extraction, and digest a 1 g sample—placed on a dry 9 cm filter paper—directly with NH_4 citrate according to the official procedure with continuous agitation. At the end of the digestion period, quickly cool the contents of the flask to room temperature, transfer to a 250 ml volumetric flask, make to vol. with H_2O, mix thoroly, and immediately filter through a dry paper. Analyze the extract for P_2O_5 by the direct volumetric procedure outlined in Method II.

SOME FACTORS INFLUENCING VOLUMETRIC DETERMINATION OF P_2O_5 IN PRESENCE OF AMMONIUM CITRATE

Preliminary experiments indicated that precipitation of phosphomolybdate in the presence of ammonium citrate is considerably influenced by the quantities of molybdate solution and HNO_3 used and by the temperature at which it is precipitated, more so than in the absence of citrate. For the volumetric determination of P_2O_5 in the presence of citrate, Allen (10) has recently recommended that the phosphomolybdate be precipitated with continuous agitation at 50°C. instead of 30–35°C. as previously specified by Allen, *et al.* (4). The advantages of precipitation at the higher temperature were confirmed in the present investigation.

Table 1 shows the effect of varying the volumes of molybdate solution and supplemental HNO_3 on the recovery of P_2O_5 from different quantities of a standard monopotassium phosphate solution, to each aliquot of which was added 10 ml of neutral ammonium citrate solution. Addition of the extra HNO_3 to the molybdate solution (5 ml/100 ml), as specified in the official volumetric procedure, was omitted, and the determinations were made as outlined in Method II.

In the absence of supplemental HNO_3, 20 ml of molybdate solution gave no precipitate with 5–30 mg of P_2O_5, and only 4–44 per cent of the P_2O_5 was recovered when the volume of the molybdate solution was increased to 30 ml; nearly theoretical results were obtained, however, with 45–75 ml of molybdate solution. In the presence of 1.5 to 5.0 ml of supplemental acid, 30 ml of molybdate solution gave nearly complete recovery of the 5- and 10 mg quantities of P_2O_5 but not of the larger quantities. With the larger volumes of molybdate solution (45–75 ml) supplemental additions of HNO_3 had little or no effect on the accuracy and precision of the results.

To insure the presence of sufficient molybdate and HNO_3 (as supplied by the unfortified molybdate solution itself) to effect complete precipitation of the P_2O_5, 60 ml portions of molybdate solution were used in the direct volumetric analysis of the fertilizer samples.

TABLE 1.—*Effect of volume of nitric acid and of molybdate solution on recovery of P_2O_5 from monopotassium phosphate in presence of ammonium citrate*

HNO_3 added[a]	P_2O_5 recovered[b]								
	ml of molybdate solution								
	20	30		45		60		75	
	average	average	deviation	average	deviation	average	deviation	average	deviation
ml	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
				5.10 mg. P_2O_5					
0	0.00	0.38	0.33	5.15	0.04	5.21	0.02	5.26	0.23
1.5	0.00	5.05	0.06	4.96	0.07	5.20	0.15	—	—
3.0	0.00	5.08	0.10	5.10	0.07	5.15	0.07	—	—
5.0	0.00	5.06	0.09	5.13	0.10	5.18	0.06	—	—
				10.20 mg P_2O_5					
0.0	0.00	0.39	0.42	10.22	0.03	10.27	0.07	10.29	0.14
1.5	—	10.15	0.06	10.28	0.04	10.23	0.04	—	—
3.0	—	10.18	0.03	10.27	0.12	10.22	0.08	—	—
5.0	—	10.11	0.04	10.25	0.12	10.34	0.10	—	—
				20.30 mg P_2O_5					
0.0	0.00	3.73	7.10	20.25	0.06	20.36	0.21	20.38	0.04
1.5	—	17.61	0.00	20.28	0.06	20.26	0.02	—	—
3.0	—	18.30	0.05	20.21	0.02	20.36	0.11	—	—
5.0	—	19.78	0.21	20.24	0.12	20.36	0.11	—	—
				30.40 mg P_2O_5					
0.0[c]	0.00	13.29	—	30.27	—	30.25	—	30.50	—
1.5	—	19.93	1.22	30.08	0.08	30.20	0.29	—	—
3.0	—	22.85	1.07	30.22	0.10	30.25	0.04	—	—
5.0	—	24.02	1.17	30.16	0.10	30.27	0.06	—	—

[a] Concd. acid, sp. gr. 1.42.
[b] Duplicate detns, except as indicated otherwise. The deviation represents the difference between the high and low results. Each pptn was made in the presence of 10 ml of neutral ammonium citrate soln, with the use of molybdate soln from which the extra HNO_3 (5 ml per 100 ml of molybdate) was omitted.
[c] Single detns.

COMPARISON OF RESULTS BY THE OFFICIAL PROCEDURE AND THE DIRECT VOLUMETRIC AND PHOTOMETRIC PROCEDURES

In the 36 comparisons, the average results of triplicate determinations by the official procedure (Method I) were higher than those by the direct volumetric procedure (Method II) and the direct photometric procedure (Method III) in each of 27 cases (Table 2). Disregarding signs, the difference between the average results on all samples by the official and the

TABLE 2.—*Available P_2O_5 by the official method and by direct volumetric and photometric analyses of the combined water and citrate extracts*

SAMPLE NO.	FERTILIZER GRADE	TOTAL P_2O_5[a]	CITRATE-INSOLUBLE P_2O_5[a,b]	AVAILABLE P_2O_5[a] BY METHOD:			DIFFERENCE IN AVAILABLE P_2O_5 BY METHOD I[e] AND METHOD:	
				I[b]	II[c]	III[d]	II	III
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
				Superphosphates				
30	Normal	20.36	0.25	20.11	19.98	20.13	0.13	−0.02
31	Normal	23.06	2.16	20.90	20.75	20.82	0.15	0.08
32	Normal	21.80	1.13	20.67	20.54	20.63	0.13	0.04
33	Normal	22.61	0.26	22.35	22.32	22.32	0.03	0.03
27	Triple[f]	47.05	1.96	45.09	44.39	44.92	0.70	0.17
28	Triple[g,h]	44.25	0.47	43.78	43.62	43.33	0.16	0.45
29	Triple[i]	50.67	2.25	48.42	49.01	48.67	−0.59	−0.25
Average[j]		—	—	—	—	—	0.27	0.15
				Mixed Fertilizers[k]				
25	6-3-6[l]	3.66	0.11	3.55	3.39	3.45	0.16	0.10
8	4-4-8	4.40	0.35	4.05	4.11	4.01	−0.06	0.04
20	4-7-5	10.56	3.07	7.49	7.38	7.33	0.11	0.16
5	17-7-0	7.35	0.06	7.29	7.42	7.41	−0.13	−0.12
22	4-8-6	11.00	0.77	10.23	10.01	9.97	0.22	0.26
6	8-8-8	9.04	1.21	7.83	7.81	7.87	0.02	−0.04
11	3-9-6[h]	10.42	0.40	10.02	10.29	10.41	−0.27	−0.39
17	6-9-12	10.19	0.71	9.48	9.51	9.74	−0.03	−0.26
23	4-10-6	12.71	1.21	11.50	11.24	11.43	0.26	0.07
21	4-10-7	11.28	0.56	10.72	10.67	10.47	0.05	0.25
9	5-10-5	11.09	0.87	10.22	10.00	10.08	0.22	0.14
18	5-10-10	9.93	0.29	9.64	9.77	9.63	−0.13	0.01
4	10-10-0	11.65	0.91	10.74	10.77	10.65	−0.03	0.09
7	10-10-10	9.87	0.18	9.69	9.53	9.63	0.16	0.06
14	2-12-6	10.67	0.31	10.36	10.17	10.77	0.19	−0.41
13	3-12-6	13.33	0.98	12.35	12.28	12.24	0.07	0.11
19	3-12-12	14.06	0.93	13.13	13.14	13.05	−0.01	0.08
10	4-12-4	14.33	1.46	12.87	12.77	12.83	0.10	0.04
12	4-12-8	13.28	1.21	12.07	12.04	11.95	0.03	0.12
2	0-14-7	16.78	0.92	15.86	15.66	15.96	0.20	−0.10
26	5-16-0[m]	18.35	2.13	16.22	16.16	16.11	0.06	0.11
24	8-16-16	14.93	0.06	14.87	14.72	14.84	0.15	0.03
15	3-18-9[h]	19.35	0.78	18.57	18.38	18.55	0.19	0.02
3	6-18-0[h]	21.75	5.11	16.64	16.31	16.31	0.33	0.33
1	0-20-20	20.02	0.07	19.95	19.92	19.78	0.03	0.17
16	6-24-12	27.82	1.97	25.85	25.52	25.71	0.33	0.14
Average[j]		—	—	—	—	—	0.14	0.14
				Products Containing Calcium Metaphosphate[n]				
35	4-16-8	17.16	1.18	15.98	15.69	15.85	0.29	0.13
34	0-20-20	18.88	0.44	18.44	18.34	18.44	0.10	0.00
36	Ca meta-phosphate	63.86	0.62	63.24	64.37	62.50	−1.13	0.74
Average[j]		—	—	—	—	—	0.50	0.29

[a] Average of triplicate detns.
[b] Official method.
[c] Volumetric detn of P_2O_5 in combined H_2O and citrate extracts.
[d] Photometric detn of P_2O_5 in combined H_2O and citrate extracts.
[e] The minus sign denotes that the official method gave the lower result.
[f] Made from western phosphate rock.
[g] Made from Tennessee brown-rock phosphate.
[h] Color of citrate extract was a dark yellow.
[i] Made from Florida land-pebble phosphate.
[j] Disregarding signs.
[k] Not contg Ca metaphosphate.
[l] Citrate extract was very cloudy.
[m] Ammoniated superphosphate prepared with anydrous NH_3.
[n] Prior to the molybdate pptn the acidified aliquots were digested overnight on the steam bath.

volumetric procedures was 0.19 per cent of P_2O_5, as compared with 0.15 per cent by the official and the photometric procedures. Omitting the triple superphosphates and the metaphosphates, each of which contained more than 43 per cent of available P_2O_5, the average differences were 0.14 and 0.12 per cent, respectively. The distribution of the samples according to the magnitude of the differences was as follows (Table 3):

TABLE 3.—*Distribution of samples according to the magnitude of the difference in results for available P_2O_5 by the official and the direct procedures*

DIFFERENCE FROM RESULTS BY OFFICIAL PROCEDURE	SAMPLES	
	DIRECT VOLUMETRIC PROCEDURE	DIRECT PHOTOMETRIC PROCEDURE
per cent	*number*	*number*
<0.05	7	10
0.05–0.14	11	14
0.15–0.29	13	7
0.30–0.50	2	4
>0.50	3	1

With the 32 samples containing less than 30 per cent of available P_2O_5, there was no definite relation between the content of available P_2O_5 and the difference in the results by the official procedure and those by either of the direct procedures. The average differences were much greater, however, with the four samples that contained 40–63 per cent of available P_2O_5. Disregarding signs, the distribution of the differences with respect to the available P_2O_5 in the samples was as follows (Table 4):

TABLE 4.—*Distribution of differences in average results for available P_2O_5 by the official and the direct procedures*

AVAILABLE P_2O_5 BY OFFICIAL PROCEDURE	SAMPLES	DIFFERENCE IN RESULTS BY OFFICIAL PROCEDURE AND DIRECT			
		VOLUMETRIC PROCEDURE		PHOTOMETRIC PROCEDURE	
		RANGE	AVERAGE	RANGE	AVERAGE
per cent	*number*	*per cent*	*per cent*	*per cent*	*per cent*
<10	8	0.02–0.16	0.10	0.01–0.26	0.10
10–15	12	0.01–0.27	0.13	0.03–0.41	0.17
15–20	7	0.03–0.33	0.17	0.00–0.33	0.12
20–30	5	0.03–0.33	0.15	0.02–0.14	0.06
44–63	4	0.16–1.13	0.64	0.17–0.74	0.40
3.5–63	36	0.01–1.13	0.19	0.00–0.74	0.15

As previously mentioned, nine of the samples of commercial mixed fertilizers (Nos. 2, 3, 4, 9, 11, 15, 17, 20, and 22) were known to contain or-

TABLE 5.—*Precision of results for available P_2O_5 by the official method and by direct volumetric and photometric analyses of the combined water and citrate extracts*

SAMPLE NO.	FERTILIZER GRADE	AVAILABLE P_2O_5[a,b]	RANGE BETWEEN HIGH AND LOW RESULTS[c] FOR AVAILABLE P_2O_5 BY METHOD:		
			I[a]	II[d]	III[e]
		per cent	*per cent*	*per cent*	*per cent*
Superphosphates					
30	Normal	20.11	0.17	0.26	0.13
31	Normal	20.90	0.18	0.20	0.22
32	Normal	20.67	0.12	0.06	0.00
33	Normal	22.35	0.08	0.15	0.19
27	Triple	45.09	0.13	0.33	0.50
28	Triple[f]	43.78	0.17	0.37	0.50
29	Triple	48.42	0.15	0.07	0.50
Average		—	0.14	0.21	0.29
Mixed Fertilizers[g]					
25	6-3-6[h]	3.55	0.15	0.29	0.12
8	4-4-8	4.05	0.06	0.03	0.04
20	4-7-5	7.49	0.19	0.13	0.10
5	17-7-0	7.29	0.06	0.16	0.18
22	4-8-6	10.23	0.13	0.13	0.20
6	8-8-8	7.83	0.08	0.10	0.12
11	3-9-6[f]	10.02	0.08	0.22	0.17
17	6-9-12	9.48	0.09	0.06	0.23
23	4-10-6	11.50	0.07	0.04	0.10
21	4-10-7	10.72	0.15	0.28	0.10
9	5-10-5	10.22	0.14	0.14	0.35
18	5-10-10	9.64	0.14	0.15	0.10
4	10-10-0	10.74	0.12	0.00	0.23
7	10-10-10	9.69	0.06	0.09	0.22
14	2-12-6	10.36	0.16	0.11	0.23
13	3-12-6	12.35	0.14	0.08	0.12
19	3-12-12	13.13	0.14	0.26	0.15
10	4-12-4	12.87	0.08	0.08	0.23
12	4-12-8	12.07	0.09	0.19	0.33
2	0-14-7	15.86	0.16	0.14	0.12
26	5-16-0[i]	16.22	0.16	0.39	0.10
24	8-16-16	14.87	0.11	0.11	0.13
15	3-18-9[f]	18.57	0.23	0.25	0.25
3	6-18-0[f]	16.64	0.13	0.15	0.19
1	0-20-20	19.95	0.11	0.13	0.23
16	6-24-12	25.85	0.14	0.26	0.25
Average		—	0.12	0.15	0.18
Products Containing Calcium Metaphosphate[j]					
35	4-16-8	15.98	0.30	0.12	0.13
34	0-20-20	18.44	0.16	0.26	0.18
36	Ca metaphosphate	63.24	0.21	0.37	1.12
Average		—	0.22	0.25	0.48

[a] Official method.
[b] Average of triplicate detns.
[c] Triplicate detns.
[d] Volumetric detn of P_2O_5 in combined H_2O and citrate extracts.
[e] Photometric detn of P_2O_5 in combined H_2O and citrate extracts.
[f] Color of citrate extract was a dark yellow.
[g] Not contg Ca metaphosphate.
[h] Citrate extract was very cloudy.
[i] Ammoniated superphosphate prepared with anhydrous NH_3.
[j] Prior to the molybdate pptn the acidified aliquots were digested overnight on the steam bath.

ganic materials of one kind or another. Disregarding signs, the results on these samples with the official procedure differed from those with the direct volumetric and photometric procedures by 0.03 to 0.33 (average 0.18) and 0.02 to 0.39 (average 0.19) per cent of P_2O_5, respectively. As indicated in Table 2, the results on three of the samples by the official procedure were lower than those by one or both of the direct procedures. Although the average differences (0.18 and 0.19 per cent) for these samples were higher than those for the other commercial mixtures, this could not be definitely attributed either to the presence of soluble phosphorus in non-*ortho*-phosphate forms or to adverse effects of the organic matter on the determinations by the direct procedures.

The precision of the three methods for available P_2O_5 is indicated in Table 5 which shows the differences between the high and low results of each of the triplicate determinations. For all of the samples the average precision was best with the official procedure and poorest with the direct photometric procedure. As compared with the official procedure, the precision was better on 31 per cent of the samples by one or both of the direct procedures. Better precision was indicated for 67 per cent of the samples by the direct volumetric procedure than by the photometric procedure.

There was no definite relation between the percentage of available P_2O_5 and the difference in the high and low results of the determinations by either of the three procedures. The trend, however, was toward wider differences at the higher levels of P_2O_5, especially with the photometric procedure on the four samples containing 44–63 per cent of available P_2O_5. The distribution of the differences, with respect to the available P_2O_5 in the samples, was as follows (Table 6):

TABLE 6.—*Distribution of differences in results of replicated determinations of available P_2O_5*

AVAILABLE P_2O_5 BY OFFICIAL PROCEDURE	SAMPLES	DIFFERENCE IN HIGH AND LOW RESULTS					
		OFFICIAL PROCEDURE		DIRECT VOLUMETRIC PROCEDURE		DIRECT PHOTOMETRIC PROCEDURE	
		RANGE	AVERAGE	RANGE	AVERAGE	RANGE	AVERAGE
per cent	*number*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
<10	8	0.06–0.19	0.10	0.03–0.29	0.13	0.04–0.23	0.14
10–15	12	0.07–0.16	0.12	0.00–0.28	0.13	0.10–0.35	0.19
15–20	7	0.11–0.30	0.18	0.12–0.39	0.21	0.10–0.25	0.17
20–30	5	0.08–0.18	0.14	0.06–0.26	0.19	0.00–0.25	0.16
44–63	4	0.13–0.21	0.17	0.07–0.37	0.28	0.50–1.12	0.65
3.5–63	36	0.06–0.30	0.13	0.00–0.39	0.17	0.00–1.12	0.22

TABLE 7.—*Effect of removing water-soluble compounds prior to the citrate extraction*

SAMPLE NO.	FERTILIZER GRADE	TOTAL P_2O_5[a]	WATER-SOLUBLE P_2O_5[b,c]	AVAILABLE P_2O_5[a] BY METHOD:			DIFFERENCE IN AVAILABLE P_2O_5 BY METHOD I[f] AND METHOD:	
				I[b]	II[d]	IV[e]	II	IV
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
				Superphosphates				
30	Normal	20.36	17.17	20.11	19.98	19.78	0.13	0.33
31	Normal	23.06	16.43	20.90	20.75	20.49	0.15	0.41
32	Normal	21.80	15.98	20.67	20.54	20.40	0.13	0.27
33	Normal	22.61	18.15	22.35	22.32	22.03	0.03	0.32
27	Triple	47.05	38.11	45.09	44.39	43.97	0.70	1.12
28	Triple	44.25	31.20	43.78	43.62	43.29	0.16	0.49
29	Triple	50.67	44.00	48.42	49.01	47.84	−0.59	0.58
Average[g]		—	—	—	—	—	0.27	0.50
				Mixed Fertilizers[h]				
20	4- 7- 5	10.56	4.12	7.49	7.38	7.32	0.11	0.17
5	17- 7- 0	7.35	5.81	7.29	7.42	7.35	−0.13	−0.06
23	4-10-6	12.71	3.48	11.50	11.24	10.82	0.26	0.68
18	5-10-10	9.93	6.23	9.64	9.77	9.49	−0.13	0.15
7	10-10-10	9.87	6.23	9.69	9.53	9.61	0.16	0.08
12	4-12- 8	13.28	5.25	12.07	12.04	11.72	0.03	0.35
26	5-16- 0[i]	18.35	9.73	16.22	16.16	15.77	0.06	0.45
3	6-18- 0	21.75	9.90	16.64	16.31	13.69	0.33	2.95
16	6-24-12	27.82	16.95	25.85	25.52	24.93	0.33	0.92
Average[g]		—	—	—	—	—	0.17	0.65

[a] Average of triplicate detns.
[b] Official method.
[c] Average of duplicate detns.
[d] Volumetric detn of P_2O_5 in combined H_2O and citrate extracts.
[e] Volumetric detn of P_2O_5 in citrate extract of sample not previously washed with H_2O.
[f] The minus sign denotes that the official method gave the lower result.
[g] Disregarding signs.
[h] Not contg Ca metaphosphate.
[i] Ammoniated superphosphate.

TABLE 8.—*Effect of nitric acid digestion of citrate extract on results for available P_2O_5 in calcium metaphosphate*

SAMPLE NO.	AVAILABLE P_2O_5 BY PHOTOMETRIC PROCEDURE	
	WITHOUT HYDROLYSIS	WITH HYDROLYSIS
	per cent	*per cent*
34	4.80, 4.90, 4.90	18.38, 18.38, 18.56
35	11.60, 11.70, 11.60	15.81, 15.94, 15.81

DIRECT CITRATE DIGESTION OF THE SAMPLE

Jacob and Tremearne (11) have shown that with the official procedure, lower results for available P_2O_5 in superphosphates, ammoniated superphosphates, mixed fertilizers, and other products containing water-soluble P_2O_5 are usually obtained when the water extraction is omitted and the sample is digested directly with citrate solution. This is due to the greater burden placed on the citrate solution by the unwashed sample, not only with respect to the P_2O_5 itself but also, and probably of even greater importance, to the presence of larger quantities of calcium salts, particularly calcium sulfate, of which substantial portions may be removed by the water extraction (11). Jacob, *et al.* (12), have pointed out that the solubility of phosphates in neutral ammonium citrate solution may be considerably depressed in the presence of such salts.

As shown in Table 7, direct determination of available P_2O_5 by the volumetric procedure with omission of the water extraction (Method IV) usually gave considerably lower results on superphosphates and mixed fertilizers than were obtained when the sample was first extracted with water and the determination was completed by either the official procedure or the direct volumetric procedure (Method II).

DIRECT DETERMINATION OF AVAILABLE P_2O_5 IN CALCIUM METAPHOSPHATE

For the direct determination of available P_2O_5 in calcium metaphosphate and its products, it is essential that the dissolved phosphorus be converted to the *ortho*-phosphate form before adding the molybdate solution or the vanadomolybdate solution. This can be done by overnight digestion of the aliquot on the steam bath with additions of 5 ml (volumetric procedure) or 2 ml (photometric procedure) of concentrated HNO_3. The effect of omitting the digestion with HNO_3 is shown by the following results obtained with the photometric procedure on two samples of mixed fertilizers (Nos. 34 and 35) containing calcium metaphosphate (Table 8):

DISCUSSION

The results obtained in this study are further evidence of the possibilities of both volumetric and photometric procedures as direct methods for the determination of available P_2O_5 in fertilizers. They appear to offer a considerable saving in the time required for this determination, especially in mass analyses where the interest is solely in available P_2O_5. The methods merit thorough investigation to determine whether, through improvements in techniques, their results can be brought more closely into line with those obtained by the official procedure, and if their applicability can be extended to a wider range of products.

With the direct volumetric method, interference of the sulfate ion in the hot precipitation of ammonium phosphomolybdate (13)—the pre-

ferred procedure for this method—appears to be eliminated by the presence of citrate. This is in accord with the results of other studies made in the writers' laboratory (14) and elsewhere.

SUMMARY

Determinations of available P_2O_5 were made on 36 samples by the official procedure and, with slight alterations, by the direct volumetric procedure of Allen, *et al.* (4), and the direct photometric procedure of Epps (7).

The samples comprised 4 normal superphosphates, 3 triple superphosphates, 1 calcium metaphosphate, 1 ammoniated superphosphate, 25 commercial mixed fertilizers, and 2 experimentally formulated mixed fertilizers containing calcium metaphosphate.

The results by the official procedure were usually higher than those by the direct procedures. Disregarding signs, the difference between the average results on all samples by the official procedure and the direct volumetric procedure was 0.19 per cent of P_2O_5, as compared with 0.15 per cent by the official and the photometric procedures. Omitting the triple superphosphates and the calcium metaphosphate, each of which contained more than 43 per cent of available P_2O_5, the average differences were 0.14 and 0.12 per cent, respectively.

For all of the samples the average precision, as indicated by the difference between the high and low results of triplicate determinations, was best with the official procedure and poorest with the direct photometric procedure. As compared with the official procedure, the precision was better on 31 per cent of the samples by one or both of the direct procedures. Better precision was indicated for 67 per cent of the samples by the direct volumetric procedure than by the photometric procedure.

Direct determination of available P_2O_5 by the volumetric procedure, omitting the water extraction, usually gave considerably lower results on superphosphates and mixed fertilizers than were obtained when the sample was first extracted with water and the determination was completed by either the official procedure or the direct volumetric procedure.

RECOMMENDATIONS

It is recommended*—

(1) That determination of citrate-insoluble P_2O_5 in calcium metaphosphate be made on samples ground to pass the 35-mesh sieve, with continuous agitation during the citrate digestion, and made official.

(2) That further study be made of methods for direct determination of available P_2O_5 in fertilizers.

(3) That study be made of the use of perchloric acid in preparation of phosphate fertilizer solutions for analysis.

* For report of Subcommittee A and action of the Association, see *This Journal*, 36, 49 (1953).

REFERENCES

(1) FRESENIUS, R., NEUBAUER, C., and LUCK, F *Z. Anal. Chem.*, **7**, 304–309 (1871).
(2) Association of Official Agricultural Chemists, *Proc. Conv. Agr. Chemists and First Ann. Meeting Assoc. Offic. Agr. Chemists*, Philadelphia, Sept. 8–9, 1884.
(3) *Methods of Analysis*, 5th Ed., A.O.A.C., Washington, D. C., 1940, p. 24.
(4) ALLEN, H. R., SWIFT, E., and HAYES, R., *This Journal*, **35**, 764–767 (1952).
(5) *Methods of Analysis*, 7th Ed., A.O.A.C., Washington, D. C., 1950, pp. 10–11.
(6) BARTON, C. J., *Anal. Chem.*, **20**, 1068–1073 (1948).
(7) EPPS, E. A., Jr., *ibid.*, **22**, 1062–1063 (1950).
(8) CLARK, K. G., and HOFFMANN, W. M., *Farm Chemicals*, **115**, No. 5, 17–19, 21, 23 (1952).
(9) CLARK, K. G., GADDY, V. L., BLAIR, A. E., and LUNDSTROM, F. O., *ibid.*, **115**, No. 6, 21, 23–26 (1952).
(10) ALLEN, H. R., Private communication, June 16, 1952.
(11) JACOB, K. D., and TREMEARNE, T. H., *This Journal*, **20**, 277–287 (1937).
(12) JACOB, K. D., RADER, L. F., JR., MARSHALL, H. L., and BEESON, K. C., *Ind. Eng. Chem., Anal. Ed.*, **4**, 25–31 (1932).
(13) JACOB, K. D., RADER, L. F., JR., and WHITTAKER, C. W., *This Journal*, **31**, 209–234 (1948).
(14) MAGNESS, R. M., and JACOB, K. D., unpublished data.
(15) Organization for European Economic Cooperation, *Fertilizers: Methods of Analysis Used in OEEC Countries*, Paris, November, 1952.

REPORT ON NITROGEN IN FERTILIZERS

BY H. A. DAVIS (New Hampshire Agricultural Experiment Station, University of New Hampshire, Durham, New Hampshire), *Associate Referee*

It was recommended at the 1951 meeting of the Association that the study of the Shuey method for the determination of nitrogen in high nitrate-high chloride mixtures be continued. Unforeseen circumstances had prevented the preparation of samples for collaborative study in 1951. The work this year was designed to check on the conclusions resulting from the 1950 collaborative program.*

SAMPLES

The Associate Referee in correspondence with Mr. Philip McG. Shuey concerning this work, requested suggestions as to samples that should be submitted to the collaborators. It was suggested that three samples be prepared:

No. 1: 5 -9-16 mixture containing about 20 mg nitrate-N per gram
No. 2: 6 -9-27 mixture containing about 26 mg nitrate-N per gram
No. 3: 12-6-14 mixture containing about 56 mg nitrate-N per gram

These mixtures were made up using Aeroprills, 34.02 per cent N; superphosphate; muriate of potash; and Hynite tankage, 9.38 per cent N (same

* *This Journal*, 34, 653 (1951).

amount of tankage in each of the three samples); so that the calculated nitrogen content of sample No. 1 was 5.04 per cent; sample No. 2, 6.06 per cent; and sample No. 3, 12.01 per cent.

The method of preparation of the samples was as follows: it was decided that 2500 grams (4–5 lbs.) would be enough for each lot. A sufficient amount of each ingredient was ground and passed a 1 mm sieve as rapidly as possible. All grinding was done in a Mikro Samplmill. Each ingredient was weighed and put in a large screw-top glass bottle which was filled about one-half full by the 2500 gram lot. After thorough mixing in the bottle, the entire sample was sieved through a 1 mm screen, returned to the bottle, and remixed. Exposure to the air was kept at a minimum to avoid loss or accumulation of moisture. The samples were packaged in 2 oz. containers directly from the bottle and packed for shipment to the collaborators.

INSTRUCTIONS TO COLLABORATORS

Instructions to collaborators included the description of each sample and requested analyses by two methods:

A. *Official Methods of Analysis*, A.O.A.C., 1950, section **2.25** or **2.26.**

B. The Shuey Method, detail enclosed. (*This Journal*, **34**, 655 (1951)).

A form for reporting results and comments was also enclosed.

Samples were mailed late in March to thirty collaborators who had expressed an interest in this work. Results were received from twenty-four collaborators in time for this report. The collaborators submitting results are listed as follows:

(1) W. R. Austin, Armour Fertilizer Works, Nashville, Tennessee.
(2) A. T. Blackwell and R. Felker, The Davison Chemical Corporation, Baltimore, Maryland.
(3) C. R. Byers and F. Stuart, Armour Fertilizer Works, Carteret, New Jersey.
(4) William Chapman, Consolidated Rendering Co., Boston, Massachusetts.
(5) E. E. Eastman and H. A. Davis, Agricultural Experiment Station, Durham, New Hampshire.
(6) M. P. Etheredge and John Brent, State College, Mississippi.
(7) W. C. Geagley and V. Thorpe, Department of Agriculture, Lansing, Michigan.
(8) C. W. Gehrke, Department of Agricultural Chemistry, University of Missouri, Columbia, Missouri.
(9) Roland W. Gilbert, Agricultural Experiment Station, Kingston, Rhode Island.
(10) Richard S. Harding, Department of Agriculture, Denver, Colorado.
(11) Gordon Hart, Chem. Div., Department of Agriculture, Tallahassee, Florida.
(12) R. C. Koch and J. B. Hulsey, Swift & Co., Hammond, Indiana.
(13) Charles V. Marshall, Chemistry Laboratory, Department of Agriculture, Ottawa, Canada.
(14) G. A. McIlveen, North American Cyanamid Ltd., Niagara Falls, Canada.
(15) Ralph D. Miller, Spencer Chemical Co., Pittsburg, Kansas.
(16) W. A. Morgan, E. I. du Pont de Nemours Co., Wilmington, Delaware.

TABLE 1.—*Determination of nitrogen by the Shuey method*

ANALYST NO.	SAMPLE NO. 1, 5-9-16			SAMPLE NO. 2, 6-9-27			SAMPLE NO. 3, 12-6-14		
	OFFICIAL (A)	SHUEY (B)	DIFF., B−A	OFFICIAL (A)	SHUEY (B)	DIFF., B−A	OFFICIAL (A)	SHUEY (B)	DIFF., B−A
1	5.00	4.92	−0.08	6.09	6.09	0.00	11.71	11.71	0.00
2	5.01	5.07	0.06	6.03	6.04	0.01	11.71	11.86	0.15
3	4.63	4.87	0.24	5.43	5.94	0.51	11.34	11.62	0.28
4	4.63	4.69	0.06	5.63	5.90	0.27	11.60	11.59	−0.01
6	4.76	5.25	0.49	5.79	6.19	0.40	10.99	11.66	0.67
7	4.71	4.77	0.06	5.72	5.83	0.11	11.61	11.62	0.01
8	5.01	5.06	0.05	6.59	6.36	−0.23	12.11	12.06	−0.05
9	4.95	4.94	−0.01	5.91	6.04	0.13	11.81	12.03	0.22
10	4.93	4.96	0.03	5.98	6.31	0.33	11.58	11.91	0.33
11	4.78	4.89	0.11	5.75	6.00	0.25	11.14	11.95	0.81
14	5.26	4.88	−0.38	5.57	5.87	0.30	11.33	11.51	0.18
15	4.56	4.69	0.13	5.52	5.89	0.37	10.88	12.54	1.66
16	5.05	5.08	0.03	6.08	6.11	0.03	11.82	12.05	0.23
17	4.97	5.46	0.49	6.12	6.60	0.48	12.02	12.47	0.45
18	4.07	4.99	0.92	4.92	6.05	1.13	10.94	11.77	0.83
19	4.58	4.99	0.41	5.50	6.39	0.89	10.12	11.80	0.68
20	5.01	4.14	−0.87	6.29	5.03	−1.26	11.85	9.56	−2.29
22	4.85	4.90	0.05	5.71	6.01	0.30	11.82	12.07	0.25
23	4.99	5.01	0.02	6.02	6.03	0.01	12.09	12.26	0.17
26	4.85	4.88	0.03	5.91	5.94	0.03	11.47	11.92	0.45
27	4.56	5.20	0.64	5.49	6.28	0.79	11.00	11.77	0.77
28	4.91	4.94	0.03	6.40	5.95	−0.45	12.29	12.29	0.00
29	5.01	5.16	0.15	6.61	6.27	−0.34	12.05	12.05	0.00
30	5.02	4.92	−0.10	6.10	5.72	−0.38	11.77	11.90	0.13
Av.	4.84	4.94	0.11	5.88	6.03	0.15	11.54	11.83	0.24
Low-High	4.07–5.26	4.14–5.46		4.92–6.61	5.03–6.60		10.12–12.29	9.56–12.47	
Range	0.89	1.32		1.69	1.57		2.17	2.91	
Std. Error of Diff.			0.06965			0.09958			0.01362
Mean Diff.			0.1067			0.1533			0.2466

(17) Owen L. Nolan and Harry J. Fisher, Connecticut Agricultural Experiment Station, New Haven, Connecticut.
(18) C. H. Perrin, Canada Packers, Ltd., Toronto, Canada.
(19) Willis Richerson, Feed & Fertilizer Laboratory, State Department of Agriculture, Oklahoma City, Oklahoma.
(20) Philip Shuey, Shuey & Co., Savannah, Georgia.
(21) A. C. Wark, New Jersey Experiment Station, New Brunswick, New Jersey.
(22) Robert W. Way, The Solvay Process, Nitrogen Division, Hopewell, Virginia.
(24) H. Williamson and O. W. Ford, Purdue University, Lafayette, Indiana.
(25) C. Young and Alan B. Lemmon, Bureau of Chemistry, Department of Agriculture, Sacramento, California.

RESULTS

Table 1, which presents the results submitted by collaborators, together with some summarizing calculations made by the author, is self-explanatory. It is disturbing to note the wide range between the high and low results by the official method as well as by the Shuey method. Collaborator 20 reported much lower results by the Shuey procedure than by the official method. Calculations show that the final conclusion is not materially changed by including these results. While most collaborators reported somewhat higher results with the Shuey method, the difference was found not to be significant in any of the three samples. The question of proper sample preparation might be raised; however, special care was used in the preparation as previously described. Further, certain collaborators reported a series of results agreeing very well when the official method was used and poor agreement with the Shuey method. Contrary results were also reported. A large number of the collaborators reported excellent agreement between replicate analyses. A close examination of Table 1 shows differences in results that cannot be explained by poor sample preparation.

Comments by collaborators are much the same as those received with the 1950 program report.* The conclusion is that the Shuey method as described is not the answer to the problem of determination of total nitrogen in fertilizers for general control work. While there may be certain conditions when higher results will be obtained than by the official method, it does not appear to be adaptable for general use. Several collaborators report essentially the same results by both methods.

A common comment is that the Shuey method is cumbersome and ties up equipment too long. These objections could be overcome if significantly better results were obtained regularly, but this does not seem to be the case. Therefore, the Associate Referee recommends that further collaborative study on the Shuey method be discontinued for the present.

OTHER ITEMS FOR ATTENTION

During the year it was brought to the attention of the Associate Referee that there appeared to be an error in *Methods of Analysis*, 7th Ed.,

* *This Journal*, **33**, 653 (1951).

Section **2.26,** in that no catalyst is mentioned. Also in Section **2.25** no reference to K_2SO_4 is made. A note in regard to this matter, as to whether the directions were specific enough as printed, was sent to each collaborator. Dr. H. J. Fisher replied that if K_2SO_4 is used in Section **2.25,** it would no longer be the Kjeldahl method, but would be the Gunning method. No change is required at this time.

In regard to Section **2.26** since the point of catalyst addition may not be entirely clear, it is recommended that "0.1–0.3 g of $CuSO_4 \cdot 5H_2O$ may also be added," be inserted following, "anhyd. Na_2SO_4," so that the sentence will read: "Add 5 g of $Na_2S_2O_3$ and heat soln 5 min. Cool, add 10 g of K_2SO_4 or anhyd. Na_2SO_4 (0.1–0.3 g of $CuSO_4 \cdot 5H_2O$ may also be added), heat very gently until foaming ceases, and continue digestion as directed under **2.23.**" This will definitely clear up the point in question.

The subject of the application of the formaldehyde titration method for nitrogen in ammonium nitrate when some ammonium sulfate is also present has been presented. The method will not apply to that situation, but is designed to determine ammonium nitrogen and is assigned to the section of *Methods of Analysis* concerned with ammonium nitrogen. The fact that it may be used in the determination of total nitrogen in NH_4NO_3 when no interfering substances are present is understood by chemists. To avoid misunderstandings and yet retain the method which serves a useful purpose, it appears to be desirable to delete reference to total nitrogen in this section. The Associate Referee recommends the deletion of the parenthetical statement "(Ammoniacal N×2=total N in NH_4NO_3)" from section **2.28.**

RECOMMENDATIONS

It is recommended*—

(1) That further collaborative work on the Shuey method for nitrogen in fertilizers be discontinued, as results obtained by it are not significantly better than those obtained by the official method.

(2) That the phrase "(0.1–0.3 g of $CuSO_4 \cdot 5H_2O$ may also be added)" be inserted in Section **2.26** of *Methods of Analysis*, page 14, line one, following "anhyd. Na_2SO_4."

(3) That the parenthetical statement, "(Ammoniacal N×2=total N in NH_4NO_3)," be deleted from section **2.28.**

(4) That consideration be given to the study of methods of sampling and analysis of liquid fertilizer mixtures.

ACKNOWLEDGEMENT

The Associate Referee wishes to express his thanks for assistance in this work as follows: to Mr. Philip McG. Shuey for suggestions of the formulas used and for the Hynite tankage; to North American Cyanamid,

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

Ltd., Niagara Falls, Ontario, for supplying a special lot of Aeroprills ammonium nitrate fertilizer for which an accurate analysis was furnished; to the Consolidated Rendering Company, Boston, Massachusetts, for supplying 45% superphosphate; to the Agronomy Department, University of New Hampshire, for supplying 20% superphosphate and muriate of potash; and finally, to the collaborators who have given of their time to analyze the samples furnished and to submit their comments.

REPORT ON POTASH

By O. W. FORD (Purdue University Agricultural Experiment Station, Lafayette, Indiana), *Associate Referee*

In accordance with the recommendations approved by the Association,* additional collaborative studies were made, using the flame photometer in comparison with the A.O.A.C. and modified Perrin wet-digestion methods for the determination of potash in fertilizers. Twenty-five chemists, representing the fertilizer industry and commercial and control laboratories, found time to do all or at least part of the work and report to the Associate Referee.

OUTLINE OF 1952 COLLABORATIVE WORK ON POTASH IN FERTILIZERS

DIRECTIONS TO COLLABORATORS

(a) Prepare a double amount of soln as follows: Weigh and transfer 5 g of each sample to a 500 ml Pyrex volumetric flask. Add 100 ml NH_4 oxalate soln and 250 ml H_2O, and digest as in A.O.A.C. method. When cool, add NH_4OH, make to vol., and filter. This should be a sufficient vol. of soln for all potash detns on a sample.

(b) Using the Official Method for Potash, make 3 individual detns on each sample on soln prepared in **(a)**.

(c) Using the slightly modified Perrin wet-combustion method (details given below), make 3 individual potash detns on each sample on soln prepared in (a).

(d) Determine potash by the flame photometer (if available).

(e) Also, make some detns as in **(b)** and **(c)**, using methyl alcohol in place of ethyl alcohol or compound 30 alcohol.

Report all results as soon as possible and not later than August 1, 1952, so that the report of the General Referee can be completed for the Fall meeting. Please list any comments or criticisms. The samples sent to you have been ground and prepared for analysis, and should be analyzed as received.

DETAILS OF THE RAPID WET-DIGESTION METHOD FOR POTASH IN FERTILIZER

METHOD

REAGENTS

(a) *Platinum soln.*—Use a Pt soln contg the equivalent of 0.5 g of Pt (1.05 g H_2PtCl_6) in every 10 ml.

(b) *Diglycol stearate soln.*—Dissolve 20 g of diglycol stearate (tech.) in one l of equal parts of benzene and ethyl alcohol.

* *This Journal*, **35**, 44 (1952).

PREPARATION OF SOLUTION

Place 2.5 g or the factor weight 2.425 g of sample in a 250 ml volumetric flask. Add 125 ml H_2O, 50 ml satd NH_4 oxalate soln, and 1 ml diglycol stearate soln when necessary to prevent foaming. Boil 30 min., add slight excess of NH_4OH, and after cooling, dil. to 250 ml. Mix and pass thru dry filter.

DETERMINATION*

Place 50 ml aliquot of soln (or 25 ml aliquot and 25 ml H_2O, if sample contains more than 20% K_2O) in a 500 ml Kjeldahl flask. Add 10 ml HNO_3 and a silica granule (about 1 cm long), previously weighed along with a prepared Gooch or medium fritted crucible (Pyrex M porosity). Boil 2 min. and add 10 ml HCl. Boil down to ca 25 ml and add 5 ml HCl and excess of Pt soln. Boil down to 10–15 ml, rotating flask occasionally, and then add 5 ml HCl. Reduce heat and boil down to 3–5 ml (depending on amt of ppt), rotating flask frequently near the end of the evapn. Remove flask from heat and swirl to dissolve any soluble residue on walls. After cooling, immediately add 25 ml of 95% alcohol so that it washes down neck of flask. Chill under tap, swirl, and allow to stand for at least 5 min. Decant into the tared crucible and transfer ppt and granule with the aid of a stream of 95% alcohol. Wash 5 or 6 times with 10 ml portions of NH_4Cl soln (**2.39 (a)**) to remove Mg and Na salts from ppt. Wash again thoroly with alcohol and dry ppt for 30 min. at 100°C. Weigh and subtract wt of crucible plus the silica granule. $K_2PtCl_6 \times 0.19376 = K_2O$.

COMPOSITION OF THE SAMPLES

Four samples of fertilizer were analyzed by three methods: (*a*) the official A.O.A.C. method, (*b*) the modified Perrin wet-digestion method, and (*c*) the flame photometer. All samples were prepared at the Purdue University laboratory in a Micro-Samplmill with the $\frac{1}{8}''$ screen, thoroughly mixed, and bottled for shipment to the collaborators. The four samples were as follows:

(1) Potassium chloride, A. R.—theoretical value, 63.17 per cent.
(2) A mixture of several manufacturers' 3-18-9 fertilizer, approximately 9.0 per cent potash.
(3) A mixture of several manufacturers' 10-10-10 fertilizer, approximately 10.0 per cent potash. This sample had sodium nitrate as an impurity.
(4) A mixture of several manufacturers' 3-9-18 fertilizer, approximately 18.0 per cent potash.

RESULTS

Results on Sample 1 showed good agreement by all three methods. Where methyl alcohol was used (in the studies) in place of ethyl alcohol or Compound 30 alcohol as a wash, in either the A.O.A.C. or the modified Perrin method, values were obtained that were in good agreement with those obtained with the alcohol commonly used. Several collaborators also commented that this was confirmed by results obtained in their laboratories over a period of years. Data are given in Tables 1 and 2.

The flame photometer continues to show promise as a rapid method for the determination of potash in fertilizers, and several collaborators

* Perrin, C. H., *Anal. Chem.*, **21**, 984 (1949), slightly modified.

TABLE 1.—*Per cent of K_2O found by the three methods*

COLLABORATOR	NO. SAMPLES	SAMPLE 1			SAMPLE 2			SAMPLE 3			SAMPLE 4		
		A.O.A.C.	MOD. PERRIN	FLAME PHOTOMETER	A.O.A.C.	MOD. PERRIN	FLAME PHOTOMETER	A.O.A.C.	MOD. PERRIN	FLAME PHOTOMETER	A.O.A.C.	MOD. PERRIN	FLAME PHOTOMETER
1	3	63.31	63.08	—	9.50	9.45	—	9.42	9.46	—	17.55	17.49	—
2	3	63.18	62.83	—	9.59	9.39	—	9.50	9.39	—	17.62	17.48	—
		63.20[a]	61.93[a]		9.48[a]	9.15[a]		9.45[a]	9.13[a]		17.47[a]	17.12[a]	
3	3	63.49	63.13	—	9.21	9.18	—	9.23	9.20	—	17.36	17.21	—
		63.75[a]			9.29[a]			9.33[a]			17.48[a]		
4	3	63.28	63.27	63.27	9.32	9.21	9.44	9.24	9.68	9.23	17.16	16.96	17.23
5	3	62.95	62.85	62.92	9.35	9.28	9.55	9.37	9.31	9.65	17.26	17.24	17.30
9	3	63.40	62.00	—	9.43	9.43	—	9.28	9.28	—	17.60	17.53	—
			59.87[a]			8.38[a]			8.87[a]			16.83[a]	
10	3	62.95	62.91	—	9.33	9.29	—	9.22	9.25	—	17.19	17.19	—
12	3	64.14	63.30	63.50	9.49	9.38	9.40	9.32	9.26	9.22	17.04	17.22	17.15
13	3	62.86	62.71	—	9.30	9.29	—	9.12	9.11	—	17.59	17.19	—
		63.01[a]	62.71[a]		9.24[a]	9.29[a]		9.01[a]	9.09[a]		17.51[a]	17.15[a]	
14	1	63.15	63.07	63.24	9.56	9.52	9.32	9.24	9.54	9.68	17.27	17.35	17.19
					9.55[a]			9.17[a]			17.25[a]		
15	3	63.19	63.32	—	9.58	9.55	—	9.33	9.33	—	17.33	17.30	—
17	3	64.69	64.47	—	9.43	9.65	9.55	9.31	9.34	9.30	17.59	17.91	18.20
		64.67[a]	64.07[a]		9.76[a]	9.65[a]		9.59[a]	9.67[a]		17.49[a]	17.90[a]	
18	3	62.95	62.59	—	9.47	9.34	—	9.35	9.21	—	17.31	17.01	—
		62.93[a]	62.65[a]		9.53[a]	9.45[a]		9.31[a]	9.18[a]		17.44[a]	17.04[a]	
19	3	62.93	62.73	62.64	9.14	9.24	9.56	8.72	9.19	9.31	17.26	17.08	17.78
22	3	63.22	63.11	63.21	9.23	9.05	9.26	9.32	9.30	9.45	17.47	17.08	17.27
23	3	63.27	62.91	63.50	9.46	9.24	10.10	9.46	9.23	9.95	17.57	17.25	18.30
		63.42[a]						9.33[a]			17.57[a]		
24	3	63.10	63.36	62.98	9.36	9.32	9.38	9.49	9.37	9.34	17.37	17.45	17.25
25	3	63.45	63.38	—	9.54	9.51	—	9.67	9.63	—	17.69	17.56	—
26	3	—	62.84	63.10	—	9.44	9.31	—	9.18	9.21	—	17.20	17.14
28	3	63.20	63.26	63.25	9.34	9.38	9.12	9.17	9.24	9.15	17.22	17.26	17.14
29	3	63.37	63.32	63.83	9.39	9.88	9.71	9.26	9.80	10.20	17.23	17.99	17.96
		63.36[a]			9.34[a]			9.23[a]			17.18[a]		
30	3	—	63.27	—	9.56	9.56	—	8.94	9.45	—	—	17.22	—
32	3	63.97	63.30	—	9.41	9.25	—	9.14	9.09	—	17.13	17.09	—
33	3	63.46	63.97	—	9.40	9.34	—	9.22	9.09	—	17.35	17.32	—
		63.46[a]			9.46[a]			9.25[a]			17.34[a]		
34	3	63.20	63.37	63.07	9.44	9.65	9.47	9.38	9.45	9.49	17.40	17.51	17.54
								9.50			17.16[a]		

[a] Methyl alcohol used in place of ethyl alcohol or compound 30 alcohol. Values obtained using methyl alcohol were not included in the averages.

TABLE 2.—*Summary of results*

METHOD	COLLABORATORS REPORTING	SAMPLE 1	SAMPLE 2	SAMPLE 3	SAMPLE 4
Theory	—	63.17	9.0	10.0	18.0
A. O. A. C.	24	63.35	9.40	9.27	17.38
Modified Perrin	25	63.14	9.38	9.33	17.34
Flame photometer	12	63.08	9.49	9.48	17.56

reported that it is being used to screen all their potash samples. The results on Sample 1 were very encouraging since they agreed closely with the theoretical value. The results on the three mixed fertilizers were slightly higher than those obtained by the other two methods. Perhaps this indicates the need for investigating further the effect of the other elements present in fertilizer on the emission intensity of potassium.

COLLABORATORS

(1) H. R. Allen and Elizabeth Swift, Kentucky Agricultural Experiment Station, Lexington, Kentucky.
(2) W. R. Austin and Madalene C. Buford, Armour Fertilizer Works, Nashville, Tennesee.
(3) H. C. Batton and B. H. Craver, Swift & Company, Plant Food Division, Norfolk, Virginia.
(4) R. C. Berry, J. H. Elder, and L. D. McKillop, Virginia Department of Agriculture, Richmond 19, Virginia.
(5) A. T. Blackwell, The Davison Chemical Corporation, Baltimore, Maryland.
(9) R. M. Ludwig, W. N. Simpson, J. A. Layton and J. F. Kilpatrick, The American Agricultural Chemical Company, New York 7, New York.
(10) M. C. Etheredge, *State Chemist*, Mississippi State Chemical Laboratory, State College, Mississippi.
(12) W. C. Geagley, Percy O'Meara, and Virginia A. Thorpe, Bureau of Chemical Laboratories, Michigan Department of Agriculture, Lansing, Michigan.
(13) Harold Gilbertson, State of Minnesota Department of Agriculture, Dairy and Food, St. Paul, Minnesota.
(14) W. B. Griem and D. N. Willett, Wisconsin Department of Agriculture, Madison 6, Wisconsin.
(15) C. Clifton Howes, *Chief Chemist*, Mixed Fertilizer Division, The Davison Chemical Corporation, Nashville 1, Tennessee.
(17) W. T. Mathis, Helen Kocaba, and Richard Botsford, The Connecticut Agricultural Experiment Station, New Haven 4, Connecticut.
(18) R. C. Koch and C. W. Schneider, Swift & Company, Plant Food Division, Hammond, Indiana.
(19) L. A. Koehler and R. T. Ottes, State Laboratories Department, Bismarck, North Dakota.
(22) Ralph D. Miller, Spencer Chemical Company, Pittsburg, Kansas.
(23) W. A. Morgan, E. I. du Pont de Nemours, Inc., Wilmington, Delaware.
(24) Eugene H. Holeman, *State Chemist*, Glenn C. Mowery, and James Lee McClaren, Department of Agriculture, Nashville, Tennessee.
(25) H. L. Moxon, *Chemist in Charge*, and S. B. Nickels, Jr., Virginia-Carolina Chemical Corporation, Richmond, Virginia.

(26) C. H. Perrin and R. M. C. King, Canada Packers Limited, Toronto 9, Canada.
(28) Stacy B. Randle, *State Chemist*, Rutgers University, New Jersey Agricultural Experiment Station, New Brunswick, New Jersey.
(29) Richard M. Smith, State of Florida Department of Agriculture, Chemical Division, Tallahassee, Florida.
(30) L. E. Sommers, Smith-Douglass Company, Inc., Streator, Illinois.
(32) Charles W. Gherke, University of Missouri, Columbia, Missouri.
(33) Harry A. Miller, North Carolina Department of Agriculture, Raleigh, North Carolina.
(34) Stanley J. Kazeniac, Department of Agricultural Chemistry, Agricultural Experiment Station, Purdue University, West Lafayette, Indiana.

COMMENTS OF COLLABORATORS

(1) Evaporation in Perrin method was conducted in Erlenmeyers. It was necessary to reduce sample aliquot to 0.125 g in order to cut down spattering at end of evaporation. We find the Perrin method for mixed fertilizers is more satisfactory if a 0.25 g sample aliquot is used for most samples.

It is noted that the potash salt by the official method was slightly above theoretical value.

(2) All alcohols and Lindo-Gladding held well below 30°C.; all aliquots were drawn at 20°C. No crucible gains over 0.002 g.

(3) As you will note, our results on Sample number 1 exceeded the theoretical figure for muriate of potash by the A.O.A.C. method, using both Formula No. 30 alcohol and synthetic methanol. It may be, of course, that this salt contains some K_2CO_3.

We believe the development of the Perrin method has now reached the point at which it is fully as reliable as the official method.

(4) The Perrin method seems to be adaptable only when quick results are desired on a limited number of samples.

The flame photometer results on Sample 3 are somewhat higher than with the other two methods. This variation is probably due to the presence of some interfering element.

(13) The methyl alcohol used in the determinations was absolute, acetone free, analytical reagent.

(14) Beckman DU spectrophotometer used. Readings were taken with the instrument set at a wavelength of 768 mμ and a slit width of 0.13 mm. with the sensitivity control at nearly counter-clockwise limit. A solution containing 25 p.p.m. K gave a transmission reading of about 50%.

Methanol used in place of ethyl alcohol in A.O.A.C. method. . . . Single determinations only for lack of more aliquots.

(15) It has been noticed in using the Perrin method that high temperature will frequently produce results with a lower trend than the official method. It may be well to emphasize chilling and standing when the final write-up of the Perrin method takes place.

(19) Sample No. 3 is Na-corrected. Samples No. 1, 2, and 4 had no apparent Na interference and no correction.

(22) We have used methyl alcohol for the determination of potash in place of ethyl alcohol for several years. Experimental work indicated that the results were the same with both reagents.

(23) Direct weights of K_2PtCl_6 were used since the precipitate appeared to be completely soluble in hot H_2O. Pt dishes were employed. All results are reported.

The flame photometer used was a 1949 Beckman with #9200 Beckman flame photometer attachment and #9230 acetylene-oxygen burner assembly.

(26) In the case of the flame photometer analyses, the standard solutions used were prepared from a fertilizer sample of known potash content. The internal standard principle was employed.

(28) The flame photometer is working nicely for us. We screen all samples using the flame photometer and check the deficiencies by the A.O.A.C. method. The Perrin method appears to be satisfactory.

(29) Although the Perrin method for potash is a good, rapid method in most instances, there seems to be certain cases where it is not reliable, depending on certain ingredients of the mixture.

Thus far our experience with the Beckman flame photometer for the past year has established this instrument as a good device for the rapid screening of routine samples but insufficiently trustworthy for final analysis on deficient or disputed samples. The samples sent this year for collaborative checking will indicate why.

The curve for translating intensity of light to percentage must be set up for a 10–50 p.p.m. range. This will handle samples with potash values up to 10%. Percentages higher than 10% must be diluted. Any slight error of reading in these small amounts is multiplied several times in bringing the percentage back to original basis.

The Beckman flame photometer gives good results in many instances where percentages do not exceed 20%. On the other hand, there are interfering substances that occur in fertilizer mixtures which cause the flame results to register higher than results by the official method.

The use of methyl alcohol (methanol) seemingly gives little difference in results in the official method.

(33) Practically the same results are obtained using either ethyl or methyl alcohol. Since the fumes of methyl alcohol are harmful, ethyl alcohol is preferred.

More time is consumed by using the rapid wet-digestion method. The use of Kjeldahls makes the transfer of precipitate awkward and tedious.

RECOMMENDATION

It is recommended*—

(1) That the modified Perrin method be adopted as an alternate A.O.A.C. method for potash in fertilizers (Final Action).

(2) That additional studies be made toward adopting the flame photometer for use in the determinations of potash in fertilizers.

ACKNOWLEDGEMENT

The Associate Referee wishes to express his thanks to the many collaborators for their cooperation and comments and to Dr. F. W. Quackenbush and Dr. E. D. Schall for their suggestions and criticisms in the development of this report.

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

REPORT ON INERT MATERIALS IN FERTILIZERS: CARBONATE CARBON OR CALCIUM CARBONATE EQUIVALENT AND ACID-INSOLUBLE ASH

By K. G. CLARK, *Associate Referee*, and V. L. GADDY (Division of Fertilizer and Agricultural Lime, Bureau of Plant Industry, Soils, and Agricultural Engineering, Beltsville, Maryland)

In 1948, the Association of Official Agricultural Chemists appointed an Associate Referee to study methods of possible use for determination of the inert material or filler content of commercial fertilizer mixtures. A report was made to the 1948 meeting of this Association (1), with the recommendation that further study be made of the subject. Subsequently, Clark, *et al.* (2) reported on the calcium carbonate equivalent and acid-insoluble ash contents determined in a survey of 425 commercial fertilizer mixtures marketed in the United States during the 1949–1950 fertilizer season.

This report presents the results of collaborative studies on methods for determination of calcium carbonate equivalent and acid-insoluble ash.

SAMPLES

The formulation of the samples submitted to the collaborators is given in Table 1, which also shows the $CaCO_3$ equivalent and acid-insoluble ash contents of the ingredient materials. Table 2 shows the estimated $CaCO_3$ equivalent and acid-insoluble ash contents of the samples based on the data of Table 1.

COLLABORATORS' DIRECTIONS

(1) Prepare samples as directed in *Methods of Analysis*, A.O.A.C., 7th Ed., 1950, p. 6, sec. **2.2.**

(2) Determine (*a*) carbonate carbon or $CaCO_3$ equivalent and (*b*) acid-insoluble ash on the prepared samples in accordance with the following methods.

TABLE 1.—*Formulation of samples for collaborative study of calcium carbonate equivalent and acid-insoluble ash contents*

MATERIAL	$CaCO_3$ EQUIV.	ACID INSOLUBLE ASH	SAMPLE NUMBER AND GRADE				
			1	2	3	4	5
			0-20-0	2-12-6	3-12-12	4-8-6	5-10-5
	per cent	*per cent*	*lbs/ton*	*lbs/ton*	*lbs/ton*	*lbs/ton*	*lbs/ton*
Ammonium sulfate	—	0.01	—	—	—	175	220
Ammoniated superphosphate	—	4.54	—	780	1165	865	1080
Normal superphosphate	—	5.11	2000	465	120	—	—
Potassium chloride	—	1.09	—	217	428	217	180
Dolomite	104.52	2.93	—	300	100	200	150
Sand	—	99.80	—	188	137	493	320
Peanut hull bran	—	4.38	—	50	50	50	50
Total	—	—	2000	2000	2000	2000	2000

TABLE 2.—*Estimated calcium carbonate equivalent and acid-insoluble ash contents of samples submitted to collaborators*

MATERIAL	SAMPLE NUMBER AND GRADE				
	1	2	3	4	5
	0-20-0	2-12-6	3-12-12	4-8-6	5-10-5
Calcium carbonate equivalent, per cent					
Dolomite	—	15.68	5.23	10.45	7.84
Acid-insoluble ash, per cent					
Ammonium sulfate	—	—	—	0.001	0.001
Ammoniated superphosphate	—	1.77	2.64	1.96	2.45
Normal superphosphate	5.11	1.19	0.31	—	—
Potassium chloride	—	1.18	2.33	1.18	0.98
Dolomite	—	0.44	0.15	0.29	0.22
Sand	—	9.38	6.84	24.60	15.97
Peanut hull bran	—	0.11	0.11	0.11	0.11
Total	5.11	14.07	12.38	28.14	19.73

METHODS

I. *Carbonate Carbon or Calcium Carbonate Equivalent*

APPARATUS

250 ml Knorr alkalimeter with guard tube filled with Ascarite (NaOH-absestos absorbent mixture), and condenser outlet connected to a CO_2 absorption train. The absorption train consists of four U-shaped glass-stoppered drying tubes, or the equivalent. The first tube removes acidic gases other than CO_2 and is charged with Ag_2SO_4-satd H_2SO_4 (1 +1) to a depth of ca ½″ above the bend. The second tube is filled with anhyd. $MgClO_4$ for moisture removal. The inlet two-thirds of the third and fourth tubes is filled with Ascarite for CO_2 absorption, and the outlet third with anhyd. $MgClO_4$. The fourth or guard tube of the train is connected with an aspirating bottle or suction source.

DETERMINATION

Aspirate a slow stream of air through the assembled apparatus until the first CO_2 absorption tube has reached a constant wt. Close off the train, remove the dry alkalimeter flask, place 5 g of fertilizer therein, replace the flask, and fill the dropping funnel with 50 ml HCl (1 + 4). Slowly add the acid to the sample while aspirating. After the reaction has substantially subsided, gently heat the flask until the soln boils, and continue boiling for 2–3 min. Discontinue heating and continue aspirating the system for 20 min. Close off the system, remove the first CO_2 absorption tube, and hang in a balance case until cool enough to weigh (usually 20–30 min.). Using a standardized procedure, wipe the tube with a dry lint-free cloth and weigh against a similarly packed tare. The increase in wt is due to CO_2. Calc. and report the result as $CaCO_3$ equivalent.

II. *Acid-insoluble Ash*

DETERMINATION

Weigh and place 5 g of fertilizer in a 250 ml beaker. Add 50 ml of hot H_2O (98–100°C.), swirl contents of beaker several times, decant, and filter the liquid (12.5 cm Whatman No. 40, or equivalent rapid filtering paper). Add 100 ml HCl (1 + 4) to residue in beaker, cover beaker with watch glass, and boil for 20 min. Decant soln thru original filter paper and transfer insoluble residue to filter with a stream of H_2O. Place folded filter paper contg residue in porcelain crucible and ignite in muffle furnace at 800°C. for 1 hr. Cool, transfer contents of crucible to original beaker, add 25 ml HCl (1 + 4), cover, and boil for 15–20 min. Filter thru a tared Gooch crucible contg an acid-washed asbestos mat on a filter paper disk. Wash the insoluble residue several times with H_2O, dry crucible 1 hr at 125°C., cool in a desiccator, and weigh. Report net increase in wt of crucible as % acid-insoluble ash.

NOTES

(*a*) Individual results for each of three replicate detns of the carbonate carbon and acid-insoluble ash contents of the samples are desired.

(*b*) In case the carbonate carbon content is determined other than in the manner described above, please indicate the equipment and reagents used. *Methods of Analysis*, 6th Ed., 1945, p. 208, sec. 17.2 and earlier editions describe procedures for the gravimetric detn of the CO_2 content of baking powders and baking chemicals with the use of liquid absorbents and reagent H_2SO_4 rather than solid absorbents and reagent HCl.

(*c*) Your comments and observations concerning the proposed analytical procedures and suggestions for their improvement are requested.

COLLABORATORS

(1) R. D. Caldwell, Armour Fertilizer Works, Atlanta, Georgia.
(2) S. J. Few, Mississippi State Chemical Laboratory, State College, Mississippi.
(3) V. L. Gaddy, Division of Fertilizer and Agricultural Lime, Bureau of Plant Industry, Soils, and Agricultural Engineering, Beltsville, Maryland.
(4) Gordon Hart, Chemical Division, Florida Department of Agriculture, Tallahassee, Florida.
(5) W. M. Hoffman, Division of Fertilizer and Agricultural Lime, Bureau of Plant Industry, Soils, and Agricultural Engineering, Beltsville, Maryland.
(6) J. B. LeClair, Bureau of Chemistry, California Department of Agriculture, Sacramento, California.
(7) F. O. Lundstrom, Division of Fertilizer and Agricultural Lime, Bureau of Plant Industry, Soils, and Agricultural Engineering, Beltsville, Maryland.

COMMENTS OF COLLABORATORS

CARBONATE CARBON OR CALCIUM CARBONATE EQUIVALENT

Collaborator 1.—We have never made CO_2 analyses and did not have the equipment needed.

Collaborator 3.—A Stetser-Norton bulb was used for CO_2 absorption. With the 5 g sample specified it was necessary to agitate the alkalimeter flask to insure adequate contact between the sample and the acid.

Collaborator 4.—Two H_2SO_4-$AgSO_4$ guard tubes were used in the determination. A Stetser-Norton tube rather than a U-tube was used for CO_2 absorption.

Collaborator 5.—Samples were ground to pass a 35-mesh sieve before analysis. No difficulties were encountered with either method.

Collaborator 6.—Prior to analysis the samples were ground to less than 20-mesh

TABLE 3.—*Calcium carbonate equivalent*

COLLABORATOR	SAMPLE NUMBER AND GRADE				
	1	2	3	4	5
	0-20-0	2-12-6	3-12-12	4-8-6	5-10-5
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
2	0.00	14.23	5.45	9.55	7.37
	0.00	14.23	5.80	9.32	7.34
	0.00	14.28	5.45	9.77	7.28
Av.	0.00	14.28	5.57	9.55	7.33
3	0.33	14.64	5.02	9.48	7.35
	0.16	14.64	5.29	9.64	7.44
	—	14.58	5.17	9.53	7.27
Av.	0.25	14.62	5.16	9.55	7.35
4	0.04	13.97	4.75	10.00	7.44
	0.08	14.02	4.21	9.62	7.23
	—	14.03	4.98	9.73	7.84
Av.	0.06	14.01	4.65	9.78	7.50
5	0.03	14.27	5.45	9.18	7.55
	0.03	14.25	5.41	9.27	7.53
Av.	0.03	14.26	5.43	9.23	7.54
6	0.11	14.62	5.39	10.19	7.48
	0.23	14.65	5.32	10.21	7.51
	0.18	14.58	5.37	10.14	7.64
	0.14	—	—	—	—
Av.	0.22	14.62	5.36	10.18	7.54
7[a]	0.02	14.56	5.14	9.57	7.60
	0.05	14.37	5.09	9.46	7.82
	0.00	14.35	5.16	9.37	7.64
	—	14.58	—	9.46	7.87
	—	14.62	—	9.26	7.89
	—	—	—	—	7.89
Av.	0.02	14.50	5.13	9.42	7.79
Group Av.	0.09	14.40	5.20	9.62	7.55
Observed Range	0–0.25	14.01–14.62	4.65–5.57	9.23–10.18	7.33–7.79
Estimated Value	0	15.68	5.23	10.45	7.84
L.S.D.[b] P=0.01	NS	0.15	0.57	0.37	0.37

[a] Two-g rather than 5-g samples were used.
[b] When necessary, missing values were estimated to insure applicability.

TABLE 4.—*Acid-insoluble ash*

COLLABORATOR	SAMPLE NUMBER AND GRADE				
	1	2	3	4	5
	0-20-0	2-12-6	3-12-12	4-8-6	5-10-5
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
1	5.18	13.91	10.57	28.16	21.65
	5.15	13.93	10.56	28.25	21.63
	5.22	13.79	10.70	28.13	21.68
Av.	5.18	13.88	10.61	28.18	21.65
2[a]	5.25	13.35	10.60	26.89	20.33
3	5.32	13.98	10.90	27.89	20.70
	5.22	13.71	10.45	28.89	21.30
	5.24	13.47	10.39	28.61	20.91
Av.	5.26	13.72	10.58	28.46	20.97
4[b]	5.22	11.37	10.54	29.17	20.59
	5.28	14.97	10.60	28.62	20.30
	5.20	13.50	10.47	28.16	20.62
	5.16	13.62	10.21	27.53[c]	21.05
Av.	5.22	13.37	10.46	28.65	20.64
5	5.30	14.20	10.34	27.77	20.68
	5.04	14.40	10.23	27.80	20.61
	5.01	14.25	10.57	27.66	20.67
Av.	5.12	14.28	10.38	27.75	20.65
6	5.14	13.08	10.41	25.95	19.89
	5.17	13.22	10.30	26.10	19.89
	5.08	13.16	10.24	25.94	20.13
Av.	5.13	13.15	10.32	26.00	19.97
7	5.29	13.46	10.93	28.00	21.89
	5.31	13.31	10.76	27.73	21.54
	5.23	13.54	10.80	28.21	21.33
Av.	5.28	13.44	10.83	27.98	21.59
7[d]	5.25	13.75	10.88	28.56	22.05
	5.16	13.53	10.93	28.30	22.10
	5.25	13.69	10.91	28.34	21.96
Av.	5.22	13.66	10.91	28.40	22.04
Group Av.	5.20	13.62	10.52	27.84	20.90
Observed Range	5.12–5.28	13.15–14.28	10.32–10.83	26.00–28.65	19.97–21.65
Estimated Value	5.11	14.07	12.38	28.14	19.75
L.S.D. P=0.01	NS	NS	0.35	0.79	0.52

[a] Results not included in analysis of variance.
[b] Fourth result for all 5 samples reported omitted from analysis of variance.
[c] Sample bumped and this value is omitted from the average.
[d] Samples digested for 2 hrs in (1+4) HCl on steam bath at 100°C. prior to ignition, and for 1 hr after ignition. Results not included in group average or analysis of variance.

in a Mikro-Samplmill fitted with a 1 mm screen. No criticism is made of either procedure.

Collaborator 7.—Two g rather than 5 g samples were used in all CO_2 determinations to (*a*) reduce the period required for complete absorption of the CO_2 at the usual rate of aspiration, and (*b*) avoid too frequent replacement of the absorbent in the absorption tubes. To conserve time, aspiration of air through the assembled apparatus was omitted between successive determinations on the same day.

ACID-INSOLUBLE ASH

Collaborator 3.—Serious bumping was observed during the acid digestion following ignition of the sample. A smaller sample might decrease this tendency.

Collaborator 4.—Sample No. 4 bumped during the second digestion on the hot plate. The initial treatment with hot H_2O could be omitted, making digestion with acid the first step in the analysis.

Collaborator 7.—Serious bumping occurred and constant attention was required when the samples were boiled with (1 + 4) HCl. These disadvantages were overcome and substantially the same results were obtained by a 2 hour digestion period on a steam bath at 100°C. before ignition and 1 hour after.

DISCUSSION OF RESULTS

Carbonate Carbon or Calcium Carbonate Equivalent.—Differences in the average values of the calcium carbonate contents reported by the collaborators were statistically significant except in the case of Sample 1, superphosphate, as shown in Table 3. In general, the results reported for Samples 2 and 4 which contained moie than 10 per cent calcium carbonate equivalent were appreciably lower than the estimated values. The results reported for the other samples, however, were generally in satisfactory agreement with the estimated values. It would appear that the method should be modified to provide a weight of sample such that the evolution of carbon dioxide would not exceed approximately 175–200 mg.

Acid-insoluble Ash.—As shown in Table 4, statistically significant differences occurred in the results reported by the several collaborators for Samples 3, 4, and 5. The values reported for Samples 2 and 3 in general were lower than the formulated values. For Samples 4 and 5 the reported values in some cases were appreciably higher and in others appreciably lower than values based on the formulation. It appears that the method should be modified to decrease the sample size and to simplify the digestion procedures prior to ignition.

RECOMMENDATION

It is recommended* that work on carbonate carbon or $CaCO_3$ equivalent and acid-insoluble ash be continued.

REFERENCES

(1) CLARK, K. G., RADER, L. F., JR., and WALLS, H. R., *This Journal*, **32**, 691–697 (1949).

* For report of Subcommittee A and action of the Association, see *This Journal*, **36**, 49 (1953).

(2) CLARK, K. G., GADDY, V. L., BLAIR, A. E., and LUNDSTROM, F. O., *Off. Pub. Assoc. Amer. Fert. Control*, Off. No. 5, 51–60 (1951); *Farm Chemicals*, 115 (6), 21, 23–26 (1951).

No reports were received on: free water, acid- and base-forming quality, sulfur, copper, and zinc.

REPORT ON COFFEE

By SABINO T. COLAMARIA (Department of Health, Education, and Welfare, Food and Drug Administration, Boston 10, Massachusetts), *Referee*

Your Referee has made a search of the literature for chemical methods of detection of adulterants in coffee. Little work seems to have been done. Lepper* has suggested that methods be developed for the determination of chlorogenic acid as an index of the degree of bitterness of coffee. An official method for the determination of moisture in coffee is also needed. Two Associate Referees have been appointed to study these problems and both have submitted reports embodying suggested methods for the determination of these two constituents.

RECOMMENDATIONS

It is recommended†—

(1) That methods of sample preparation of coffee be studied further as suggested by the Associate Referee on chlorogenic acid in coffee.

(2) That the method proposed by the Associate Referee on chlorogenic acid be submitted to collaborative study.

(3) That the method proposed by the Associate Referee on moisture in roasted coffee be submitted to collaborative study.

(4) That the method for the determination of moisture in green coffee be studied as recommended by the Associate Referee.

REPORT ON MOISTURE IN COFFEE

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

The methods for moisture in coffee in the Sixth Edition of *Methods of Analysis* (1) for drying in ovens or *in vacuo* have been in use for many years (2) although they were never subjected to collaborative study. It was perhaps this absence of collaborative work which led Committee C to delete them from the Seventh Edition of *Methods of Analysis* and to recommend that more modern methods be developed.

* *This Journal*, 33, 523 (1950).

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

Three methods for moisture determination were considered by the Associate Referee: the oven method, the distillation method, and the Karl Fischer reagent method (3).

Although the Karl Fischer method is a very rapida nd useful method, in routine work, it does not lend itself very readily to the occasional user because the reagent has many drawbacks. It is expensive and unpleasant to prepare; it deteriorates rapidly and therefore requires frequent standardization.

Loomis (4) discussed moisture methods for coffee and compared various oven and vacuum methods at different temperatures. It was therefore decided that a comparative study of the air oven method and the toluene distillation method would be made in this laboratory.

METHOD

The oven method was essentially the same as that in the Sixth Edition except that covered metal dishes were used. A series of determinations was run on a standard brand of coffee for various lengths of time and the results are shown in Table 1.

TABLE 1.—*Results by air oven method at 105°C.*

TIME, HOURS	MOISTURE
	per cent
	2.34
2	2.37, 2.35
	2.51
4	2.56, 2.54
	2.58
5	2.58, 2.58
	2.59
6	2.60, 2.60
	2.62
8	2.57, 2.60

In the toluene distillation method, the difficulty of drops of water clinging to the condenser and sides of the receiving tube and the formation of emulsions was eliminated by a final rinse of the glassware with 0.5 *N* alcoholic KOH (5, 6).

Table 2 shows a comparative study of the two methods on 5 standard brands of roasted percolator ground coffees.

In all the above determinations, although the recovery of moisture was usually complete in 1½ to 2½ hrs, the coffee was refluxed for a minimum of 3 hours.

The method for air oven drying was that of *Official Methods of Analysis*, 18.5, (1): Dishes used were ca 70 mm in diam. and 30 mm in depth, with tight fitting covers. For the distillation procedure, method 28.2 (7) was used. A minimum of 100 g of sample and a one l distilling flask were used. All glass joints were standard taper.

TABLE 2.—*Results by toluene distillation and air oven at 105°C.*

BRANDS	PER CENT MOISTURE	
	TOLUENE DISTILLATION	AIR OVEN AT 105°C.
A	2.52 2.54, 2.53	2.34 2.34, 2.34
B	2.80 2.79, 2.80	2.71 2.73, 2.72
C	2.08, 2.08	1.80 1.80, 1.80
D	2.50 2.53, 2.52	2.33 2.33, 2.33
E	1.73 1.76, 1.75	1.72 1.70, 1.71

RECOMMENDATIONS

It is recommended*—

(1) That the above methods for determining moisture in roasted coffee be subjected to collaborative study.

(2) That the determination of moisture in green coffee be studied.

REFERENCES

(1) *Official Methods of Analysis*, 6th Ed., Association of Official Agricultural Chemists, Washington, D. C., 1945, 18.5.
(2) *Official and Provisional Methods of Analysis*, Bulletin 107, Association of Official Agricultural Chemists, Washington, D. C., 1908.
(3) MITCHELL, J., *Anal. Chem.*, **23**, 1058 (1951).
(4) LOOMIS, H. M., *This Journal*, **3**, 498 (1920).
(5) ALFEND, S., *ibid.*, **24**, 675 (1941).
(6) DE LOUREIO, J. A., *ibid.*, **21**, 645 (1938).
(7) *Official Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Washington, D. C., 1950, **28.2**.

REPORT ON CHLOROGENIC ACID IN COFFEE

By LOUIS C. WEISS (Department of Health, Education, and Welfare, Food & Drug Administration, Los Angeles 15, California),
Associate Referee

Lepper (1) has pointed out that need for a method of determining chlorogenic acid, a major constituent of coffee, has arisen as a result of advertising claims made concerning the alleged effect of this substance on the flavor of the beverage. A review of the published methods for the

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

analysis indicated that the ultraviolet absorption method of Moores, *et al.* (2), was the most promising both as to accuracy and ease of manipulation. It consists of the preparation of a water extract of the ground coffee and the determination of the light absorption of the solution at 324 mμ. Green coffees must be dried and defatted prior to extraction. The absorption of roasted coffee extracts must be determined both before and after precipitation of the chlorogenic acid by basic lead acetate. The following is essentially the method of Moores and his co-workers with certain modifications.

METHOD

APPARATUS

Glass stirrers.—Prepare special glass stirrers for use with mechanical stirrers. The shaft must be long enough to reach to the bottom of a 200 ml Pyrex volumetric flask and yet be gripped by the chuck of the stirrer. A button of such size that it will pass the neck of the flask should be fastened to the bottom of the shaft by heating until soft and pressing on a flat surface. Radial flutes can be fashioned by reheating the button and pressing with the edge of a 3-cornered file.

REAGENTS

(a) *Petroleum ether.*—Either the 20–40°C. or 30–60°C. reagent grade is suitable.

(b) *Potassium acetate solution.*—Prepare a satd soln from reagent grade crystals.

(c) *Basic lead acetate solution.*—Sp. gr. 1.25. Soln 29.121 may be used, or a soln may be prepared from reagent grade dry powder.

PREPARATION OF SAMPLE

Grind sample to pass through 30-mesh sieve and store in tightly-stoppered bottle. Avoid overheating during grinding as this may result in destruction of part of the chlorogenic acid.

DETERMINATION

Weigh a 1 g portion of ground green coffee into a 50 ml centrifuge tube. Add 25 ml of per. ether, mix thoroly, centrifuge, and decant the supernatant liquid. Repeat this extn twice more. Dry the residue in a gentle stream of air and transfer to a 500 ml Erlenmeyer flask with a small amount of H_2O. Roasted coffee need not be defatted but may be weighed and transferred directly into a 500 ml flask. Add ca 400 ml of boiling H_2O to the flask, reheat quickly to boiling, and continue to boil gently for 15 min. Cool quickly under the tap to room temp, transfer to a 500-ml volumetric flask and make to vol. Filter thru retentive filter paper, discarding first 25–50 ml of filtrate. If the filtrate is cloudy, it will be necessary to filter with suction thru a fine porosity fritted glass disk. Filter aids may not be used.

Transfer 10 ml of filtrate to a 100 ml volumetric flask and dil. to vol. with H_2O. Det. the absorbance of the soln at 324 mμ.

The following additional procedure is necessary for the analysis of roasted coffee extracts:

Transfer 100 ml filtrate to a 200 ml Pyrex volumetric flask. Add 2 ml satd K acetate soln and 10 ml of basic Pb acetate soln with swirling. Place flask in boiling H_2O bath for 5 min., remove, cool under the tap, and then place in an ice H_2O bath. Stir mechanically for 1 hr with flask continually immersed in the ice H_2O bath. Remove from bath, wash down stirrer, bring the mixt. to room temp. and dil. to vol. with H_2O. Filter thru a fluted filter paper. Discard the first 25–50 ml of filtrate and collect ca 25 ml. Det. the absorbance of the soln immediately without diln at 324 mμ.

The amount of chlorogenic acid present may be calcd using an $A^{1\%}_{1cm}$ value of 526 for anhydrous chlorogenic acid. A more convenient factor is 0.01901 which, when multiplied by the absorptivity of the soln at 324 mμ gives the concn of chlorogenic acid in mg/ml.

$$\text{Chlorogenic Acid (mg/ml)} = \frac{\text{absorbtivity}}{526} \times 0.01 \times 1000 = \text{absorbtivity} \times 0.01901$$

The concn thus found in the filtrate from the Pb treatment must be corrected for the solubility of lead chlorogenate by deducting 0.002 mg/ml. To obtain the true concn in the 1 + 9 diln of the roasted coffee extract, one-fifth of the corrected concn in the Pb filtrate (itself a 1 +1 diln) is subtracted from the apparent concn in the untreated soln.

EXPERIMENTAL

On attempting to prepare a saturated solution of basic lead acetate from C. P. dry powder as described by Moores, a concentration of over 1.4 g of the salt per ml of solution was attained without saturation. This appeared to provide entirely too great an excess of lead over that required to precipitate the chlorogenic acid. The solution commonly used in sugar work was substituted and has been satisfactory. The absorbance of the lead-containing solution must be determined quickly after filtration as a precipitate of lead carbonate soon forms. This could be avoided by acidifying with acetic acid but would add an unnecessary step to the procedure.

Moores recommends milling the coffee samples to a particle thickness of 50–100 μ on a chocolate or cereal flaking mill, followed by extraction of the chlorogenic acid with cold water. Inasmuch as such mills are relatively rare, it was decided to seek an alternative method of size reduction and extraction that would be equally effective, but employ more generally available equipment. Results are shown in Table 1.

A sample of a green Ecuador coffee was selected for this work. One portion was repeatedly passed through a laboratory burr mill until it all

TABLE 1.—*Extraction of chlorogenic acid from ground green coffee*

SAMPLE NO.	SIEVE SIZE	PER CENT CHLOROGENIC ACID
1	− 30	8.16
2	− 30	8.12
3	− 30	8.11
4	− 30	8.15
5	− 30	8.12
6	− 30	8.12
7	−150	7.97
8	− 30	7.97
9	− 30	7.91
10	− 30	7.95
11	− 30	7.93
12	− 30	7.92

passed through a 30 mesh sieve. Another portion was ground in a laboratory hammer-type mill in a single pass to −150 mesh. Portions of each of these materials were dried *in vacuo* at 70° for 15 hours. Samples were then weighed and analyzed for chlorogenic acid by the above method. The results are presented in Table 1.

From this it would seem that more efficient extraction was obtained from the more coarsely ground material. A more likely explanation is that local and general heating effects during the grinding of the fine material may have resulted in a destruction of part of the chlorogenic acid. Further investigation of this phenomenon must be made. Considerable heat was generated during the grinding of the coffee in each of two impact mills (Raymond and Mikro Samplmill), even when dry ice was fed with the beans. Multiple passes with progressively finer screens rather than a single pass through the finest screens might reduce the heating.

The difficulty in attaining the degree of size reduction recommended by Moores suggested that a more severe extraction of less finely ground material might give comparable results. A sample of pure chlorogenic acid in water (0.1572 mg/ml) was refluxed continuously for several hours and samples were withdrawn at intervals for analysis. A similar experiment was performed with a portion of −30 mesh green coffee. The data obtained are presented in Fig. 1.

From this it is evident that there is no appreciable destruction of pure chlorogenic acid during the first 30 minutes under reflux conditions. Also,

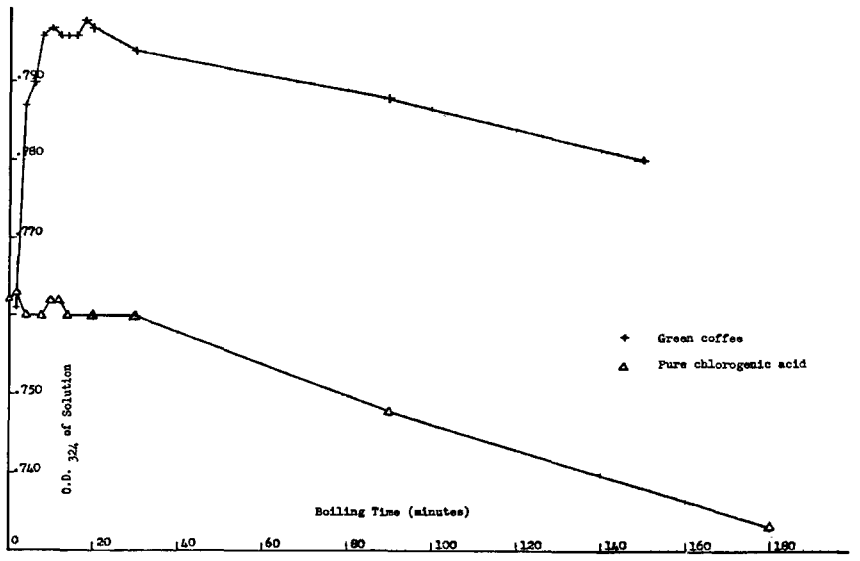

FIG. 1.—Effect of boiling on efficiency of extraction of chlorogenic acid and on its decomposition. + Green coffee; △ Pure chlorogenic acid.

under similar conditions the concentration of chlorogenic acid extracted from green coffee reaches a maximum in about 10 minutes and remains essentially constant for an additional 10 minutes. Thus it appears feasible to submit ground coffee to a 15 minute extraction with boiling water followed by rapid cooling to room temperature.

Several samples of ground green coffees were analyzed for chlorogenic acid by both cold and hot extraction techniques. The results are shown in Table 2.

TABLE 2.—*The effectiveness of hot water and cold water extraction of chlorogenic acid from ground green coffees*

SAMPLE NO.	SIEVE SIZE	PER CENT CHLOROGENIC ACID (DRY BASIS)	
		COLD EXTRACTION	HOT EXTRACTION
13	− 30	7.71	7.93
14	−150	7.74	8.02
15	−30, +100	8.13	8.30
16	−100	7.49	7.62

These data clearly demonstrate that hot water extraction recovers a significantly greater amount of chlorogenic acid than cold water extraction.

Roasted coffee extracts, unlike green coffee extracts, contain substances other than chlorogenic acid which exhibit appreciable absorption at 324 μ. To compensate for this, absorbance readings are obtained on roasted coffee extracts before and after precipitating the chlorogenic acid with basic lead acetate.

Extensive experiments were conducted with ion exchange resins with a view to eliminating the lead precipitation step. Chlorogenic acid is readily removed from solution by anion exchangers, but no method of quantitatively regenerating the acid was found. Acids and salts do not displace all the chlorogenic acid; it is decomposed by alkalis above *p*H 9.9. The chlorogenic acid content of roasted coffee extracts can be determined by measuring the absorbance before and after passage through a column of anion exchanger. The process, however, is more time-consuming than the lead precipitation method, but does serve to confirm its specificity.

A portion of a roasted coffee extract was subjected to lead precipitation and another to deionization with an anion exchange resin. The filtrate from the lead treatment was deleaded with hydrogen sulfide and the excess removed with nitrogen gas. Absorbancies of all these solutions were determined at various wavelengths, and the curves obtained are shown in Fig. 2. The similarity of the curves of the two solns receiving widely different treatment strongly indicates that the same constituent or constituents are being removed by each process. Since the resin (Am-

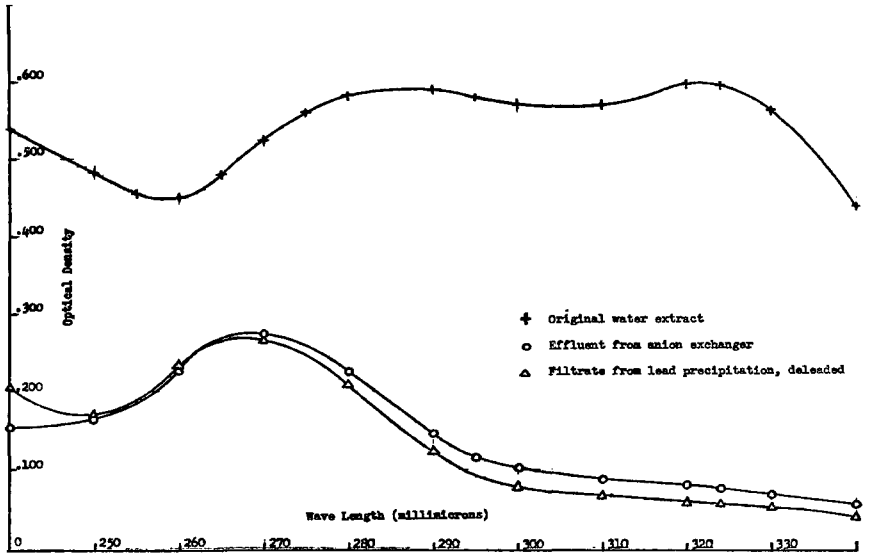

FIG. 2.—Effect of removal of chlorogenic acid on ultraviolet absorption of roasted coffee extract. + Original water extract; ○ Effluent from anion exchanger; △ Filtrate from lead precipitation, deleaded.

berlite IR-4B) used was a weakly basic exchanger, only relatively strong acids could be removed. This eliminates phenols and tannins as constituents of the lead precipitate, which have appreciable absorption at 324 mμ.

Table 3 contains the results of analysis of a roasted coffee by both methods.

TABLE 3.—*Analysis of roasted coffee for chlorogenic acid by lead precipitation and ion exchange techniques*

SAMPLE NO.	PER CENT CHLOROGENIC ACID	
	BY LEAD PRECIPITATION	BY ANION EXCHANGE
17	5.34	5.18
18	5.38	5.25
19	5.32	5.41
20	5.31	5.44

Two analyses of the same roasted coffee, using a hot water extraction followed by lead precipitation, gave chlorogenic acid values of 5.60 per cent and 5.51 per cent, demonstrating the greater effectiveness of this method of extracting the substance from roasted as well as green coffee.

Three commercial coffee concentrates purchased from stock in a local

market were analyzed by the proposed method. Two were dry powders, one containing added dextrose and dextrins, the other only coffee extractives. The third was a frozen liquid. Clear solutions were obtained with each in cold water, so no heating or filtration was required. For purposes of comparison, the results were calculated to the chlorogenic acid concentration in a normal cup of coffee beverage prepared from each according to directions. The figure for brewed coffee was calculated, assuming a chlorogenic acid content of 5 per cent in the ground coffee and the preparation of 36 cups ($\frac{1}{2}$ pint each) from one pound. The results are shown in Table 4.

TABLE 4.—*Chlorogenic acid content of coffee beverages prepared from ground coffee and coffee concentrates*

PRODUCT	APPROXIMATE CHLOROGENIC ACID CONCENTRATION IN CUP (MG/ML)
Powder, coffee extractives only	0.53
Powder, with added dextrose and dextrins	0.67
Liquid, concentrated extractives only	1.23
Brewed coffee	2.67

All optical measurements in this work were made with a Beckman DU spectrophotometer, using 1 cm quartz cells and a slit width of approximately 0.54 mm.

DISCUSSION

Further study must be made of methods of grinding the samples, both green and roasted whole beans and commercially ground roasted coffee. Because of apparent destruction of chlorogenic acid during grinding, the effect of particle size on efficiency of extraction is obscured. The superiority of hot water extraction to cold water extraction appears definitely established. Additional work should be done to confirm the specificity of the method for chlorogenic acid, particularly when applied to coffee concentrates.

RECOMMENDATIONS

It is recommended*—

(1) That methods of sample preparation be studied further.
(2) That the proposed method be submitted to collaborative study.
(3) That the topic be continued.

ACKNOWLEDGEMENT

The Associate Referee wishes to thank Dr. R. G. Moores of the General Foods Corporation, who kindly supplied the two samples of pure chlorogenic acid which were used in this work.

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

REFERENCES

(1) LEPPER, H. A., *This Journal*, **33**, 523 (1950).
(2) MOORES, R. G., MCDERMOTT, D. L., and WOOD, T. R., *Anal. Chem.*, **20**, 620 (1948).

REPORT ON ALCOHOLIC BEVERAGES

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

The report on the collaboration between the A.O.A.C. and the A.S.B.C., and the reports on phosphates and tannins, on trace elements in beer, and on non-volatile acids in wines need no extensive comment. Certain subjects recommended for study last year by the A.O.A.C., *viz.*, those on color and turbidity in beer and degassing of beer, are being conducted by the A.S.B.C. and arrangements will be made to see that the results are made available to readers of the A.O.A.C. publications. It is your Referee's understanding that this is one of the chief functions of the liaison Associate Referee. Mr. Tenney, who served in this capacity for the past year, has demonstrated the need for such an official.

No work was conducted on the application to cordials and liqueurs of the method for citric acid made official last year for fruits and fruit products. We are continuing this recommendation and expect to do more collaborative work on it. Members of the Laboratory Division of the Alcoholic Tax Unit advised the Referee that they have a problem in connection with color in berry wines which involves both the evaluation of the color of different lots and the detection of foreign coloring matter which makes the wines appear better or of greater value than they are. We are including a recommendation for work along this line next year.

The Referee's recommendations for work next year, including those made by the Associate Referees, follow:

It is recommended*—

A. MALT BEVERAGES, BREWING MATERIALS AND ALLIED PRODUCTS

(1) That the study of methods for the determination of essential oils and resins in hops be continued.

(2) That the method for the determination of yeast-fermentable sugar in beer by A. P. Mathers and J. E. Beck be studied collaboratively.

(3) That work on methods for turbidity and color in beer and for the degassing of beer be discontinued, as the A.S.B.C. is working on these methods.

(4) That the study of the wet-ash *o*-phenanthroline method for iron be continued and be submitted to collaborative study after completion.

* For report of Subcommittee D and action of the Association, see *This Journal*, **36**, 60 (1953).

(5) That the direct, non-ash *o*-phenanthroline procedure for iron adopted as First Action last year be further studied by the Associate Referee.

(6) That collaborative studies on copper be postponed until work on iron has been completed, or present work on copper in the A.S.B.C. is completed.

(7) That the methods of Stone and of Kuznetsov and Bender for the determination of tin be studied.

(8) That collaborative work on tin be postponed until work on iron and copper has been completed.

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

(10) That collaborative studies of methods for the determination of ash, ether extract, crude fiber, and crude carbohydrate in yeast be postponed until the studies on moisture, total nitrogen, and P_2O_5 are completed.

B. WINES

(1) That collaborative work be continued on the method for the determination of non-volatile acids in wines by paper chromatography.

(2) That collaborative work be continued on the first action spectrophotometric method for determining phosphates in wines and spirits.

(3) That collaborative work be continued on the spectrophotometric method for tannin in wines and whiskeys.

(4) That work be conducted on the determination of color in wines.

C. DISTILLED LIQUORS

(1) That the official method, **9.29,** for methanol by the immersion refractometer method be studied in the light of the findings of Beyer and Reeves.*

(2) That study be continued on the methods for methanol in distilled liquors and drugs with a view to their correlation.

(3) That the investigation of methods for the determination of higher alcohols in distilled spirits by chromatography be continued.

D. CORDIALS AND LIQUEURS

(1) That the method for citric acid in fruits and fruit products, *This Journal,* **34,** 74 (1951), be studied collaboratively on samples of cordials and liqueurs.

* *This Journal,* **28,** 800 (1945).

REPORT ON ACTIVITIES OF THE AMERICAN SOCIETY OF BREWING CHEMISTS

By ROBERT I. TENNEY (Wahl-Henius Institute, Chicago, Ill.), *Associate Referee*

The collaborative studies conducted by the American Society of Brewing Chemists have been of considerable help to the A.O.A.C. in the development of methods which are capable of inter-laboratory agreement. This group draws its members from the brewing and malting industries, including suppliers of other commodities used by brewers. Its interest in analytical precision parallels our own and its members have given generously of laboratory time in collaborative tests. The A.S.B.C. *Book of Methods* has, with editorial changes, been largely adopted within the present A.O.A.C. *Methods of Analysis.*

The most active cooperation between the two societies is currently centered in studies of trace elements in beer. Mr. A. L. Brandon of Anheuser-Busch, Inc., is the Associate Referee for this topic and is also chairman of the A.S.B.C. committee studying this problem. This committee is comprised of the collaborators for the A.O.A.C. work and has contributed original research as well. A report on this work appears elsewhere on the program of this meeting.

Mr. Brandon is also Associate Referee concerning yeast analyses and has drawn many of his collaborators from the A.S.B.C. membership. The dual interests served are of distinct advantage to both groups.

Because of questions relating to the fundamental accuracy of chemical analyses of hops, and due to lack of generally accepted interpretive policies, work on hops in both societies has been devoted chiefly to physical tests. Both societies are awaiting further results from investigators in this field and are not now planning collaborative studies. The present A.O.A.C. Methods are also those of the A.S.B.C. and are capable of good agreement among laboratories. The methods are lengthy, however, and have been subjected to much criticism by those who must analyze large numbers of samples. Colorimetric and spectrophotometric methods, as well as solvent distribution techniques have been applied by a number of researchers whose work is discussed within A.S.B.C. circles.

The annual meetings of the American Society of Brewing Chemists are held in the spring of each year and their collaborative studies are completed in time for spring reports. That places them either six months ahead or six months behind reports to our organization; however, this has many advantages for both societies. Each has half a year to anticipate or prepare for the other. Proceedings of the A.S.B.C. meeting held in May of this year in Toronto are being published coincident with this meeting.

In the reported work is a method for the determination of α-amylase

in malt which has evolved from considerable collaborative study under the chairmanship of Dr. Sutten Redfern of the Fleischmann Laboratories. It is the opinion of the Associate Referee that the A.O.A.C. may adopt this method without further collaborative study as Official, First Action.

Mr. Frank E. Connery of P. Ballentine & Sons, as chairman of the subcommittee on hops, reported on broken cone determinations. Mr. Connery is also Associate Referee on hops for this society. No new methods were recommended.

Mr. Brandon gave a report on the determination of copper in beer and recommended that collaborative studies be continued.

Mr. Morrow C. Miller of Johns Manville Company reported on beer turbidity measurements and attempts to obtain a physical standard of turbidity. Until this A.S.B.C. committee defines measurements and recommends methods for collaborative study, it is pointless for this Society to adopt any turbidity methods.

Mr. Irwin Stone of Wallerstein Laboratories reported upon measurement of color in wort, using the spectrophotometric method now adopted by both societies for beer. This topic is being studied further.

Twenty-three original papers were presented at the meeting. Those members of A.O.A.C. who are interested in beer and who have not been aware of the A.S.B.C. and its work are urged to examine the proceedings of that Society, available from the Executive Secretary, Dr. W. J. Olson, P. O. Box 2146, Madison, Wisconsin.

In view of the overlapping of interests of these two Societies, the excellent collaborative type of investigations carried out, and the precedent of having adopted A.S.B.C. methods in part of our methods, it is recommended*—

1. That close cooperation between the two Societies be fostered through the mechanism of appointing an Associate Referee on Malt Beverages, Sirups, Extracts and Brewing Materials from the membership of the A.S.B.C. who is also a member of its Technical Committee or of the Executive Committee. Such qualification would insure adequate familiarity with current work in the entire field.

2. That Associate Referees for separate topics be appointed as in the past but those who are serving as chairmen of corresponding committees within the A.S.B.C. may also be requested to serve.

3. That method for α-amylase in malt as developed by A.S.B.C. be adopted as First Action.

4. That studies concerning methods for examination of hops and for determination of color and turbidity in beer as well as trace elements be continued through the mechanism suggested in 1 and 2, above.

* For report of Subcommittee D and action of the Association, see *This Journal*, 36, 60 (1953).

REPORT ON TRACE ELEMENTS IN BEER

By A. L. BRANDON (Anheuser-Busch, Inc., St. Louis, Missouri),
Associate Referee

IRON

In last year's report (1), the Associate Referee recommended (*a*) that the direct, non-ash procedure for iron in beer be adopted First Action, and that it be further studied by the Associate Referee; and (*b*) that the wet-ash *ortho*-phenanthroline method be further studied by the Associate Referee before submitting it to collaborative study.

No further studies have been conducted during the past year on the non-ash procedure, but a program of study is being planned for the coming year.

In studying the collaborators' comments in last year's report, it appeared that the following changes in method No. 1 (1) should be studied: (*a*) the effect of removing the excess perchloric acid or the substitution of another wet-ash procedure, (*b*) the effect of filtering the insoluble residue from the acid digest, and (*c*) the effect of titrating the acid digest to *p*H 3.5 with ammonium hydroxide instead of buffering with sodium acetate.

In studying the first change, two digestion procedures were tried, one using a mixture of nitric, sulfuric, and perchloric acids, and the other using a mixture of nitric and sulfuric acids and 30% hydrogen peroxide.

DIGESTION METHODS

METHOD NO. 1

REAGENT

Acid mixture.—Mix 125 ml concd H_2SO_4, 290 ml concd HNO_3, and 85 ml 70–72% $HClO_4$.

DETERMINATION

Transfer 50–100 ml of thoroly degassed beer to a 300 ml or 500 ml Kjeldahl flask. Support the flask in an inclined position on an asbestos-centered wire gauze having a hole 1 in. in diam. Heat the flask, cautiously at first until danger of foaming has passed, then concentrate the sample to a thick syrup and cool. Add 20 ml of acid mixt. to the flask and cautiously heat to start the reaction. After the reaction has subsided, heat over a low flame and add HNO_3 dropwise when the digest shows evidence of charring. Continue addn of HNO_3 dropwise until $HClO_4$ fumes appear. Continue heating until SO_3 fumes appear and H_2SO_4 recondenses on the walls of the flask. Cool and continue with Method No. 1 in last year's report, starting with "Cool the digests . . . ."

METHOD NO. 2

REAGENTS

(a) *Nitric Acid.*—Concentrated.
(b) *Sulfuric Acid.*—Concentrated.
(c) *Hydrogen Peroxide.*—30%.

DETERMINATION

Concentrate the sample as in Method No. 1 above. Add 10 ml of concd HNO_3 and heat gently to start reaction. After reaction has subsided, add 2 ml of concd H_2SO_4 if 50 ml of beer was used, and 3 ml if 100 ml of beer was used. After reaction has again subsided, heat the flask gently over a low flame until digest begins to char. Add H_2O_2 dropwise until digest clears and SO_3 fumes fill the flask. Cool and continue with Method No. 1 in last year's report, starting with "Cool the digests . . . ."

Both digestion procedures produce digests that are water clear and free of perchloric acid. The photometric data obtained by subjecting the digests from the above procedures to the method of color development used last year were very erratic and showed poor reproducibility. It is apparent that the presence of perchloric acid in the digests is not a cause of erratic results and thus it would seem that the presence of the insoluble residue in the digest or the use of sodium acetate as a buffering agent is the cause of erratic results.

COPPER

During the past year no collaborative work was done on copper.

In 1950, a method for the determination of copper in beer proposed by Stone (5) was studied collaboratively by the Subcommittee on Copper in Beer of the American Society of Brewing Chemists (7). This study was continued last year (8) after substituting a wet-ashing procedure for the dry-ashing used in Stone's method. Copper studies are being continued this year by the A.S.B.C. with emphasis on direct methods. Three methods, one of which will be studied collaboratively, are being reviewed at the present time. These methods include (*a*) the use of diethyldithiocarbamate added directly to beer (8); (*b*), the use of dithizone as proposed by Kratz, Lewis, and Feldman (2) for the determination of copper in carbonated beverages and sugar syrups; and (*c*) the use of zinc dibenzyldithiocarbamate as proposed by Martens and Githens (4) for the determination of small amounts of copper in dyes and rubber chemicals. The advantage of using zinc dibenzyldithiocarbamate is that iron, which is the main interference encountered in beer, does not interfere in amounts up to 50 mg.

No collaborative work is planned for the coming year pending the outcome of the studies being conducted by the A.S.B.C.

TIN

After an extensive literature survey, two chemical methods for the determination of tin have been found that may be applied to this determination in beer. In 1941, Stone (6) proposed the use of dithiol (1-methyl-3,4-dimercaptobenzene) for the determination of traces of tin in malt beverages. The use of anthraquinone-1-azo-4-dimethylaniline for the determination of small amounts of tin was proposed by Kuznetsov and Bender (3)

in 1940. These methods will be studied and evaluated for possible collaborative study.

RECOMMENDATIONS

It is recommended*—

(1) That the study of the wet-ash *o*-phenanthroline method for iron be continued by the Associate Referee and after completion be submitted to collaborative study.

(2) That the direct, non-ash *o*-phenanthroline procedure for iron adopted First Action last year, be studied further by the Associate Referee and be modified to conform with the present official method of the A.S.B.C.

(3) That collaborative studies on copper be postponed until work on iron has been completed, or present work on copper in the A.S.B.C. is completed.

(4) That the methods of Stone and of Kuznetsov and Bender for the determination of tin be studied.

(5) That collaborative work on tin be postponed until work on iron and copper has been completed.

REFERENCES

(1) BRANDON, A. L., *This Journal*, **35**, 243 (1952).
(2) KRATZ, P. D., LEWIS, J. I., and FELDMAN, A., *Anal. Chem.*, **24**, 524 (1952)
(3) KUZNETSOV, V. I., and BENDER, I. M., *J. Applied Chem. (U.S.S.R.)*, **13**, 1724 (1940).
(4) MARTENS, R. I., and GITHENS, R. E., SR., *Anal. Chem.*, **24**, 991 (1952)
(5) STONE, I., *Ind. Eng. Chem., Anal. Ed.*, **14**, 479 (1942).
(6) ———, *ibid.*, **13**, 791 (1941).
(7) Subcommittee on Copper in Beer, *Am. Soc. Brew. Chem. Proc.*, 147 (1951).
(8) Subcommittee on Copper in Beer, *ibid.*, in press.

REPORT ON CHROMATOGRAPHY OF WINES AND DISTILLED SPIRITS

By ALEX P. MATHERS (Alcohol and Tobacco Tax Division Laboratory, Bureau of Internal Revenue, Washington 25, D.C.),
Associate Referee

DISTILLED SPIRITS

An investigation of fusel oil constituents at the University of California indicates that chromatographic separation of the higher alcohols in fusel oil is feasible. No collaborative work has yet been attempted in this field.

WINES

Tests were conducted based on the method outlined in the Associate Referee report of 1951. Collaborators were able to separate and identify lactic, tartaric, citric, and malic acids in wine when the concentrations

* For report of Subcommittee D and action of the Association, see *This Journal*, **36**, 60 (1953).

of these components were 0.1 g per 100 ml or higher. It was difficult to detect the presence of one of the acid constituents when its concentration was low, which is the case in some admixtures of wine. It will apparently be necessary to use ion exchange columns or other methods of partial purification before resorting to paper chromatography, since other materials in the wine tend to obscure the acid patterns.

RECOMMENDATIONS

It is recommended*—

(1) That the separation and determination of the higher alcohols in distilled spirits by chromatography be studied further.

(2) That collaborative work be continued on detecting and identifying non-volatile acids in wine by paper chromatography.

No reports were received on: hops, distilled spirits, phosphates in wines and spirits, tannins in whiskies and wines, cordials and liqueurs, and methanol.

REPORT ON FLAVORS AND NON-ALCOHOLIC BEVERAGES

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

No reports were submitted this year on the following projects:

(1) Isopropyl alcohol in lemon and orange flavors.
(2) Essential oil in emulsions.
(3) Essential oil in citrus juices and other beverages.
(4) β-Ionone when small amounts are present.
(5) Propylene glycol in vanilla.

The Associate Referee on vanilla extracts and imitations has completed the work on the photometric methods for vanillin and coumarin, and these methods are now being recommended for official status. The photometric method for vanillin has been shown to be superior to the Folin and Denis colorimetric method which is now being recommended for deletion, Final Action.

The question has arisen as to whether or not the gravimetric method for vanillin and coumarin should be dropped. It is expected that a study of this procedure will be made soon.

In addition to work on the photometric method, this Associate Referee has also made a study of the ultraviolet absorption method for determining vanillin, ethyl vanillin, and coumarin. Thus far, no fully satisfactory method has been established for the determination of vanillin and ethyl vanillin in mixtures of these two flavoring ingredients.

* For report of Subcommittee D and action of the Association, see *This Journal*, 36, 60 (1953).

The data submitted by Mr. Ensminger indicate that his procedure is capable of determining coumarin and the sum of vanillin and ethyl vanillin with a fair degree of accuracy. However, the estimation of the proportion of ethyl vanillin in the vanillin-ethyl vanillin mixture involves the use of three simultaneous equations with the attendant difficulties, and the results do not warrant its application. In the method, Mr. Ensminger uses the clarified diluted extracts directly for his measurement of the ultraviolet absorption in acid and in alkaline solution. The Referee believes that some sort of isolation procedure must first be employed before the ultraviolet absorption method can be successfully applied to this problem.

Recently our attention has been directed toward the factors used to calculate lemon oil content of oil base flavors from the polarization methods **19.32.** A manufacturer stated that he was unable to meet the government specification for a 20 per cent mixture of lemon oil in cottonseed oil because the only lemon oil available to him had a rotation of 58° (circular).

The specification in question calls for a vehicle of a fatty oil and requires a lemon oil content of 20 per cent as determined by the polarization method. If the oil used has a low polarization, the specification may be met by building the oil content up to the point where the polarization of the mixture meets the requirement. Since experience may have shown that lemon oils with high polarizations are preferable, the right of an agency to fix such a specification cannot be denied. It would compel manufacturers to supply a product containing an oil of high polarization or use a larger proportion of oil having a lower polarization to meet the specification.

However, the present problem has to do with the accuracy of the method in determining the oil content of the flavor.

Six authentic samples of commercial cold pressed California lemon oil all produced this year were available. On determining the polarization, rotation was found to be 66.15° and the minimum, 55.72°. The factors given in **19.32** vary slightly with the oil solvent used so that it is not certain that the rotation of such a flavor is exactly proportional to the rotation of the essential oil adjusted to the dilution. Assuming that the rotation of the essential and fixed oil solution is proportional to the dilution, the circular degrees were converted to the international scale, this rotation multiplied by the dilution and the length of tube, and the theoretical reading divided by the factor 3.7 for lemon oil in cottonseed oil. The oil with maximum rotation would show an oil content of 5.16 per cent for 5 per cent of oil by volume in the mixture and 20.65 per cent for a 20 per cent mixture. The oil with minimum rotation would show an oil content of 4.08 per cent for 5 per cent of oil by volume in the mixture and 17.4 per cent for a 20 per cent mixture.

The Referee expects to investigate further these factors to decide if some adjustment should be made to increase the accuracy of the method.

RECOMMENDATIONS

It is recommended*—

(1) That the First Action photometric method for vanillin, *This Journal*, **34,** 72 (1951) and **35,** 77 (1952) be made official for vanilla, vanilla containing added vanillin and coumarin, and for imitation vanilla.

(2) That the First Action photometric method for coumarin, *This Journal*, **34,** 73 (1951) and **35,** 77 (1953) be made official for vanilla, vanilla containing added coumarin and/or vanillin, and for imitation vanilla.

(3) That studies be continued on method **19.4** and **19.5.**

(4) That the official colorimetric method for vanillin **19.6** and **19.7** (p. 306) be deleted, Final Action.

(5) That collaborative studies be continued on the following:

(a) First action method for isopropyl alcohol in lemon and orange flavors, *This Journal*, **35,** 77 (1952).

(b) First action method for essential oil in emulsions, *This Journal*, **35,** 78 (1952).

(c) First action method for essential oil in citrus juices and other beverages, *This Journal*, **35,** 79 (1952).

(d) β-Ionone when small amounts are present.

(e) Propylene glycol in vanilla extracts, *This Journal*, **35,** 103 (1950).

REPORT ON VANILLA EXTRACTS AND IMITATIONS

A. DETERMINATION OF VANILLIN, ETHYL VANILLIN, AND COUMARIN BY ULTRAVIOLET ABSORPTION

By LUTHER G. ENSMINGER (Food and Drug Administration, Department of Health, Education, and Welfare, Cincinnati, Ohio), *Associate Referee*

The Associate Referee has reported collaborative studies on the determination of vanillin and coumarin in vanilla flavors by colorimetric methods in 1950 and 1951.† The methods have worked well for determining these two constituents only.

Since vanillin, ethyl vanillin, and coumarin are aromatic compounds the Associate Referee felt that ultraviolet spectra might reveal the type of flavor as well as the concentrations of these flavoring constituents. Vanilla extracts are actual vegetable extractives which may contain other radiation-absorbing compounds.

Alkaline water solutions of vanillin, ethyl vanillin, and coumarin,

* For report of Subcommittee D and action of the Association, see *This Journal*, 36, 63 (1953).
† *This Journal*, 34, 330 (1951); 35, 264 (1952).

containing 0.5 mg of each and 2 ml 0.1 *N* NaOH per 100 ml, were prepared The ultraviolet spectra were obtained to determine absorption characteristics (Fig. 1). Vanillin and ethyl vanillin produced practically coincident maxima and minima, but a small yet noticeable shift of the ethyl vanillin spectrum to longer wavelengths was observed. This was to be expected because of the influence of the larger ethoxy group on the ethyl vanillin molecule. The vanillins gave pronounced absorption maxima at 348 mμ and secondary maxima at 248 mμ. Minima occurred at 228 and 271 mμ. Coumarin gave low absorption maxima at 264 and 322 mμ and minima at about 256 and 296 mμ.

In a like manner, absorption spectra were obtained on acidic water solutions of the three aromatics (Fig. 2). The acidic spectra of all three are very similar and are influenced principally by the benzene ring, whereas in alkaline solutions, the formation of salts (especially by the vanillins) is the characterizing force.

Mixtures of the aromatics gave acidic and basic spectra as shown in Fig. 3.

Fig. 4 shows ultraviolet spectra for two authentic vanilla extracts (Madagascar and Tahiti) in both acidic and basic solutions. Preliminary

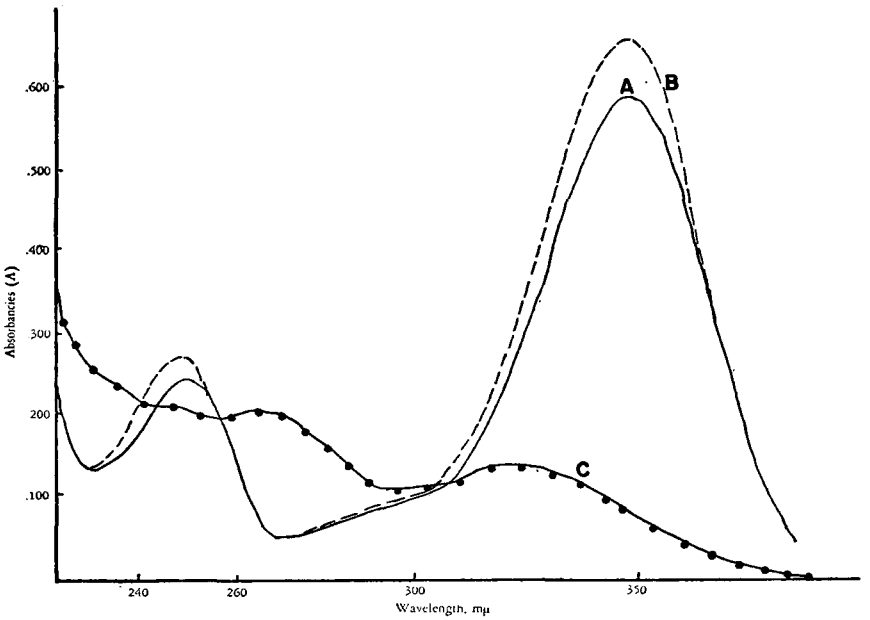

FIG. 1.—Ultraviolet spectra for vanillin, coumarin, and ethyl vanillin in alkaline solution against water blank. Key: A, ethyl vanillin; B, vanillin; C, coumarin.

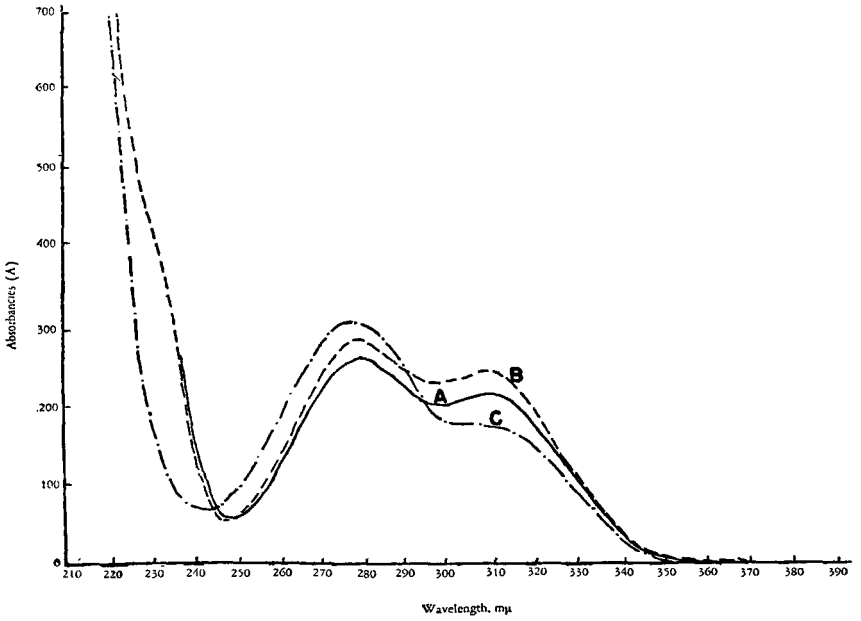

FIG. 2.—Ultraviolet spectra for vanillin, coumarin, and ethyl vanillin in acid solution against water blank. A, ethyl vanillin; B, vanillin; C, coumarin.

clarification was obtained with lead acetate solution. The minima are larger with respect to the maxima (especially for the acidic spectra) than for a mixture of equal amounts of vanillin, ethyl vanillin, and coumarin. The larger minima-to-maxima ratios indicate that the extracts have a considerable background absorbance at shorter wavelengths. Curves made on authentic imitation vanilla flavors exhibited little, if any, background absorbance.

A study of the spectra indicated three possible combinations for a two-component analysis of vanillin (also ethyl vanillin) and coumarin:

(1) 270-basic solution *vs.* 348-basic solution,
(2) 279-acid solution *vs.* 348-basic solution,
(3) 309-acid solution *vs.* 348-basic solution.

In addition, 247 mμ acidic solution and 270 mμ basic solution were selected as points for the study of background absorbancies.

Before actual analyses were made, stabilities of the three aromatics were determined at each of the above points. It was found that all were completely stable at 247, 279, and 309 mμ acidic solution for a period of five hours. However, in alkaline solutions, only the two vanillins reached complete stability immediately and retained it. Coumarin was quite unstable in freshly prepared solutions, and absorbancies increased,

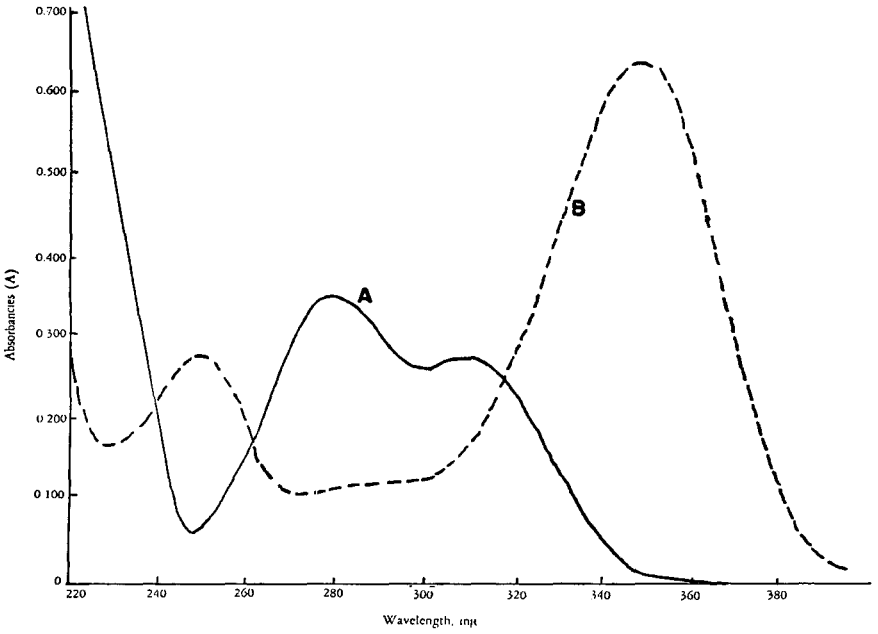

FIG. 3.—Ultraviolet spectra for a mixture of vanillin, coumarin, and ethyl vanillin (0.2 mg vanillin, 0.2 mg ethyl vanillin, and 0.12 mg coumarin in 100 ml). Key: A, acid soln; B, alkaline soln.

especially at shorter wavelengths. They reached maxima after a period of about 3.5 hours, remained stable about 1.5 hours, and then slowly declined. Hence the alkaline solutions require an optimum stabilizing period of 4 hours before reading, while acid solutions can be read at any time.

Vanillin, ethyl vanillin, and coumarin standards with final concentrations of 0.1 to 0.8 mg/100 ml were checked for absorptivity at 247, 279, and 309 mμ, acidic solution, and at 270 and 348 mμ, basic solution. Absorption was found to be a linear function of concentration at all mentioned wavelengths and the absorptivity was unchanged by a change in concentration for each case. A concentration of 0.5 mg/100 ml was selected for each compound for determining absorptivities in the method.

Observing the above precautions, eight vanilla extracts and imitations were analyzed for vanillin and coumarin by the three two-component methods. It was found that all work equally well for imitations, but that the No. 3 method (309 mμ-acidic solution *vs.* 348-mμ basic solution) gives best results for extracts. (Concentrations of vanillin and coumarin in extracts were predetermined by colorimetric methods.) Background absorb-

ancies of extracts are greatest at the shorter wavelengths for both acidic and basic solutions, and decline as longer wavelengths are reached. They become quite nominal at 309 mμ acidic solution and 348 mμ basic solution and this makes the 309–348 mμ combination best for extracts.

A. VANILLIN AND COUMARIN COMBINATION

When a flavoring is known to be a pure vanilla extract or imitation containing only vanillin and coumarin, proceed as follows:

REAGENTS

(a) *Lead acetate solution.*—Dissolve 50 g each of neutral and basic Pb acetate in hot H_2O, mix, dil. to one l, cool, and filter. Keep stoppered.

(b) *Standard vanillin solution.*—Weigh 0.2000 g vanillin and transfer to a 200 ml volumetric flask. Dissolve in 10 ml ethyl alcohol, dil. to mark with H_2O, and mix (1 ml = 1 mg).

(c) *Standard coumarin solution.*—Weigh 0.2000 g coumarin and transfer to a 200 ml volumetric flask. Dissolve in 20 ml ethyl alcohol, dil. to mark with H_2O, and mix (1 ml = 1 mg). (Caution: keep in dark.)

DETERMINATION OF ABSORPTIVITIES

(a) *For vanillin.*—Pipet 10 ml vanillin standard soln into a 200 ml volumetric flask, add 100 ml H_2O, and mix. Add 2 ml Pb acetate soln, mix, dil. to mark, and

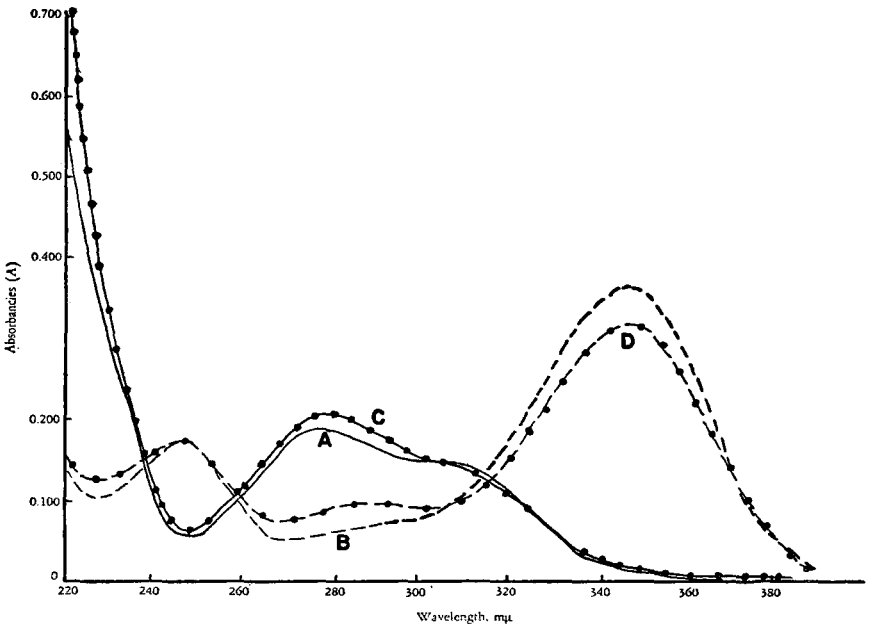

FIG. 4.—Ultraviolet spectra for authentic vanilla extracts (sample dilution—1000×). Key: A, Madagascar, acidic soln; B, Madagascar, basic soln; C, Tahiti, acidic soln; D, Tahiti, basic soln.

filter thru a fine quality filter paper, discarding the first 20 ml filtrate. (18.5 cm size Whatman No. 12, folded filter papers have been found satisfactory for all filtrations.)

Pipet 10 ml of clear filtrate into each of two 100 ml volumetric flasks and add ca 70 ml H_2O to each flask. Add exactly 2 ml 0.1 *N* NaOH to one flask and 2 ml 0.1 *N* H_2SO_4 to the other. Dil. flasks to mark with H_2O and mix. Hold the alk. soln exactly 4 hrs in the stoppered flask. In the same manner, prepare acid and basic reagent blanks against which sample solns may be read.

Filter the acidic soln thru a fine grade folded filter paper, discarding the first 20 ml of filtrate, and immediately after filtering, obtain absorbancies, A, at 247 and 309 mμ against an acidic reagent blank, using 1 cm silica cells. Before reading, adjust the spectrophotometer for maximum sensitivity to enable use of the narrowest possible slit width.

After the alk. soln has stood 4 hrs, filter, discarding the first 30 ml, and at once obtain its absorbancies, A, at wavelengths of 270 and 348m μ against an alk. reagent blank.

Calc. the absorptivities for vanillin at each of the above wavelengths, using the following equation: $a = A/bc$, where a = absorptivity, b = cell thickness (cm), c = concentration (g/l), and A = absorbance. (Note: Final standard solns have a concn of 0.5 mg/100 ml or 0.005 g/l.)

(b) *For coumarin.*—Det. the absorptivities for coumarin in the same manner and at the same wavelengths as for vanillin.

SAMPLE ASSAY

When a sample has a vanillin concn of less than 0.5 g/100 ml, pipet 5 ml of flavoring into a 25 ml volumetric flask, carefully dil. to mark with H_2O, and mix well. Pipet 10 ml of dild sample into a 200 ml volumetric flask, add 100 ml H_2O, and mix. Continue as for the vanillin standard above, beginning: "Add 2 ml Pb acetate soln, mix, dil. to mark, and filter. . . . " When flavorings have vanillin concns of 0.5 to 1.0 g/100 ml, transfer 5 ml of sample to a 50 ml volumetric flask, dil. to mark, mix, take a 10 ml aliquot for clarification with Pb acetate, and proceed as before. With samples having vanillin concns higher than 1.0 g/100 ml, proceed as for the 0.5 to 1.0 g/100 ml flavorings, but take only a 5 ml aliquot of dild sample for clarification.

CALCULATIONS

(a) *Vanillin and coumarin concentrations.*—Det. vanillin and coumarin concns in the two-component system by the following simultaneous equations:

$$100A_{348} = V.a_{v348} + C.a_{c348}$$
$$100A_{309} = V.a_{v309} + C.a_{c309}$$

where A_{348} and A_{309} are absorbancies of sample at 348 mμ alk. soln and 309 mμ acidic soln respectively, V and C are the unknown final concns (mg/100 ml) of vanillin and coumarin respectively, a_{v309} and a_{v348} are the absorptivities for vanillin at 309 mμ acidic soln and 348 mμ alk. soln respectively, and a_{c309} and a_{c348} are the absorptivities for coumarin at 309 mμ acidic soln and 348 mμ alk. soln respectively. (Substituting averages of a values, the equations are simplified as follows:

$$V = 0.819A_{348} - 0.535A_{309}$$
$$C = 2.331A_{309} - 1.352V)$$

Multiply the concns V and C by the appropriate factor to give concns of vanillin and coumarin in g/100 ml of sample.

(b) *Qualitative check to indicate whether flavoring may be an extract or imitation.*—

Calc. the combined absorbance due to vanillin and coumarin at 247 mμ in the final acidic soln, using the *a* values for vanillin and coumarin at 247 mμ and the concns V and C which are in terms of mg/100 ml. Subtract this combined absorbance from the total absorbance for the sample at 247 mμ to obtain background absorbance. Reduce this background absorbance to the basis of a 1000-fold diln of sample from an original 100 ml vol. of flavoring. (For a 1000-fold diln, no change is necessitated in the background absorbance of the final acidic soln, but for 2000- and 4000-fold dilns, multiply the background absorbance by 2 and 4 respectively.)

In the same way det. the background absorbance of the sample (total absorbance of sample minus the combined absorbance of vanillin and coumarin) at 270 mμ alk. soln. Obtain the average of the backgrounds at 247 and 270 mμ by adding them and dividing by 2. An average background absorbance of 0.020 or more indicates that the flavoring is an extract, while an average of less than 0.020 indicates an imitation. (Note: if a negative background is obtained at 247 or 270 mμ, retain the sign in determining the average.) When advisable, chemical tests should be performed for confirmation of extract or imitation.

B. ETHYL VANILLIN AND COUMARIN COMBINATION

Det. the concns and background absorbancies of the flavoring as for the vanillin-coumarin combination, but use the *a* values of an ethyl vanillin standard (instead of those from vanillin) prepared and run in the same manner as the vanillin standard.

RESULTS ON AUTHENTIC VANILLA FLAVORINGS AND IMITATIONS

The Associate Referee analyzed 11 authentic vanilla extracts and 2 authentic imitation vanilla flavors by ultraviolet absorption. Results are given in Table 1 where columns (1) and (2) give the sample numbers and types of flavoring respectively. Sample 52-5 is an imitation containing only ethyl vanillin and coumarin, and was determined by the ethyl vanillin-coumarin method. All other samples were determined by the vanillin-coumarin method.

In columns under heading (3) are compared the recoveries of vanillin or ethyl vanillin and coumarin with those obtained on extracts by colorimetric methods,* and with the actual formulas of the imitations. Upon comparing the vanillin-ethyl vanillin components found with those determined by colorimetric methods, it is noted that the greatest variance is 0.05 g/100 ml (sample 52-14). Also, the ultraviolet method gave higher results on 7 of 10 of the extracts numbered 52-11 to 52-20, especially on the extracts with the greater concentrations of vanillin. Samples 52-11 to 52-20 are authentic extracts which are several years old, and changes in molecular structure may account for this. The extract 52-1 and imitations 52-5 and 52-7 were prepared this year (1952). The ultraviolet method gave excellent recoveries of vanillin and ethyl vanillin on the imitations.

The coumarin recoveries are especially good on samples 52-1, 52-5, and 52-7—the fresh flavorings. On the rest (extracts), the largest variance between the methods was 0.025 g/100 ml.

Regarding column (4), it is noted that the two imitations gave average background absorbancies of less than 0.020, while all the extracts gave

* *This Journal*, **34**, 72 (1951).

TABLE 1.—*Ultraviolet determination of vanillin, ethyl vanillin, and coumarin and background absorbancies of vanilla extracts and imitations*

(1)	(2)	(3) RECOVERY (G/100 ML)				(4)
SAMPLE NUMBER	TYPE OF FLAVORING	VANILLIN-ETHYL VANILLIN COMPONENT		COUMARIN COMPONENT		AV. OF BACKGROUND ABSORBANCIES AT 247 AND 270 M/μ
		ULTRAVIOLET METHOD	COLOR. METHOD (OR AMOUNT ADDED)	ULTRAVIOLET METHOD	COLOR. METHOD (OR AMOUNT ADDED)	
52-1	Bourbon-Tahiti Extract	0.179	0.19	0.031	0.03	0.056
52-5	Imitation	0.715	0.70	0.100	0.10	0.015
52-7	Imitation	0.405	0.40	0.139	0.14	−0.001
52-11	Mexican Extract	0.053	0.06	0.035	0.03	0.027
52-12	South America Extract	0.195	0.15	0.028	0.04	0.038
52-13	Java Extract	0.184	0.16	0.074	0.06	0.028
52-14	Marquette Splits Extract	0.217	0.17	0.035	0.06	0.022
52-15	Comores Extract	0.180	0.14	0.027	0.05	0.024
52-16	Puerto Rico Extract	0.091	0.10	0.059	0.04	0.028
52-17	Tahiti Extract	0.090	0.10	0.027	0.05	0.069
52-18	Madagascar Extract	0.132	0.12	0.039	0.05	0.029
52-19	Bourbon Extract	0.149	0.12	0.037	0.04	0.024
52-20	Nossi Be Extract	0.096	0.09	0.047	0.05	0.025

backgrounds over 0.020. This supports the procedure for identification of unknown flavorings as vanilla extracts or imitations.

Absorptivities, (*a*), obtained by the Associate Referee for vanillin, ethyl vanillin, and coumarin and used to determine the results in Table 1, are given in Table 2.

TABLE 2.—*Absorptivities of vanillin, ethyl vanillin, and coumarin at selected wavelengths*

WAVELENGTH (mμ)	NATURE OF SOLUTION	ABSORPTIVITY (a)		
		VANILLIN	ETHYL VANILLIN	COUMARIN
270	Basic	10.3	8.8	42.0
348	Basic	161.3	144.7	25.6
247	Acidic	10.6	9.8	13.0
309	Acidic	58.6	51.8	42.2

The coefficients of ethyl vanillin are less than those for vanillin because the latter has a lower molecular weight.

COLLABORATIVE RESULTS ON AUTHENTIC VANILLA EXTRACTS AND IMITATIONS

The Associate Referee diluted a Bourbon extract to half its strength and enriched it with pure vanillin (sample 52-2), enriched a second pure Bourbon extract with ethyl vanillin (sample 52-3), and also prepared an imitation vanilla extract (sample 52-6). This first series of samples were analyzed by four analysts in this laboratory and the results obtained are given in Table 3.

Since these results were satisfactory, a second series of vanilla flavorings was distributed to seven collaborators (including the four above), and Table 4 summarizes their results. Two imitation vanilla extracts (52-4 and 52-8), a Bourbon extract (52-21), and the latter extract enriched with ethyl vanillin (52-22) comprised the second series. The procedure employed by all collaborators was the ultraviolet method given above for the vanillin and coumarin combination. Recoveries are compared to those by the colorimetric methods or to amounts of aromatics actually added in column 5 of Tables 3 and 4. Also, column 4 of Tables 3 and 4 gives the per cent vanillin in the vanillin-ethyl vanillin component to clarify the tabulation of recoveries of vanillins in column 5. Column 6 gives average background absorbancies of samples based on a 1000-fold dilution.

DISCUSSION

A. Vanillin and Ethyl Vanillin.—Table 5 lists a statistical analysis of the collaborative results for the vanillin-ethyl vanillin component given in Tables 3 and 4. The arithmetic mean and average recovery by sample, determined by the color method or from the amount added, are also given.

TABLE 3.—*Results of collaborative analyses of authentic vanilla extracts and imitations (first series)*

(1)	(2)	(3)	(4)	(5)				(6)
SAMPLE NUMBER	TYPE OF FLAVORING	COLLABORATOR	VANILLIN IN VANILLIN-ETHYL VANILLIN COMPONENT	RECOVERY (G/100 ML)				AVERAGE OF BACKGROUND ABSORBANCIES AT 247 AND 270 Mμ
				VANILLIN-ETHYL VANILLIN		COUMARIN		
				ULTRAVIOLET METHOD	COLOR. METHOD (OR AMOUNT ADDED)	ULTRAVIOLET METHOD	COLOR. METHOD (OR AMOUNT ADDED)	
			per cent					
52-2	Diluted Extract Enriched	1	100	0.277	0.262	0.068	0.072	0.021
52-2	Diluted Extract Enriched	2	100	0.250	0.262	0.075	0.072	0.024
52-2	Diluted Extract Enriched	3	100	0.255	0.262	0.063	0.072	0.019
52-2	Diluted Extract Enriched	4	100	0.237	0.262	0.089	0.072	0.011
52-3	Enriched Extract	1	39	0.458	0.488	0.131	0.155	0.067
52-3	Enriched Extract	2	39	0.447	0.488	0.146	0.155	0.045
52-3	Enriched Extract	3	39	0.448	0.488	0.152	0.155	0.042
52-3	Enriched Extract	4	39	0.445	0.488	0.150	0.155	0.050
52-6	Imitation	1	33	0.824	0.900	0.011	0	−0.008
52-6	Imitation	2	33	0.832	0.900	−0.002	0	−0.003
52-6	Imitation	3	33	0.862	0.900	−0.024	0	−0.002
52-6	Imitation	4	33	0.832	0.900	0.021	0	0.007

TABLE 4.—*Results of collaborative analyses of authentic vanilla extracts and imitations (second series)*

(1) SAMPLE NUMBER	(2) TYPE OF FLAVORING	(3) COLLABORATOR	(4) VANILLIN IN VANILLIN-ETHYL VANILLIN COMPONENT	(5) RECOVERY, (G/100 ML)				(6) AVERAGE OF BACKGROUND ABSORBANCIES AT 247 AND 270 Mμ
				VANILLIN-ETHYL VANILLIN		COUMARIN		
				ULTRAVIOLET	COLOR. METHOD (OR AMOUNT ADDED)	ULTRAVIOLET	COLOR. METHOD (OR AMOUNT ADDED)	
			per cent					
52-4	Imitation	1	43	0.352	0.350	0.131	0.130	0.012
52-4	Imitation	2	43	0.341	0.350	0.138	0.130	0.006
52-4	Imitation	3	43	0.341	0.350	0.116	0.130	0.002
52-4	Imitation	4	43	0.333	0.350	0.161	0.130	0.001
52-4	Imitation	5	43	0.348	0.350	0.110	0.130	0.007
52-4	Imitation	6	43	0.374	0.350	(0.063)	0.130	0.030
52-4	Imitation	7	43	0.356	0.350	0.121	0.130	0.013
52-8	Imitation	1	50	1.860	2.000	0.116	0.120	0.002
52-8	Imitation	2	50	1.856	2.000	0.123	0.120	−0.016
52-8	Imitation	3	50	1.872	2.000	0.144	0.120	−0.020
52-8	Imitation	4	50	1.875	2.000	0.140	0.120	−0.014
52-8	Imitation	5	50	1.875	2.000	0.116	0.120	−0.010
52-8	Imitation	6	50	1.796	2.000	(0.238)	0.120	−0.036
52-8	Imitation	7	50	1.908	2.000	0.120	0.120	−0.012
52-21	Extract	1	100	0.197	0.195	0.018	0.020	0.030
52-21	Extract	2	100	0.184	0.195	0.025	0.020	0.024
52-21	Extract	3	100	0.196	0.195	0.013	0.020	0.026
52-21	Extract	4	100	0.188	0.195	0.023	0.020	0.024
52-21	Extract	5	100	0.190	0.195	0.014	0.020	0.028
52-21	Extract	6	100	0.194	0.195	(0.008)	0.020	0.026
52-21	Extract	7	100	0.196	0.195	0.020	0.020	0.047
52-22	Enriched Extract	1	39	0.452	0.495	0.123	0.120	0.028
52-22	Enriched Extract	2	39	0.440	0.495	0.103	0.120	0.034
52-22	Enriched Extract	3	39	0.455	0.495	0.108	0.120	0.024
52-22	Enriched Extract	4	39	0.436	0.495	0.115	0.120	0.028
52-22	Enriched Extract	5	39	0.457	0.495	0.100	0.120	0.032
52-22	Enriched Extract	6	39	0.445	0.495	(0.054)	0.120	0.040
52-22	Enriched Extract	7	39	0.459	0.495	0.120	0.120	0.032

TABLE 5.—*Analysis of collaborative results for vanillin-ethyl vanillin component and for coumarin*[a]

(1) SAMPLE	(2) ARITHMETIC MEAN	(3) AVERAGE RECOVERY	(4) STANDARD DEVIATION	(5) PROBABLE ERROR		(6) ACTUAL RANGE
				OF SINGLE DETERMINATION	OF ARITHMETIC MEAN	
Vanillin						
		per cent				
52-2 (Diluted Extract)	0.255	102.0	0.017	0.011	0.006	0.237–0.278
52-3 (Enriched Extract)	0.450	92.2	0.006	0.004	0.002	0.445–0.458
52-4 (Imitation)	0.349	99.7	0.013	0.009	0.003	0.333–0.374
52-6 (Imitation)	0.838	93.1	0.016	0.011	0.006	0.824–0.862
52-8 (Imitation)	1.863	93.2	0.034	0.023	0.009	1.796–1.908
52-21 (Extract)	0.192	98.5	0.005	0.003	0.001	0.184–0.197
52-22 (Enriched Extract)	0.449	90.7	0.009	0.006	0.002	0.436–0.459
Coumarin[b]						
52-2 (Diluted Extract)	0.074	102.4	0.011	0.007	0.004	0.063–0.089
52-3 (Enriched Extract)	0.149	93.5	0.009	0.006	0.003	0.131–0.152
52-4 (Imitation)	0.130	100.0	0.018	0.012	0.005	0.110–0.161
52-6 (Imitation)	0.001	—	—	—	—	−0.024–0.021
52-8 (Imitation)	0.127	105.8	0.012	0.008	0.003	0.116–0.144
52-21 (Extract)	0.019	95.0	0.005	0.003	0.001	0.013–0.025
52-22 (Enriched Extract)	0.111	92.5	0.009	0.006	0.002	0.100–0.123

[a] Data in columns (2), (4), (5), and (6) are in terms of grams per 100 ml of sample.
[b] Coumarin results by Collaborator 6 are not included in the analysis as all were computed to be outside the random limit of error for the method.

The quantitative determination of vanillin and combined vanillin-ethyl vanillin appears to be very satisfactory for all samples, with average recoveries ranging from 90.7 per cent in the case of an ethyl vanillin-enriched extract to 102.0 per cent for a diluted extract enriched with vanillin. The grand average recovery is 95.6 per cent. The lowest average recoveries are those for samples enriched with ethyl vanillin. Use of vanillin absorptivities to determine ethyl vanillin results in low recoveries due to the larger molecular weight of ethyl vanillin.

Columns (4), (5), and (6) of Table 5, give the measures of variation of the vanillin-ethyl vanillin results by sample. The very rich imitations gave the most variation. To obtain absorbancies, imitations 52-6 and 52-8 were diluted 2000 and 4000 times respectively as compared to 1000 times for the other samples, and these larger dilutions are reflected in the variability. In general, the ultraviolet procedure gave a much smaller range of results than did the colorimetric method in collaborative studies. The standard deviations and probable errors of single determinations and of means are of the same order as those found by colorimetric studies. But since the number of degrees of freedom considerably affect the measures of variation (except the range), results indicate that, if many determinations were made on a given sample, the precision of the ultraviolet method would be considerably better than for the color method previously studied. The high absorptivities and stability of vanillin and ethyl vanillin at 348 mμ basic solution enhance the precision of the method.

All results for the vanillins were within the random limits of error for the method.

B. Coumarin.—The coumarin results are listed in Tables 3 and 4 according to collaborator and sample, and a statistical analysis is given in Table 5. Only one sample contained no coumarin (sample 52-6). Two collaborators found no coumarin in 52-6 while the other two found 0.011 and 0.021 g/100 ml, which is insignificant compared to the high content of vanillins.

By statistical determination it was found that all coumarin results by collaborator 6 were outside the random limits of error for the method. It appeared from studying the absorbancies submitted by collaborator 6 that he encountered interference from some cloudiness in solutions during reading which considerably affect the coumarin results, yet affect the vanillin results but little. Consequently, these coumarin determinations were not included in Table 5. (Very fine filter papers must be used for filtrations.)

The average recovery of coumarin ranged from 92.5 to 105.8 per cent. The grand average recovery is 98.2 per cent which is good, considering the small amount of coumarin present. However, the measures of variation (standard deviation, probable errors, and range) compare well with the colorimetric procedure for coumarin,* but the precision of the

* *This Journal*, 34, 73 (1951).

ultraviolet method is slightly less. To indicate the accuracy of the method, for all samples except 52-4 a future determination of coumarin has more than a 50 per cent chance of falling within the range *mean plus or minus 0.01 g/100 ml* of flavoring.

C. Identification of Extract or Imitation.—In applying the criterion (formerly determined) that pure vanilla extracts give average absorbancies at 247 mμ (acidic) and 270 mμ (basic) of more than 0.020 while imitations give less, it is noted that in only one out of 36 determinations would the collaborators have been wrong in judging the samples to be an extract (or extract base) or imitation. (See column (6) in Tables 3 and 4 for the average background absorbancies.) Backgrounds of extracts were usually well over 0.020 while those of imitations approached zero. Sample 52-2 was an extract diluted with water to half strength and enriched with

TABLE 6.—*Average absorptivities for vanillin, ethyl vanillin, and coumarin at selected wavelengths (seven collaborators)*

WAVELENGTH (mμ)	NATURE OF SOLUTION	AVERAGE ABSORPTIVITY (*a*)		
		VANILLIN	ETHYL VANILLIN	COUMARIN
270	Basic	10.0	8.3	40.8
348	Basic	159.9	144.3	28.0
247	Acidic	10.1	9.3	13.6
309	Acidic	58.0	51.6	42.9

vanillin and coumarin, and hence is not considered a fair test of the method above. Two collaborators found its average background absorbance to be more than 0.020 and the other 2 found it less; the average of their averages is 0.019, which is fairly high for imitations.

D. Absorptivities.—The averages of all absorptivities (based on concentrations in grams per liter) submitted by the seven collaborators are given in Table 6. There was very little variance among the coefficients found. Coumarin standards in clear plastic vials were sent to three collaborators, and absorptivities obtained from these standards were completely out of line with those of others. Coumarin was again sent to the same collaborators, but this time in foil-wrapped glass vials; the latter standards gave proper values.

SUMMARY

The ultraviolet method gave excellent precision in the quantitative analysis of the vanillin-ethyl vanillin component, but running mixtures of the two vanillins by the vanillin-coumarin method gave low recoveries for the vanillins. The procedure was fairly accurate for coumarin; however, a high ratio of the vanillins to coumarin (15 and over to 1) tends to decrease accuracy. Determination of the type of flavoring (whether extract or imitation) by utilizing background absorbancies was correct in

35 out of 36 collaborative analyses. This method offers means for quick and economical assays of vanilla flavorings.

B. COLORIMETRIC DETERMINATION OF VANILLIN AND COUMARIN

Collaborative studies by colorimetric methods have been made on imitation vanilla, true vanilla, and enriched vanilla flavorings with vanillin contents of 1 gram per 100 ml and less. (*This Journal*, **34**, 330 (1951); **35**, 264 (1952)). This year, flavorings having 1.6 grams vanillin and about 0.14 to 0.16 grams coumarin per 100 ml have been studied by the same colorimetric methods, as revised; **35**, 77, 271 (1952). These methods have been adopted, First Action, for both coumarin and vanillin in true and imitation vanilla flavorings.

An authentic vanilla extract (Tahiti and Bourbon blend), which had vanillin and coumarin contents of 0.201 and 0.020 grams per 100 ml respectively *as determined by the colorimetric methods,* was obtained from a local manufacturer. This extract was enriched with 1.400 grams vanillin and 0.140 grams coumarin per 100 ml and was put into one ounce bottles designated "Sample I." Sample II, prepared as an imitation vanilla flavor, was made up to volume with a sugar-alcohol-water solution to contain 1.600 grams vanillin, 0.140 grams coumarin and 2 grams caramel per 100 ml. The imitation was put in one ounce bottles labeled "Sample II."

COLORIMETRIC DETERMINATION OF VANILLIN

Results of Collaborators.—Nine collaborators analyzed Samples I and II, reporting vanillin contents in duplicate. The neutral wedge photometer was used to determine the results listed in Table 7. In addition, Chemist 6 read the vanillin solutions, diluted as required, in the Beckman DU spectrophotometer at a wavelength of 610 mμ, and obtained the following results:

SAMPLE	VANILLIN (GRAMS/100 ML)	AVERAGE RECOVERY (%)
I	1.605, 1.613	100.5
II	1.605, 1.605	100.3

Chemist 4 had one high standard point (0.5 mg per 100 ml) which led to low recoveries. When the error was corrected, the vanillin contents of the samples were raised by 0.06 to 0.07 grams per 100 ml, and these corrected values are given in Table 7.

Discussion of Results on Vanillin.—Each collaborator obtained relatively close checks on duplicates for both samples with differences ranging from 0.0 to 0.07 grams per 100 ml for each sample. It is noted that Chemist

TABLE 7.—*Determination of vanillin in collaborative samples*

CHEMIST	VANILLIN (G/100 ML)		CHEMIST	VANILLIN (G/100 ML)	
	SAMPLE I	SAMPLE II		SAMPLE I	SAMPLE II
1	1.56	1.54	6	1.61	1.61
	1.57	1.55		1.61	1.61
2	1.60	1.60	7	1.54	1.56
	1.60	1.60		1.58	1.63
3	1.65	1.60	8	1.56	1.62
	1.65	1.61		1.56	1.64
4	1.52	1.49	9	1.58	1.60
	1.52	1.49		1.65	1.55
5	1.62	1.58			
	1.66	1.60			

2 obtained exact duplicates and the correct amounts of vanillin in each sample. The chemists who obtained low results on one sample usually did the same for the second sample. Results of chemists 7 and 9 were the most variable for both samples, but their average recoveries were excellent.

An arithmetic statistical analysis of the data in Table 7 is given in Table 8. Samples I and II contained 1.601 and 1.690 grams vanillin per 100 ml, respectively, (Sample I was the enriched extract and II the imitation). With these almost identical concentrations in the two different types of flavoring, very similar results were obtained. The average recovery of vanillin obtained for Sample I, 99.4 per cent, was only 0.5 per cent higher than that for Sample II. In addition, the measures of variation

TABLE 8.—*Analysis of collaborative results, vanillin (Table 7)*[a]

(1) SAMPLE NO.	(2) ARITHMETIC MEAN	(3) AVERAGE RECOVERY (%)	(4) STANDARD DEVIATION	(5) PROBABLE ERROR, SINGLE DETERMINATION
I	1.591	99.4	0.045	0.030
II	1.582	98.9	0.047	0.032
	(6) PROBABLE ERROR OF ARITHMETIC MEAN	(7) COEFFICIENT OF VARIATION	(8) RANGE	(9)[b] RANGE PERMITTED
I	0.007	2.83	1.52–1.66	1.47–1.71
II	0.008	2.97	1.49–1.64	1.45–1.71

[a] Data in columns 2, 4, 5, 6, 8, and 9 are in terms of grams per 100 ml of sample.
[b] Calculated on basis of arithmetic mean plus or minus 4 times the probable error of a single determination.

check well. For both samples, a single future determination has a 50 per cent chance of falling within the range, ±0.03 grams per 100 ml, and should another series of results be obtained for each sample, the arithmetic mean would have a 50 per cent chance of yielding a value varying less than 0.01 gram per 100 ml from the present mean. The coefficients of variation are 2.83 for Sample I and 2.97 for Sample II, disclosing a high consistency for the method on the two types of samples. These coefficients have the same magnitude as those obtained on less rich vanilla extracts and imitations in previous years.

It should be noted that the measures of variation (standard deviation, probable error of a single determination, probable error of arithmetic mean, and permitted range) for these high vanillin flavorings are approximately double those obtained previously on samples having less than 1.0 gram vanillin per 100 ml. The reason for this is the necessity for diluting samples having more than the above amount of vanillin to twice the volume with water before removing an aliquot for analysis, and in calculating the concentrations of vanillin in the samples a factor of 2 is employed.

COLORIMETRIC DETERMINATION OF COUMARIN

Results of Collaborators.—The same nine collaborators who analyzed the vanillin also determined the coumarin concentrations in duplicate. Chemist 2 employed the Coleman Spectrophotometer, and all other collaborators used the neutral wedge photometer for measuring color intensities. The results are tabulated in Table 9.

Discussion of Results on Coumarin.—Duplicate determinations were

TABLE 9.—*Determination of coumarin in collaborative samples*

CHEMIST	COUMARIN (G/100 ML)		CHEMIST	COUMARIN (G/100 ML)	
	SAMPLE I	SAMPLE II		SAMPLE I	SAMPLE I
1	0.17	0.16	6	0.16	0.14
	0.16	0.15		0.16	0.14
2	0.16	0.15	7	0.15	0.14
	0.16	0.15		0.15	0.14
3[a]	0.18	0.19	8	0.16	0.14
	0.19	0.19		0.16	0.14
4	0.15	0.14	9	0.15	0.14
	0.15	0.14		0.15	0.14
5	0.16	0.14			
	0.15	0.14			

[a] Results of Chemist 3 are outside the limits of random error.

within 0.01 gram per 100 ml of each other in all cases. The high results for coumarin by chemist 3 are outside the limits of the normal curves of distribution. Hence, they are listed in Table 9 but were eliminated in deriving the statistical summary of Table 10.

TABLE 10.—*Analysis of collaborative results, coumarin (Table 9)*[a]

(1) SAMPLE NO.	(2) ARITHMETIC MEAN	(3) AVERAGE RECOVERY (%)	(4) STANDARD DEVIATION	(5) PROBABLE ERROR, SINGLE DETERMINATION
I	0.156	97.6	0.007	0.005–
II	0.143	102.1	0.007	0.005–
	(6) PROBABLE ERROR OF ARITHMETIC MEAN	(7) COEFFICIENT OF VARIATION	(8) RANGE	(9)[b] RANGE PERMITTED
I	0.001	4.75	0.15–0.19	0.14–0.17
II	0.001	4.78	0.14–0.19	0.13–0.16

[a] Data in columns 2, 4, 5, 6, 8, and 9 are in terms of grams per 100 ml of sample.
[b] Calculated on basis of arithmetic mean plus or minus 4 times the probable error of a single determination.

Chemist 3 was last to report, and had retained the standard coumarin for seven months. He made ultraviolet absorption readings on the standard solutions of coumarin and obtained higher than normal values. The Associate Referee has checked this observation and has found that the ultraviolet absorption spectrum of a basic solution shifts for coumarin standards retained in the light for a period of days. This shows that the coumarin molecular structure changes, probably with the opening of the second ring. Such a change would reduce color intensities by the colorimetric method and produce a lower standard curve and higher recoveries. It is concluded the 0.18 to 0.19 gram per 100 ml recoveries for Samples I and II are not representative of the coumarin method here studied and are beyond the limits for random error as determined by statistical methods.

The average recovery of coumarin in Sample I (the enriched extract) was 97.6 per cent compared to 102.1 per cent in Sample II, the straight imitation. For both samples the measures of variation are relatively small and agree closely with each other and with those obtained in previous studies.

The results for Sample I ranged from 0.15 to 0.19 and those for Sample II from 0.14 to 0.19 gram per 100 ml. The limits of random error (permitted range) are 0.14 to 0.17 gram per 100 ml for Sample I and 0.13 to 0.16 gram per 100 ml for Sample II, which necessitated, as stated before, the elimination of the results only of Chemist 3. The limits of random error for these two distributions of coumarin results also compare favorably with those found previously on vanilla extracts and imitations.

CONCLUSIONS

The colorimetric procedures for vanillin and coumarin as revised and adopted, First Action, have worked equally well for an enriched vanilla extract and an imitation vanilla flavoring, each having 1.6 grams vanillin per 100 ml and about one-tenth as much coumarin. Also, the collaborative results gave normal distributions for both vanillin and coumarin, and gave measures of variation for coumarin comparable in magnitude to those obtained by previous collaborative studies. The measures of variation for vanillin this year are approximately double those obtained before, which is a reflection of the dilution of the samples to double volume before removing aliquots for analysis and the multiplication of results obtained by 2. The average recoveries for both vanillin and coumarin were in the range 97.6 to 102.1 per cent.

RECOMMENDATIONS

It is recommended*—

(1) That the colorimetric method for vanillin, *This Journal*, **34,** 72 (1951), as revised, *This Journal*, **35,** 271 (1952), and adopted, First Action, for vanilla imitations, extracts, and enriched vanilla flavors, be made Official.

(2) That the colorimetric method for coumarin, *This Journal*, **34,** 73 (1951), as revised, *This Journal*, **35,** 271 (1952), and adopted, First Action, for vanilla imitations, extracts, and enriched vanilla flavors, be made Official.

(3) That studies be continued on methods **19.4** and **19.5.**

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Charles H. Eisenberg. Cincinnati
Raymond H. Johnson, Portland
F. C. Minsker, Philadelphia

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

No reports were received on *beta*-ionone, peel oils in citrus juices, organic solvents in flavors, emulsion flavors, maple flavor concentrates and imitations, or propylene glycol.

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*

(1) *Rutin in tablets.*—The Associate Referee submitted a comprehensive report covering collaborative work by nineteen laboratories on forty-two preparations. The method depends on the quantitative extraction of rutin from tablets by acidified alcohol and the measurement of the rutin by the spectrophotometer at 352.5 mμ. The method was considered acceptable by all of the collaborators and the Associate Referee recommends that it be adopted as official, First Action. The Referee concurs in this recommendation.

(2) *Quinine and strychnine.*—The Associate Referee submitted to collaborators a modified method of the former tentative method for the separation of quinine and strychnine. This method involved essentially the precipitation of strychnine by potassium ferrocyanide (quinine is not precipitated) and the subsequent gravimetric determination of the two alkaloids. In general, satisfactory results were obtained for both alkaloids.

The report includes directions for treatment of a sample if the quinine and strychnine are to be determined in tablets, liquid preparations, and if iron is present. It is noted that the collaborative samples consisted of appropriate quantities of strychnine and quinine alkaloid weighed individually into glass vials. Undoubtedly these samples were analyzed by the collaborators within a relatively short time.

It is the recollection of the Referee from discussion with others who have concerned themselves with this problem in the past and from experience in analyzing samples of quinine and strychnine in combination in tablet form, that for some unexplained reason, if the product was two or three years or more old, unreliable results were obtained. It is not known just what the limits of this age factor are, nor can it be explained in any rational manner; nevertheless it appears to exist. It may be a deterioration of the alkaloids due to contact with certain excipients used in tablets, or a combination with other ingredients not readily broken up in the assay process, which gives different solubility properties to the alka-

* For report of Subcommittee B and action of the Association, see *This Journal*, 36, 52 (1953).

loids. Whatever the cause (if in fact it is a factor in the determination) it is believed that some study should be made before the method is adopted as official. It is suggested that study be applied to collaborative samples prepared by adding known quantities of quinine and strychnine to appropriate quantities of the usual tablet excipients and assaying these samples periodically over a period of months sufficient to establish the effect, if any, of age. This study should also be applied to a liquid preparation. It is recommended that the subject be continued along the lines outlined.

(3) *Aminopyrine, ephedrine, and phenobarbital.*—No report was received. The Referee recommends that the subject be continued.

REPORT ON RUTIN IN TABLETS

By ARTHUR TURNER, JR. (Eastern Regional Research Laboratory, Bureau of Agricultural and Industrial Chemistry, U. S. Department of Agriculture, Philadelphia 18, Pennsylvania), *Associate Referee*

A method for the determination of rutin in pharmaceutical tablets was included in the 9th Edition of the National Formulary (1). This method was derived from the tentative spectrophotometric procedure of Porter, *et al.* (2).* A revision of the method, made to detect and allow for non-rutin absorption, has now been given a collaborative test in 19 laboratories and is the subject of this report. Two progress reports have been made previously to the A.O.A.C. (3).

The method tested collaboratively depends on the quantitative extractability of rutin from tablets by acidified ethanol, and on the fact that the maximum at 352.5 mμ in the characteristic absorption spectrum can provide a precise measure of the rutin concentration. The solvent chosen for the extraction was 50 per cent ethanol containing 5 per cent acetic acid. The inclusion of acid in the extraction reagent is important because rutin decomposes in alkaline media and solutions of many of the tablet preparations are alkaline. Figure 1 shows the absorption spectrum of rutin in acidified water. The curve, with two absorption maxima, is characteristic of the flavonols. In the case of rutin these maxima are at 255 and 352.5 mμ. The location and intensity of the maxima, especially the long wavelength maximum, depend on both solvent and *p*H. The long wavelength maximum moves toward longer wavelengths and increases in intensity when the *p*H exceeds 6. Repeated analyses of rutin† at this laboratory have established its absorptivity at 352.5 mμ as 26.3 at low *p*H in water solution. Under the conditions of the procedure to be outlined, the absorption by rutin follows the Lambert-Beer Law.

* A modification of the method of Porter appeared in *J. Pharm. Pharmacol.*, 1, 323 (1949) by R. V Swann.

† The rutin used in this study was specially prepared by Dr. J. Naghski. The purity of the sample was established by repeated crystallizations and subsequent analysis by ultraviolet absorption (2).

To detect absorption by contaminants, the solution absorbance, defined by $A = \log (1/T)$, where T is the transmittance relative to solvent, is measured at the wavelength of maximum absorption by rutin, 352.5 mμ, and at two wavelengths, 338.5 mμ and 366.5 mμ, equidistant from the maximum. Absorbance ratios $R_1 = A_{338.5}/A_{352.5}$, and $R_2 = A_{366.5}/A_{352.5}$ are then determined. A consideration of Figure 2 will explain this approach. When observations are made on pure rutin the ratios are $R_1 = 0.914$ and $R_2 = 0.842$. If R_1 is increased and R_2 is decreased an interfering absorption of negative slope is indicated. If the change in the ratios is reversed it indicates that the interfering absorption has a positive slope. If both ratios are increased it indicates a non-selective or nearly uniform interference. Quercetin is a natural contaminant in rutin preparations and is

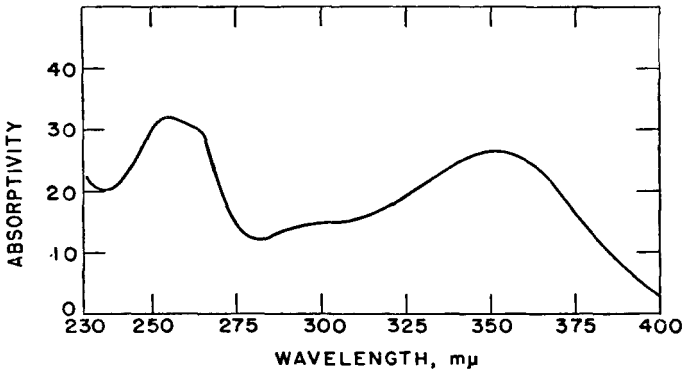


FIG. 1.—Absorption spectrum of pure rutin in acidified water.

permissible up to a level of 5 per cent (1). Structurally, quercetin differs from rutin only in that it lacks the glucose-rhamnose residue attached to the rutin molecule. Its absorption spectrum differs from that of rutin in that the long wavelength maximum is at 366.5 instead of 352.5 mμ (which impelled the choice of wavelengths used in R_2) and its absorptivity is approximately twice as great. With quercetin as the interfering constituent, R_1 is decreased and R_2 is increased. The quantities of rutin and quercetin present may be calculated by solving simultaneous equations based on known constants of rutin and quercetin and absorptivities observed at two wavelengths. Limits were calculated for R_1 and R_2, assuming wavelength errors of ± 0.2 mμ and an observational error of ± 0.002 absorbance units. The calculated range was ± 0.009. However, the collaborative study indicates that the values should be: for R_1, ± 0.009 and for R_2, ± 0.013.

The method was applied to the analysis of 42 preparations supplied by

20 tablet manufacturers.* The results may be summarized as follows: 23 preparations (55 per cent) had ratios indicative of essentially pure rutin; 12 preparations (29 per cent) had ratios indicating the presence of quercetin; in 6 preparations (14 per cent) R_1 exceeded its limits, whereas R_2 remained within its limits. The last condition was interpreted as indicating an interfering absorption with a negative slope, which could be ignored. Figure 2 illustrates this type of interference and the magnitude of the error created by neglecting it. Of the 42 preparations analyzed, only one had ratios outside both allowable limits. Thirty-four preparations (81 per cent) had a rutin content within the ± 7.5 per cent of the labeled amount of rutin permitted by the National Formulary (1). Four preparations gave values below 92.5 per cent of the labeled amount of rutin. Recovery experiments on synthetic samples, which included the usual tablet excipients, gave values within ± 2 per cent of those anticipated. Believing that no simple method could be expected to cover completely all the possibilities of tablet formulation, but that the procedure proposed would indicate the majority of significant interferences, the Associate Referee offered the method for criticism at the 1951 Association meeting. Since there were no major criticisms, the method was subjected to collaborative study.

THE COLLABORATIVE STUDY

The main points to be determined by collaborative study were the validity of the value, 26.3, as the absorptivity of rutin at 352.5 mμ, the values of the ratios, R_1 and R_2, and their limits, and the precision and accuracy to be expected from the procedure.

A single weighed sample of pure rutin was supplied to each collaborator, with instructions for its transfer and dilution. No extraction manipulations were required. This sample was included to provide a check on the wavelength and photometric scales. Should any data submitted be definitely irregular, these two important factors could be evaluated and the suspected data interpreted in the light of these facts.

The second section of the study required the recovery of known amounts of pure rutin. This step called for the extraction of rutin from a powdered preparation which included some of the usual tablet excipients (lactose, starch, calcium phosphate). Efforts to prepare a synthetic tablet or powder of known rutin content were abandoned after repeated attempts produced preparations which gave low recoveries. This difficulty was

* The following manufacturers supplied tablet samples: Abbott Laboratories, North Chicago, Ill.; Boyle and Co., Los Angeles; S. F. Durst & Co., Inc., Philadelphia; Eli Lilly & Co., Indianapolis; Keith-Victor Pharmacal Co., St. Louis; The S. E. Massengill Co., Bristol., Tenn.; Pitman-Moore Co., Indianapolis; Premo Pharmaceutical Laboratories, Inc., South Hackensack, N. J.; Schenley Laboratories, Inc., New York; Sharp & Dohme, Inc., Philadelphia; E. R. Squibb & Sons, Brooklyn; R. J. Strasenburgh Co., Rochester, N. Y.; U. S. Vitamin Corp., New York; Buffington's, Worcester, Mass.; Paul B. Elder Co., Bryan, Ohio; Empire Chemical Co., New Brunswick, N. J.; National Drug Laboratories, Inc., Chicago; Richlyn Laboratories, Inc., Philadelphia; Raymer Pharmacal Co., Philadelphia; and Standard Chemical Co., Des Moines, Iowa.

traced to the preferential adsorption of rutin on the walls of the containers used. The added rutin could easily be recovered if the entire sample (including container washings) was analyzed, but if portions of the whole were used, the results were low. To overcome this difficulty, samples of pure (unknown to the collaborator) rutin were supplied, with instructions for weighing and admixture of excipients. Enough rutin and excipients was supplied for quadruplicate analyses. This step was to check the absorptivity value, the ratios and their limits, and to indicate the precision and accuracy to be expected.

The third section of the study called for the analysis of a commercial tablet. The tablet chosen contained ascorbic acid as an additional active ingredient. The rutin used in the preparation of the tablet showed evidence of quercetin. The source of the tablet was unknown to the collaborators but the labeled value of 20 mg per tablet was given. The analysis of this tablet provided a test of the method under more extreme conditions than in the preceding section.

Instructions, data sheets, and a questionnaire were supplied to collaborators in addition to samples for analysis. The instruction sheet described the procedure and calculations and also requested the analysis of other commercial samples. The data sheets had designated blanks for the recording of original data and the intermediate and final calculations. This form of reporting allowed recomputation of the results submitted. The questionnaire included questions on wavelength calibration, absorption cells, grade of glassware used, and questions on the utility and acceptability of the method. Twenty-three sets of samples were distributed to the 14 manufacturers and 9 government laboratories that cooperated.* Four collaborators had to withdraw from the study due to personnel changes or instrumental difficulties.

METHOD

EQUIPMENT AND REAGENTS

(a) *Centrifuge tubes.*—Conical, 50 ml.

(b) *Centrifuge.*—With head accommodating 50-ml tubes.

(c) *Glass stirring rods.*—Of small enough diam. to dislodge material from the tips of 50 ml conical centrifuge tubes.

(d) *Glass funnels.*—Approx. 45 mm diam., short stem.

(e) *Water bath.*—70–80°C.

(f) *Flasks.*—Volumetric; 100 ±0.4 ml or better; 250 ±1.0 ml or better; 500 ±2.0 ml or better.

(g) *Transfer pipets.*—10 ±0.04 ml or better.

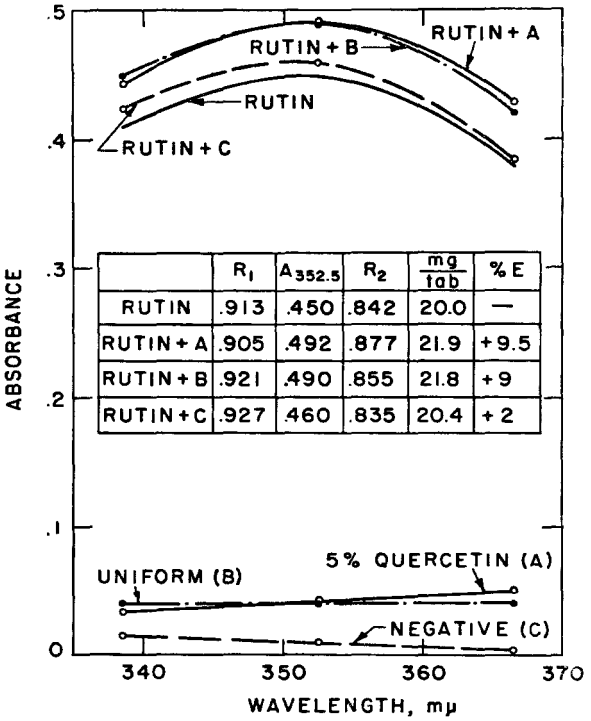
(h) *Spectrophotometer.*—Capable of isolating the following wavelengths: 338.5 mμ, 352.5 mμ, and 366.5 mμ.

* The following laboratories participated in the collaborative study: Abbott Laboratories, North Chicago, Ill.; Boyle and Co., Los Angeles; Eli Lilly and Co., Indianapolis; S. E. Massengill Co., Bristol, Tenn.; Premo Pharmaceutical Laboratories, Inc., South Hackensack, N. J.; Schenley Laboratories, Inc., New York; Sharp and Dohme, Inc., Philadelphia; R. J. Strasenburgh Co., Rochester, N. Y.; U. S. Vitamin Corp., New York; The Upjohn Co., Kalamazoo, Mich.; Food & Drug Administration Laboratories in Philadelphia, San Francisco, Cincinnati, Denver, Chicago and New York; Department of National Health and Welfare, Food and Drug Divisions, Ottawa, Canada; and Eastern Regional Research Laboratory, U. S. Dept. of Agriculture, Philadelphia.

(i) *Absorption cells.*—Matched, 1 cm.
(j) *Analytical balance.*—Accurate to ±0.5 mg.
(k) *Acetic acid.*—Glacial, A.C.S.
(l) *Ethanol.*—U.S.P., 95%.
(m) *Acid-alcohol reagent.*—Prepared with above reagents; 550 ml 95% ethanol plus 50 ml glacial acetic acid dild to one l with distd H_2O.

DETERMINATION

Extraction.—Weigh directly into a 50 ml centrifuge tube the number of tablets required to give 0.05 to 0.50 g of rutin (not less than 5 tablets). Record the number and wt. (If tablets are coated, after weighing dissolve coating with distd H_2O, discard the aq. washings, and transfer the rutin-contg core to centrifuge tubes.) Add 20 ml acid-alcohol reagent and, by means of the stirring rod, break up tablets. After tablets are thoroly disintegrated, heat mixt. in H_2O bath maintained at



	R_1	$A_{352.5}$	R_2	mg/tab	%E
RUTIN	.913	.450	.842	20.0	—
RUTIN + A	.905	.492	.877	21.9	+9.5
RUTIN + B	.921	.490	.855	21.8	+9
RUTIN + C	.927	.460	.835	20.4	+2

FIG. 2.—Effect of absorbing impurities on the absorbance curve of rutin near the maximum at 352.5 mμ. The lower group of curves illustrate background absorbance where: (A) has a positive slope, as exampled by quercetin; (B) is uniform with wave length; and (C) has a negative slope. The upper group of curves show how these impurity absorptions modify the absorbance curve of rutin. The table gives numerical data on the absorbance by pure rutin and the three impure rutin preparations; the ratios R_1 and R_2; the computed weight of rutin in mg per tablet; and, in the last column, the per cent error brought about by ignoring the effect of the absorbing impurities.

70–80°C. for 10 min. During this period resuspend the material occasionally by stirring. At the end of this period remove stirring rod, rinse with acid-alcohol reagent, and centrifuge mixt. at ca 2000 r.p.m. for 15 min. After centrifugation, decant supernatant into 250 ml volumetric flask. Use funnel, decant with one smooth motion, and allow tube to drain for ca 10 sec. While still inverted, rinse mouth of tube with acid-alcohol reagent. Ext. twice more, starting with "Add 20 ml acid-alcohol reagent. . . . " After the third extn, dil. combined supernatants to 250 ml with acid-alcohol reagent. Any insol. material may be removed by filtration after diln if the first portions of filtrate are discarded. Depending on the original wt of rutin taken, make a diln with distd H_2O to give a final concn of 0.01–0.03 g/l of rutin. Precipitates forming during this aq. diln may be removed by filtration if the first portions of filtrate are discarded. Discarding the first 15–20 ml of filtrate guards against concn changes due to adsorption.

ABSORPTIMETRY

Det. the absorbance of this aq. diln at 338.5, 362.5, and 366.5 mμ against a distd H_2O blank. Calc. the following:

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

where a = absorptivity; A = absorbance; b = cell length, cm; and c = concn in g/l (at the final diln, assuming the tablet to be completely soluble).

$R_1 = \frac{A_{338.5}}{A_{352.5}}$, the ratio of the absorbancies at 338.5 and 352.5 mμ.

$R_2 = \frac{A_{366.5}}{A_{352.5}}$, the ratio of the absorbancies at 366.5 and 352.5 mμ.

A sample calculation, using typical data, is as follows:

No. of tablets, 5. Wt. of 5 tablets, 812 mg. Av. wt. of tablet, 162.4 mg. Sample wt., 0.812 g. Final diln, 10–200 ml. Cell length $b = 1.004$ cm. Concn $c = 0.812 \times 4 \times 10/200 = 0.1624$ g/l.

$$A_{338.5} = 0.490 \qquad R_1 = \frac{0.490}{0.537} = 0.913$$

$$A_{352.5} = 0.537$$

$$A_{366.5} = 0.453 \qquad R_2 = \frac{0.453}{0.537} = 0.844$$

$$a_{352.5} = \frac{0.537}{1.004 \times 0.1624} = 3.293$$

CALCULATION OF RUTIN CONTENT

If $R_1 = 0.914 \pm .009$ and $R_2 = 0.842 \pm 0.013$, the extd material can be considered pure rutin and the wt of rutin per tablet can be calcd by means of the following equation:

$$\text{mg rutin per tablet} = \frac{a_{352.5}}{26.3} \times \text{av. wt. of tablet (mg)}$$

Using the data given in the above sample calculation,

$$\text{mg rutin per tablet} = \frac{3.293}{26.3} \times 162.4 = 20.3$$

A value of R_1 beyond its upper limit while R_2 remains within its range indicates an interfering absorption which diminishes rapidly enough to be ineffective at 352.5

mμ. Under this condition the absorbance observed at 352.5 mμ is accepted as correct and the rutin content calcd as for pure rutin. An increase in R_2 while R_1 remains within or below its limits usually indicates the presence of quercetin. The amount of rutin and quercetin may be calcd as follows:

$$\text{mg rutin per tablet} = [0.1452a_{352.5} - 0.1273a_{366.5}] \times \text{av. wt. of tablet (mg)}$$

$$\text{mg quercetin per tablet} = [0.05099a_{352.5} + 0.06057a_{366.5}] \times \text{av. wt. of tablet(mg)}$$

The value of $a_{366.5}$ is calcd in the same manner as $a_{352.5}$ except that $A_{366.5}$ is used. The above equations are based on $a_{352.5}$ and $a_{366.5}$ for rutin as 26.31 and 22.15, respectively, and for quercetin, 55.29 and 63.06, respectively.

A simultaneous increase or decrease of both ratios beyond their respective limits indicates an invalidating condition. This condition could be due to an interfering absorption or it may indicate destruction of the rutin in the tablet formulation. Interpretation of results requires that the analyst use reasonable judgment based on all the facts; the above limiting conditions are intended only as guides.

RESULTS OF THE COLLABORATIVE STUDY

The results of the study are collected in Table 1. All the results reported were recomputed by the Associate Referee and then computed independently by a colleague. Where errors in calculations were discovered, the specific point was investigated and the result reported in the table was verified.

The Standard Sample.—This was the sample of pure rutin used to test the instrument. Since only single determinations were made on weighed samples, only the average values for the 19 analysts are reported: $R_1 = 0.907$, $R_2 = 0.845$, and $a_{352.5} = 26.47$. No significant errors were demonstrated by the data submitted on this sample.

The Recovery Sample.—This was the sample which required weighing and extraction. The data indicate that the mean of quadruplicate determinations for $a_{352.5}$ will fall within 26.31, ± 0.52, or that 95 times in 100 the error should not exceed 2 per cent. The most probable values for R_1 and R_2 are 0.914 ± 0.009, and 0.842 ± 0.013, respectively, where the limits represent two times the standard deviation of the means for R_1 and R_2. Some indication of the accuracy of the method can be obtained by comparison of the two values for the absorptivity at 352.5 mμ. The value 26.47 was obtained by simple dilution and 26.31 by extraction.

The Tablet Sample.—The factors evaluated by this sample are the constancy of the tablet weight, the limits for R_1 and R_2, and the combined precision of the tablet composition and the method. The tablet weights were constant to ± 0.5 per cent. The limits for R_1 and R_2 are, as they should be, essentially the same as determined from the Recovery Sample. The precision is not as high as in the Recovery Sample, the standard deviation being 2.7 per cent as compared to 1 per cent. Inhomogeneity of sample rules out commercially prepared tablets as standards for evaluating precision. This variation from tablet to tablet is reflected in the generally higher standard deviations of the individual analysts. The precision of the method is better evaluated by the Recovery Sample because, under

TABLE 1.—*Compilation of data submitted by collaborators*[a]

COLLABORATOR NO.	RECOVERY SAMPLE						TABLET SAMPLE							
	R_1		R_2		s_{R_1,R_2}		AV. WT TABLETS		R_1		R_2		MG/TAB	
	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
15	9172	19	8432	28	2610	24	1639	7	9075	13	8635	34	1942	16
17	9134	26	8432	41	2661	13	1641	11	8969	20	8667	12	1941	25
18	9171	16	8358	7	2595	6	1644	4	9117	20	8610	40	2064	7
34	9157	23	8293	34	2650	51	1632	5	9092	16	8459	33	2080	24
35	9121	16	8399	10	2666	8	1639	10	9055	34	8627	18	1961	28
36	9081	32	8451	3	2584	7	1644	11	9000	24	8659	30	1924	21
37	9146	18	8428	41	2638	7	1641	4	9067	15	8574	10	2023	10
55	9194	42	8441	38	2633	16	1639	9	9015	9	8616	20	1994	25
56	9121	13	8476	16	2645	27	1636	11	9054	8	8683	7	1941	17
57	9063	16	8457	12	2605	14	1643	1	9045	4	8675	4	1918	4
73	9176	18	8399	31	2628	10	1637	3	9037	2	8636	23	1946	13
74	9217	31	8435	42	2665	8	1635	6	9144	9	8640	8	1970	6
75	9108	16	8513	36	2645	16	1640	4	9040	7	8751	0	1915	2
76	9108	14	8297	24	2580	8	1644	7	9052	20	8516	29	2031	36
77	9229	59	8441	53	2633	25	1636	0	9096	29	8617	8	1970	9
93	9057	13	8370	9	2618	6	1630	7	9163	25	8564	95	1975	51
95	9119	33	8541	17	2632	20	1648	6	9010	35	8718	30	1897	26
96	9127	51	8440	42	2655	17	1644	4	9072	44	8712	36	1884	36
97	9148	34	8365	24	2640	6	1640	8	9095	10	8583	17	2025	12
$\bar{\bar{x}}$	9139		8419		2631		1640		9063		8634		1968	
$s_{\bar{x}}$	47		63		26		5		49		71		55	

[a] Values given to the nearest integer. Standard deviation, s; $s = \sqrt{\frac{\sum (x - \bar{x})^2}{n - 1}}$; x = individual values; $\bar{x}$ = mean of the individual values; n = number of analyses (in this case, $n = 4$); $\bar{\bar{x}}$ = average of the means; $s_{\bar{x}}$ = standard deviation of the means ($n = 19$). For ease in calculations, decimal points have been shifted. See sample calculation for magnitude.

the conditions established, the problem of homogeneity of sample was avoided.

The Questionnaire.—The replies to questions concerning the instrument used may be summarized as follows: all the collaborators used the Beckman DU spectrophotometer; 10 used the tungsten lamp plus filter; 9 used the hydrogen lamp; nearly all checked the wavelength scale by means of a mercury lamp; the majority used high sensitivity settings and slit widths in the neighborhood of 0.3 mm.

None of the collaborators felt the method required equipment that was not already part of their general laboratory equipment. No operation was considered particularly troublesome. A few collaborators made suggestions for minor changes, e.g. "warming tablets hastens disintegration," "use sintered glass funnels instead of centrifugation," "use glass stoppered centrifuge tubes," "use sand as a diluent to avoid gumming." A few were confused by the calculations and asked for elucidation. All felt that the qualitative indications of interference made evident by the values of R_1 and R_2 were worth while. The method was considered acceptable by all the collaborators and nearly all who tried the method on their own preparations or other commercial preparations reported success. The exception was a complex tablet preparation containing four active ingredients plus dyes.

RECOMMENDATION

It is recommended* that the proposed method be adopted, First Action.

ACKNOWLEDGMENT

The Associate Referee would like to thank the manufacturers and collaborators for their fine cooperation and to acknowledge the technical assistance of Miss Mary Ann Morris and Mr. Charles S. Fenske. Thanks are due Dr. William L. Porter, previous Associate Referee and author of the National Formulary procedure, for advice during the early phases of the work, and Dr. Joseph Naghski for supplying the pure rutin sample.

REFERENCES

(1) The National Formulary, 9th Ed., Mack Publishing Co., Easton, Pa., 1950, pp. 440–441.

(2) PORTER, W. L., BRICE, B. A., COPLEY, M. J., and COUCH, J. F., U. S. Dept. of Agriculture, Bur. of Agricultural and Industrial Chemistry, AIC-159 (July 1947).

(3) TURNER, A., JR., *This Journal*, **34**, 565 (1951); **35**, 566 (1952).

* For report of Subcommittee B and action of the Association, see *This Journal*, **36**, 52 (1953)

REPORT ON QUININE

SEPARATION OF QUININE AND STRYCHNINE

By David J. Miller (Food and Drug Administration, Department of Health, Education, and Welfare, Buffalo, New York), *Associate Referee*

In 1949 (1) it was recommended that the tentative A.O.A.C. method for the separation of quinine and strychnine be deleted and further study made of that subject. In that report three alternative methods of separation were discussed: the Herd method (2), a modification of the A.O.A.C. method, and a spectrophotometric method. Although the Associate Referee had used the Herd method and found it reliable, the modified A.O.A.C. method appeared to be equally accurate and required considerably less manipulation. Accordingly, the Herd method was not offered for collaborative study. The modified A.O.A.C. method was tested by collaborators. The spectrophotometric method was studied but not tested collaboratively. The latter two methods are discussed separately below.

(A) MODIFIED A.O.A.C. (GRAVIMETRIC) METHOD

Before submission of the method to collaborators, strychnine ferrocyanide was studied with respect to its solubility, composition, and stability at 100°C. Using the spectrophotometric method described later, the solubility of strychnine ferrocyanide in the sulfuric acid-potassium ferrocyanide solution in which it is precipitated (5 ml of 4% $K_4Fe(CN)_6$ and 50 ml of 20% sulfuric acid) was found to be less than 0.01 mg/100 ml. In 1+99 HCl the solubility was 0.8 mg/100 ml.

On a stoichiometric basis the reaction between strychnine and potassium ferrocyanide in sulfuric acid solution should result in a compound having the formula $C_{21}H_{22}N_2O_2 \cdot K_4Fe(CN)_6$, containing 60.76% strychnine. Cumming and Brown (3), by assay for iron, found air-dried strychnine ferrocyanide to have the composition shown above plus 2 molecules of water. The Associate Referee, by assay for strychnine, found strychnine ferrocyanide dried at 100°C. to have the composition of the anhydrous salt essentially as shown above.

Strychnine-ferrocyanide precipitates, dried for 1, 2, 3, and 4 hours at 100°–105°C., showed total losses of only 0.1 to 0.3 mg after the first hour.

Portions of N. F. strychnine, previously dried at 100°C., assaying 99.8 per cent strychnine by titration, and of quinine, twice recrystallized from benzene and dried at 125°C., were individually weighed into vials and submitted to collaborators. Weights and ratios of the two alkaloids were chosen so as to give approximately a 50-to-1 ratio of quinine and strychnine and an actual weight of strychnine of 20–25 mg. In order to insure as much uniformity as possible, a small amount of recrystallized potassium ferrocyanide was furnished each collaborator for use as a reagent, and the request made that the final filtration of the strychnine ferrocyanide be

made the same day as the separation was started. The following method was furnished.

QUININE AND STRYCHNINE

REAGENTS

(a) *Potassium ferrocyanide,* $K_4Fe(CN)_6 \cdot 3H_2O$.—Recrystallize from hot H_2O and dry at room temp. Prepare a 4% soln in H_2O as needed.

(b) *Sulfuric acid,* 20%.—114 ml of concd H_2SO_4 to one liter.

(c) *Sulfuric acid,* 40%.—228 ml of concd H_2SO_4 to one liter.

(d) *Sulfuric acid-potassium ferrocyanide wash solution.*—Add 10 ml reagent (a) to 100 ml 20% H_2SO_4. Prepare freshly as needed.

DETERMINATION

Strychnine.—Dissolve the mixed alkaloids in 50 ml 20% H_2SO_4, warming on steam bath to aid soln if necessary. Cool, add 5 ml 4% $K_4Fe(CN)_6$ dropwise with stirring, and allow the resultant ppt to stand for 3 hrs, stirring occasionally. Filter on a 7 cm retentive filter (S&S 589 blue or equivalent), and wash with 15 ml H_2SO_4-$K_4Fe(CN)_6$ wash soln, catching the filtrate and washing in a 250 ml volumetric flask. Remove filter paper and wash funnel with 10–15 ml H_2O. Transfer ppt on the filter paper to the original beaker with ca 25 ml H_2O. Add 10% NaOH dropwise until soln clears and then add ca 10 drops (0.5 ml) more. Add ca 25 ml 40% H_2SO_4 gradually, followed by 2 ml of 4% $K_4Fe(CN)_6$ added dropwise. Allow the resultant ppt to stand 3 hrs and filter on a fine sintered glass filter with suction. It is convenient to catch filtrate and washings in same volumetric flask used for first filtration, using a bell jar for the filtration. Transfer ppt with minimal amount H_2SO_4-$K_4Fe(CN)_6$ wash soln (15–20 ml). Wash ppt on filter portionwise with 15 ml of ice cold 1% HCl. Wash finally with 2 ml ice-cold H_2O and suck dry. Dry ppt at 100°C. (2 hrs is usually sufficient) and weigh.

Wt. ppt $\times$ 0.6076 = wt strychnine.

Quinine.—Make soln of quinine in volumetric flask to 250 ml and transfer aliquot contg ca 0.2 g quinine to a 250 ml separatory funnel. Neutralize with 30% NaOH (approximate neutrality is reached when quinine ppts out). Add 1 ml alkali in excess and ext quinine completely with $CHCl_3$ (6–50 ml portions are usually sufficient). Wash each extn successively through 10 ml of H_2O contg 2 drops NH_4OH. Filter extracts through cotton, evap. to small vol., transfer to suitable weighing dish, and evap. to dryness. Dry residue at 120°C. (2 hrs. usually sufficient) and weigh as anhyd. quinine.

Results obtained are shown in Table 1.

COMMENTS BY COLLABORATORS

Collaborator 3: Believe that directions for the 1+99 HCl wash might well read, "Wash ppt on filter with five 3 ml portions of ice-cold 1+99 HCl, sucking ppt dry between washes. Washes are hardly enough to wash properly and adequately the sides of the filtering crucibles which are . . . 30 ml size."

DISCUSSION

Quinine recovery by all collaborators is considered quite good. With the exception of Collaborator No. 3, the strychnine recovery may also be considered acceptable. Correspondence with Collaborator No. 3 revealed there was some question of complete removal of sulfuric acid-potassium

TABLE 1.—*Assay of strychnine and quinine by modified A.O.A.C. method*

COLLABORATOR	QUININE IN SAMPLE, G	QUININE REPORTED, G	PER CENT RECOVERY	STRYCHNINE IN SAMPLE, G	STRYCHNINE REPORTED, G	PER CENT RECOVERY	RATIO QUININE/ STRYCHNINE
1	1.1042	1.0925	98.9	0.0223	0.0228	102.2	50–1
	1.3480	1.3420	99.6	0.0246	0.0245	99.6	55–1
2	1.0779	1.065	98.8	0.0219	0.0219	100.0	49–1
	1.2139	1.215	100.1	0.0250	0.0255	102.0	49–1
3	1.2058	1.2130	100.6	0.0248	0.0289	116.5	49–1
	1.0678	1.0805	101.2	0.0203	0.0205	101.0	53–1
	0.9971	1.0060	100.9	0.0196	0.0218	111.2	51–1
	1.0841	1.0910	100.6	0.0220	0.0229	104.1	49–1
4	0.9158	0.9175	100.2	0.0203	0.0199	98.0	45–1
	1.1674	1.1645	99.8	0.0250	0.0245	98.0	47–1
5	1.1515	1.1565	100.4	0.0251	0.0252	100.4	46–1
	1.0311	1.0330	100.2	0.0214	0.0217	101.4	48–1
6	1.0069	1.0230	101.6	0.0202	0.0196	97.0	50–1
	1.1036	1.0740	97.3	0.0229	0.0219	95.6	48–1
7[a]	0.5022	0.5043	100.4	0.0250	0.0245	98.0	20–1
	0.4863	0.4901	100.8	0.0242	0.0239	98.8	20–1
	0.4933	0.4957	100.5	0.0247	0.0247	100.0	20–1
	0.9307	0.9370	100.7	0.0232	0.0219	94.4	40–1
	0.9946	0.9905	99.6	0.0256	0.0250	97.7	39–1
	0.9231	0.9265	100.4	0.0231	0.0225	97.4	40–1
Av. (not including strychnine results for No. 3)			100.1			98.8	

[a] The Associate Referee.

ferrocyanide solution because of the large size crucibles used. While the method is not "micro," it does require the use of some semi-micro apparatus. The directions therefore should be modified to limit the size of the crucibles and to amplify washing directions. Such modification could be accomplished by the insertion in the directions of the parenthetical phrase "(15 ml size or smaller)" after the words "sintered glass filter," and substitution of the words "with five 3 ml portions" for the words "portionwise with 15 ml."

The procedure for the separation of quinine and strychnine requires a prior isolation of the mixed alkaloids in relatively pure form. In general this isolation can be obtained by well-recognized alkaloidal extraction methods. The present official A.O.A.C. methods for strychnine in liquid preparations and strychnine in tablets will serve to isolate quinine as well.

Iron, frequently present in combination with quinine and strychnine, can be kept from precipitating by the use of citric acid. The method, as submitted to collaborators, should therefore be modified by including general directions for the isolation of the mixed alkaloids. If nux vomica is present instead of strychnine, brucine will also be extracted and will stay with quinine in the separation procedure. The method, therefore, is not suitable for preparations containing nux vomica, unless strychnine only is desired, and appropriate note should be so made. Such modification could take the following form:

SEPARATION OF QUININE AND STRYCHNINE*

(ISOLATION OF QUININE AND STRYCHNINE)

Tablets.—Proceed as in **32.62**, using sufficient sample to yield 20 mg of strychnine. If residue appears impure, or if appearance of ext. or composition of tablet indicates that substances other than alkaloids are being extd, shake out alkaloids completely from $CHCl_3$ soln with 5% H_2SO_4 or HCl. Make soln alk. with NH_4OH and repeat operation beginning with "Extract 5 times with $CHCl_3$. If Fe is present, add sufficient citric acid (ca 1 g for each 0.2 g of iron present) to prevent the pptn of iron when soln is made alk. Treat mixed alkaloids as described below under "strychnine."

Liquid preparations.—Take sufficient sample to yield at least 20 mg of strychnine. If alcohol is present, make ca neutral, add an equal vol. of H_2O, and evap. to ca the original vol. Transfer to separatory funnel and proceed as in **32.62**, beginning "To remove all strychnine. . . . " If Fe is present, or if impurities appear to be present in $CHCl_3$ ext., follow precautions noted under *Tablets*. Treat mixed alkaloids as described below under "strychnine."

(B) SPECTROPHOTOMETRIC METHOD

In some pharmaceuticals the quantity of strychnine present is very small and the gravimetric procedure described above would require the use of inordinately large samples. In addition, the gravimetric procedure is not suitable if both quinine and strychnine are to be determined in preparations containing nux vomica since brucine, not being precipitated as the ferrocyanide, would be weighed as quinine. Accordingly, a spectrophotometric procedure requiring only 4 mg of strychnine and 10 mg of quinine, and not subject to interference by brucine, has been studied but has not been tested collaboratively. However, enough preliminary investigation has been made to warrant describing the method.

As in the gravimetric method, a preliminary isolation of the mixed alkaloids is necessary. The alkaloids are dissolved in alcohol to a definite volume and separate aliquots are used for quinine and strychnine. Quinine is determined in the evaporated aliquot by its absorption at 347.5 mμ as in the A.O.A.C. *Methods of Analysis*, 7th Ed., **32.59.**

Strychnine is measured by the intensity of the red color produced following reduction with zinc amalgam and treatment with dilute $NaNO_2$

* Not applicable in the presence of other alkaloids, nor for preparations containing nux vomica unless strychnine only is desired.

solution. Allen and Allport (4) use this reaction, originally attributed to Malaquin and modified by Denigé, as a basis for the determination of strychnine in nux vomica; Allport and Jones (5) used the reaction for determining strychnine in hypodermic tablets and injections. In an unpublished study, T. J. Klayder, Denver District, Food and Drug Administration, has more carefully defined the conditions for optimum color development, measurement, and stability and also studied interfering alkaloids. Quinine present in quantities less than the strychnine does not interfere. A study of the impure strychnine ferrocyanide precipitate obtained when strychnine is precipitated in the presence of quinine revealed that the quantity of quinine coprecipitated with the strychnine was usually less than half the quantity of strychnine present, even with quinine present in ratios of 50:1. (Thus with 40 mg portions of strychnine, quantities of quinine ranged from 15–20 mg. With 5 mg portions, there was approximately 3 mg of quinine and with 2 mg portions of strychnine approximately 1 mg of quinine was coprecipitated.) Large excesses of quinine definitely interfere.

The final red color obtained was found to have a maximum absorption at 530 mμ, and in concentrations from 0.05 to 0.20 mg per 25 ml to conform essentially to Beer's law.

Briefly, the procedure involves a preliminary separation of the strychnine from the bulk of the quinine by precipitation as strychnine ferrocyanide. The precipitate is dissolved in dilute alkali, and strychnine (plus a small amount of non-interfering quinine) is extracted. The residue is dissolved in 1+1 HCl, heated on a steam bath with zinc amalgam, cooled, decanted, and the red color developed with 0.1 per cent sodium nitrite solution. Absorbance at 530 mμ, is compared with standard solutions.

Quinine recoveries were excellent, ranging (eight determinations) from 99.1 to 100.3 per cent. Strychnine recoveries were poorer, ranging from 90.1 to 95.2 per cent. Some further modifications in details may result in increased recoveries; therefore actual operating instructions are not being reported pending a final revision and collaborative study.

ACKNOWLEDGMENT

The Associate Referee is grateful for the collaboration of the following chemists, all of the Food and Drug Administration: J. T. Welch, Buffalo; T. N. Bennett, New York; W. C. Woodfin, Buffalo (Pittsburgh Sub-District); H. D. Silverberg, St. Louis; T. J. Klayder, Denver; Charles E. Beisel, Baltimore.

RECOMMENDATIONS*

It is recommended that the gravimetric method for the separation of quinine and strychnine, as submitted to collaborators, be modified by in-

* For report of Subcommittee B and action of the Association, see *This Journal*, **36**, 52 (1953).

sertion of preliminary directions for separation of total alkaloids and cautions as to size of crucible and washing directions and the method be adopted as First Action.

It is also recommended that the spectrophotometric method be studied further.

REFERENCES

(1) MILLER, D. J., *This Journal*, **33**, 192 (1950).
(2) HERD, R. L., *J. Am. Pharm. Assoc.*, **31**, 9 (1942).
(3) CUMMING, W. M., and BROWN, D. G., *J. Soc. Chem. Ind.*, **44**, 110T (1925).
(4) ALLEN, J., and ALLPORT, N. L., *Quart. J. Pharm. Pharmacol.*, **13**, 252 (1940).
(5) ALLPORT, N. L., and JONES, N. R., *ibid.*, **15**, 245 (1942).

No report was received on aminopyrine, ephedrine, and phenobarbital.

REPORT ON SYNTHETIC DRUGS

By F. C. SINTON (Food and Drug Administration, Department of Health, Education, and Welfare, New York, N. Y.), *Referee*

RECOMMENDATIONS*

Tuinal ®.—The Associate Referee has submitted a report which includes a collaborative study. While the results for Amobarbital Sodium and Secobarbital Sodium varied significantly from the theoretical in a few instances, they were generally satisfactory, considering the mixture involved. The Associate Referee has recommended that the method be adopted as official, First Action, and that the subject be dropped. The Referee concurs in this recommendation.

Propadrine hydrochloride.—The Associate Referee was unable to do any work on this topic but feels he will be able to do so in the coming year. The Referee recommends that the subject be continued.

Spectrophotometric methods.—A spectrophotometric method was studied for the determination of isonicotinyl hydrazide in tablets. The Associate Referee has recommended that the proposed method be submitted to collaborative study. The Referee concurs.

Diphenhydramine hydrochloride (Benadryl ®) and tripelennamine hydrochloride (Pyribenzamine ®).—No report. The Referee recommends that the subject be continued.

Sulfanilamide derivatives.—No report. The Referee recommends that the subject be continued.

Amphetamines.—The Associate Referee has indicated that a report will

* For report of Subcommittee B and action of the Association, see *This Journal*, **36**, 53 (1953).

be submitted on methods for the determination of total amphetamines which were studied collaboratively with good results. He has further indicated that he is recommending that the subject be continued with respect to several phases that need further investigation. The Referee concurs.

Methylene blue.—No report. The Referee recommends that the subject be continued.

Synthetic estrogens.—No report. The Referee recommends that the subject be continued.

REPORT ON DEXTRO AND RACEMIC AMPHETAMINES

By LLEWELLYN H. WELSH (Division of Pharmaceutical Chemistry, Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C.), *Associate Referee*

Although *d*-amphetamine has been employed therapeutically for several years, the drug does not yet have an official status, and there are no official methods for the analysis of *d*-amphetamine preparations. Racemic (*dl*) amphetamine base, sulfate, and tablets of the sulfate are U.S.P. items, the monographs of which include identification tests and methods of assay (1).

The U.S.P. methods for the determination of racemic amphetamine are titrimetric, and to whatever extent they are satisfactory for the determination of that substance they should be equally satisfactory for the determination of total amphetamine in a preparation of the *d*-form. In the examination of tablets of *d*-amphetamine salts by the Food and Drug Administration, the U.S.P. method for the determination of *dl*-amphetamine in tablets has been employed to obtain a measure of the total amphetamine salt present. In several cases, supplementary examination showed that, although the total amount of amphetamine salt present agreed reasonably well with that declared, the *l*-isomer had been partially substituted for the *d*-form. This fact was established primarily by the lengthy procedure of extracting the amphetamine sulfate with dilute acid from a large sample of the tablets, extracting the basified aqueous solution with ether, extracting the ethereal solution of amphetamine base with somewhat less than the calculated amount of standard sulfuric acid, and determining the specific rotation of the dried amphetamine sulfate ultimately obtained. In many respects the extraction scheme is similar to that of the U.S.P. method of assay. The properties of the benzoyl derivative obtained by application of the U.S.P. identification test for racemic amphetamine failed to detect the adulteration, since, in the two recrystallizations of the derivative specified in the monograph, the *l*-isomer, when present in an amount substantially less than that of the *d*-form, is fractionated into the mother liquors (which also contain some

d-form), and the crystalline product which results is virtually pure benzoyl-*d*-amphetamine.

The A.O.A.C. method (2) for the determination of racemic amphetamine presupposes that an aqueous solution containing 50–100 mg of amphetamine is at hand and, like the U.S.P. identification test, involves the reaction of benzoyl chloride with amphetamine base in the presence of alkali. After the reaction, the system is allowed to stand with occasional shaking for two hours to hydrolyze excess benzoyl chloride, and the benzoyl amphetamine is extracted with chloroform and ultimately weighed. For identification purposes, such a procedure might conveniently supplement titrimetric methods, like those in the U.S.P., by being applied to the titrated solution. Thus the necessity of separating amphetamine from an additional portion of sample would be eliminated. Since the procedure is designed to be quantitative, the benzoylation product as isolated should be representative of the composition of the amphetamine present in a preparation. However, doubt as to the feasibility of applying the procedure in its present form was raised by the information that occasionally there are obtained residues of benzoyl amphetamine which are contaminated with benzoyl chloride that escaped hydrolysis after completion of the benzoylation reaction. It appeared necessary, therefore, to investigate means of eliminating this difficulty in the A.O.A.C. method, or to explore the possibility of quantitatively converting amphetamine to another derivative by employing a reagent an excess of which is easily destroyed after the desired reaction is complete. It was decided to pursue initially the latter course. This decision was influenced by the experience of the Associate Referee in effecting the quantitative acetylation of a number of amino and hydroxy compounds in aqueous solution (3) under experimental conditions in which excess acetic anhydride is completely destroyed in a few minutes. However, for the acetylation reaction to be useful in the present problem, the acetyl derivative must have certain characteristics. The optically active forms of the derivative should exhibit a specific rotation which is great enough to allow, without requiring the use of a high precision instrument, a reasonably accurate polarimetric determination of stereochemical composition of the amphetamine present in the same sample used for the estimation of total amphetamine. In 2 per cent chloroformic solution, acetyl-*d*-amphetamine has a specific rotation of $-44°$, which is numerically about twice that of the sulfate in water.* In addition, in order to allow a measure of the stereochemical composition by thermal analysis, the derivative must not decompose at the temperature of melting, and the temperature-composition diagram of mixtures of the *d*- and *l*-forms of the derivative should have certain characteristics which are discussed below.

* The rotatory power of the acetyl derivative in chloroform is numerically about two-thirds that of the benzoyl derivative in ethanol or methanol (4).

TEMPERATURE-COMPOSITION DIAGRAMS OF ENANTIOMORPHS

There are several types of temperature-composition diagrams which may be formed by systems of enantiomorphs.

In Figs. 1, 2, and 3 are represented different types of continuous series of solid solutions. Fig. 1 represents a rare type, of which only two examples are cited in the standard texts (5, 6), that of *d*- and *l*-carvoxime, and of *d*- and *l*-monobornyl malonate. The *dl* substance is a racemic compound with a melting point higher than that of the active forms, and forms a series of solid solutions with the active forms. The composition represented by *c* melts over the range *t*–*t'* with liquid phase first appearing at *t* and a completely liquid system finally resulting when the temperature is raised to *t'*. In the case of the *dl* composition, the initial and final temperature of melting are identical.

Fig. 2 is an example of a not uncommon type, characteristic of the systems of the *d*- and *l*-forms of camphor, camphor oxime, borneol, camphoric anhydride, camphene, and bornyl acid phthalate. All mixtures of the enantiomorphs show the same melting point (119° in the case of camphor oximes).

Apparently no system of enantiomorphs has as yet been found to yield the type of diagram represented in Fig. 3, in which the racemic material is a solid solution and has a melting point lower than that of the enantiomorphs.

Only a few diagrams of the type shown in Fig. 4 have been demonstrated to date. *d*- and *l*-Benzoyl hydratropic acid form this type of diagram in which the *dl*-composition is a eutectic, has the lowest melting point in the phase diagram, and is a racemic mixture of the *d*- and *l*-forms. In all mixtures the beginning of melting occurs at the same temperature (*t*, eutectic point), but the final temperature of melting varies with the composition. A system of composition *c* shows a melting range of *t*–*t'*. In contrast, in systems characterized by diagrams shown in Figs. 1 and 3, all compositions differ with respect to both initial and final temperatures of melting.

In systems exhibiting a type of temperature-composition diagram shown in Figs. 5 and 6, a racemic compound exists and forms a eutectic mixture with each of the active forms. The melting point of the *dl*-form is higher in Fig. 5 and lower in Fig. 6 than that of the optically active forms. This type of diagram seems to be the one most commonly exhibited. The diagram in Fig. 5 is characteristic of the dimethyl tartrates; that in Fig. 6 is characteristic of *d*- and *l*-mandelic acids.

It is obvious that if a derivative of amphetamine is to be useful in thermal analysis, the enantiomorphic forms should not yield a diagram of the type represented in Fig. 2. Consideration of Figs. 5 and 6 shows that it is possible, in a substantial portion of the diagram, to have as many as four different compositions exhibiting the same melting range. For example (Fig. 5), compositions represented by *c*, *c'*, *c''*, and *c'''*, all exhibit the

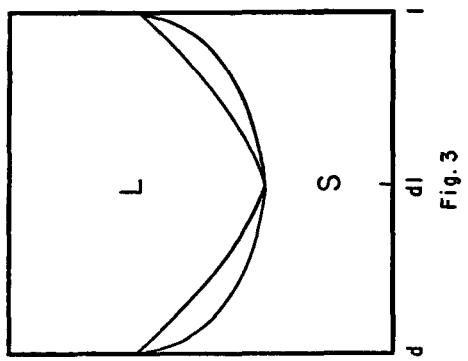


Fig. 3

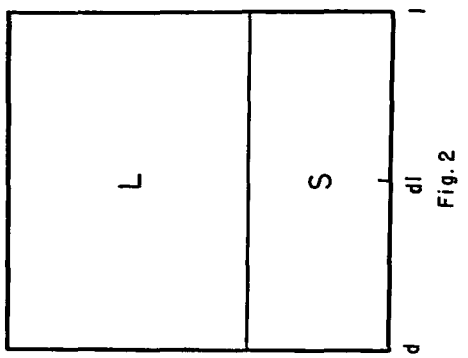


Fig. 2

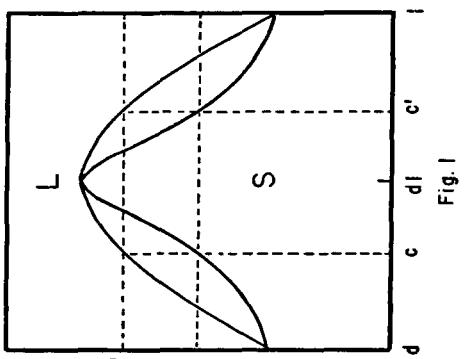


Fig. 1

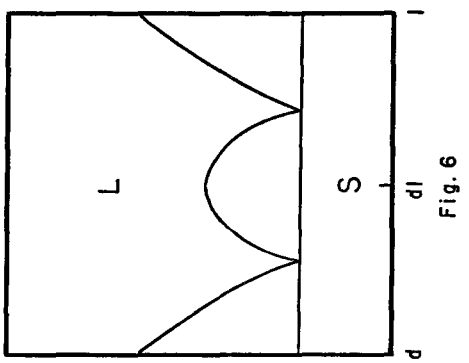


Fig. 6

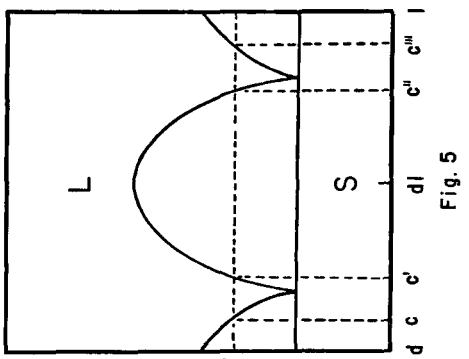


Fig. 5

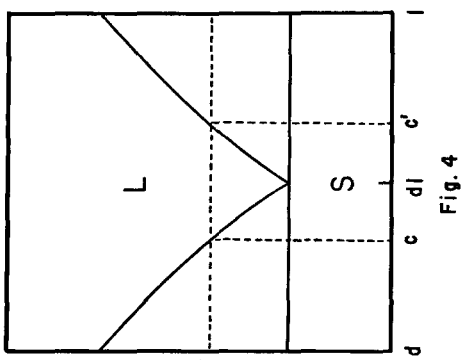


Fig. 4

FIGS. 1–6.—Temperature-composition diagrams of enantiomorphs. Temperature axis vertical; composition axis horizonal; L, liquid phase; S, solid phase.

melting range t–t'. Consideration of Figs. 1 and 4 shows that a maximum of two different compositions (c and c') correspond to a given melting range (t–t'). From these considerations it is obvious that thermal analysis is considerably simplified and rendered more practicable if the enantiomorphic derivatives form the type of diagram represented in Figs. 1 or 4. It is also obvious that the accuracy obtainable by such analysis is increased by having a relatively large spread between the melting points of the racemic material and the optically active forms.

The acetyl amphetamines were found to exhibit a diagram of the type shown in Fig. 4. The eutectic racemic material melts at 93° and the optically active forms at 124°, without decomposition.

COLLABORATIVE STUDY

The U.S.P. method for the determination of amphetamine sulfate in tablets is prolonged by the requirements of (*a*) agitating the powdered tablets two hours with acidulated water in order to extract the amphetamine salt, and (*b*) concentrating a 50 ml aliquot of the extract to a small volume on the steam bath in order that the volume will be 20 ml after the concentrate is quantitatively transferred to a separatory funnel before adding alkali and extracting the amphetamine with ether. It seemed desirable to attempt a modification of this procedure in order to eliminate or substantially diminish the time consumed in these operations.

The properties of acetylamphetamine appeared to warrant exploring on a collaborative basis the possibility of using an acetylation procedure as part of a method for determining stereochemical composition. It was necessary to limit this aspect of the present collaborative study to a method involving a polarimetric measurement, since it was not possible at a sufficiently early date to obtain the fundamental data or to define the experimental conditions necessary for the application of thermal analysis to the problem at hand.

The sample sent to collaborators contained 2 per cent of a mixture of amphetamine sulfates having the composition 50 per cent *dextro* plus 50 per cent racemic, which is the equivalent of 75 per cent *d* and 25 per cent *l*. The *d*-sulfate had $[\alpha]_D^{20}+23.5°$ (H_2O, $c=5$ g/100 ml), and was obtained by recrystallizing from ethanol the acid salt of *d*-amphetamine and dextrorotatory tartaric acid until the sulfate obtained from that salt exhibited constant rotation. 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; stearic acid, 1 per cent. Collaborators were sent the following instructions.

METHOD A

Using a 5 g sample, proceed according to the U.S.P. XIV method for amphetamine sulfate tablets until the main ether extracts have been combined with the ether used to extract the wash water. At this point, filter the combined ether extracts

through a pledget of cotton fixed in a funnel, rinse the cotton and funnel well with ether, and proceed according to the revision described on p. 1, Third Supplement to the U.S.P. XIV.

Quantitatively combine the titrated solns from duplicate assays in a 250 ml separatory funnel, acidify with 1–2 drops of 0.1 *N* H_2SO_4, and extract with three 10 ml portions of CCl_4. To the aq. soln which has been separated from as much CCl_4 as practicable, add 4.10 g of $NaHCO_3$ and swirl the funnel until the salt has mostly dissolved. Rapidly introduce into the funnel 1.0 ml acetic anhydride, A.C.S. reagent grade, by blowing the reagent in from a 1 ml pipet. Immediately stopper the funnel securely and shake it vigorously until evolution of CO_2 has nearly ceased (release pressure in the funnel frequently during the shaking by opening the stopcock). Add another 1.0 ml portion of anhydride, and continue to shake the funnel until the evolution of CO_2 has ceased (5–10 min. after addn of the second portion of anhydride). Allow the mixt. to stand 5 min. and extract the acetylamphetamine by 4 shakeouts with 50 ml portions of $CHCl_3$. Filter the extracts through a pledget of cotton, concentrate the filtrate to a small vol. on the steam-bath in a current of air, quantitatively transfer the concentrate to a tared 50 ml beaker, and continue the evapn until no odor of $CHCl_3$ is evident. Heat the beaker in an oven (not forced-draft type) at 80° for 1 hr, cool in a desiccator, and weigh. Acetylamphetamine $\times 1.0395$ = amphetamine sulfate. Calc. the per cent amphetamine sulfate in the duplicates.

Powder the acetylamphetamine finely and det. the corrected melting range of a small portion in a capillary tube.

Accurately weigh the remainder (ca 90 mg) of the acetylamphetamine, transfer it quantitatively to a 5 ml volumetric flask, and make to vol with U.S.P. $CHCl_3$. Det. the optical rotation of the soln in a 2 dm. tube at the same temperature at which the soln was made to vol.

In measuring the rotation with a polariscope, take 10 readings on the soln and calc. the average to 0.001°. In the same way, det. the average reading with the same tube filled with U.S.P. $CHCl_3$, and use the average zero-point reading thus obtained to correct the average reading given by the soln.

If a saccharimeter is used instead of a polariscope, estimate all readings to 0.05 division, calc. the averages to 0.01 division, correct for zero-point, and multiply the value so obtained by 0.3468 to obtain the 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 dm.

METHOD B

In a 100 ml beaker stir a 6.3 g sample, accurately weighed, for 15 min. with a mixt. of 10 ml of 0.1 *N* H_2SO_4 and 5 ml of H_2O. With the aid of a rod or policeman, transfer as much of the suspension as possible to a fritted glass funnel (one with a medium porosity 40 mm disk is convenient), and filter by means of suction into a suitable vessel. Break the suction and, with portions of H_2O totaling 5 ml, rinse as much adhering material as possible from the beaker into the funnel. Triturate the mixt. in the funnel until a uniform paste results and reapply suction. Make the transfer and filtration quantitatively by repeating the washing process 4 addl times. With the aid of small portions of H_2O, quantitatively transfer the filtrate to a 50 ml volumetric flask, make up to vol., and mix. Transfer a 20 ml aliquot to a 125 ml separatory funnel, add 1 ml of 10% NaOH, and extract with six 15 ml portions of ether. Wash the combined ether extracts with two 5 ml portions of H_2O and extract

the combined washings with two 10 ml portions of ether. Combine the ethereal washings with the main ethereal extract, filter through a pledget of cotton into a 125 ml separatory funnel, and rinse the filter with ether. Extract the filtrate with exactly 20 ml of 0.02 *N* H_2SO_4, and drain the acid extract into a 200 ml Erlenmeyer flask. Wash the ether with 10, 5, and 5 ml portions of H_2O, combine the washings with the acid extract, and heat the whole on the steam bath until dissolved ether is expelled. Cool, and titrate the soln with 0.02 *N* NaOH in the presence of methyl red indicator. Calc. the % amphetamine sulfate in the sample.

In Method A, the combined ether extracts are filtered to eliminate the possibility, which exists with the U.S.P. method, that traces of unremoved alkali will be titrated along with amphetamine. In the course of preliminary work it was determined that the water used to wash the combined ether extracts contained, after washing with ether, the equivalent of about 2.5 ml of 0.02 *N* alkali.

Results of collaborators are listed in Table 1.

DISCUSSION OF RESULTS

The recoveries by Method A range from 95.5 to 98.0 per cent and average 96.8 per cent; those by Method B are within the range of 95.5 to 99.5 per cent, and average 97.1 per cent. The results show there is scarcely any significant difference between the apparent recoveries obtainable by the two methods. However, recoveries by both methods are consistently low, 3 per cent on the average, and further work is necessary to explain and eliminate the discrepancy. The recoveries by Method A are really about 1 per cent lower than they appear, since it was determined that when the mixture of the collaborative sample and acidulated water is made up to 100 ml volume, slightly more than 1 ml of volume is occupied by insoluble matter. Therefore, the 50 ml aliquot taken from the filtered extract represents 50.5 per cent of the sample weighed instead of exactly 50 per cent. The nature of the error is inherent in the U.S.P. method, but its value is dependent on the composition of the particular tablet mixture on hand.

Estimation of the stereochemical composition by determining the specific rotation of the acetyl derivative gave results which ranged from 95 to 100 per cent of the theoretical, and averaged 97.9 per cent. The percentage of *d*-amphetamine was calculated by use of the equation

$$\% \, d = 50 + 50\frac{[\alpha]}{44}$$

in which $[\alpha]$ is the specific rotation of the acetyl derivative from the sample, 44 is the specific rotation of pure derivative, and the sign of rotation has been ignored. The Associate Referee is of the opinion that the results are of such a nature as to warrant a First Action status for the method, but, in view of the incomplete nature of the entire problem related to the amphetamines, he does not now formally recommend that such a status be accorded the method.

TABLE 1.—*Results of collaborators*

COLLABORATOR	METHOD	AMPHETAMINE SULFATE FOUND	PER CENT RECOVERY	ACETYL DERIV.		PER CENT *d*-ISOMER FOUND	PER CENT RECOVERY
				$[\alpha]_D$	M.P. °C.		
1. C. F. Bruening, U. S. Food and Drug Administration, Chicago, Ill.	A	1.94	97.0				
		1.94	97.0				
	B	1.95	97.5				
		1.95	97.5				
	Acetylation	1.94	97.0	−20.8°		74	99
		1.94	97.0	−21.9°		75	100
2. G. McClellan, U. S. Food and Drug Administration, Baltimore, Md.	A	1.93	96.5				
		1.91	95.5				
	B	1.91	95.5				
	Acetylation	1.92	96.2	−18.3°	94	71	95
3. T. N. Bennett, U. S. Food and Drug Administration, New York, N. Y.	A	1.93	96.5				
		1.96	98.0				
	B	1.99	99.5				
		1.93	96.5				
	Acetylation	1.95	97.5	−19.7°	106–110	72	96
				−20.0°		73	97
4. Associate Referee	A	1.93	96.5				
		1.94	97.0				
	B	1.95	97.5				
		1.91	95.5				
	Acetylation	1.88	94.0	−21.9°	93–120.5	75	100

The close agreement between the recoveries of total amphetamine by the titrimetric methods and by acetylation indicate that the latter method should be useful as a check on the former.

The wide divergence between melting points of acetyl derivative reported by the collaborators demonstrates the impossibility of relating stereochemical composition to melting point data as ordinarily obtained. The divergence is chiefly due to the absence of equilibrium conditions between solid and liquid phases in the melting point tube when the determination is carried out in the usual manner.

RECOMMENDATIONS

It is recommended*—

(1) That study of the subject be continued in order to improve the titrimetric method, to test collaboratively the application of thermal analysis to the determination of stereochemical composition, and to investigate the possibility of eliminating difficulties associated with the A.O.A.C. gravimetric determination of amphetamine as its benzoyl derivative.

(2) That investigation be undertaken for the purpose of developing a titrimetric method for total amphetamine in which the drug is separated from tablet mixtures by distillation.

REFERENCES

(1) *The United States Pharmacopeia*, Fourteenth Edition, Mack Publishing Co., Easton, Pa., 1950.
(2) *Official Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Washington, D. C., 1950.
(3) WELSH, L. H., *J. Am. Pharm. Assoc.*, **36**, 373 (1947); **41**, 545 (1952); *J. Am. Chem. Soc.*, **74**, 4967 (1952).
(4) LEITHE, W., *Ber.*, **65**, 660 (1932).
(5) RICCI, J. E., *The Phase Rule and Heterogeneous Equilibrium*, D. Van Nostrand Co., Inc., New York, N. Y., 1950.
(6) FINDLAY, A., *Phase Rule and Its Applications*, 9th Ed., Dover Publications, Inc., New York, N. Y.

REPORT ON THE DETERMINATION OF ISONICOTINYL HYDRAZIDE IN TABLETS BY ULTRAVIOLET SPECTROPHOTOMETRY

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

Isonicotinyl hydrazide (INH) has recently attracted widespread attention as a new therapeutic agent for the treatment of certain types of tuberculosis. At present its efficacy is being tested in sanitariums throughout the country. A number of distinctly different analytical methods are

* For report of Subcommittee B and action of the Association, see *This Journal*, **36**, 53 (1953).

being used by the various manufacturers of this drug in their control laboratories. Some of the methods used are:

(1) Titration with standard sodium nitrite solution, using starch-iodide as an outside indicator.
(2) Titration with standard potassium iodate solution, using the disappearance of iodine color in chloroform as the end point.
(3) A colorimetric estimation of the color formed with *p*-dimethyl aminobenzaldehyde (max. 450 mμ).
(4) Direct ultraviolet absorption.
(5) Polarographic.

Consideration of the reactions involved in each led to the conclusion that the ultraviolet absorption method was as specific, or more so, than any of the others. Moreover, it was rapid and very easily carried out. As used by several of the laboratories, absorption readings were made in either neutral or acid solution. It was found that changing the solvent from 0.1 *N* HCl to 0.1 *N* NaOH caused a shift in the λ_{max} from 266 mμ to 298 mμ as shown by the spectra in Fig. 1. It will be noted also that λ_{min} shifts from 234 mμ to 250 mμ and that a shoulder appears at 275–285 mμ

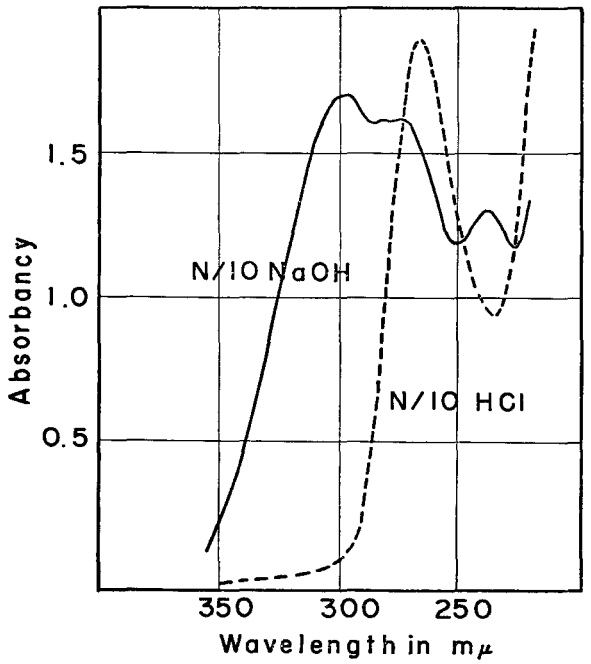


FIG. 1.—The absorption spectra of isonicotinyl hydrazide in 0.1 *N* NaOH and 0.1 *N* HCl. Concentration, 50 mmg/ml.

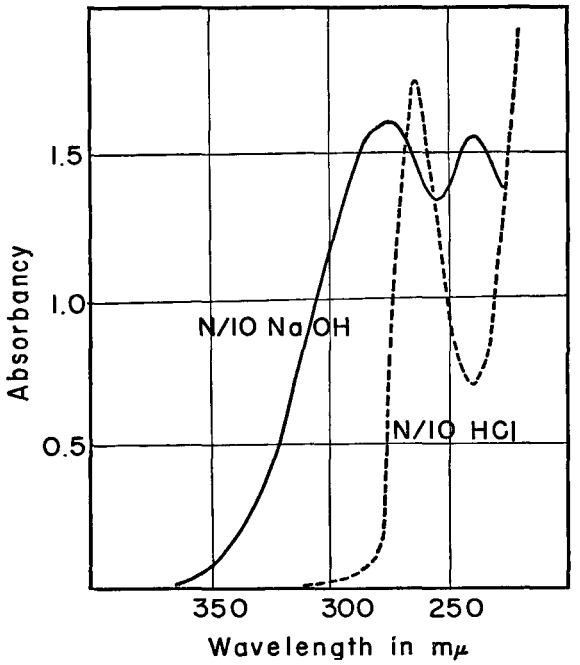


FIG. 2.—The absorption spectra of nicotinyl hydrazide in 0.1 *N* NaOH and 0.1 *N* HCl. Concentration, 50 mmg/ml.

on the curve made in alkaline solution. The Beer-Lambert law is followed by both acid and alkaline solutions at all the measuring wavelengths. In the method below, advantage is taken of the change in absorption spectra to check the identity of the sample solution. To illustrate the specificity of these spectra, the spectra of solutions of nicotinyl hydrazide, made under the same conditions, are shown in Fig. 2 for comparison. The absorption curves in Figs. 1 and 2 were made with a Cary recording ultraviolet spectrophotometer, using solutions containing 50 mmg/ml of isonicotinyl hydrazide. Using a Beckman DU quartz spectrophotometer, absorbance measurements are best made with a concentration of about 20 mmg/ml of isonicotinyl hydrazide.

METHOD

Accurately weigh an amount of the finely powd. tablets sufficient to contain about 100 mg isonicotinyl hydrazide. Transfer to a 100 ml volumetric flask, add 75 ml H_2O, and shake for 5 min. Make to vol. and filter thru a dry filter paper, discarding the first 10 ml of filtrate. Using a pipet, transfer 5 ml aliquots of filtrate to each of two 100 ml volumetric flasks. Make one to vol. with 0.1 *N* HCl and the other with 0.1 *N* NaOH. Det. the absorbance spectrum of each soln relative to the appropriate blank soln. Very few absorbance readings will be necessary to prove identity

if the spectra are compared with standards containing 20 mmg of isonicotinyl hydrazide in 0.1 *N* HCl and 0.1 *N* NaOH. The concn of isonicotinyl hydrazide should be computed from the absorbancy readings in both acid and alk. solns and the two concns should check closely. Any appreciable variation will indicate the presence of substances other than isonicotinyl hydrazide in the sample soln.

Using the above procedure, analysis was made of two commercial samples labeled to contain 50 mg of isonicotinyl hydrazide and of a synthetic tablet mixture of lactose, starch, magnesium stearate, talc, and isonicotinyl hydrazide. These results are shown in Table 1.

TABLE 1.—*Analysis of tablets containing isonicotinyl hydrazide*

	DECLARED	FOUND (CALCD. FROM)	
		ACID SOLN.	ALKALINE SOLN.
Tablet 1	50 mg	51.2 mg	52.0 mg
Tablet 2	50 mg	51.1 mg	50.8 mg
Synthetic Mixture			
1	26.8%	27.0%	26.8%
2	10.5%	10.5%	10.5%
3	7.5%	7.48%	7.49%

SUMMARY AND CONCLUSIONS

The ultraviolet spectrophotometric procedure for the determination of isonicotinyl hydrazide in tablets when carried out in both acid and alkaline solutions is capable of giving very accurate results. Identity of a sample is easily checked by comparing the shift of λ_{max} when changing from acid to alkaline solution with that of authentic isonicotinyl hydrazide.

RECOMMENDATION

It is recommended* that the proposed method be submitted to collaborative study.

REPORT ON TUINAL ®

AMOBARBITAL SODIUM AND SECOBARBITAL SODIUM

By GEORGE E. KEPPEL (Department of Health, Education, and Welfare, Food and Drug Administration, Minneapolis 1, Minnesota), *Associate Referee*

Tuinal ® consists of capsules of a powdered mixture of two barbiturates, amobarbital sodium, N.F., and secobarbital sodium, N.N.R., together with excipient.

A search of the available literature failed to reveal a suitable method for

* For report of Subcommittee B and action of the Association, see *This Journal*, 36, 53 (1953).

this barbiturate mixture. Garratt (1), however, states that "allobarbitone" may be determined by bromination, using the Koppeschaar method, wherein bromine forms an additive compound with the unsaturated allyl groups. Since secobarbital also contains one allyl group, it was thought that bromination would be feasible. This was confirmed by experiments on authentic secobarbital sodium, following the method given below.

EXPERIMENTAL

An aqueous solution of secobarbital sodium in an iodine flask was mixed with an excess of 0.1 *N* bromine and 5 ml HCl. After stoppering, the flask was allowed to stand 15 minutes with occasional shaking. Potassium iodide was added and the liberated iodine titrated with 0.1 *N* thiosulfate. From the bromine consumed, the amount of secobarbital was calculated. It was assumed that two moles of bromine were added to one mole of secobarbital sodium. Based on molecular weights of 260.27 for secobarbital sodium and 79.92 for bromine, the factor is 13.01 mg/ml of 0.1 *N* bromine. Results for known amounts of secobarbital sodium alone and in the presence of amobarbital, sucrose, and lactose are shown in Table 1.

TABLE 1.—*Results obtained by bromination of sodium secobarbital alone, and in presence of other substances*

NO.	PRESENT, MG				SODIUM SECOBARBITAL	
	SODIUM SECOBARBITAL	AMOBARBITAL	SUCROSE	LACTOSE	FOUND, MG	RECOVERY, PER CENT
1	65.5	—	—	—	61.5	93.9
2	—	83.7	—	—	0.3	—
3	110.6	103.4	—	—	111.2	100.5
4	93.4	118.2	100.0	—	90.8	97.2
5	101.5	89.5	—	100.0	100.4	98.9

Since no specific method is available for amobarbital, it would be necessary to calculate its value by difference, provided total barbiturates are known. This value may be determined by the published method for barbiturates (2) which consists in extracting the acidified mixture with chloroform and weighing the extracted barbiturates. A more convenient method was devised, based on absorption, at a wavelength of 244 mμ, of the barbiturates in alkaline solution. This method has been successfully used on single barbiturate preparations such as pentobarbital and secobarbital. It was noted that these substances both had absorption maxima at about 244 mμ. Experiments showed that this was also true for amobarbital. From this it appears that absorption is a function of the barbituric acid group and is not affected by the substituted alkyl radicals of the above barbiturates. This means that the total barbiturates of a mixture of amobarbital and secobarbital may be obtained in terms of their common

component, namely, barbituric acid. A standard curve is prepared using either amobarbital or secobarbital, or a mixture of both. Absorbance is plotted against equivalent weights of barbituric acid, calculated from known weights of the barbiturate used for standard. Since secobarbital is known from bromination, this value may be converted to equivalent barbituric acid, subtracted from total barbiturates as barbituric acid, and the difference calculated to amobarbital sodium.

METHOD

REAGENTS

(a) *NaOH.—1 N.*

(b) *KBr-KBrO₃, 0.1 N.*—39.18.

(c) *Sodium thiosulfate, 0.1 N.*—39.35.

(d) *KI soln.*—10%.

(e) *Secobarbital Sodium, N.N.R.*—Assay by N.N.R. method to obtain per cent secobarbital sodium.

Standard Curve.—Weigh out ca 250 mg secobarbital sodium and transfer to 500 ml volumetric flask. Add H_2O to mark and mix well. Calc. the equivalent barbituric acid per ml as follows:

$$\text{mg barbituric acid per ml} = \frac{\text{wt Na Secobarbital}}{500} \times \frac{\%\text{Na Secobarbital (from assay)}}{100} \times \frac{128.05}{260.27}$$

(128.05 = mol. wt. of barbituric acid; 260.27 = mol. wt of Na Secobarbital.)

Transfer 1, 2, and 5 ml aliquots of the standard soln to 100 ml volumetric flasks. To each flask add 10 ml *N* NaOH, dil. to mark with H_2O, and mix. Prepare blank soln by diluting 10 ml *N* NaOH to 100 ml in volumetric flask.

Transfer solns to 1 cm quartz cells and det. absorbance of the standards at 244 mμ and a slit width of 0.5 mm with the blank set to read zero absorbance. Plot absorbancies against mg barbituric acid.

Total Barbiturates.—Accurately weigh sufficient sample to represent ca 200 mg combined Na Amobarbital and Na Secobarbital. Transfer to 100 ml volumetric flask. Add H_2O to mark, mix, and filter thru dry folded filter paper.

Transfer 1 ml of filtered soln to 100 ml volumetric flask. Add 10 ml *N* NaOH, dil. to mark with H_2O and mix. Measure absorbance at 244 mμ as in preparation of standard curve, using same blank. Obtain total barbiturates as barbituric acid by reference to curve, and calc. as % barbituric acid in sample.

Secobarbital Sodium.—Transfer 50 ml of filtered soln to I flask. Add 10 ml of 0.1 *N* KBr-$KBrO_3$ soln. and 5 ml HCl. Stopper at once and let stand 15 min. with occasional swirling. Carefully add 10 ml KI soln; stopper flask and shake vigorously. Remove stopper and rinse with H_2O. Titrate at once with 0.1 *N* sodium thiosulfate.

1 ml 0.1 *N* KBr-$KBrO_3$ soln = 13.01 mg Na secobarbital.

Calc. as % Na secobarbital in sample.

Amytal Sodium.—This component is obtained by difference. Calc. barbituric acid equivalent of the secobarbital sodium (% in sample × 0.492). Subtract this value from total barbiturates (calcd as % barbituric acid) to obtain barbituric acid equivalent of amobarbital sodium. Calc. to % amobarbital sodium (per cent ×1.939).

Results obtained by applying the proposed method to known mixtures

are given in Table 2. Table 3 shows results on a number of Tuinal ® samples.

TABLE 2.—*Application of method to known mixtures*

NO.	PRESENT				FOUND			
	Na SECOBARBITAL, MG	AMOBARBITAL, MG	SUCROSE, MG	LACTOSE, MG	Na SECOBARBITAL		AMOBARBITAL	
					MG	PER CENT	MG	PER CENT
1	110.6	103.4			111.2	100.5	103.0	99.6
2	93.4	118.2	100	100	90.8	97.2	118.9	100.6
3	101.5	89.5		100	100.4	98.9	89.4	99.9

TABLE 3.—*Application of method to Tuinal ® samples*[a]

SAMPLE NO.	Na SECOBARBITAL, GR	Na AMOBARBITAL, GR
153	0.75	0.75
158	0.75	0.73
172	0.72	0.75
183	0.76	0.73
185	0.75	0.77
186	0.74	0.73
190	0.75	0.75
595	0.79	0.76
151	0.76	0.79
152	0.76	0.77

[a] Declared: 0.75 gr. Na amobarbital, 0.75 gr. Na secobarbital.

COLLABORATIVE STUDY

A powdered mixture was prepared, containing 30 per cent sodium amobarbital and 30 per cent sodium secobarbital, with corn starch excipients. Assays were made on each of the barbiturates (by N.N.R. or N.F. methods) and weights calculated to obtain the above concentrations. After thorough mixing by shaking in a closed container, portions were placed in vials. A second vial contained secobarbital sodium for preparation of the standard curve. This substance contained 99.8 per cent sodium secobarbital by N.N.R. assay, and collaborators were instructed to use this value in their calculations. Results obtained by collaborators are listed in Table 4.

The results show rather wide variation from theory in some cases. Since amobarbital is determined indirectly, results will reflect analytical errors to some extent.

No adverse comments were received from any of the collaborators.

COLLABORATORS

The Associate Referee wishes to express his appreciation for the help

TABLE 4.—*Collaborative results*

COLLABORATOR	Na SECOBARBITAL		Na AMOBARBITAL	
	FOUND	RECOVERY	FOUND	RECOVERY
	per cent	*per cent*	*per cent*	*per cent*
1	30.4	101.3	30.6	102.0
	31.6	105.3	29.9	99.7
2	31.0	103.3	29.2	97.3
	31.0	103.3	29.2	97.3
3	29.6	98.7	32.2	107.3
	30.2	100.7	32.8	109.3
	30.0	100.0	32.2	107.3
	30.2	100.7	32.4	108.0
4	32.4	108.0	30.5	101.7
	32.4	108.0	30.6	102.0
5	29.8	99.3	28.7	95.7
	30.1	100.3	29.9	99.7
	30.6	102.0	30.1	100.3
6	31.7	105.7	29.0	96.7
	31.7	105.7	29.0	96.7

of the following collaborators:

E. J. Hughes, Eli Lilly and Company, Indianapolis, Indiana
Janice C. Bloomingdale, Food and Drug Administration, Chicago, Illinois
J. E. Yarnall, Food and Drug Administration, Kansas City, Missouri
Thompson N. Bennett, Food and Drug Administration, New York, New York
Juanita E. Breit, Food and Drug Administration, Minneapolis, Minn.

RECOMMENDATIONS

It is recommended* that the method be adopted as First Action, and that the subject be dropped.

ACKNOWLEDGMENT

Appreciation is expressed to Eli Lilly and Company for furnishing the amobarbital sodium used in this work.

REFERENCES

(1) GARRATT, D. C., *Drugs and Galenicals*, John Wiley and Sons, Inc., New York, N. Y., 1937, p. 38.
(2) *Official Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Washington, D. C., 1950, **32.116.**

* For report of Subcommittee B and action of the Association, see *This Journal*, **36**, 53 (1953).

No reports were received on methylene blue, sulfanilamide derivatives, propadrine hydrochloride, di- and triphenhydramine hydrochloride, or synthetic estrogens.

REPORT ON MISCELLANEOUS DRUGS

By I. SCHURMAN (Food and Drug Administration, Department of Health, Education, and Welfare, Chicago 7, Illinois), *Referee*

RECOMMENDATIONS*

Microscopic Tests for Alkaloids and Synthetics.—The Referee recommends that the subject be continued.

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

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

Alkali Metals.—The Associate Referee has resigned from the Food and Drug Administration. The Referee recommends that subject be reassigned and continued.

Glycols and Related Compounds.—The Referee concurs with the recommendation that the method be adopted, First Action.

Preservatives and Bacteriostatic Agents in Ampul Solutions.—No report was received. The Referee recommends that subject be continued.

REPORT ON MICROSCOPIC TESTS FOR ALKALOIDS AND SYNTHETICS

By WILLIAM V. EISENBERG (Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C.), *Associate Referee*

Work on this subject during the past year departed from the usual technique reported in previous years which consisted of microscopic tests based principally on characteristic crystal habits. This is in accordance with the recommendation made last year in which partial data on microscopic-crystallographic properties were introduced to supplement the microscopic tests based on reactions producing characteristic crystalline precipitates.

As a start, the Associate Referee with the collaboration of Dr. Albert

* For report of Subcommittee B and action of the Association, see *This Journal*, 36, 53 (1953).

H. Tillson is presenting a table of microscopic-crystallographic data for most of the crystalline sulfonamides and barbiturates reported in the literature. The data in Table 1 have been compiled from the literature and unpublished data in the files of the microanalytical laboratory of the Food and Drug Administration. For the most part, the measurements are determined from mounts in the common organic immersion liquids given below:

Immersion Liquid	*Index Range*
Kerosene—mineral oil mixtures	1.430–1.470
Mineral oil—halowax oil (α-monochlornaphthalene) mixtures	1.470–1.630
Halowax oil—methylene iodide	1.630–1.74
Methylene iodide—sulfur	1.740–1.78

Under "remarks" in Table 1 are included the most distinctive data for diagnostic purposes. The intermediate index, *beta*, may be easily determined in all cases where interference figures are obtained. Where an acute bisectrix figure is common, *alpha* and *beta* are readily determined if the optic sign is positive and *beta* and *gamma* if the sign is negative. Where optic axis figures are common, *beta* is the distinctive index.

In the case of sulfadiazine, sulfamerazine, and sulfathiazole, two sets of data are given. The second set 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 orientation of the crystal. In all cases the principal indices may be obtained from the recrystallized melt.

In analytical work with these substances the microscopic-crystallographic data should prove very useful in conjunction with microchemical tests. It may be well to combine both sets of data in determinative tables.

RECOMMENDATIONS*

For next year's work the Associate Referee recommends the study and preparation of determinative tables of antihistamine compounds based on microscopic-crystallographic properties.

* For report of Subcommittee B and action of the Association, see *This Journal*, **36**, 53 (1953).

(*Table I is given on the following pages*)

TABLE 1.—*The identification of crystalline sulfonamides and barbiturates by means of microscopic-crystallographic properties*[a]

Barbiturates

COMPOUND	α	β	γ	OPTIC SIGN	EXTINCTION	ELONGATION	2 V	REMARKS
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	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
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
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_i 1.500	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

Sulfa Drugs

COMPOUND	α	β	γ	OPTIC SIGN	EXTINCTION	ELONGATION	2 V	REMARKS
Sulfacetamide	1.559	1.564	1.727	+	s		21°	———
Sulfadiazine	1.596	1.675	1.830	+	p, i	±	76°	
"	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
"	1.587		1.675					
Sulfamethazine	1.584	1.623	>1.778		p	—		Rods
Sulfamidazole (sulfanilamide+sulfathiazole—double crystal[b]	1.661	1.678	>1.733	+			small	Bx. ac. fig.
Sulfanilamide	1.558	1.675	>1.733	−				n_β common
Sulfanilamide HCl	1.540	1.655	1.690		p	−		Rods
Sulfapyridine	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	1.674	1.685	>1.733	+			small	α & β common
"	1.695	n_i 1.733	>1.733		(apparently intermediate indices)			
Sulfathiazole sodium sesquihydrate	1.596		1.621					

[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.
[b] Equimolecular proportions.

REPORT ON GLYCOLS AND RELATED COMPOUNDS

DETERMINATION OF PROPYLENE GLYCOL IN MEDICINAL MIXTURES

By HARRY ISACOFF (Food and Drug Administration, Department of Health, Education, and Welfare, New York, New York), *Associate Referee*

This year's collaborative study was undertaken for the purpose of obtaining more collaborative results and for determining the suitability of the method. Accordingly, two samples were prepared, each containing different amounts of propylene glycol and glycerin, and were sent to each of five collaborators. They had the following composition (Table 1):

TABLE 1.—*Composition of mixtures containing glycerol and propylene glycol*

COMPOUND	1	2
Propylene glycol	46.16 g	56.05 g
Glycerol	46.23 g	36.06 g
Phenobarbital	1.00 g	1.00 g
Alcohol	35.00 ml	35.00 ml
Amaranth soln (1%)	2.50 ml	2.50 ml
Oil of orange	0.25 ml	0.25 ml
Syrup	37.50 ml	37.50 ml
Water, to make	250.00 ml	250.00 ml

The method submitted was essentially the same as that used in last year's collaborative study.* The following directions were given to the collaborators:

A sample aliquot of 4 ml will be convenient for the separation of the propylene glycol. An aliquot of 4 ml of the aqueous glycol solution drawn from the receiver (diluted to 200 ml volumetric solution) will be convenient for the periodate oxidation. Several determinations are desired.

Apply a correction for any acidity in the 0.02 *M* KIO_4 solution by titrating 25 ml with 0.02 *N* NaOH, using bromcresol purple indicator. Report the correction used.

The results obtained by the various collaborators are shown in Table 2.

No adverse comments were received from any of the collaborators and it is assumed that no difficulties were encountered in the application of the method.

ACKNOWLEDGMENT

The Associate Referee wishes to express his appreciation to Luther G. Ensminger, Cincinnati District; T. N. Bennett, New York District;

* *This Journal*, **35**, 579 (1952).

TABLE 2.—*Collaborative results*

COLLABORATOR	PROPYLENE GLYCOL RECOVERED, G/5 ML	PROPYLENE GLYCOL ADDED, G/5 ML	PER CENT RECOVERED
	Sample 1		
A	1.021	0.092	110.9
	1.019		110.7
B	0.97		105.4
	0.99		107.6
	0.965		104.9
	0.97		105.4
C	0.92		100.0
	0.912		99.1
	0.88		95.7
	0.94		102.2
D	0.955		103.8
	0.945		102.7
E	0.927		100.7
	0.954		103.7
	0.948		103.0
	0.930		101.1
	Sample 2		
A	1.156	1.12	103.2
	1.148		102.5
B	1.14		101.8
	1.17		104.4
	1.14		101.8
	1.13		100.9
C	1.08		96.4
	1.08		96.4
	1.096		98.2
	1.12		100.0
D	1.14		101.8
	1.145		102.2
E	1.15		102.6
	1.17		104.4

Robert L. Herd, St. Louis District; Abram Kleinman, Chicago District, all members of the U. S. Food and Drug Administration, for their collaborative work with the proposed method.

RECOMMENDATIONS

It is recommended* that the proposed general method for the determination of propylene glycol in medicinal mixtures be adopted, First Action.

No reports were received on mercury compounds, organic iodides, alkali metals, or preservatives and bacteriostatic agents in ampul solutions.

* For report of Subcommittee B and action of the Association, see *This Journal*, **36**, 53 (1953).

TUESDAY—AFTERNOON SESSION

REPORT ON PRESERVATIVES AND ARTIFICIAL SWEETENERS

By MARGARETHE OAKLEY (State Department of Health, Bureau of Chemistry, Baltimore, Maryland), *Referee*

The progress of the chapter is encouraging. It took a long time to bring the chapter up to date, principally because many of the subjects were so outdated no one was interested in spending any time on them. Committee D helped by eliminating two compounds which are no longer in use.

The problems of the chapter are now twofold: first, in regard to the subjects which should be included, and secondly, which of the new subjects should be studied.

For the first problem we recommended study of antioxidants, which are now in the chapter with the oils, and propionates, which are in the cereal chapter. With formic acid now among the other volatile acids, and the quantitative fluoride determination included with poisonous residues, this chapter has many cross references and many qualitative tests. Since the question about a preservative or artificial sweetener is usually "Is it present?", qualitative tests are very useful in this chapter.

The second problem, the choice of subjects for study with the limited number of available Associate Referees, is conditioned by the activity in the field of preservatives and artificial sweeteners. For example, last year a request was received from Canada for information on a method for peracetic acid, a problem which is not being studied.

Bacteriocidal ices are being suggested for the preservation of sea food, and all of the known preservatives and antioxidants are being tried singly and in combinations. The mold inhibitors are being suggested and used—dehydroacetic acid for fungistatic wrappers, and ethanolamine, formed by treatment with ethylene and ammonia gases, for corn. There is a qualitative test for Tweens (1) which may be applicable to food products, such as bread and chocolate. Two articles on preservatives in dairy products have appeared: one from the University of Milan on hydrogen peroxide and a patent for the treatment of milk with 200 p.p.m. of soluble chlorites (2). Antisprouting compounds are beginning to find favor; methyl-1-naphthalene acetate has been patented (3). The Japanese have published several more articles on the efficacy of various preservatives for soy sauce.

Although the National Canners Association states that antibiotics cannot be relied upon in preserving foods, interest in this class of compounds is still strong. Since the Swiss tried a plant-derived antibiotic on milk, there have been a large number of patents issued to Swift and Co. on antibiotics for use as food preservatives, including antibiotics

from avocado trees, rhatany, grapevine, broadleaf gum plant, pareira, bittersweet, osage orange tree, common agrimony, white cedar, purple prairie clover, spring avens, St. John's wort, Iceland moss, butternut tree, and galingale root (4).

In addition to antibiotics, the subject of irradiation is being considered. A recent article (5) indicates that milk treated with X-ray dosages of 36,000–73,000 *r* kept salably fresh about twice as long as the controls.

A patent for protective coatings of hams includes methyl cellulose or similar products, together with antibacterial material and mold inhibitors.

In a patent to a Netherland organization, mold inhibitors, chiefly gallates, are proposed for use in a variety of foods ranging from dairy products through smoked fish to chocolate. An interesting patent concerns the rendering of metal surfaces antiseptic (6) by anodizing them and then immersing them in a solution of 8-quinolinol or one of its salts.

This past year our Associate Referees have shown a tremendous amount of activity. According to the Associate Referee on Artificial Sweeteners, newly developed methods for dulcin and P-4000 are ready for collaborative study. A spectrophotometric method for dehydroacetic acid has been studied collaboratively. A spectrophotometric method for quantitative estimation of benzoic acid is in progress and shows possibilities for adaptation to hydroxybenzoates. The qualitative test for hydroxybenzoates must still be studied. A qualitative method for the detection of the fluoride ion by its ability to quench the fluorescence of aluminium oxinate was investigated and is ready for collaborative study. Further work was done on quaternary ammonium compounds with the recommendation that the bromphenol blue method be adopted as a qualitative test for these compounds in milk. The Referee on thiourea continues to watch for the use of this compound but recommends discontinuing further study of the method which has already been adopted, since it is adequate for present needs. The Referee on monochloroacetic acid and on sucaryl recommends further work on both of these substances.

ACKNOWLEDGMENT

The Referee wishes especially to thank the Associate Referees who, because of pressure of their regular duties, have had to use their own time to work on their preservative and artificial sweetener problems and who, despite this handicap, have accomplished most laudable results.

RECOMMENDATIONS

It is recommended*—

(1) That the colorimetric method for the detection of P-4000 be submitted to collaborative study.

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

(2) That the colorimetric method for the determination of P-4000 be submitted to collaborative study.

(3) That study of methods for the determination and identification of Sucaryl® be continued.

(4) That further work be done on the determination of monochloracetic acid in fruit juices other than orange juice.

(5) That collaborative study of the method for monochloracetic acid in beverage bases containing weighting oils (*This Journal*, **34**, 345 (1951)) be continued.

(6) That the method "Quaternary Ammonium Compounds in Milk, Qualitative," as reported by the Associate Referee this year (1952) be adopted as First Action.

(7) That the test for purity of the Bromphenol Blue reagent as described in the Associate Referee's report on Quaternary Ammonium Compounds, be added to the paragraph **27.34** entitled "REAGENTS."

(8) That collaborative study be continued on the Reineckate Methods for the determination and identification of Quaternary Ammonium Compounds.

(9) That collaborative study be continued on the Bromphenol Blue methods for Quaternary Ammonium Compounds (*This Journal*, **29**, 318 (1946)) to include methods for fruit juices, bottled sodas, milk, mayonnaise, pickles, and shrimp.

(10) That further study of a spectrophotometric method for the quantitative determination of benzoic acid be made.

(11) That the study of methods for the detection of benzoates and hydroxybenzoates be continued.

(12) That the method for the detection of the fluoride ion by the quenching of aluminium fluorescence be submitted to collaborative study.

(13) That study of thiourea be discontinued.

(14) That the qualitative test and the spectrophotometric method for dehydroacetic acid in cheese be adopted, First Action.

(15) That Associate Referees be appointed to work on the following subjects:

(a) Hydrogen peroxide in dairy products.
(b) Peracetic acid in fruit juices.
(c) Gallates with 8–12 carbon atoms in oily foods, fish, meat, or baked goods.

REFERENCES

(1) NEWBURGER, S. H., *This Journal*, **34**, 109 (1951).
(2) HANSEN, F. F., U. S. Pat. 2,570,231, Oct. 9, 1951, thru *Chem. Abstr.*, **46**, 1666*c* (1952).
(3) GUNDEL, W., MEYER, E., OFFERMAN, W., and FUCHS, H., to Henkel & Cie., G.m.b.H., U. S. Pat, 2,570,664, Oct. 9, 1951, thru *Chem. Abstr.*, **46**, 1666*g* (1952).

(4) *Chem. Abstr.*, **45**, 7724 (1951).
(5) GADEN, E. L., JR., HENLEY, E. J., and COLLINS, V. P., *Food Technol.*, **5**, 506 (1951).
(6) RINGK, W. F., and FREEMAN, S. K., to Benzol Products Co., U. S. Pat. 2,574,225, thru *Chem. Abstr.*, **46**, 694*g* (1952).

REPORT ON THIOUREA IN CHEMICAL SPRAYS

By W. O. WINKLER (Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C.),
Associate Referee

Although there have been no reports of the use of thiourea as a food preservative in the United States during the past year, food officials must still be alert to discover instances where it might be used. The effective fungicidal properties of thiourea make it a very tempting agent for the control of rot in various foods, particularly fruits. Imported foods, in which it might be used, should be given careful scrutiny.

Reports that chemical sprays were being used in a South American country to control stem-end rot in citrus fruit led to the collection of a sample of the spray and a portion of this liquid was analyzed. The material was a yellowish emulsion with an odor resembling carbon disulfide. The principal constituents found were a yellow, resinous, waxy solid, soluble in chloroform, and a white solid, soluble in water and alcohol, which proved to be thiourea. The waxy solid was evidently added as an adhesive to aid the spray in sticking to the fruit. No other active fungicide in addition to thiourea was found.

The reported use of thiourea sprays in other countries is cause to maintain vigilance against the importation of food contaminated with the toxic chemical.

The First Action methods have sufficed to detect thiourea in all cases coming to the attention of the Associate Referee and although the recoveries may not be total, they appear adequate for present needs. The Associate Referee does not believe it necessary to continue the work on methods at the present time.

RECOMMENDATION

It is recommended*—

That the study of methods for thiourea in foods be discontinued for the present.

* For report of Subcommittee D and action of the Association, see *This Journal*, **36**, 60 (1953).

REPORT ON QUATERNARY AMMONIUM COMPOUNDS

TEST FOR PURITY OF BROMPHENOL BLUE

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

During the past year further investigation was made of the method for quaternary ammonium compounds in milk. Food and Drug Administration chemists in Washington, D. C., and in Denver, Colorado, confirmed the findings reported at the 1947 meeting,* i.e., that when about 2 p.p.m. of quaternary is present a positive test (blue color) is obtained. In that report all six collaborators obtained a recognizable blue color when the milk contained 2.3 p.p.m. of lauryldimethylbenzylammonium chloride. The amounts found varied from 0.4 to 1.2 p.p.m. with an average of 0.7 p.p.m.

In view of the fact that for action under the provisions of the Federal Food, Drug, and Cosmetic Act, only the presence of these chemicals need be established, the Associate Referee feels that the work reported justifies the adoption of the method given below as a qualitative method for quaternary ammonium compounds in milk.

For reagents and procedure to be followed, see *This Journal*, **36**, 81–82 (1953).

The 1950 report† on this subject contains a "Test for Purity" of the bromophenol blue to establish the suitability of any particular lot for use as a reagent in determining quaternary ammonium compounds. As the Associate Referee had several requests for reprints of that report during the year, it seemed advisable to make a survey of the various brands of bromophenol blue on the market and test them by the procedure given.

Accordingly, lots of bromophenol blue were purchased from each of five supply houses and tested. When all but one of these lots gave an off color, the procedure was extended to determine the extent of the impurities present.

The indicators were purchased from the following suppliers: Bacto-Bromphenol Blue, Difco Laboratories, Detroit, Michigan; Tetrabromophenolsulfonphthalein 752, Eastman Kodak Co., Rochester, N. Y.; Bromphenol blue (for *p*H work), Fisher Scientific Co., Silver Spring, Maryland; Bromphenol blue 5017, The Matheson Co., Inc., Joliet, Illinois; Tetrabromphenolsulfonphthalein, National Aniline Division, Allied Chemical & Dye Corp., New York, N. Y.

The original test consists of shaking 30 mg of the indicator with a mixture of 50 ml ethylene chloride and 5 ml 1 per cent Na_2CO_3 in a separatory

* *This Journal*, **31**, 483 (1948).
† *This Journal*, **34**, 343 (1951).

funnel until complete solution is achieved. After standing, the mixture separates into two layers; the lower (ethylene chloride) should be colorless and the upper should have the purple color of alkaline bromophenol blue. Ten ml of a solution containing 0.1 mg of DC 12 or other quaternary is added, the mixture is again shaken for 1 minute, and allowed to stand until clear. The lower layer, which should have a clear blue color, is drawn off into a glass-stoppered flask, dried with granular anhydrous sodium sulfate, and examined in a photometer (to eliminate the personal equation). The absorption should be greatest at about 608 mμ.

When the above procedure was applied to the five lots listed above, only the Eastman product gave a clear blue ethylene chloride layer. With this product, a second portion of ethylene chloride was added to the separated top layer and 20 ml of solution containing 0.2 mg of DC 12 was added for a second test. This addition also produced a clear blue solution and when depth of color was measured with the neutral wedge photometer in a 1 inch cell, using a filter centering at 610 mμ, the readings were 37 mm and 73 mm, respectively.

In the case of the four other lots, the addition of 0.1 mg of DC 12 gave a nearly colorless ethylene chloride layer. The addition of a second 0.1 mg portion of DC 12 produced a slightly greenish-yellow solution, while the addition of a third 0.1 mg of DC 12 gave a definite green to blue-green color in the ethylene chloride layer. In one case, a fourth 0.1 mg of DC 12 was added, producing a dark green color.

After standing until clear, the lower layers were drawn off, and dried over sodium sulfate; color density was read in the wedge photometer. The readings were 30, 40, 50, and 50 mm, corresponding to 0.080, 0.108, 0.164, and 0.164 mg of DC 12 combined with bromophenol blue. The remaining DC 12 in each case (0.220, 0.192, 0.236, and 0.136 mg) was apparently combined with some impurity which reacts more readily with the DC 12 than does bromophenol blue, and produces a yellowish compound.

In each case the top layer was superimposed on fresh ethylene chloride, and 0.1 mg DC 12 and 5 ml 1 per cent Na_2CO_3 solution were added. The readings in these cases were 37, 41, 26, and 32 mm, similar to the values given by the Eastman product with that amount of DC 12. A second test, using 0.2 mg of DC 12, gave the readings 72, 72, 73, and 65 mm, also near those given by the Eastman product with 0.2 mg of DC 12.

While all of these four lots appear suitable for indicators, their use as reagent for quaternary ammonium compounds would lead to low results. Thus, only the lots of bromophenol blue which pass the proposed test may be used as reagents for quaternary ammonium compounds. It also appears from these experiments that the procedure for purification given in the previous report (loc. cit.) can be expected to yield a suitable product when applied to these lots of bromophenol blue.

A set of samples of quaternaries for the collaborative study of the

reineckate method for determination and identification of certain quaternary ammonium compounds has been prepared and analyzed by the author with the cooperation of A. H. Tillson. Certain of these samples have beeen sent to two of the laboratories of the Food and Drug Administration which expected to have proper equipment available for the identification procedure by the end of the summer. Since no report is forthcoming at this time, we expect to call upon a third laboratory, which will probably be equipped soon for this work, with the hope that the results will be on hand for the 1953 meeting.

RECOMMENDATIONS

It is recommended*—

(1) That the method "Quaternary Ammonium Compounds in Milk (Qualitative)" as given in this year's report† be adopted, First Action.

(2) That under "**27.34**, Reagents", the following statement be added:

(a) *D.C. 12.*—Lauryldimethylbenzylammonium chloride, or other solid quaternary ammonium compound.

(b) *Bromophenol Blue.*—Tetrabromophenolsulfonaphthalein, which passes the "Test for Purity" given in *This Journal*, **36**, 81 (1953).

(3) That collaborative study be continued on the reineckate methods for the determination and identification of quaternary ammonium compounds.

(4) That collaborative study be continued on the bromophenol blue methods for quaternary ammonium compounds as follows: *This Journal*, **29**, 318 (1946).

(a) Method for Fruit Juices
(b) Shorter method for Fruit Juices
(c) Method for Bottled Sodas, increasing reagent to 5–10 ml
(d) Method for Milk
(e) Method for Mayonnaise, Salad Dressings, and Sandwich Spreads
(f) Method for Pickles and Relishes
(g) Method for Shrimp, *ibid.*, **33**, 670 (1950)

REPORT ON QUALITATIVE TEST FOR FLUORINE

By MARY C. HARRIGAN (Food and Drug Administration, Department of Health, Education, and Welfare, Boston, Massachusetts), *Associate Referee*

Of all the tests the Associate Referee examined, one which seemed most likely to prove of value in the examination of foods for traces of fluorine was Feigl's method of the quenching of aluminum oxine fluorescence (1).

When aluminum oxinate is treated with a solution containing fluoride ions, the fluorescence disappears. The aluminum oxinate reacts with the fluoride ion to form aluminum fluoride or the hexafluoraluminate ion. A

* For report of Subcommittee D and action of the Association, see *This Journal*, **36**, 64 (1953).
† Published in *Changes in Methods*, *This Journal*, **36**, 81–82 (1953).

piece of filter paper is dipped in a chloroform solution of aluminum oxinate. After drying, the fluorescent paper is exposed to hydrogen fluoride vapor. The surface that comes in contact with the hydrogen fluoride does not fluoresce in ultraviolet light.

EXPERIMENTAL

To check the sensitivity of the test, 3 drops of 6 *N* H_2SO_4 and 1 drop of NaF soln were placed in a tiny crucible covered with a piece of filter paper of a diam. sufficiently wider than the crucible so that the exposed and unexposed areas could be easily compared. The crucible covered with the paper was heated to 50–60°C. for 5 min., the paper was spotted with $CHCl_3$ soln of oxine (0.5 mg/ml) and observed under ultraviolet light. The limit of identification of 0.05 mg of fluorine corresponds to a concn limit of $1:10^6$. The sensitivity of the fluoride test is somewhat lower in the presence of boric acid.

The Al-8-hydroxyquinoline salt was made by the Associate Referee according to the method of Hillebrand and Lundell (2) as follows:

Warm a soln of 2.22 g of $AlNH_4(SO_4)_2 \cdot 12H_2O$ and 3 drops HCl in 250 ml H_2O to 50–60°C. and add an excess of an acetic acid soln of the reagent (5% 8-hydroxyquinoline in 2 *N* acetic acid). One ml of reagent is equivalent to ca 5 ml of the Al soln. Add a 2 *N* soln of NH_4 acetate slowly until a permanent ppt is formed and then 20–25 ml addnl to insure complete pptn. Allow ppt to settle, filter thru fritted glass crucible, wash with cold H_2O, dry at 120–140°C., and store in desiccator. The 8-hydroxyquinoline reagent keeps for several weeks without change.

The fluoride test of Gettler and Ellerbrook (3), the production of faintly pink hexagonal crystals of sodium fluosilicate, was tried. The amount and size of the crystals were so small, however, that the test was not considered conclusive as the quenching of the oxine fluorescence.

RECOMMENDATION*

The Associate Referee recommends that this test be submitted to collaborative study.

REFERENCES

(1) Feigl, F., *Anal. Chim. Acta*, **3**, 561 (1949).
(2) Hillebrand, W. F., and Lundell, G. E., *Applied Inorganic Analysis*, John Wiley and Sons, Inc., New York, N. Y., 1929, p. 116.
(3) Gettler, A. O., and Ellerbrook, L., *Am. J. Med. Sci.*, **197**, 625 (1939).

REPORT ON DEHYDROACETIC ACID IN CHEESE

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

The packaging of sliced cheeses represents a definite and substantial trend in the cheese industry, but it poses a serious problem for the packager; the cheese must be handled under carefully controlled conditions to prevent mold contamination. As a possible solution to the prob-

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

lem, the use of a wrapper impregnated with the antimycotic dehydroacetic acid (DHA) has been suggested. This antimycotic affords good protection against mold, but the compound diffuses into the cheese. The present study was undertaken, therefore, to evaluate the reliability of an available quantitative method for DHA in cheese (1) and to work out a confirmatory qualitative test.

Two procedures were developed by Woods, *et al.* (2), for the quantitative determination of DHA in biological materials: a spectrophotometric method based upon the absorption of DHA in chloroform solution and a colorimetric method based upon the reaction of DHA with salicylaldehyde in alkaline solution to produce a red-orange color. Hogan and DeLong (1) developed a method for DHA in cheese similar to the spectrophotometric method of Woods, which, with certain minor changes, was found satisfactory by the Associate Referee. The salicylaldehyde method was modified and adapted to cheese by the Associate Referee as a confirmatory qualitative test for DHA. A previous attempt to use the presence of a second absorption peak at 225 mμ (which is of the same order of magnitude as the peak measured at 307 mμ in the quantitative method) as a means of identification of DHA in cheese resulted in ambiguity and false positive tests; extracts of some cheeses containing no DHA showed high absorption in this region and occasionally, a definite peak at 225 mμ.

The quantitative and qualitative methods studied were described in *This Journal*, **36**, 83 (1953).

INSTRUCTIONS TO COLLABORATORS

The quantitative method was subjected to collaborative study, the following specific instructions being sent to the collaborators: to 50 g of cheese in a Waring blendor add 100 p.p.m. of DHA (5 ml of the standard stock soln required for the standard curve in the method), and make a detn in accordance with the method. Run a blank (single detn) on the cheese. Report the kind of cheese used, the cheese blanks, and the recoveries in duplicate (uncorrected). Also report the absorbance found in the recovery experiments at 307 mμ and at 225 mμ (with no correction for cheese blank).

The qualitative test was also subjected to collaborative study. The collaborators were instructed to try the test on (*a*) one cheese known to be free of DHA; (*b*) one cheese to which ca 20 p.p.m. of DHA had been added; and (*c*) any available samples analyzed by the quantitative method and found to contain DHA.

RESULTS AND DISCUSSION

In general, the results of the collaborative study of the quantitative method, summarized in Table 1, are quite satisfactory. Several collaborators, in commenting on the method, noted turbidity in the final 500 ml solution, of which a portion is transferred to a cell for the spectrophotometric reading. They usually eliminated the turbidity by refiltering through paper, asbestos, or sintered glass. One analyst, however, recommended the use of a filter aid. He transferred the aerated chloroform-free

TABLE 1.—*Recovery results for dehydroacetic acid added to cheese at the level of 100 p.p.m.*

ANALYST	KIND OF CHEESE	CHEESE BLANK, PPM APPARENT DHA	PER CENT RECOVERY (CORRECTED FOR BLANK)
1	Process American	2.1	97, 99
2	Process Cheddar	—	65, 69
	Sharp Cheddar	5.3, 7.9	78, 95
3	Proc. American	0.8	95, 96
4	Sliced Cheddar	1.0	98, 98
5	Cheddar	4.8	94, 94
6	Cheddar	4.0	94, 94
7	Cheddar	5.6	93, 93
8	Cheddar	1.0	94, 96
9	Process American	—	95, 100
	Swiss+American	—	83, 83
10	Edam	2.3	81, 86
11	Cheddar	1.1, 2.0	94, 100
12	American	1.1	97, 97
13	Semisoft	4.1	88, 89
14	Cream cheese with pimento	3.1	59, 62
	Cheddar	1.9	64, 66
	American	1.3	92
	Pimento	1.3	92
15	Cheddar	4.2	95, 95
16	Cheddar	—	98, 103
17	Cheddar	5.9	97, 99
18	Cheddar	6.3	90, 91
19	Sliced Cheddar	1.0	96
	American	5.0	93
Maximum		7.9	103
Minimum		0.8	59
Average		3.17	89.7

solution to a 500 ml volumetric flask, diluted to the mark with water. added 1–2 g of Celite, mixed well, and filtered. Analyst 18, on the other hand, reported that a diatomaceous filter aid gave a positive blank reading. Analyst 2 attributed the low recoveries of 65 and 69 per cent (Table 1) to the water added to the cheese with the DHA which caused the cheese to become swollen and gummy; when the DHA was added as the solid, recoveries of 78 and 95 per cent were obtained. Analyst 14 also attributed the low recoveries of 59, 62, 64, and 66 per cent (Table 1) to the presence of appreciable amounts of water in the cheese.

Other comments and suggestions by the collaborators: for the preparation of the standard curve, a fresh DHA solution should be used, since lower readings are obtained from older solutions (2 per cent lower after 10 days' standing); low recoveries are obtained when the chloroform is

TABLE 2.—*Results of the collaborative study of the qualitative test*

ANALYST	KIND OF CHEESE	DHA, PPM	TEST
1	Cheese Food, (a)	None	Negative
	Cheese Food, (b)	None	Negative
	Brick	None	Negative
	Cheddar	None	Negative
	Cheddar	20, added	Positive
	Process Sliced American	43, by quant. method	Positive
	Process Sliced American	100, by quant. method	Positive
	Process Sliced American	150, by quant. method	Positive
	Process Sliced American	150, by quant. method	Positive
	Process Pimento, Sliced	80, by quant. method	Positive
2	Cheddar	None	Negative
	Cheddar	None	Negative
	Cheddar	20, added	Positive
3	Process American Sliced	None	Negative
	Process American Loaf	None	Negative
	Process American Loaf	20, added	Positive
4	Cheddar	None	Negative
	Cheddar	None	Negative
	Cheddar	20, added	Positive
5	Swiss	None	Negative
	Cheddar	None	Negative
	Process Sliced American	None	Negative
	Process Swiss, Sliced	None	Negative
	Process Sliced American	None	Negative
	Process Sliced Pimento	None	Negative
	Process Sliced American	20, added	Positive
	Process Swiss & Cheddar, Sliced	103, by quant. method	Positive
	Process Sliced Pimento	216, by quant. method	Positive

not completely removed by aeration; and a smaller Waring blendor cup should give a better chloroform extraction. Where feasible, these suggestions have been incorporated in the method. It was proposed that the aerated chloroform-free solution be made to a 500 ml volume, Celite added, and the solution then filtered, but the Associate Referee has not had the opportunity to try out the proposal.

The results of the collaborative study of the qualitative test, summarized in Table 2, are entirely satisfactory. No analyst obtained a positive test on cheese known to be free of DHA; all analysts obtained a positive test on cheese to which 20 p.p.m. DHA was added; and all analysts obtained a positive test on cheese found to contain DHA by the quantitative method. One analyst did report, however, that one lot of

ether believed to contain aldehydes or peroxides was unsuitable for the extraction because it gave a false positive test.

RECOMMENDATIONS

It is recommended*—

(1) That the qualitative and quantitative methods described above for dehydroacetic acid in cheese be adopted, First Action.

REFERENCES

(1) HOGAN, D., and DELONG, R. F., *Analysis of Dehydroacetic Acid in Cheese*, Mimeograph Issue (M-763), Marathon Corp., Menasha, Wis., September 25, 1950.

(2) WOODS, L. A., SHIDEMAN, F. E., SEEVERS, M. H., WEEKS, J. R., and KRUSE, W. T., *J. Pharmacol. Exptl. Therap.*, **99**, 84 (1950).

REPORT ON BENZOATES AND HYDROXYBENZOATES

DETERMINATION OF BENZOIC ACID IN FOODS

By H. E. GAKENHEIMER (Food and Drug Administration, Department of Health, Education, and Welfare, Baltimore, Maryland), *Associate Referee*

The development of a spectrophotometric method for the quantitative determination of minute amounts of benzoic acid in oleomargarine, catsup, jellies, jams, etc., is in progress.

The procedure, based on the method of Jones (1), consists of extraction of the acid with ether, nitration, and reduction of the compound with hydroxylamine hydrochloride. The red color, which develops within a few minutes, is compared with a standard, using a Beckman DU spectrophotometer at 510 mμ.

Work is in progress on extraction of benzoic acid from various food products and adaptation of the method for quantitative determination. It is believed that, after a spectrophotometric method for this acid has been developed, hydroxybenzoates can be similarly analyzed. When the Associate Referee considers the procedure entirely satisfactory, samples will be furnished for collaborative study.

RECOMMENDATIONS

It is recommended†—

That the method be further studied.

REFERENCE

(1) JONES, A. J., *Pharm. J.*, **115**, 144 (1925).

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

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

REPORT ON ARTIFICIAL SWEETENERS

METHODS FOR THE DETECTION AND DETERMINATION OF P-4000 (PROPOXY-2-AMINO-4-NITROBENZENE)

By WILLIAM S. COX (Food and Drug Administration, Department of Health, Education, and Welfare, Atlanta, Georgia), *Associate Referee*

During the past year, an attempt has been made by the author to develop suitable color reaction methods for the detection and determination of the intensely sweet compound, P-4000. The methods outlined in this paper appear to be acceptable, but no collaborative studies have been made.

As reported in *This Journal*, **35**, 86 (1952), an organoleptic method for the detection of P-4000 was adopted, First Action. This method may not always be satisfactory, due to the personal factor and to the possible extraction of other materials which might be identified as P-4000.

During the search for a color reaction method for the detection of P-4000, it was discovered that, by reducing the sweetener with stannous chloride and then brominating the reduction product with strong bromine water, a deep burgundy-colored solution is formed. This reaction does not occur with dulcin or saccharin, the common artificial sweeteners. Since Sucaryl ® (cyclohexyl sulfamate) is not extractable with organic solvents, it was not used in these tests.

METHOD

With 10% NaOH, make alk. (*p*H 7.5–8.0) 200 ml of liquid food or aq. ext. of 200 g of solid food or semi-solid product, **27.41(c)**, and ext. 3 times with 25 ml portions of petr. ether. Wash combined petr. ether ext. once with 5 ml of H_2O, transfer to a small beaker or dish, and allow ether to evap. spontaneously to ca 5 ml. Transfer the remainder to a 25 ml test tube, and evap. to dryness. Add 2 ml HCl (1+1), then 2 ml satd $SnCl_2$ soln. Heat 15 min. in boiling H_2O bath, cool, and add strong Br water dropwise to excess. If P-4000 is present, the soln will change from nearly colorless to burgundy, and finally to yellow if a considerable excess is added. (Note: if the $SnCl_2$ soln of the suspected P-4000 is deeply colored, it should be dild so that it is nearly colorless, and the strong Br water added dropwise to ca 5 ml of the dild soln.)

Several methods for the determination of P-4000 are found in the literature (1–3). A procedure developed by Gialdi (1) appeared to be the most promising and a brief study of this method was made. The method involves the diazotization of the P-4000 at 15° and coupling with α-naphthol. As developed by Gialdi, the resulting colored solution was read on a Pulfrich photometer, using an S53 filter.

Several modifications were made during the study: it was found that the developed color could be completely extracted with isoamyl alcohol and that the alcohol solution of the color had an absorption maximum at 515 mμ, using a Coleman Junior spectrophotometer. Known concentra-

tions of the sweetener in the range 0–7 p.p.m. were used in constructing a standard curve, and several knowns were determined with excellent results.

The modified method, which is to be collaboratively studied during the coming year, is as follows:

MODIFIED METHOD

REAGENTS

(a) *Sodium nitrite solution.*—0.5 g in 100 ml H_2O.
(b) *Hydrochloric acid.*—0.1 *N*.
(c) *α-Naphthol solution.*—0.1 g α-naphthol in one l of 1% Na_2CO_3 soln.

DETERMINATION

Ext. the P-4000 from an alk. liquid or aq. ext., using petr. ether, and dil. the resulting ether soln so that 10 ml contains 0.02–0.10 mg. Transfer 10 ml to a small beaker and evap. the ether spontaneously. Dissolve residue in 10 ml hot H_2O. Cool to 15°. Add 0.25 ml $NaNO_2$ soln and mix. Add 0.25 ml 0.1 *N* HCl, and mix. After 45 sec., add 4.5 ml α-naphthol soln, and mix. Ext. developed color with 15 ml isoamyl alcohol in a separatory funnel, draw off the aq. layer, and filter the alcohol soln thru a pledget of cotton. Read, within one hr, the per cent transmission at 515 mμ. Run a blank detn on reagents and use this soln to set the colorimeter scale to read 100% transmission. From the standard curve previously prepd, obtain the amount of P-4000 which corresponds to the per cent transmission of the sample being analyzed.

RECOMMENDATION

It is recommended* that the proposed methods for the detection and determination of P-4000 be studied collaboratively.

REFERENCES

(1) GIALDI, F., *Farm Sci. e tec.* (*Pavia*), **3**, 44 (1948), thru *Chem. Abstr.*, **42**, 4714 (1948).
(2) MÖHLER, K., *Z. Lebensm. Untersuch. u. -Forsch.*, **91**, 124 (1950), thru *Chem. Abstr.*, **44**, 10603 (1950).
(3) HOEKE, F., *Chem. Weekblad.*, **43**, 283 (1947), thru *Chem. Abstr.*, **41**, 5058 (1947).

No reports were received on monochloracetic acid, or cyclamate sodium.

REPORT ON SPICES AND OTHER CONDIMENTS

By E. C. DEAL (Food and Drug Administration, Department of Health, Education, and Welfare, New Orleans, La.), *Referee*

The report (1) of Subcommittee C† on Recommendations of Referees outlined certain topics relating to spices and other condiments for study during the year 1952. Associate Referees were appointed on the subjects:

* For report of Subcommittee D and action of the Association, see *This Journal*, **36**, 64 (1953).
† *This Journal*, **35**, 49 (1952).

Vinegar; Volatile Oil in Spices; Sugar, Ash, and Pungent Principles in Mustard; Preparation of Sample of French Dressing; Seeds and Stems in Ground Chili; and Sorbitol. The Referee received reports from only two of the Associate Referees.

VOLATILE OIL IN SPICES

Associate Referee Aubrey Carson resumed work on this subject after having to forego studies last year. He reviewed the work done in 1949 on the volatile oil loss on storage of certain ground spices and investigated the factors that might cause variations in results obtained by Method **28.16,** employing the Clevenger trap.

As a further check on the above method, the Associate Referee sent out samples of three ground spices for collaborative study. Results obtained by the collaborators were promising but somewhat variable, probably due to a variety of causes. One of the spices (allspice) gave particular difficulty due in part to the differences in gravities of various fractions of the volatile oil. The Associate Referee is studying different types of traps in an effort to develop a satisfactory application of the method to this and other spices.

PREPARATION OF SAMPLE OF FRENCH DRESSING

Last year the Association adopted, as a procedure, a method* of preparation of samples of french dressing of the separable type as developed by the Associate Referee and tried out by his collaborators on duplicate samples from two different batches of laboratory-prepared french dressings. While results of analysis by this method checked well, a question arose as to the possible loss of moisture or other volatile principles due to heating of the sample during the blending with the emulsifying agent.

Experimental work was carried out by the Associate Referee to determine any effect in the composition of french dressings subjected to preparation by the approved procedure. Moisture analyses on five different triplicate batches showed that while there is a rise in temperature, due to blending, no significant change in composition of the samples occurs.

While not mentioned in the Associate Referee's report, certain difficulties have been reported in the preparation for analysis of large size containers (1 gallon or larger) by the procedure. The Associate Referee plans to study this problem during the coming year.

RECOMMENDATIONS

It is recommended†—

(1) That studies on the determination of volatile oil in spices be continued.

* *Ibid.*, 35, 86 (1952).
† For report of Subcommittee C and action of the Association, see *This Journal*, 36, 59 (1953).

(2) That studies on the preparation of samples of french dressing be continued, with attention being given to the large size containers.

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

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

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

(6) That methods for the determination of sorbitol be continued.

(7) That studies of methods for the determination of ash and sugars in prepared mustards be continued.

(8) That studies of methods for the determination of pungent principles in prepared mustard and mustard flour be continued.

(9) That methods for the detection of seeds and stems in ground chili be further studied and submitted to collaborative trial.

REPORT ON VOLATILE OIL IN SPICES

By N. AUBREY CARSON (Food and Drug Administration, Department of Health, Education, and Welfare, St. Louis, Missouri), *Associate Referee*

The old tentative method for volatile oil in spices, with some modifications, was submitted for collaborative study (1) and adopted as First Action in 1949 (2, 3). Four spices with a range in volatile oil content of 0.3–17 per cent were studied by this method. A collaborative study of additional spices was needed for the adoption of the method as official. Star anise, nutmeg, and allspice were chosen for this work. Their volatile oil content was substantial and some difficulty had been encountered in their analysis.

EXPERIMENTAL

Clevenger showed (4–7) that certain spices lost oil when held unrefrigerated any length of time. Distillations by the Associate Referee were made in 1949 immediately after grinding the four spices. The yield of volatile oil was again determined when the spices were sent to the collaborators. Results are given in Table 1.

TABLE 1.—*Volatile oil losses of stored ground spices*

DATE	CLOVES	CARAWAY	FENNEL	CORIANDER
	VOL. OIL, PER CENT (v/w)	VOL. OIL, PER CENT (v/w)	VOL. OIL, PER CENT (v/w)	VOL. OIL, PER CENT (v/w)
2/25/49	17.87	6.4	1.7	0.50
8/10/49	17.20	5.4	1.6	0.28

Another factor that might give low results in the distillations is the use of tinfoil covered cork joints. To determine the extent of this loss, three different sets of distillations were made: (*a*) with tinfoil-wrapped dry corks throughout; (*b*) with tinfoil-wrapped water-soaked corks throughout; (*c*) with standard taper glass joints throughout. The results are given in Table 2.

TABLE 2.—*Recovery of volatile oils using cork and glass connections*

	DRY CORK	WET CORK	GLASS JOINTS
	VOL. OIL, PER CENT (v/w)	VOL. OIL, PER CENT (v/w)	VOL. OIL, PER CENT (v/w)
Star Anise	6.00	6.50	6.60
Nutmeg	6.30	7.40	7.80
Allspice	3.90	4.00	4.25

COLLABORATIVE WORK

Star anise, nutmeg, and allspice were ground in an impact mill to pass a 20 mesh screen and portions of the well-mixed ground spices were sent to 7 collaborators. All collaborators were requested to keep the samples refrigerated until used and to complete all analyses within three weeks (specific instructions were given as to the amount of sample and the size of the flask to use for each spice). All were required to determine the amount of volatile oil in each spice, the specific gravity and refractive index of each oil, and the amount of eugenol in the oil of allspice. The methods used were those of the *Methods of Analysis*, 7th Ed., **28.16–28.19.**

Special instructions were given for allspice, because the volatile oil contains two fractions, one heavier and one lighter than water, and no trap was available for handling such an oil in the usual manner. Collaborators were asked to use a lighter-than-water trap, to trap off the oil into a 10 ml graduate as it came down, and to use ice water in the condensers if available. If not, they were to report the temperature of the water entering and leaving the condensers every hour since some of the lighter fractions of the oils might be lost if the condensing water were too warm.

The method specifies tinfoil-wrapped cork connections but collaborators were permitted to use all glass connections, if they were available.

The results of all collaborators are listed in Tables 3, 4, and 5. The results of Collaborators 7 and 8 are not used in computing the averages and the standard deviations. Both of these analysts kept samples unrefrigerated for more than a month through no fault of their own. Collaborator 6 kept his samples unrefrigerated for 9 days. This should not have caused any appreciable loss in volatile oil. Collaborator 5 reported that his oil baths caused such excessive scorching that he could not finish his distillations. He made check determinations two months later and his samples

were kept refrigerated during this interval. Glas-col heating mantles were used by the Associate Referee. No scorching occurred, no beads were necessary, nor was any antifoam agent needed.

As the samples were all run in the winter or early spring, the collaborators reported no temperature over 17°C. in the water leaving the condensers. Most of the collaborators encountered difficulty in measuring the oil from the allspice.

TABLE 3.—*Collaborative results on star anise*

COLLABORATOR	VOLATILE OIL, PER CENT (v/w)	REF. IND., 20°C.	SP. GR., 25/25°C.	TYPE OF CONNECTIONS
1	6.6	1.5528	0.9774	Glass joints
	6.6	1.5530	0.9783	
	6.6	1.5535	0.9761	
2	6.39	1.5519	0.9764	Glass joints[b] and tinfoil
	6.39	1.5513	0.9759	wrapped corks
3	6.6	1.5536	0.974	Glass joints[b] and tinfoil
	6.6	1.5538	0.975	wrapped corks
4	6.8	1.5522	0.9777	Glass joints
	6.7	1.5522	0.9772	
	6.9	—	—	
5	6.6	1.5524	0.9771	Tinfoil wrapped corks
6	6.70	1.5527	0.9749	Glass joints[b] and tinfoil
	6.90	1.5528	0.9749	wrapped corks
7	5.20	1.5526[a]	0.9788[a]	Glass joints[b] and tinfoil
	5.26			wrapped corks
8	6.16	1.5556	0.9766	Glass joints
	6.14	1.5558	0.9767	
Av.	6.64	1.5528	0.9763	
Stand. Dev.	0.21 or 3.2%	0.0007	0.0012	

[a] Determination made on a composite of two oils.
[b] Glass joint connection used between flask and trap and corks used between traps and condensers.

CONCLUSIONS

The yields of volatile oil on star anise were in reasonable agreement between analysts. The variations in volatile oil content were very wide on nutmeg and allspice. Variations in yields were probably caused by several factors, as follows:

1. The difference in the time of analyses by different collaborators.

TABLE 4.—*Collaborative results on nutmeg*

COLLABORATOR	VOLATILE OIL, PER CENT v/w	REF. IND. AT 20°C.	SP. GR., 25°/25°C.	TYPE OF CONNECTIONS
1	7.8	1.4890	0.9269	Glass joints
	7.8	1.4898	0.9280	
	7.8	1.4889	0.9198	
2	6.9	1.4859	0.9142	Glass joints[b] and tinfoil
	6.7	1.4831	0.9147	wrapped corks
3	7.9	1.4885	0.921	Glass joints[b] and tinfoil
	8.0	1.4887	0.922	wrapped corks
4	7.8	1.4882	0.9241	Glass joints
	7.8	1.4885	0.9241	
5	7.2	1.4880	0.9220	Tinfoil wrapped corks
	7.9	1.4891	0.9280	
	7.0	1.4858	0.9133	
6	7.0	1.4881	0.9215	Glass joints[b] and tinfoil
	7.0	1.4886	0.9215	wrapped corks
7	6.36	1.4836[a]	0.9034[a]	Glass joints[b] and tinfoil
	6.34			wrapped corks
8	6.56	1.4921[a]	0.9322[a]	Glass joints
	6.54			
Av.	7.47	1.4879	0.9215	
Stand. Dev.	0.45 or 6.0%	0.0018	0.0052	

[a] Determination made on a composite of two oils.
[b] Glass joint connections used between flask and trap and corks used between traps and condensers.

2. Excessive scorching caused by too hot an oil bath.
3. The use of tinfoil covered cork joints in part or all of the connections. From the results in Tables 2, 3, 4, and 5, all glass connections would be preferable.
4. The difficulty of measuring the oil in allspice and the possibility of losing some of the oil by so many manipulations.

No conclusions were drawn from the results for refractive index, specific gravity, or the determination of eugenol because a variation in the yield of oil causes similar variations in the physical and chemical constants.

Several types of traps have been tried in an effort to overcome the difficulties encountered with allspice. One trap studied shows promise.

TABLE 5.—*Collaborative results on allspice*

COLLABORATOR	VOL. OIL, PER CENT v/w	REF. IND. AT 20°C.	SP. GR. AT 25°/25°C.	EUGENOL	TYPE OF CONNECTIONS
1	4.22	1.5292	1.026	78.0	Glass joints
	4.25	1.5292	1.027	79.0	
	4.25	1.5284	1.022	80.5	
2	3.8	1.5286	1.026	78.0	Glass joints[b] and tinfoil wrapped corks
	3.7	1.5279	1.026	76.5	
3	3.9	1.5286	1.043	85.0	Glass joints[b] and tinfoil wrapped corks
	4.0	1.5285	1.042	85.0	
4	4.16	1.5286	1.025	77.0	Glass joints
	4.45	1.5285	1.025	79.0	
	4.45	—	—	—	
5	3.60	1.5275	1.0238	78.0	Tinfoil wrapped corks
	4.0	1.5295	1.0294	79.0	
	4.0	1.5282	1.0257	79.0	
6	4.4	1.5283	1.0234	82.5	Glass joints[b] and tinfoil wrapped corks
	4.6	1.5284	1.0253	83.0	
7	3.80	1.5283[a]	1.0291[a]	82.5[a]	Glass joints[b] and tinfoil wrapped corks
	3.75	—	—	—	
8	—	—	—	—	———
Av.	4.12	1.5285	1.0279	80.1	
Stand. Dev.	0.29 or 7.0%	0.0005	0.0058	2.9 or 3.6%	

[a] Determinations made on a composite of two oils.
[b] Glass joint connections used between flask and tap and corks used between traps and condensers.

ACKNOWLEDGMENTS

Grateful appreciation is extended to the following collaborators who participated in this study:

Theodore S. Smith, Food and Drug Administration, Kansas City, Missouri.
Theodore E. Byers, Food and Drug Administration, Cincinnati, Ohio.
James H. Cannon, Food and Drug Administration, St. Louis, Missouri.
Helen T. Hyde, Food and Drug Administration, San Francisco, California.
Juanita E. Breit, Food and Drug Administration, Minneapolis, Minnesota.
Ernest C. Deal, Food and Drug Administration, New Orleans, Louisiana.
Alin L. Suslam, Food and Drug Administration, Boston, Massachusetts.

RECOMMENDATIONS

It is recommended*—

* For report of Subcommittee C and action of the Association, see *This Journal*, **36**, 59 (1953).

(1) That further work be done on the method.
(2) That further work be done on a trap for all types of spices.

REFERENCES

(1) CARSON, N. A., *This Journal*, **33**, 575 (1950).
(2) Report of Subcommittee C, *ibid.*, **33**, 57 (1950).
(3) ALFEND, S., *ibid.*, **33**, 571 (1950).
(4) CLEVENGER, J. F., *ibid.*, **18**, 611 (1935).
(5) ———, *ibid.*, **21**, 109 (1938).
(6) ———, *ibid.*, **22**, 683 (1939).
(7) ———, *ibid.*, **17**, 283 (1934).

REPORT ON SORBITOL IN VINEGARS

By F. A. ROTONDARO (Food and Drug Administration, Department of Health, Education, and Welfare, Philadelphia, Pennsylvania), *Associate Referee*

The occurrence of sorbitol in certain fruits (apples, plums, cherries, etc.) in contrast to the traces or negligible amounts of this substance in grapes and some berries facilitates the detection of adulterations of wines, vinegars, fruit juices, jams, etc.

The first report, based on a survey of available literature, indicates that suitable methods may be developed for the practical investigation of the initial problem: sophistication of wine vinegar with apple or other sorbitol-bearing fruit vinegars.

The problem will require two main lines of investigation:

(1) A survey of the "normal" varieties of vinegars available on the open market, such as apple cider, white, red and white wine, and flavored types. This phase of the work will require an examination of purification steps to separate extraneous substances from residual sugars, acids, and/or sorbitol.

(2) Determination of sorbitol in the purified extract by qualitative and quantitative methods. Present indications are that sorbitol may be effectively separated from the residual sugar by yeast fermentation and chromatography and that sorbitol may be determined by periodate oxidation and formation of derivatives such as phenylhydrazine, benzaldehyde, benzoylchloride, and acetic anhydride.

Any satisfactory procedure developed for vinegar may be easily adapted for other food and drug products. The easy availability of sorbitol, together with its many desirable properties will undoubtedly increase its use and consequent need for determination in a great variety of products.

RECOMMENDATIONS

It is recommended* that work be undertaken on a method for determining sorbitol.

* For report of Subcommittee C and action of the Association, see *This Journal*, **36**, 59 (1953).

REPORT ON PREPARATION OF SAMPLE AND SAMPLING OF FRENCH DRESSING

MOISTURE DETERMINATION

By ALDRICH F. RATAY (Department of Health, Education, and Welfare, Food and Drug Administration, Cincinnati, Ohio), *Associate Referee*

A method for preparing french dressing of the separable type for sampling was submitted to the Association and recommendation for adoption as a procedure was approved.

The method* requires the use of a Waring blendor and that the french dressing be stirred for a period of five minutes.

A question arose as to a possible moisture loss caused by the heating up of the mass during blending, and hence a series of experiments were conducted to determine whether or not there is any moisture loss or other changes in composition (volatile acid) during the preparation of the sample.

Moisture determinations were made on different days of five different triplicate batches of laboratory-prepared french dressing. The formula of all the subdivisions was the same except that subdivisions 1 and 2 were prepared, using catsup that had a greater solids content than subdivisions 3, 4, and 5.

TABLE 1.—*Per cent moisture in samples of french dressing*

SUB. NO.	TIME, MIN.	MOISTURE, PER CENT	AVERAGE
1A	3	46.67, 46.70, 46.66	46.68
1B	5	46.68, 46.66, 46.68	46.68
1C	7	46.72, 46.72, 46.73	46.73
2A	3	46.59, 46.61, 46.55	46.58
2B	5	46.55, 46.56, 46.56	46.56
2C	7	46.60, 46.61, 46.66	46.62
3A	3	48.43, 48.54, 48.34	48.44
3B	5	48.43, 48.43, 48.43	48.42
3C	7	48.45, 48.46, 48.47	48.46
4A	3	48.21, 48.28, 48.22	48.24
4B	5	48.24, 48.25, 48.24	48.24
4C	7	48.19, 48.18, 48.17	48.18
5A	3	48.19, 48.23, 48.27	48.23
5B	5	48.16, 48.20, 48.21	48.19
5C	7	48.18, 48.26, 48.24	48.23

* *This Journal*, 35, 86 (1952).

Each subdivision of the dressing was stirred for 3, 5, or 7 minutes. Upon completion of stirring, a determination was made in triplicate by method **28.39.** Results are shown in Tables 1–3.

TABLE 2.—*Average results*

SUB NO.	3 MIN.	5 MIN.	7 MIN.	VARIATION
1	46.68	46.68	46.73	0.05
2	46.58	46.56	46.62	0.06
3	48.44	48.42	48.46	0.04
4	48.24	48.24	48.18	0.06
5	48.23	48.19	48.23	0.04

TABLE 3.—*Temperature rise in °C.—starting temp. 25°C.*

SUB NO.	3 MIN.	5 MIN.	7 MIN.
1	11	18	18
2	18	18	21
3	14	14	18
4	9	9	21
5	9	15	22
Average	12	15	20

Although there is a rise in temperature due to stirring, there is no significant loss of moisture caused by the heating up of the mass when preparing a sample of french dressing as directed by the method described in *This Journal*, **35,** 86 (1952).

RECOMMENDATION

It is recommended* that the method for preparation of samples of french dressing be adopted.

No reports were received on vinegar; sugar, ash, and pungent principles in mustards; or seeds and stems in ground chili.

REPORT ON CEREALS

By V. E. MUNSEY (Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C.), *Referee*

It is recommended†—

(1) That the study on the determination of starch in raw and cooked cereals be continued.

* For report of Subcommittee C and action of the Association, see *This Journal*, **36,** 59 (1953).
† For report of Subcommittee D and action of the Association, see *This Journal*, **36,** 61 (1953).

(2) That the method for sugar in baked products be further studied as recommended by the Associate Referee (essentially **13.30–13.32** inclusive) on flour.

(3) That the method for the determination of lactose in bread, *This Journal*, **35**, 697 (1952), be studied further.

(4) That the study of methods on soybean flour for moisture, ash, nitrogen, crude fiber, and oil be continued.

(5) That the method referred to in *This Journal*, **25**, 83–84 (1942), for the determination of unsaponifiable matter and sterols in noodles, be studied for its application to bakery products containing eggs.

(6) That the method for water-soluble protein-nitrogen precipitable by 40% alcohol (albumen) in cereal products, *This Journal* **35**, 75 (1952) be adopted as official and **13.34** be deleted.

(7) That the study on the determination of bromates in flour be continued.

(8) That the method in *This Journal*, **35**, 284–296 (1951) under III. APPARATUS, p. 287 (*b*) be changed to read as set forth below and that this study be discontinued.

"Chromatographic tubes ca 15×250 mm or ca 15×450 mm constricted at lower end to ca 4 mm i. d." and under CHROMATOGRAPHIC SEPARATION (*a*) Preparation of partition column, p. 288, add the following new paragraph at the end of (*a*) "Where the amount of propionic acid approaches 20 mg in the column and a definite band is observed below the propionic acid band, the long chromatographic tube (450 mm) and ca 10 g of silicic acid should be employed. In this case, double the amounts of water, indicator and NH_4OH."

(9) That the Official Method for lipoid, Sec. **13.35** and lipoid P_2O_5, Sec. **13.36** be deleted, First Action.

(10) That the procedure for lipoids and Method I for lipoid P_2O_5 reported this year by the Referee, be adopted, First Action, and the study continued.

(11) That the method for choline in noodles reported by the Referee this year be further studied.

REPORT ON LIPOIDS AND LIPOID P_2O_5 IN NOODLES

By V. E. MUNSEY (Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C.), *Referee*

Last year's collaborative comparison of methods for lipoid and lipoid P_2O_5 in noodles (Sec. **13.110, 13.35** and **13.36**, *Methods of Analysis*) and a modified procedure were published in *This Journal*.* The procedures varied essentially in the following respects: (1) use of alcoholic NaOH

* *This Journal*, **35**, 693 (1952).

in place of alcoholic KOH; (2) substitution of Pyrex beaker for platinum dish; and (3) molybdate solution **20.46,** was used for the solution under **2.8.** The same procedure was used again this year for the lipoid P_2O_5. The lipoid directions, **13.35,** were modified by prescribing a filtration through an extraction tube containing an asbestos mat covered with sand instead of through a cotton pledget in a funnel. This modification seemed to offer less possibility of flour particles being ashed with the lipoids.

Egg noodles were prepared in the laboratory from fresh whole eggs and yolk. The yolk noodle, No. 1, contained 5.69 per cent yolk solids and the whole egg noodle, No. 2, contained 5.34 per cent whole egg solids on the moisture free basis. The semolina, whole egg, and yolk were analyzed for solids, lipoid, and lipoid P_2O_5.

A solution containing a known amount of KH_2PO_4 was also sent along with the noodles to the collaborators. The purpose of the phosphate solution was to check the actual determination of P_2O_5 apart from any variation in isolation. Instructions were to take 10 ml portions of the phosphate solution, add 10 ml HNO_3 (1+9), dilute to 60 ml, add 20 ml NH_4NO_3, and proceed as under Method II. Also, if time permitted, collaborators were to report P_2O_5 in solution by Method I. They were to determine blank on reagents and report as mg P_2O_5 per 10 ml and to report lipoid, lipoid P_2O_5, and egg solids on moisture free basis by the formula given in *This Journal,* **7,** 407 (1924).

METHODS

For adopted methods, see *This Journal,* **36,** 76 (1953). These are designated as Method I. A further modification (Method II) is described below:

Method II

Wash the sides of the Pyrex beaker with 10 ml $CHCl_3$ to dissolve the lipoids, and then with 10 ml alcohol, NaOH soln, **16.10(b),** evap. to dryness (cautiously) on steam bath, and ash 1 hr at 500°. Cover beaker with watch glass, add 15 ml HNO_3 (1+9) to make soln definitely acid, heat on steam bath ca 10 min., and filter into 300 ml Erlenmeyer flask. Wash residue and filter with ca 25 ml of hot H_2O. Make soln slightly alk. to litmus paper with NH_4OH from Mohr pipet, then slightly acid with HNO_3 (1+9), and add 10 ml HNO_3 (1+9). Keep vol. to ca 60 ml. Proceed under **20.47,** line 4: " . . . add 20 ml of NH_4NO_3 soln . . . " and use 20 ml of molybdate prepared as under **20.46.**

RESULTS AND DISCUSSION

Results for lipoid, lipoid P_2O_5, and calculated egg content were received from 10 chemists as tabulated in Table 1 for yolk noodle and Table 2 for whole egg noodle. Table 3 lists their results on the pure phosphate solution.

The results on yolk noodles in Table 1 are in fair agreement and range from 5.3 to 6.6 with an average of 5.8 per cent yolk solids by Method I and from 5.1 to 6.2 with an average of 5.6 per cent by Method II. The results are also in general agreement with the actual amount of 5.69 per

TABLE 1.—*Collaborative results on per cent lipoid, lipoid P_2O_5 and egg content (m.f.b.) on sample I—yolk noodle containing 5.69% yolk solids (m.f.b.)*

COLLABORATOR	PROCEDURE 1			PROCEDURE 2		
	LIPOIDS	LIPOID P_2O_5	EGG CONTENT	LIPOIDS	LIPOID P_2O_5	EGG CONTENT
1	6.45	0.142	5.8	6.62	0.139	5.7
	6.23	0.141	5.8	6.62	0.139	5.7
	Av. 6.34	0.142	5.8	6.62	0.139	5.7
2	5.68	0.137	5.6	5.70	0.136	5.5
	5.66	0.136	5.5	5.66	0.135	5.4
	Av. 5.67	0.137	5.6	5.68	0.136	5.5
3	6.52	0.145	6.0	6.41	0.141	5.8
	6.54	0.143	5.9	6.46	0.142	5.8
	Av. 6.53	0.144	6.0	6.44	0.142	5.8
4	6.40	0.135	5.5	6.42	0.134	5.4
	6.43	0.134	5.4	6.49	0.135	5.5
	6.44	0.134	5.4	6.40	0.135	5.5
	6.43					
	Av. 6.43	Av. 0.134	5.4	6.43	0.135	5.5
5	6.08	0.142	5.9	5.99	0.134	5.4
	5.97	0.137	5.6	5.89	0.131	5.2
	Av. 6.03	0.140	5.8	5.94	0.133	5.3
6	5.92	0.129	5.1	5.85	0.131	5.2
	6.03	0.135	5.5	5.92	0.128	5.0
	Av. 5.98	0.132	5.3	5.89	0.130	5.1
7	6.30	0.152	6.5	6.29	0.155	6.2
	6.25	0.151	6.4	6.27	0.155	6.2
	Av. 6.28	0.152	6.5	6.28	0.155	6.2
8	6.07	0.134	5.3	6.08	0.132	5.2
	6.15	0.133	5.3	6.06	0.131	5.2
	Av. 6.11	0.134	5.3	6.07	0.132	5.2
9	6.48	0.152	6.5	6.46	0.138	5.6
	6.47	0.153	6.6	6.44	0.139	5.7
	Av. 6.48	0.153	6.6	6.45	0.139	5.7
10	6.06	0.153	6.6	6.11	0.145	6.1
	5.94	0.150	6.4	6.14	0.145	6.1
Max.			6.6			6.2
Min.			5.3			5.1
Av.			5.8			5.6

TABLE 2.—*Collaborative results on per cent lipoid, lipoid P_2O_5 and egg content (m.f.b.) on sample II—whole egg noodle containing 5.34% egg solids (m.f.b.)*

COLLABORATOR	PROCEDURE 1			PROCEDURE 2		
	LIPOIDS	LIPOID P_2O_5	EGG CONTENT	LIPOIDS	LIPOID P_2O_5	EGG CONTENT
1	5.02	0.109	4.9	5.20	0.105	4.6
	5.08	0.110	5.0	5.14	0.106	4.7
	Av. 5.05	0.110	5.0	5.17	0.106	4.7
2	4.36	0.112	5.2	4.36	0.111	5.1
	4.32	0.113	5.2	4.38	0.112	5.2
	Av. 4.34	0.113	5.2	4.37	0.112	5.2
3	4.70	0.104	4.5	4.80	0.106	4.6
	4.83	0.105	4.6	4.84	0.103	4.4
	Av. 4.77	0.105	4.6	4.82	0.105	4.5
4	5.02	0.094	3.6	5.06	0.095	3.8
	5.21	0.094	3.6	4.89	0.094	3.6
	4.95	0.091	3.4	5.00	0.095	3.7
	5.18					
	Av. 5.09	0.093	3.6	4.98	0.095	3.7
5	4.62	0.109	4.9	4.67	0.104	4.5
	4.50	0.106	4.7	4.61	0.103	4.4
	Av. 4.56	0.108	4.8	4.64	0.104	4.5
6	4.29	0.089	3.3	4.42	0.091	3.4
	4.40	0.090	3.3	4.54	0.088	3.2
	Av. 4.35	0.090	3.3	4.48	0.090	3.3
7	4.78	0.121	5.9	4.81	0.125	6.3
	4.73	0.122	6.0	4.78	0.125	6.3
	Av. 4.76	0.122	6.0	4.80	0.125	6.3
8	4.65	0.097	3.9	4.66	0.086	3.0
	4.76	0.097	3.9	4.58	0.091	3.4
	Av. 4.71	0.097	3.9	4.62	0.089	3.2
9	4.97	0.119	5.8	4.82	0.103	4.4
	4.93	0.119	5.8	4.79	0.102	4.3
	Av. 4.95	0.119	5.8	4.80	0.103	4.4
10	4.35	0.108	4.8	4.45	0.100	4.2
	4.47	0.109	4.9	4.55	0.099	4.1
Max.			6.0			6.3
Min.			3.3			3.2
Av.			4.7			4.4

TABLE 3.—*Collaborative results on P_2O_5 in KH_2PO_4 solution containing 5 mg P_2O_5 per 10 ml*

COLLABORATOR	PROCEDURE 1		PROCEDURE 2	
	MG/10 ML	PER CENT RECOVERY	MG/10 ML	PER CENT RECOVERY
1	5.06 4.94 4.89		5.16 4.95 4.95	
	Av. 4.96	99.2	5.02	100.3
2	4.91 4.87		4.87 4.84	
	Av. 4.89	97.8	4.86	97.2
3	5.10 5.07		5.06 5.06	
	Av. 5.09	101.8	5.06	101.2
4	5.09 5.06		5.15 5.13	
	Av. 5.08	101.6	5.14	102.8
5	5.18 5.19		5.05 5.05	
	Av. 5.19	103.8	5.05	101.0
6	5.08 5.11		5.01 4.99	
	Av. 5.10	102.0	5.00	100.0
7	5.04 5.04		5.07 5.07	
	Av. 5.04	100.8	5.07	101.4
9	5.43 5.39		5.07 5.13	
	Av. 5.41	108.2	5.10	102.0
Max.		108.2		102.8
Min.		97.8		97.2
Av.		101.9		100.7

cent yolk (m.f.b.) added. The results in Table 2 on whole egg noodles are in poor agreement, ranging from 3.3 to 6.0 per cent by Method I and 3.2 to 6.3 per cent by Method II, and only Nos. 4 and 2 of the 10 collaborators obtain fair agreement with the actual amount of the whole egg solids added.

The results in Table 3 indicate that all the collaborators accurately determined the P_2O_5 with either molybdate solution.

Apparently, the disagreement on the noodles is in the isolation of the phosphate. Although the samples were well mixed and should be uniform, the Referee requested one collaborator who reported low values to return the remaining portion of the sample. The analysis of the Referee on the returned portion was in agreement with the original analysis, indicating that the low results reported by the collaborator were not due to sample variation. This same collaborator reported satisfactory results on yolk noodle on a replacement sample but no marked improvement on the whole egg noodle. It is obvious that care must be taken after ashing to extract all the lipoids and leach out all the phosphate with sufficient acid.

One possible explanation for low results is that all the phosphate may not be in the *ortho* form, although generally the excess alkali during ashing may be expected to leave it in this form. To be sure of conversion to the *ortho* form, the residue after ashing should be heated considerably longer than the 5 minutes specified in Method I. While this may be an explanation for the low results, the Referee has not been able to confirm it by experiment.

Four equal aliquots of the lipoids in chloroform from each of these noodles were handled, after ashing in Pt dishes and Pyrex beakers by heating as specified in the procedures, and also with stronger HNO_3 (1 + 3) and longer heating. The heating consisted of boiling 30 minutes in the beakers and heating 1 hour on a steam bath in the Pt dishes. The extra periods of heating with stronger acid did not increase the recovery of P_2O_5. It should be cautioned that the increase in acid strength and longer heating results in considerable loss of Pt, amounting to 142 mg in extreme cases.

Comparing the values by the two procedures, there is a trend (which was also shown in last year's results) toward a higher value by Method I. Several collaborators have expressed preference for the use of Pt dishes. In connection with either method, too much care cannot be taken in the determination of the blank which could account for as much as 0.5 to 0.75 per cent egg content, depending on type of egg used.

The procedures for lipoids and lipoid P_2O_5 studied over the past two years are more specific in detail than the present ones under sec. **13.35** and **13.36.**

COLLABORATORS

The assistance of the following collaborators is acknowledged with much appreciation:

J. J. Winston, Jacobs-Winston Laboratory, Inc., New York, New York.

John E. Despaul, Quartermaster Subsistence Testing Laboratory, Chicago, Illinois.

Mary Zenk, U. S. Department of Agriculture, Grain Branch, Washington, D. C.

L. W. Ferris, Sylvia Shendleman, Catherine G. Cunningham, John F. Mallon, and E. F. Steagall, all of the Food and Drug Administration.

RECOMMENDATIONS

It is recommended*—

(1) That the Official Method for lipoids, Sec. **13.35**, and lipoid P_2O_5, Sec. **13.36**, be deleted, First Action.

(2) That the method for lipoids and Method I for lipoid P_2O_5 be adopted, First Action.

(3) That the study be discontinued.

REPORT ON CHOLINE IN EGG NOODLES

By V. E. MUNSEY (Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C.), *Referee*

The determination of choline, even after split from the lecithin molecule, offers another means for the estimation of egg content in noodles. An additional advantage is the relatively small amount of actual working time. A disadvantage is a rather large correction. However, the correction for choline in semolina or durum flour is almost exactly the same as the lipoid P_2O_5 in these products. The choline is relatively constant in flour and eggs and the conversion factors for calculation of egg content are significantly less than for lipoid P_2O_5 conversion. Considering these factors to be of some merit, a method for the determination of choline has been applied to flour, eggs, and egg noodles of known composition. The precision and accuracy of the method indicated that a collaborative study was warranted. Accordingly, the same collaborators in the report on lipoids and lipoid P_2O_5† were requested to analyze a sample of yolk and whole egg noodles. Unknown to the collaborators, these were the same noodles sent out for the lipoid and lipoid P_2O_5 study, containing 5.69 per cent yolk solids and 5.34 per cent whole egg solids. They were also submitted a portion of the standard choline solution prepared by the Referee for their standard curve and sufficient reineckate salt for these determinations. The following method was submitted with instruction to report percentage choline on an "as is" basis.

* For report of Subcommittee D and action of the Association, see *This Journal*, **36**, 61 (1953).
† *This Journal*, **36**, 760 (1953).

METHOD

Weigh 5 g of flour, semolina, noodle, or macaroni (ground to 20 mesh or finer), add 2 g filter cel, mix well, and transfer to Soxhlet extn thimble (ca 30×77 mm). Cover with cotton pad, place in Soxhlet extractor, add ca 150 ml methyl alcohol, ext. 8 hrs on hot plate, filter through extn tube ca 20 mm diam. × ca 11 cm long with stem ca 10 cm long, contg asbestos pad covered with ca 10 mm layer of sand, into 200 ml Erlenmeyer flask under bell jar with suction. Rinse flask and tube with methyl alcohol. Evap. on steam bath to dryness with aid of air blast, rinse down sides with 2 ml methyl alcohol, add 20 ml HCl (5+3), and boil under reflux condenser for 1 hr. Wash condenser with 5 ml hot H_2O, cool, add ca 2 g Nuchar C 190 N or equivalent decolorizing carbon, mix well, add ca 4 g filter cel, and mix. Prepare Büchner type medium porosity fritted glass funnel of ca 60 ml capacity by adding with suction a suspension of ca 4 g filter cel and 25 ml H_2O to form uniform layer in the funnel. Filter with suction, under bell jar, into 125 ml Erlenmyer flask previously marked at 40 ml. Rinse digestion flask and funnel with H_2O to 40 ml mark. Neutralize with KOH (1+1) to litmus paper (ca 12 ml required, turbidity and ppt forms just before litmus turns blue), add 20 ml cold (10°C.) satd $Ba(OH)_2$ soln (18 g $Ba(OH)_2 \cdot 8H_2O$ dissolved in 400 ml warm H_2O, cooled, and made to 500 ml. Allow excess $Ba(OH)_2$ to settle and store at 10°C. in stoppered flask in refrigerator), add 1–2 drops of 1% alc. thymolphthalein, acidify with drops of glacial acetic acid until blue just disappears, and bring back to very faint tinge of blue with $Ba(OH)_2$ soln. Cool in refrigerator ca 1 hr or more, add ca 2 g filter cel to flask, mix, filter on the same type Büchner funnel into 250 ml beaker previously marked at 100 ml level, and wash with H_2O up to 100 ml mark. Add 6 ml 2% ammonium reineckate in CH_3OH and leave overnight in refrigerator. Collect the pink choline reineckate with aid of suction in a Knorr extraction type tube (20 mm diam. × 11 cm long, stem 10 cm long) on an asbestos mat 3–4 mm thick, covered with ca 10 mm layer of sand. Wash with 4–5 ml portions of ice cold H_2O and finally 3 times with 2.5 ml portions of *n*-propyl alcohol. Discontinue suction when sand is dry. Dissolve ppt with 5 ml portions of acetone, draining without suction into 25 ml volumetric flask. Make to vol. with acetone and read on neutral wedge photometer using #52 filter or in a spectrophotometer at 526 mμ. From a standard curve, calc. amount of choline in mg/10 g from the photometer reading or convert to per cent.

STANDARD CURVE

Dissolve pure choline chloride in H_2O to give ca a 2% soln (11.7 g/500 ml). Standardize by detn of Cl content. Dilute 50 ml of above soln to 1000 ml (ca 1 mg choline per ml). Measure, from 10 ml buret, 2, 4, 6, 8, and 10 ml portions of dild soln into 250 ml beaker, add H_2O to make 100 ml, add 6 ml 2% ammonium reineckate, stir, and proceed as above, beginning "Leave in refrigerator. . . . "

RESULTS

The results from 8 chemists, given in Table 1, show percentage of choline and estimated egg content on the moisture free basis (m.f.b.). The egg content was calculated from the actual analysis of the materials used. The semolina contained 0.053 per cent choline (m.f.b.), whole egg 1.54 per cent, and yolk 2.31 per cent. The formulas used for calculation on m.f.b. are:

$$\text{Per cent yolk solids} = \frac{\%\ \text{choline (m.f.b.)} - 0.053}{2.31 - 0.053};$$

$$\text{Per cent whole egg solids} = \frac{\%\ \text{choline (m.f.b.)} - 0.053}{1.54 - 0.053};$$

TABLE 1.—*Collaborative results on moisture free basis for choline and egg content on yolk and whole egg noodles*

COLLABORATOR	YOLK NOODLE		WHOLE EGG NOODLE	
	CHOLINE	EGG CONTENT	CHOLINE	EGG CONTENT
	per cent	*per cent*	*per cent*	*per cent*
1	0.140	3.9	0.091	2.6
	0.137	3.7	0.091	2.6
2	0.183	5.8	0.141	5.9
	0.189	6.0	0.139	5.8
3	0.150	4.3	0.110	3.8
	0.150	4.3	0.116	4.2
4	0.188	6.0	0.128	5.0
	0.185	5.8	0.132	5.3
5	0.174	5.4	0.103	3.3
	0.172	5.3	0.103	3.3
6	0.184	5.8	0.139	5.8
	0.184	5.8	0.139	5.8
7	0.188	6.0	0.154	6.8
	0.188	6.0	0.154	6.8
8	0.182	5.7	0.088	2.3
	0.168	5.1	0.102	3.3

COMMENTS

The results on the yolk noodles are much better than on the whole egg noodles. On the yolk noodle 6 of the 8 chemists and on the whole egg noodle 4 of the 8 chemists were in fairly close agreement with the amount of egg added. Some of the collaborators obtained very low recoveries but the majority reported good recoveries, and it is believed the method warrants further consideration.

One collaborator with low results stated that the 10 mg standard reineckate precipitate did not appear to dissolve completely in the acetone. He also mentioned that the directions were not clear at the point where the method states "same type of Büchner funnel." Directions have now been changed to insert "to flask" after "add ca 2 g filter cel."

COLLABORATORS

The assistance of the following collaborators is gratefully acknowledged.

J. J. Winston, Jacobs-Winston Laboratory, Inc., New York, New York.

Charles H. Coleman, Quartermaster Subsistence Testing Laboratory, Chicago, Illinois.

Frank J. Kokoski, Department of Agriculture and Markets, Albany, New York.

F. W. Ferris, Catherine C. Cunningham, Sylvia Shendleman, and Edward F. Steagall, all of the Food and Drug Administration.

RECOMMENDATION

It is recommended* that the study be continued.

REPORT ON ACETIC AND PROPIONIC ACID MOLD INHIBITORS IN BREAD

By LEWIS H. McROBERTS (Food and Drug Administration, Department of Health, Education, and Welfare, San Francisco, California), *Associate Referee*

In the previous reports on this subject (1—3, 4, 6, and 7), two methods were described for the determination of acetic and propionic acids in bread. The main purpose has been to provide means for the detection and estimation of propionates added as mold inhibitors. While acetic acid is normal in bread, the presence of excessive amounts would be indicative of the addition of vinegar or of chemicals such as sodium diacetate.

Following the adoption of the method described in the 1950 report (5, 6), the Referee on cereal products recommended that additional collaborative studies be made with breads other than white bread; milk bread and whole wheat bread with added propionate and sodium diacetate were suggested. Attention was also called to the possible presence of butyric acid which might interfere with the chromatographic separation of propionic acid. He also referred to the fact that lactic acid may be used in canned bread—primarily intended for Armed Services rations—and asked that experiments be conducted to determine any interferences from this source.

This report covers the requested collaborative study of the method (6) for the determination of propionic and acetic acids in milk bread and whole wheat bread. Results of the seven collaborators are reported and are found to be in good agreement. These data substantiate findings of previous studies on white bread. While there was some indication of trace amount of acids higher than propionic acid, the presence of butyric acid was not confirmed (8). To determine if interferences are caused by butyric

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

or lactic acids, the Associate Referee conducted experiments in which mixtures of formic, acetic, propionic, butyric, and lactic acids were added to authentic control bread. There was no evidence of interference from lactic acid. Butyric acid interferes in the efficient separation of butyric and propionic acids where the concentration of propionic acid approaches 20 mg (about 1.6 times the usual amount of propionic acid in baked bread containing added propionate) in the 5 gram silicic acid chromatographic column. Under these conditions, the threshold volume for propionic acid is within a few ml of the preceding butyric acid. Where propionic acid is present in the amount indicated and where there are indications of butyric or other higher acids—i.e., visible bands below the propionic acid band—it is recommended that a longer tube with about a 10 gram charge of silicic acid be employed. A sufficient spread in threshold volumes between butyric and propionic acids is thus obtained to allow efficient separation.

COLLABORATIVE ANALYSES

The bread samples were prepared by the Referee on cereal products and transmitted to San Francisco by air express. The following information was supplied:

"Based on the weight of flour used (310–315 g per loaf) the formula was: 3 per cent shortening, 2 per cent salt, 2 per cent yeast, 3 per cent sugar, and 4 per cent dry skim milk (only in the wheat samples). Whole liquid milk was used for the milk breads. Calcium propionate and sodium diacetate were added in the amount of 0.63 g per loaf (0.20 per cent in the flour or 0.13 per cent in the finished bread). These breads were fermented, proofed, and baked by common procedure. Baking was at 425°F. for 32 minutes."

The weight loss during the one day shipping period was from 2.5 to 4.2 per cent. The breads used were:

(1) Milk Bread with propionate: 4 loaves; (2) Whole Wheat Bread, with propionate: 4 loaves; (3) Whole Wheat Bread, with sodium diacetate: 4 loaves. Two loaves of each kind were reserved for controls.

The loaves in each category were sliced and divided into two portions by taking alternate slices. One portion was prepared for analysis on the fresh basis and the other was air dried (6). One pint subdivisions of the prepared fresh breads were preserved with washed chloroform as directed in the method (6) and were submitted to six collaborators. They were instructed as follows:

"Each jar contains about 5 ml of washed chloroform and should be kept tightly closed before the initial opening for the analyses. Roll the closed jar to mix the sample thoroughly and break up any clumps of bread particles.

"Analyze the three samples for acetic and propionic acids as outlined in the method given in *This Journal*, **34**, 284 (1951).

"It should be stressed that the threshold volume for propionic acid will vary

widely over the range of concentration found in bread and that identification of this acid by threshold volume alone must be based on previous determinations of several known concentrations similar to those described on page 289 (See footnote No. 5, pages 289 and 290.)

"There is some possibility that butyric acid may be present in amounts not large enough to provide a visible band on the chromatographic column. However, a titration of the forerun eluate that is greater than the blank titration is an indication of traces of higher acids. If time is available, the analyst is requested to determine if butyric acid is present by the formation of mercurous butyrate crystals [*This Journal*, **28**, 644 (1945)].

"Analyze each sample in duplicate and report the following data:

(1) Blank titration, 20 ml CB 1 eluate
(2) Forerun titration (to first band) CB 1 eluate
(3) Number of bands observed
(4) Butyric acid (indicate if present)
(5) Propionic acid
    Threshold volume in ml
    Mg/100 g
(6) Acetic acid
    Threshold volume in ml
    Mg/100 g
(7) Formic acid
    Threshold volume in ml

"For best comparison of threshold volumes, the analysts are requested to change the mobile solvent from CB 1 to CB 10 at 40 ml of CB 1. This is about midway between the propionic and acetic thresholds."

The collaborative analyses of the three samples of fresh breads preserved with chloroform are tabulated in Tables 1, 2, and 3. Those of the Associate Referee are listed under Collaborator No. 1. The names of the others and corresponding numbers are given in the section devoted to collaborator comments.

In addition to the above-described analyses, the Associate Referee analyzed samples Nos. 1, 2, and 3 and control breads when they were first prepared fresh—without addition of chloroform, air dried portions of the same, and samples of the calcium propionate and sodium diacetate that were added previous to baking. Additional data were thus provided for comparison of methods of sample preparation. The results of these bread analyses are tabulated in Table 4.

The calcium propionate and sodium diacetate were analyzed by the following described procedures:

(a) *Calcium propionate.*—A weighed portion of 2.5 g was dissolved in H_2O, made to 250 ml, and an aliquot of 20 ml was made to 100 ml. Acetic acid (ca 1 ml 0.1 *N*), was then added to 10 ml of the final diln. The mixt. was made alk. to phenolphthalein and evapd to dryness. Propionic acid was then separated on the silicic acid column as described in the bread procedure, using the added acetic acid as the following acid. Two bands were observed and identified as propionic and acetic acids by threshold volumes. A strong positive test was obtained for calcium in the ashed sample.

(b) *Sodium diacetate.*—A weighed portion of 2.5 g was dissolved in H_2O, made to 250 ml, and an aliquot of 30 ml made to 100 ml. Formic acid (ca 1 ml 0.1 *N*)

TABLE 1.—*Acetic and propionic acids in fresh authentic milk bread preserved with $CHCl_3$ (with added propionate)—Collaborative sample No. 1*

COLLABORATOR NO.	1	2	3	4	5	6	7	(AV.)
Blank titration, ml 0.01 *N* $Ba(OH)_2$	0.05	0.05	0.10	0.03	0.05	0.05	0.05	
Forerun titration, ml 0.01 *N* $Ba(OH)_2$	0.45	0.45	0.86	0.34	0.27	0.32	0.32	
No. bands observed	3	3	4	4	3	4	3	
Butyric acid	None	None	Present[c]	None[d]	None	None[d]	None	
Propionic acid								
Threshold vol. (ml CB1)[a]	18	17	15	19	15	14	15	16
mg/100 g	74.8	67.8	77.9	74.7	72.8	71.1	77.9	
mg/100 g	78.2	72.4	77.8	74.2	73.0	71.8	76.5	
mg/100 g (Av.)	76.5	70.1	77.9	74.5	72.9	71.5	77.2	74.4
Acetic acid								
Threshold vol. (ml CB1+CB10)[b]	50	51	49	53	48	47	50	50
mg/100 g	34.2	36.8	31.3	36.4	34.4	44.7	29.6	
mg/100 g	35.2	36.8	34.9	30.6	35.2	44.8	31.3	
mg/100 g (Av.)	35.7	36.8	33.1	33.5	34.8	44.8	30.5	35.6
Formic acid								
Threshold vol. (ml CB1+CB10)[b]	66	70	65	73	68	59	68	68

[a] CB1 (1% *n*-butyl alcohol in $CHCl_3$); [b] CB10 (10% *n*-butyl alcohol in $CHCl_3$); [c] Trace (1–2 mg/100 g) identified by threshold volume; [d] Slight band preceding propionic band—not identified.

TABLE 2.—*Acetic and propionic acids in authentic fresh whole wheat bread preserved with $CHCl_3$ (with added propionate)—Collaborative sample No. 2*

COLLABORATOR NO.	1	2	3	4	5	6	7	(AV.)
Blank titration, ml 0.01 *N* $Ba(OH)_2$	0.05	0.05	0.10	0.03	0.05	0.05	0.05	
Forerun titration, ml 0.01 *N* $Ba(OH)_2$	0.33	0.25	0.70	0.24	0.28	0.25	0.21	
No. bands observed	3	3	4	3	3	3	3	
Butyric acid	None	None	Present[c]	None	None	None[d]	None	
Propionic acid								
Threshold vol. (ml CB1)[a]	17	18	16	20	15	17	13	17
mg/100 g	79.3	76.1	82.7	76.7	76.0	78.5	82.2	
mg/100 g	79.7	69.2	82.9	77.2	74.5	76.3	81.9	
mg/100 g (Av.)	79.5	72.7	82.8	77.0	75.3	77.4	82.1	78.1
Acetic acid								
Threshold vol. (ml CB1+CB10)[b]	50	51	50	53	50	48	48	50
mg/100 g	38.5	42.8	39.9	37.1	43.2	38.4	38.9	
mg/100 g	38.2	44.7	42.7	37.4	44.6	39.4	36.6	
mg/100 g (Av.)	38.4	43.8	41.3	37.3	43.9	38.9	37.8	40.2
Formic acid								
(Threshold vol. (ml CB1+CB10)[b]	65	70	67	71	67	60	66	67

[a,b,c,d] See footnotes to Table 1.

TABLE 3.—*Acetic acid in fresh authentic whole wheat bread preserved with $CHCl_3$ (with added sodium diacetate)—Collaborative sample No. 3*

COLLABORATOR NO.	1	2	3	4	5	6	7	(AV.)
Blank titration, ml 0.01 *N* $Ba(OH)_2$	0.05	0.05	0.10	0.03	0.05	0.05	0.05	
Forerun titration, ml 0.01 *N* $Ba(OH)_2$	0.60	0.33	0.42	0.61	0.35	0.52	0.34	
No. bands observed	2	3	3	2	2	3	2	
Butyric acid	None	None[b]	None[b]	None	None	None[b]	None	
Propionic acid	None	None[b]	None[b]	None	None	None[b]	None	
Acetic acid								
Threshold vol. (ml CB1+CB10)[a]	46	44	49	50	49	45	45	47
mg/100 g	133.0	129.5	118.2	116.9	120.0	114.4	119.2	
mg/100 g	127.0	132.0	121.3	110.2	119.0	116.7	125.7	
mg/100 g (Av.)	130.0	130.8	119.8	113.6	119.5	115.6	122.5	121.7
Formic acid								
Threshold vol. (ml CB1+CB10)[a]	64	66	65	73	69	59	68	66

[a] CB10 (10% *n*-butyl alcohol in $CHCl_3$); [b] Slight band preceding acetic band—not identified.

TABLE 4.—*Acetic and propionic acids in authentic breads (mg/100 g—fresh basis)—Associate Referee analyses*

SAMPLE DESCRIPTION	FRESH BREAD				FRESH BREAD+$CHCl_3$				AIR DRIED BREAD (CALCD. FRESH BASIS)					
	PROPIONIC ACID		ACETIC ACID		PROPIONIC ACID		ACETIC ACID		PROPIONIC ACID			ACETIC ACID		
	T. VOL.,[a] ML	MG[c]	T. VOL., ML	MG[c]	T. VOL., ML	MG[a]	T. VOL., ML	MG[a]	T. VOL., ML	MG[a]	LOSS[a] PER CENT	T. VOL., ML	MG[a]	Loss,[b] PER CENT
Milk Bread (Control)	None	None	50	34	None	None	50	35	None	None	—	50	26	26
Milk Bread (with added calcium propionate)	18	81	50	32	18	75	50	34	20	58		50	29	
	19	78	51	31	18	78	50	35	20	59		51	28	
(Av.)	19	80	51	32	18	77	50	35	20	59	25	51	29	15
Whole Wheat Bread (Control)	None	None	49	39	None	None	50	39	None	None	—	49	33	18
Whole Wheat Bread (with added calcium propionate)	18	84	50	37	18	79	50	39	19	57		51	32	
	17	84	49	40	17	80	50	38	—	—		—	—	
(Av.)	18	84	50	39	18	80	50	39	19	57	31	51	32	18
Whole Wheat Bread (with added sodium diacetate	None	None	48	127	None	None	47	133	None	None		48	101	
	None	None	45	127	None	None	45	127	—	—		—	—	
(Av.)	None	None	47	127	None	None	46	130	—	—	—	48	101	22

[a] Threshold volume. [b] Loss due to air drying. [c] Mg/100 g.

was then added to 10 ml of the final diln. The mixt. was made alk. to phenolphthalein and evapd to dryness. Acetic acid was then separated on the silicic acid column, making use of the added formic acid as the following acid. No propionic acid was detected. Two bands were observed and identified as acetic and formic acids by threshold volumes.

(c) As a check analysis, 10-ml aliquots of the final dilns of (a) calcium propionate and (b) sodium diacetate were combined with ca 1 ml of 0.1 *N* formic acid. Three bands were then observed and identified as propionic, acetic, and formic acids.

PROPIONATE AND DIACETATE ANALYSES

Calcium propionate

Propionic acid: chromatographic separation, 73.6 and 72.5%; average, 73.1%.
Propionic acid: calculated to $Ca(CH_3CH_2CO_2)_2 \cdot H_2O$, 100.7%.

Sodium diacetate

Free acidity: direct titration, 39.3 and 39.3%; average, 39.3% as acetic acid.
Total available acid: chromatographic separation, 78.8 and 78.5%; average 78.6% as acetic acid.
Combined acid: by difference, 39.3% as acetic acid; calculated as sodium acetate, 53.7% as acetic acid.
Total calculated: 93.0%.

BAKING LOSSES OF PROPIONIC AND ACETIC ACIDS

The following baking losses were calculated from the averages of collaborator analyses:

(1) *Milk bread with added propionate*

Propionic acid, calculated from formula:	94 mg/100 g
Propionic acid, determined:	74 mg/100 g
Propionic acid, baking loss:	21%

(2) *Whole wheat bread with added propionate*

Propionic acid, calculated from formula:	94 mg/100 g
Propionic acid, determined:	78 mg/100 g
Propionic acid, baking loss:	17%

(3) *Whole wheat bread with added sodium diacetate*

Acetic acid, calculated as added plus normal	144 mg/100 g
Acetic acid, determined:	122 mg/100 g
Acetic acid, baking loss:	15%

COLLABORATOR COMMENTS

V. E. Munsey (No. 2): "Mobile solvent was changed from CB 1 to CB 10 at 40 ml of CB 1. No definite band indications of higher acid were noted; a propionic band on No. 3 was faint. The forerun titration seems too small to be of significant interpretive value."

George E. Keppel (No. 3): "An attempt was made to identify the propionic acid and butyric acid fractions of the bread samples by crystal formation (*This Journal*, **28**, 644 (1945)), but was not successful. Even with authentic acids, only with propionic and acetic acids did the crystals resemble those in the illustrations. The butyric acid fractions of the bread (samples 1 and 2) gave crystals similar to those obtained using *n*-butyric acid but neither resembled those illustrated.

"The threshold volume of the faint bands noted in samples 1 and 2 was 9–10

ml. This agrees approximately with the threshold volume of 8–10 ml found for butyric in a mixture of four acids. This was the same work done February, 1951, using the same batch of silica gel. Based on the threshold volume, I am therefore reporting butyric acid present in samples 1 and 2."

Louis C. Weiss (No. 4): "For sample No. 1, a narrow, barely visible band preceded the propionic acid. The amount of acid present in the band was so small that no attempt was made to identify it—in the case of sample No. 3, no bands were observed before the acetic acid zone.

"The technique for the preparation of the slurry suggested by Mitchell* was used throughout the work and was found to be very satisfactory. I prefer it to the grinding method.

"Bollinger's* method of releasing the acids from their salts and their subsequent transfer to the column was used. This too was found to be very satisfactory, as indicated by excellent recoveries on standardization runs.

"Some difficulty was experienced in the distillation procedure unless considerable care was exercised to prevent charring during the period before the charge in the distillation flask reached the boiling point."

Douglas D. Price (No. 5): "A negative test was obtained for butyrates in sample 2; other samples not tested."

Luther G. Ensminger (No. 6) " . . . No special difficulty was encountered in the procedure . . .

"In preparation of the column, I found that 0.5 ml (9 drops) of "RNH_4" indicator soln and 1 drop of 1 *N* NH_4OH added to the silicic acid gave a column easiest to follow the acid front. One ml of the indicator made the column too dark. 1.80 ml of H_2O was used to saturate the surfaces of the silicic acid particles. Three pounds pressure was used to drive the column.

"I used 25 ml beakers in place of the test tubes to complete evaporation of the distillate. Evaporation is faster, and by using a small glass stirring rod it is very easy to get all the residue quickly acidified and extracted with the CB 1 solvent."

Herman O. Fallscheer (No. 7): "No butyric acid band was observed on any of the runs."

DISCUSSION

The results obtained by the collaborators are considered to be in good agreement. Together with those of the previous report on white bread (6), they provide assurance that the method is adequate for general use in bread analysis in the detection and estimation of added propionates. Acetic acid has been definitely proved to be a normal constituent in all of the bread analyses. The amounts determined in control breads must be taken into account in judging whether acetic acid has been added.

The forerun titrations of the eluates to the thresholds of the first definite bands are always higher than a blank titration of about 25 ml of CB1 (1 per cent *n*-butanol in chloroform) that has passed through the column without addition of acids. This indicates the presence of traces of higher acids. We were especially interested in the possibility of detecting butyric acid in the milk bread. One collaborator identified butyric acid by threshold volume as present in trace amounts in both the milk bread and whole wheat bread. The others reported none or trace amounts too small to determine. No butyric acid was detected in the analysis of the control

* Collaborator Comments, *This Journal*, 34, 291 (1951).

breads. No definite mercurous butyrate crystals were obtained from the forerun eluates previous to propionic acid in samples Nos. 1 or 2 (Associate Referee analyses).

Data in addition to that provided in the previous report have been supplied on baking losses of propionic and acetic acids. Substantial losses are also obtained in the air drying of bread in preparation for analysis.

No appreciable differences are noted in comparing analyses of the fresh breads and the fresh breads preserved with chloroform (Table 4).

The following experiments were conducted to determine if small amounts of butyric or lactic acids might interfere in the determination of acetic or propionic acids by the method (6) used in this study (Table 5):

TABLE 5.—*Direct chromatographic separation of pure acid mixtures without distillation*

MIX NO.	LACTIC ACID	FORMIC ACID	ACETIC ACID	PROPIONIC ACID	BUTYRIC ACID	NO. OF BANDS
(1) Ml 0.01 *N* added	—	9.40	7.05	15.95	—	3
Ml 0.01 *N* recovered	—	—	6.96	15.28	—	
% recovered	—	—	99	98	—	
Threshold vol., ml[a]	—	65	50	16	—	
(2) Ml 0.01 *N* added	—	9.40	14.10	26.58	—	3
Ml 0.01 *N* recovered	—	—	14.14	25.92	—	
% recovered	—	—	103	98	—	
Threshold vol., ml[a]	—	65	47	13	—	
(3) Ml 0.01 *N* added	—	9.4	14.10	26.58	4.30	4
Ml 0.01 *N* recovered	—	—	14.20	27.29	2.49	
% recovered	—	—	101	103	58	
Threshold vol., ml	—	64	48	14	10	
(4) Ml 0.01 *N* added	—	9.4	7.05	—	4.30	3
Ml 0.01 *N* recovered	—	—	6.78	—	4.33	
% recovered	—	—	96	—	101	
Threshold vol., ml[a]	—	64	49	—	12	
(5) Ml 0.01 *N* added	—	9.4	7.05	5.32	4.30	4
Ml 0.01 *N* recovered	—	—	6.96	5.46	4.05	
% recovered	—	—	99	102	94	
Threshold vol., ml[a]	—	65	50	23	12	
(6) Ml 0.01 *N* added	4.30	9.40	7.05	5.32	4.30	5
Ml 0.01 *N* recovered	—	3.60	7.15	6.11	4.09	
% recovered	—	38	101	115	95	
Threshold vol., ml[a]	—	65	50	22	12	

[a] (Changed from "CB1" to "CB10" at 40 ml CB1).

The acids are eluted from the column in the order of butyric, propionic, acetic, formic, and lactic acids. Lactic is definitely above formic and moves very slowly even with the "CB10" mobile solvent. It could not

be used as a following acid in the determination of formic, for it seemed to merge with the formic band so that a clear-cut separation from formic acid was not possible.

With comparatively high amounts of propionic as compared to butyric acid (as in mixture 3) the threshold volume for propionic acid is within 4 ml of the preceding butyric acid. The recovery of butyric acid was definitely low and the separation of the two bands was not sharp. The separation of this same mixture was repeated with a longer column, making use of 10 g of silicic acid instead of the 5 g specified in the method. The following recoveries were then obtained: acetic acid, 101%; propionic acid, 98%; and butyric acid, 104%.

In general, the recoveries from the mixture described in Table 5 were considered good. Lactic and butyric acids had no effect on the acetic acid recovery. Where the propionic acid concentration approaches 20 mg, it may be necessary to increase the length of the column to afford efficient separation of propionic and butyric acids.

The following recovery experiments were made with addition of acid mixtures to the authentic whole wheat control bread. A chromatographic tube of 18 in. length and $\frac{1}{2}$ in. internal diameter was used to afford sufficient space for 10 g of silicic acid. The usual mixture of 5 g silicic acid, 1 ml H_2O, 1 ml "RNH_4" indicator and 1–2 drops 0.5 *N* NH_4OH was doubled for each ingredient in the column mixture (Table 6):

TABLE 6.—*Recovery of acid mixtures added to 10 g of air-dried authentic whole wheat control bread (complete method (6))*

MIX NO.	LACTIC ACID	FORMIC ACID	ACETIC ACID	PROPIONIC ACID	BUTYRIC ACID	BANDS NO.
(1) Ml 0.01 *N* added	40.0	9.50	13.70	10.78	4.37	4
Ml 0.01 *N* recovered	—	—	21.75	11.15	4.35	
% recovered	—	—	105[b]	103	100	
Threshold vol. ml[a]	—	85	61	39	23	
(2) Ml 0.01 *N* added	80.0	9.50	13.70	21.56	4.37	4
Ml 0.01 *N* recovered	—	—	21.25	21.40	4.25	
% recovered	—	—	101[b]	99	97	
Threshold vol. ml[a]	—	86	62	34	23	
(3) Ml 0.1 *N* added	100	9.50	13.70	21.56	8.74	4
Ml 0.01 *N* recovered	—	—	21.70	21.20	8.50	
% recovered	—	—	105[b]	98	97	
Threshold vol. ml[a]	—	87	62	36	22	
(4) Ml 0.01 *N* added	200	9.50	13.70	43.12	4.37	4
Ml 0.01 *N* recovered	—	—	22.75	41.50	4.00	
% recovered	—	—	113[b]	97	92	
Threshold vol. ml[a]	—	86	61	28	21	

[a] Changed from "CB1" to "CB10" at 40 ml "CB1."
[b] Calcd after correction for acetic acid detd in the bread (Table 4).

The lactic acid was apparently separated from the volatile acids during the steam distillation, for only four bands were noted on the columns. The amounts investigated varied from 36 to 180 mg or 0.36 to 1.8 per cent in the air dried bread.

The use of the longer column with 10 g of silicic acid gave a spread of about 15 ml between the threshold volumes of propionic and butyric acids, thereby affording a better separation of these acids.

CONCLUSIONS

The collaborative study conducted this year has demonstrated that the method is adequate for general use in bread analysis.

There was no definite proof of the presence of butyric acid in the authentic milk bread.

Lactic acid in amounts of 0.4 to 1.8 per cent does not interfere in the determination of acetic, propionic, or butyric acids.

Where the amount of propionic acid approaches 20 mg in the silicic acid column and where efficient separation from butyric acid is desired, a longer chromatographic tube and 10 g of silicic acid should be used.

ACKNOWLEDGMENT

The Associate Referee acknowledges with appreciation the assistance of the collaborators from the Food and Drug Administration whose names are listed under Collaborator Comments. He also wishes to thank the Referee on Cereal Products, V. E. Munsey, for his guidance and assistance throughout this project.

RECOMMENDATIONS*

It is recommended that the following directions be inserted in the method as it is now described in *This Journal*, **34**, 284–296 (1951):

(1) Under III. APPARATUS, p. 287, change (*c*) to read: "chromatographic tubes ca 15×250 mm or ca 15×450 mm constricted at lower end to ca 4 mm i. d.

(2) Under CHROMATOGRAPHIC SEPARATION (*a*) *Preparation of partition column*, p. 288, add the following as a new paragraph at the end of (*a*):

Where the amount of propionic acid approaches 20 mg in the column and where a definite band is observed below the propionic acid band, the long chromatographic tube (450 mm) and ca 10 g of silicic acid should be employed. The amounts of water, indicator, and NH_4OH are then double those found applicable for 5 g silicic acid.

It is recommended that this Associate Refereeship be discontinued.

REFERENCES

(1) McROBERTS, L. H., *This Journal*, **31**, 489 (1948).
(2) ———, *ibid.*, **31**, 99 (1948).
(3) ———, *ibid.*, **32**, 496 (1949).
(4) McROBERTS, L. H., *ibid.*, **33**, 677 (1950).

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

(5) ———, *ibid.*, **33**, 86 (1950).
(6) ———, *ibid.*, **34**, 284 (1951).
(7) ———, *ibid.*, **34**, 64–68 (1951).
(8) PATTERSON, W. I., and RAMSEY, L. L., *ibid.*, **28**, 644 (1945).

REPORT ON METHODS OF YEAST ANALYSIS

By A. L. BRANDON (Anheuser-Busch, Inc., St. Louis, Missouri),
Associate Referee

For some time there has been a definite need for standard methods of yeast analysis. Without such methods, laboratories involved in the analyses of yeast have had to develop their own methods or adapt them from standard methods used in the analysis of related materials. Often such methods are adequate for one laboratory, but when data obtained by these methods is compared with data from other laboratories using different methods, discrepancies are apt to be noted.

To eliminate differences in methods, an attempt is being made to set up standard procedures through collaborative study.

The methods to be studied first are moisture, total nitrogen, P_2O_5, ash, ether extract, crude fiber, and crude carbohydrate. At a later date, studies of other methods including mineral constituents, protein, fat, and carbohydrate will be attempted. Since yeast is generally analyzed for moisture, total nitrogen, and P_2O_5, methods for determining these constituents will be studied first.

The proposed study will consist in analyses by collaborators of a set of four dried yeast samples by methods they are now using or by methods of their own choice. The data obtained by these analyses will be studied, using the statistical analysis of variance or the control chart in light of the methods submitted with the data. A second set of samples will then be analyzed by the best method evolving from the statistical study of the first set of data.

The choice of yeasts for this study was influenced by the use of yeast as an adjunct to cereal or animal foods and as a pharmaceutical or used in pharmaceutical preparations. With this in mind, four yeasts consisting of a primary baker's yeast, a non-debittered brewer's yeast, a debittered brewer's yeast, and a Torula yeast grown on sulfite waste liquor were selected. The yeasts were obtained in 100 lb quantities and were stored at 40°F. to keep down or prevent insect infection. The samples were packaged at 40°F. in clean and dry 4-oz. round, screw cap bottles which had been stored at 40°F. for two days, and were then sealed with a wax to prevent any moisture uptake in the sample.

A set of the four yeast samples and a copy of the instructions were sent to each of seventeen collaborators who had expressed a desire to

participate in the study. Each collaborator was requested to analyze the samples in duplicate for moisture, total nitrogen, and P_2O_5 by the method they are now using or by a method of their own choice if they did not regularly analyze yeast for these constituents. In order to guide them in their choice of method and also to retain present A.O.A.C. methods where possible, the following methods were suggested from the U. S. Pharmacopeia, Fourteenth Revision, or the A.O.A.C. *Methods of Analyses*, 7th Ed., 1950:

1. *Moisture* (U. S. P.)—Page 777, VII, "Water Method for Drugs Containing No Constituents Volatile at 100°."
2. *Total Nitrogen* (U. S. P.)—Page 740, "Nitrogen (Total) by the Kjeldahl Method (Method I)," or "Nitrogen (Total) by the Semi-Micro Kjeldahl Method (Method II)," or A.O.A.C.—**2.22, 2.23,** or **2.24.**
3. P_2O_5—A.O.A.C., **2.8** to **2.10, 2.11** to **2.13, 20.46,** or **20.48** to **20.50.**

In addition, each collaborator was requested to include all pertinent values such as weighing, titrations, etc. and a detailed description of the methods used when reporting their data.

At the writing of this report only eight of the seventeen collaborators have completed and reported their analyses. When all of the data has been reported, it will be evaluated statistically and the samples again analyzed by the method designed from the statistical evaluation.

Collaborative studies of the methods of analysis of the other constituents will not be started until the methods for moisture, total nitrogen, and P_2O_5 are complete.

COLLABORATORS

The collaborators who have expressed a desire to participate are:

(1) LaMar N. BeMiller, Mead Johnson & Co., Evansville, Indiana
(2) Stanley A. Bobrowski, Jr., Yeast Plant #2, Anheuser-Busch, Inc., Old Bridge, New Jersey
(3) E. F. Budde, Quaker Oats Co., Research Laboratories, Chicago, Illinois
(4) H. Delo, Gerber Products Co., Fremont, Michigan
(5) J. Fetkovich, H. J. Heinz Co., Pittsburgh, Pennsylvania
(6) L. S. Gamer, Olympia Brewing Co., Olympia, Washington
(7) F. W. Handelong, National Biscuit Co., New York, New York
(8) H. A. Jett, Central Research Division, Anheuser-Busch, Inc., St. Louis, Missouri
(9) E. O. Krueger, Abbott Laboratories, North Chicago, Illinois
(10) W. E. Maynard, The Fleischmann Laboratories, New York, New York
(11) M. Nishimura, The Griffith Laboratories, Inc., Chicago, Illinois
(12) D. A. Overbye, Brooklyn Quality Control Laboratories, E. R. Squibb & Sons, Brooklyn, New York
(13) G. E. Reinhardt, Yeast Plant #1, Anheuser-Busch, Inc., St. Louis, Missouri
(14) F. J. Rudert, Red Star Yeast and Products Co., Milwaukee, Wisconsin
(15) A. Sigal, Pabst Brewing Co., Milwaukee, Wisconsin
(16) E. K. Spotts, Ward Baking Co., Bronx, New York
(17) Miss E. Stegemeyer, Laboratories Division, The Kroger Food Foundation, Cincinnati, Ohio

RECOMMENDATIONS

It is recommended*—

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

2. That collaborative studies of methods for the determination of ash, ether extract, crude fiber, and crude carbohydrate in yeast be postponed until the studies on moisture, total nitrogen, and P_2O_5 are completed.

No reports were received on starch in cereals, milk solids and butterfat in bread, soybean flour, sugars in baked products, moisture, bromates in flour, or albumin in noodles and macaroni products.

REPORT ON BAKING POWDER

By V. E. MUNSEY (Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C.), *Referee*

It is recommended†—

(1) That the work as reported in *This Journal*, **34**, 60 (1951) on the neutralizing value of phosphate be continued.

REPORT ON COSMETICS

By G. ROBERT CLARK (Division of Cosmetics, Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C.), *Referee*

The Referee recommends continuance of the following topics:

(1) Deodorants and Anti-Perspirants
(2) Cold Permanent Wave Preparations
(3) Cosmetic Creams
(4) Mascara, Eyebrow Pencil, and Eyeshadow
(5) Hair Dyes and Rinses

The Referee further recommends that the topic Sun Tan Preparations be discontinued.

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* For report of Subcommittee D and action of the Association, see *This Journal*, **36**, 61 (1953).
† For report of Subcommittee D and action of the Association, see *This Journal*, **36**, 61 (1953).
‡ For report of Subcommittee B and action of the Association, see *This Journal*, **36**, 53 (1953).

REPORT ON HAIR DYES AND RINSES

ANALYSIS OF MIXTURES OF *p*-AMINOPHENOL AND *p*-PHENYLENEDIAMINE OR 2,5-DIAMINOTOLUENE

By S. H. NEWBURGER, *Associate Referee*, and J. H. JONES (Division of Cosmetics, Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D.C.)

An analytical method for the analysis of mixtures of *p*-aminophenol with 2,5-diaminotoluene or *p*-phenylenediamine is desirable because these combinations are widely used in blonde-mahogany or chestnut-red hair dyes.

The procedure outlined by Shupe (1) for the analysis of diamine-aminophenol mixtures did not prove satisfactory when applied to samples of hair dyes.

Ether will quantitatively extract the two diamines and the aminophenol from a concentrated salt solution buffered with sodium bicarbonate. An ether solution of *p*-aminophenol when treated with acetic anhydride in the presence of water yields the monoacetyl derivative; in the presence of pyridine the diacetyl derivative is obtained. With either procedure, the diamines form diacetyl derivatives.

The ultraviolet spectra of diacetyl *p*-aminophenol, N-acetyl-*p*-aminophenol, diacetyl *p*-phenylenediamine, and diacetyl 2,5-diaminotoluene were determined in 0.1 *N* HCl, 0.1 *N* NaOH, and alcohol. Although the spectra of the two aminophenols differ in dilute acid and in alcohol, they are identical in dilute alkali. Apparently, the diacetyl derivative hydrolyzes to N-acetyl-*p*-aminophenol in 0.1 *N* NaOH.

It was apparent from the spectra in dilute alkali that it should be possible to analyze spectrophotometrically a mixture containing either of the diamines and aminophenol.

METHOD

REAGENTS

(a) *Alcohol, 95%.*—The alcohol used should have a high transmission from 220 to 325 mμ.

(b) *Sodium hydroxide, 10% (w/v).*—Prepare fresh by dissolving 20 g NaOH in H_2O and diluting to 200 ml. Dilute in the proportion of 4 ml/100 for ca 0.1 *N* NaOH.

(c) *Standard solutions of the diacetyl derivatives.*—Dissolve 100 mg of the purified diacetyl derivative in exactly 100 ml alcohol.

APPARATUS

A spectrophotometer capable of isolating a wave band of 5 mμ, or less, in the region 230–325 mμ. (A Cary recording spectrophotometer Model 11 was used.)

ISOLATION OF AMINES FROM AQUEOUS SOLUTION

Dil. sample to 25 ml with H_2O, acidify slightly with HCl; dissolve 5 g NaCl in the soln, transfer to a continuous extractor and add 25 ml of a 20% NaCl soln contg 50 mg Na_2SO_3 and 1 g $NaHCO_3$. Ext. for 4 hrs with ether. Test for complete

extn by extracting for 15 min. with a fresh portion of ether. (The ether when filtered through cotton should show no appreciable residue upon evapn.) Filter the ether ext. thru a cotton plug into a tared beaker. Wash the extn flask and cotton plug with several small portions of $CHCl_3$. Add 3 ml acetic anhydride and 1 ml pyridine to filtrate. Evap. the volatile solvents on the steam bath. Treat residue with acetic anhydride and pyridine, again evap. and dry the residue of diacetyl derivatives to constant wt at 105°C.

SPECTROPHOTOMETRIC DETERMINATION

Dissolve the diacetyl derivatives isolated from the sample in a measured amt. of alcohol to give a concn of ca 1 mg/ml.

Prepare the following solns from sample soln and the standard solns of diacetyl derivatives. The final solns should be ca 0.1 *N* NaOH (4 ml 10% NaOH/100 ml.) contg 10% of ethyl alcohol. Det. the absorbancies of these solns at the indicated wavelengths, using H_2O as the blank.

(1) The solvent (250, 260, and 300 mμ).
(2) A soln of 10 mg/l of diacetyl *p*-phenylenediamine (260 mμ).
(3) A soln of 100 mg/l of diacetyl *p*-phenylenediamine (300 mμ).
(4) A soln of 10 mg/l of diacetyl 2,5-diaminotoluene (250 mμ).
(5) A soln of 100 mg/l of diacetyl 2,5-diaminotoluene (300 mμ).
(6) A soln of 10 mg/l of diacetyl *p*-aminophenol (250, 260, and 300 mμ).
(7) A soln of 20 mg/l of diacetyl *p*-aminophenol (300 mμ).
(8) A soln of 10 mg/l of the sample (250, 260, and 300 mμ).
(9) A soln of the sample whose absorbance at 300 mμ is 0.30 to 0.60. The concn of diacetyl *p*-aminophenol in this soln will be 20–40 mg/l.

Correct the observed absorbances of the sample and standards for the blank and convert each absorbance to that which the soln would have at a standard concn (100 mg/l is convenient).

Calc. the composition of the sample from the absorbances of the sample and standards by the method of simultaneous equations or by the use of successive approximations. The composition of the diacetyl *p*-phenylenediamine-diacetyl *p*-aminophenol mixt. are calcd, using the absorbances at 260 and 300 mμ; the composition of the diacetyl 2,5-diaminotoluene-diacetyl *p*-aminophenol mixt. from the absorbances at 250 and 300 mμ.

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

The diacetyl derivatives of *p*-phenylenediamine and 2,5-diaminotoluene were prepared as described in an earlier paper (2).

Diacetyl *p*-aminophenol (Eastman) was recrystallized from benzene. The product after drying at 105°C. was a white crystalline powder; m.p. 153.5°C.

To prepare N-acetyl-*p*-aminophenol, *p*-aminophenol was dissolved in ethyl acetate previously saturated with water. Acetic anhydride was added, the solvents partially evaporated on the steam bath, a little more water added, and the solution evaporated to dryness on the steam bath. The product was dissolved in hot ethyl acetate, treated with charcoal, and filtered; an equal volume of hot toluene was added to the

filtrate and the solution chilled in an ice bath. The crystals of N-acetyl-*p*-aminophenol were filtered off and dried at 105°C. The product was a white crystalline powder, m.p. 168.5 to 169.5°C.

Solutions of diacetyl *p*-phenylenediamine, diacetyl 2,5-diaminotoluene, and diacetyl *p*-aminophenol in 0.1 *N* NaOH containing 10% alcohol were found to obey Beer's law to within ±1%. The absorbance curves are shown in Fig. 1.

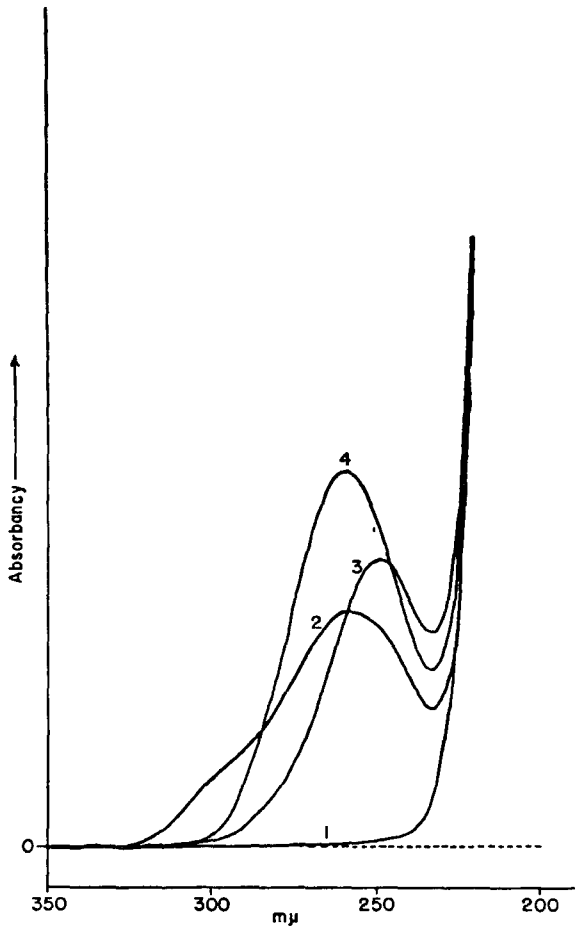


FIG. 1.—Ultraviolet absorbance curves. Solvent: 0.1 *N* NaOH containing 10% alcohol; blank: water; concentration: 10 mg per liter; cells: 1 cm. Curve 1.—Solvent. Curve 2.—Diacetyl *p*-aminophenol. Curve 3.—Diacetyl 2,5-diaminotoluene. Curve 4.—Diacetyl *p*-phenylenediamine.

The absorbance curves of mono- and diacetyl *p*-aminophenol in alcohol are given in Fig. 2. Results obtained by the described extraction procedure are tabulated in Table 1.

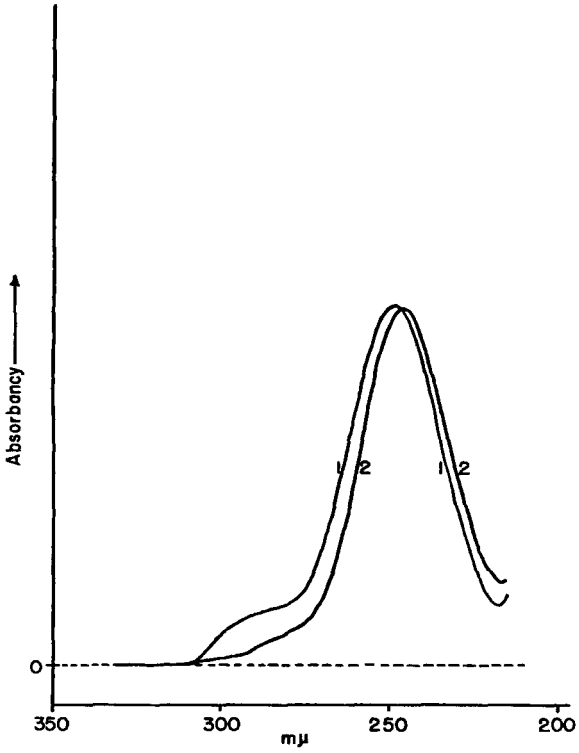


FIG. 2.—Ultraviolet absorbance curves of acetyl derivatives of *p*-aminophenol. Solvent: alcohol; concentration: 10 mg per liter; cells: 1 cm. Curve 1.—N-acetyl-*p*-aminophenol. Curve 2.—Diacetyl *p*-aminophenol.

Mixtures of the diacetyl derivatives were analyzed by the proposed spectrophotometric method. The results are given in Table 2.

DISCUSSION

The acetyl derivatives of the amine are prepared because the free amine bases are unstable.

Although the absorbance measured is actually that of N-acetyl-*p*-aminophenol the diacetyl derivative is used as a standard because it can be prepared with greater certainty and ease from *p*-aminophenol.

The directions for the spectrophotometric determination may seem rather complicated. However, the procedure and calculation are the same

TABLE 1.—*Extraction of diamines and p-aminophenol*

EXP. NO.	COMPOSITION OF SAMPLE[a]		WT OF RECOVERED ACETYL DERIVS. PREPD. TO YIELD DIACETYL DIAMINE AND N-ACETYL-*p*-AMINOPHENOL	WT OF RECOVERED ACETYL DERIVS. PREPD. TO YIELD DIACETYL DIAMINE AND DIACETYL *p*-AMINOPHENOL
		mg	*mg*	*mg*
1	*p*-Phenylenediamine	93.1	—	305.3
	p-Aminophenol	77.0	—	(Th. 301.6)
2	*p*-Phenylenediamine	96.4	276.6	—
	p-Aminophenol	73.9	(Th. 273.7)	—
3	2,5-Diaminotoluene	97.4	—	298.8
	p-Aminophenol	75.5	—	(Th. 298.1)
4	2,5-Diaminotoluene	97.4	272.0	—
	p-Aminophenol	74.4	(Th. 267.5)	—

[a] The compounds, added as the hydrochlorides, were calcd as the free bases.

TABLE 2.—*Analysis of mixtures of diacetyl diamines and diacetyl p-aminophenol*

EXP. NO.	DIACETYL *p*-AMINOPHENOL		DIACETYL *p*-PHENYLENEDIAMINE		2,5-DIAMINOTOLUENE	
	ADDED	FOUND	ADDED	FOUND	ADDED	FOUND
	mg	*mg*	*mg*	*mg*	*mg*	*mg*
1	20.0	20.0	20.0	19.8	—	—
2	10.0	10.6	40.0	39.7	—	—
3	20.0	20.0	5.0	4.4	—	—
4	20.0	19.4	—	—	20.0	19.7
5	10.0	9.7	—	—	40.0	40.3
6	20.0	20.0	—	—	5.0	4.5

as for any two-component mixture although it is necessary to use two concentrations of the standards and reference compounds so that the absorbance readings fall in a suitable range for instrumental accuracy.

The absorbance ratios for the two sets of compounds are such that calculations of the results by successive approximations is simpler than the use of simulaneous equations. Either method will give the same result.

The entire ultraviolet spectrophotometric curve of the sample at about 10 mg per liter should be obtained. If the sample of isolated diacetyl derivatives is a mixture of only two of the three compounds, the qualitative composition will be apparent from the curve. Comparison of the entire curve with the curve which a mixture of the calculated composition would produce will also serve as a check on the possible presence of other compounds in the sample.

The spectrophotometric determination should give fairly accurate results for mixtures containing 10 per cent or more of the minor component.

Interfering materials such as soaps and resorcinol can be removed by a preliminary extraction with ether from acid solution.

SUMMARY AND RECOMMENDATIONS

p-Phenylenediamine, 2,5-diaminotoluene, and *p*-aminophenol are quantitatively extracted by ether from a concentrated salt solution buffered with sodium bicarbonate.

After conversion to the diacetyl derivatives, a mixture containing *p*-aminophenol plus either (but not both) *p*-phenylenediamine or 2,5-diaminotoluene can be analyzed spectrophotometrically with reasonable accuracy.

Under the specified conditions, diacetyl *p*-aminophenol is readily hydrolyzed to N-acetyl-*p*-aminophenol by dilute alkali.

It is recommended* that work on hair dyes and rinses be continued.

REFERENCES

(1) SHUPE, I. S., *This Journal*, **23**, 721 (1940).
(2) NEWBURGER, S. H., and JONES, J. H., *This Journal*, **33**, 374 (1950).

REPORT ON MASCARAS, EYEBROW PENCILS, AND EYE SHADOWS

DISPERSING AGENTS

By PAUL W. JEWEL (Max Factor & Co., Hollywood 28, Calif.),
Associate Referee

The method for mascara, reported last year, has been given further study, particularly with a view to adapting it for use with cream mascaras.

The cream mascaras on the market present such a variety of compositions that it may prove impractical to devise one method for all types. Those which contain triethanolamine soap as a dispersing agent offer little difficulty. It is necessary simply to dry the sample to constant weight, record the amount of water found, and proceed according to Method A, as hereinafter described. If mineral soaps are used as dispersing agents, the sample must be given a preliminary acid treatment, since these soaps are not soluble in ordinary wax solvents.

There is another type in which turpentine is used as a thinner for the waxes, and this of course requires a completely different approach. There are a few brands beginning to appear in which the dispersing agent is not a soap but one of the many non-ionic dispersing agents.

The problem is complicated by the fact that many brands are changing to carbon black in place of bone black. These blacks are so small in particle size that it is almost impossible to remove them by filtration or by centrifuging from solvents.

* For report of Subcommittee B and action of the Association, see *This Journal*, **36**, 53 (1953).

SAMPLE PREPARATION

Weigh contents of one tube of cream mascara into a tared beaker. Evap. to dryness on the steam bath. Add 20 ml absolute alcohol, and again evap. to dryness. Dry to constant wt at 100°C. Report the loss in wt as H_2O.

METHOD A

(a) To the residue in the beaker add 20 ml of $CHCl_3$ and boil gently for a few min. If the entire mass disintegrates and appears to dissolve, it probably contains a triethanolamine soap. If so, add 20 ml benzene, boil gently for a few min, allow to settle, and filter into a tared beaker. Repeat the extn two more times, evap. the solvents, add a few ml of alcohol, again evap, and finally dry to constant wt. Report this wt as total base.

(b) Disperse the base in 20 ml hot alcohol, add 25 ml 0.50 *N* H_2SO_4, heat on the steam bath for 10 min., add 50 ml cold H_2O, and chill in the icebox for at least two hrs. Filter, and titrate the filtrate with aq. 0.50 *N* NaOH using methyl red as an indicator. From the amount of acid used, calc. the amount of triethanolamine present. Seven ml of 1.0 *N* acid is equivalent to one g of the amine.

(c) Transfer the waxes from the filter to the original beaker with stream of alcohol from wash bottle, and dry to constant wt. This is the total base minus the triethanolamine.

(d) Dissolve the base thus obtained in 50 ml boiling absolute alcohol and titrate with 0.50 *N* alcoholic KOH, using phenolphthalein as an indicator. Calc. the acid thus found as stearic acid, using 208 as the acid number, and report as total stearic acid.

If the sample does not disintegrate as indicated in (a) or if the pigments peptize and go thru the filter, take a new sample and proceed as in Method B.

METHOD B

To the dried sample add 10 ml 95% alcohol, and heat until the mass is well dispersed. Then add 5 ml of concd HCl, followed by 35 ml H_2O, and heat on the steam bath until the waxes melt and float to the top. Chill in the icebox, filter, and wash several times with H_2O. Transfer the waxes from the filter back to the original beaker with a stream of alcohol from a wash bottle, evap. the alcohol, and dry to constant wt. This will be the total base plus color, minus triethanolamine or mineral alkalis. Then proceed as in section (a) of Method A. Then skip (b) and (c) and continue with (d).

In the filtrate from the detn of triethanolamine in Method A or the acid ext. from Method B will be found any glycerol or glycols, and all of the alkali elements which may have been present. If Method B is used, triethanolamine must be detd in this filtrate by total N. Glycerin or glycol, if present, may be detd by the Malaprade method as modified by Shupe (1).

Evap. the soln from section (d) of Method A to dryness, and ext. several times with boiling petr. ether. Filter the ether through a dry filter paper into a tared dish. Evap. the solvent and weigh. This will give all of the hydrocarbons, and beeswax, together with small amounts of candelilla wax.

Dissolve residue in H_2O and ext. with $CHCl_3$, using several successive amounts of solvent. Filter these extracts into a tared dish, evap. solvent, and weigh. This will give all of the candelilla wax and the carnauba wax.

Make the extracted soln acid with HCl and ext. with petr. ether. Filter the ether extracts into a tared dish, evap. the solvent, and weigh. This will give all of the fatty acids originally present as soap.

Each of these fractions can then be subjected to such analytical procedures as the operator deems necessary. Usually it is desirable only to characterize roughly the

various types of waxes, since with this information, any chemist skilled in such preparations can readily arrive at a very close duplication of the original.

RECOMMENDATION*

It is recommended that this project be continued and that it be submitted to collaborative study as soon as the Associate Referee feels that the method is ready for such study.

REFERENCE

(1) SHUPE, I. S., *This Journal*, **26**, 249–256 (1943).

REPORT ON DEODORANTS AND ANTI-PERSPIRANTS

DETERMINATION OF BORIC ACID

By JOHN E. CLEMENTS (Division of Cosmetics, Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C.), *Associate Referee*

Boric Acid. The determination of boric acid in anti-perspirants and deodorants is troublesome because of interferences from zinc salts, aluminum salts, and organic buffers. The methyl borate distillation is unwieldy. A double precipitation of interfering cations, proposed by Taylor (1), seemed promising, but some adverse results were reported (2). Recently, Martin and Hayes (3) described a procedure which separates boric acid from cations by an ion exchange resin. A *p*H meter is used in titrating the boric acid with alkali.

In this investigation the method of Martin and Hayes was successfully modified for boric acid in anti-perspirants and deodorants. Organic matter is destroyed by a preliminary ashing, and the boric acid is titrated in the usual manner with indicators, mannitol, and standard alkali.

METHOD

REAGENTS

(a) *Methyl red indicator solution.*—**2.46(c).**

(b) *Phenolphthalein indicator solution.*—**4.6(a).**

(c) *Sodium hydroxide*, 0.1 *N.*—**39.32.**

(d) *Sodium hydroxide.*—10% (freshly prepared).

(e) *Mannitol.*

(f) *Amberlite IR 120 (H), analytical grade.*—This material may be regenerated after using by transferring the accumulated resin from a number of runs to a large glass tube and washing with 10% HCl soln until the issuing liquid gives a negative test for the adsorbed ions. The HCl is then removed from the resin by H_2O washing.

APPARATUS

Preparation of ion exchange column.—A glass tube 23″ long ×3/4″ in diam., provided with a stopcock and outlet tube. Tamp a 1″ glass wool plug into the bottom

* For report of Subcommittee B and action of the Association, see *This Journal*, 36, 53 (1953).

of the tube, fill the tube with H_2O, and add the ion exchange resin slowly until a column 18" in length is formed. Before using the column, wash with 100 ml of 10% HCl soln, and follow with 50 ml portions of H_2O until the effluent gives a negative test for Cl ions.

DETERMINATION

Place sample contg 50–200 mg boric acid in a 250 ml casserole, add 2 drops of phenolphthalein indicator soln, and make alk. with 10% NaOH soln. Evap. soln to dryness on a steam bath under a gentle jet of air, dry the residue at 140°C. in an oven for 1 hr., and then ash for 1 hr at 550°C. Cool casserole to room temp., add ca 50 ml of hot H_2O, cautiously acidify with concd HCl and filter the hot soln thru a quantitative filter paper into a 250 ml beaker. Wash the filter paper with a little hot H_2O and reserve filtrate (the filtrate may be slightly cloudy).

Transfer filter paper to the same casserole, make alk. by wetting with ca 10 ml of H_2O and a few drops of 10% NaOH soln. Evap. the soln to dryness on steam bath, dry 1 hr at 140°C., and ash at 550°C. for 2 hrs. Cool the casserole, add ca 50 ml of hot H_2O, acidify with concd HCl and filter into previously reserved filtrate. Wash the casserole and filter paper thoroly with hot H_2O. Discard filter paper. The total vol. of soln should be ca 200 ml.

Cool soln, add concd NH_4OH until barely alk. to litmus paper or until a flocculent ppt appears. Reacidify with concd HCl until slightly acid to litmus paper or until ppt just redissolves. Pass this soln thru the ion exchange column into a one l flask at a rate of flow requiring 10–15 min. for passage. Follow the sample soln with several 50 ml portions of H_2O until the effluent is only slightly acid to *p*H test paper. Add 5 drops methyl red indicator to the soln, make alk. with 10% NaOH soln, and then barely acid with concd HCl. Connect the flask to a H_2O-cooled reflux condenser and boil for 5 min. Wash down condenser with a little H_2O and cool soln to room temp. by placing the flask under running tap H_2O. Neutralize soln to methyl red indicator with 0.1 *N* NaOH, add 4–5 g mannitol and ca 0.5 ml phenolphthalein indicator soln. Titrate with 0.1 *N* NaOH to appearance of the phenolphthalein pink color, add more mannitol, and if pink color disappears, continue titration until it reappears. Repeat the addn of mannitol until there is no further change in color.

Run a blank in the following manner: to ca 350 ml of H_2O add an amount of 10% NaOH soln equal to that required to neutralize the sample after passing thru the column. Barely acidify this alk. soln with concd HCl and proceed as described above beginning with, "Connect the flask to a H_2O-cooled reflux condenser. . . . " Subtract the blank titration from the sample titration and calc. the boric acid content of the sample:

1 ml of 0.1 *N* NaOH = 0.00619 g boric acid

EXPERIMENTAL

The following master solution containing a number of ingredients likely to be found in anti-perspirants and deodorants was prepared.

Aluminum sulfate, C.P.	20 g
Zinc chloride, C.P.	20 g
Magnesium chloride	20 g
Urea, C.P.	20 g
Glycerol, U. S. P.	20 g
Zinc phenolsulfonate, N. F.	20 g
Hydrochloric acid, concd	12.5 ml.
Water	500 ml.

The Associate Referee analyzed 5, 10, and 20 ml aliquots of the master solution for boric acid by the described procedure. None was found. Varying amounts of boric acid were then added to 10 ml portions of the master solution. Results of analyses by the proposed procedure are given in Table 1.

TABLE 1.—*Recoveries of boric acid*

WEIGHT OF BORIC ACID ADDED	RECOVERIES	
mg	*mg*	*per cent*
50.45	50.8	100.7
50.45	50.8	100.7
50.45	50.8	100.7
100.9	100.3	99.4
100.9	100.9	100.0
201.8	199.9	99.0
201.8	199.9	99.0
201.8	198.1	98.2
201.8	198.7	98.5
	Average	99.5

SUMMARY AND RECOMMENDATIONS

An analytical method for boric acid, involving an ion exchange resin, has been successfully modified for the determination of boric acid in antiperspirants and deodorants.

RECOMMENDATIONS

It is recommended*—

(1) That the procedure be submitted for collaborative study.

(2) That study of the topic be continued.

REFERENCES

(1) TAYLOR, D. S., *This Journal*, **32**, 422 (1949).
(2) TYSON, G. N., *ibid.*, **33**, 288 (1950).
(3) MARTIN, J. R., and HAYES, J. R., *Anal. Chem.*, **24**, 182 (1952).

REPORT ON COLD PERMANENT WAVES

DITHIODIGLYCOLLIC ACID

By JOHN E. CLEMENTS (Division of Cosmetics, Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C.), *Associate Referee*

Dithiodiglycollic Acid.—The Cosmetic Laboratory of the Division of Cosmetics determines dithiodiglycollic acid by a method which is essen-

* For report of Subcommittee B and action of the Association, see *This Journal*, **36**, 53 (1953).

tially that described in the TGA Standard for thioglycollic acid (1). This procedure was submitted for collaborative study.

Results were submitted by the following collaborators:

(1) J. L. Thomson, Cosmetic and Colour Section, Department of National Health and Welfare, Ottawa, Ontario, Canada.
(2) R. T. Merwin, The Connecticut Agricultural Experiment Station, New Haven, Connecticut.
(3) J. P. Traynor, Food and Drug Administration, Baltimore, Maryland.
(4) J. C. Bloomingdale, Food and Drug Administration, Chicago, Illinois.
(5) E. G. McDonough, Evans Research and Development Corporation, New York, New York.
(6) M. C. Staves, Food and Drug Administration, Washington, D. C.

Recoveries of the added material varied from 88.5 to 103.4 per cent. Since the results were not as satisfactory as had been expected, it is believed that the deviations are due to inadequate directions in the procedure.

RECOMMENDATION*

The Associate Referee recommends that the procedure be resubmitted for collaborative study with more detailed directions for determining the dithiodiglycollic acid.

REFERENCE

(1) The Toilet Goods Association Specification No. 31 (TGA Method No. 33).

No reports were received on cosmetic creams or suntan preparations.

REPORT ON COAL-TAR COLORS

By KENNETH A. FREEMAN (Division of Cosmetics, Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C.), *Referee*

The Referee concurs with the recommendation† of the Associate Referee on subsidiary dyes in FD&C colors that the proposed method for the determination of lower sulfonated dyes in FD&C Blue No. 1 be submitted to collaborative study and that the topic be continued.

The Referee also concurs with the recommendation of the Associate Referee on paper chromatography of coal-tar colors that collaborative work be undertaken and that the topic be continued.

The Referee further concurs with the recommendation of the Associate Referee on the topic boiling range of amines derived from coal-tar

* For report of Subcommittee B and action of the Association, see *This Journal*, 36, 53 (1953).
† For report of Subcommittee B and action of the Association, see *This Journal*, 36, 53–54 (1953).

colors that the method for the determination of the boiling range of xylidines obtained from FD&C Red No. 32 be restudied and that the topic be continued.

The Referee recommends that no action be taken regarding the method proposed for the determination of subsidiary dyes in D&C Red No. 35 until the Associate Referee on subsidiary dyes in D&C colors has had an opportunity to follow his suggestion that a spectrophotometric method be compared with the chemical method.

The Referee recommends that the following additional topics be continued:

Inorganic Salts in Coal-Tar Colors.
Ether Extracts in Coal-Tar Colors.
Halogens in Halogenated Fluoresceins.
Identification of Coal-Tar Colors.
Volatile Amine Intermediates in Coal-Tar Colors.
Unsulfonated Phenolic Intermediates in Coal-Tar Colors.
Non-Volatile Unsulfonated Amine Intermediates in Coal-Tar Colors.
Intermediates Derived from Phthalic Acid.
Lakes and Pigments.
Spectrophotometric Testing of Coal-Tar Colors.
Determination of Arsenic and Antimony in Coal-Tar Colors.
Determination of Heavy Metals in Coal-Tar Colors.
Sulfonated Phenolic Intermediates in Coal-Tar Colors.
Intermediates in Triphenylmethane Dyes.

REPORT ON BOILING RANGE OF AMINES DERIVED FROM COAL-TAR COLORS

XYLIDINE FROM FD&C RED NO. 32

By LEE S. HARROW (Division of Cosmetics, Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C.), *Associate Referee*

The method for the determination of the boiling range of pseudocumidine, which was adopted, First Action, by the Association (1), was used by the Associate Referee for the determination of the boiling range of xylidine obtained by the reduction of FD&C Red No. 32. The results obtained were considered satisfactory.

Two composite samples of FD&C Red No. 32 were prepared and subsamples of these were submitted to collaborators for analysis. The results obtained by the collaborators varied widely, both in the determination of the initial boiling point and in the determination of the final boiling point. From these results, it appears that the method should be studied further.

ACKNOWLEDGMENT

The author wishes to thank the following collaborators for their assistance in this study.

A. T. Schramm, National Aniline Division, Allied Chemical and Dye Corporation, Buffalo, New York

W. H. Kretlow, Wm. J. Stange and Company, Chicago, Illinois

Anna Bartruff, The Hilton-Davis Chemical Company, Cincinnati, Ohio

I. Hanig, H. Kohnstamm and Company, Inc., Brooklyn, New York

Charles H. Brown, Bates Chemical Company, Inc., Lansdowne, Pennsylvania

G. A. Allen, Calco Chemical Division, American Cyanamid Company, Bound Brook, New Jersey

RECOMMENDATION

It is recommended* that the method for the determination of the boiling range of xylidines derived from FD&C Red No. 32 be studied further and that the topic be continued.

REFERENCE

(1) HARROW, L. S., *This Journal*, **34**, 405 (1951).

REPORT ON SUBSIDIARY DYES IN D&C COLORS

(4-TOLUENE-AZO-2-NAPHTHOL IN D&C RED NO. 35)

By LOUIS KOCH (H. Kohnstamm and Co. Inc., Brooklyn, New York), *Associate Referee*

D&C Red No. 35, toluidine toner, an azo dye synthesized by coupling 2-naphthol to diazotized 2-nitro-4-methyl aniline, may simultaneously form a subsidiary dye, 4-toluene-azo-2-naphthol, if 4-methyl aniline is present in the diazo component. Its structural similarity to 4-toluene-azo-2-naphthol-3-carboxylic acid, a subsidiary dye found in D&C Red No. 7, suggested the possible use of a method reported in *This Journal*, (1). Difficulty was encountered, however, when a side reaction caused the formation of an unknown constitutent which caused erroneous results in the bromate procedure for the estimation of 4-methyl aniline from the scission of the subsidiary dye.

Numerous investigative experiments and correspondence with Dr. K. A. Freeman, of the Color Certification branch of the Food and Drug Administration finally disclosed that the difference in the solubilities of the primary and subsidiary dyes in chloroform could effect a separation which eliminated the formation of the undesirable constituent during the hydrogenation and permitted the bromate analysis to run a normal course. Many assays by the Associate Referee indicated the validity of the proposed method, and dye samples prepared from known quantities of primary and subsidiary diazo compounds were submitted for collaborative work. Results are shown in Table 1.

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

TABLE 1.—*Collaborative results: subsidiary dye found, per cent*

ANALYST	SAMPLE			
	A	B	C	D
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
Assoc. Referee	0.42	0.57	1.10	2.11
Calcd.[a]	0.35	0.61	1.21	2.42
Calcd.[b]	0.46	0.71	1.32	2.52

[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.

METHOD

REAGENTS

(a) *Chloroform.*—Redistilled.
(b) *Sodium Hydrosulfite.*—Solid.
(c) *Potassium Bromide-Bromate Solution.*—**34.77(a).**
(d) *Sodium Thiosulfate Solution.*—About 0.05 *N*, **34.77(b).**
(e) *Starch Indicator.*—0.5% soln.
(f) *Potassium Iodide.*—Solid.

DETERMINATION

Place 2 g of the coloring matter in a Soxhlet app. fitted with a 125 ml flask, and ext. with $CHCl_3$ until the leachings are colorless. Cool ext. to room temp., filter, and wash with 50 ml alcohol. Evap. filtrate to ca 10 ml and dil. the hot solvent with 25 ml alcohol.

Cool, filter into a one l round bottom flask, and wash residue with ca 50 ml alcohol. Attach flask to a Kjeldahl-type app. so arranged that H_2O can be run into the distn flask thru a dropping funnel. Lead the tip of the adapter below the surface of 30 ml of a 1+5 HCl soln; add 10 ml 2.5% NaOH soln and 1 g Na hydrosulfite to the alc. soln of the dye, and apply heat to effect simultaneous reduction and steam distn.

When ca 50 ml of distillate has been collected, allow 250 ml of H_2O to drip into the heated vessel at a rate equal to that of the distn. Continue heating after the final H_2O addn, until the receiving flask contains at least 325 ml of distillate.

Concentrate the liquid to ca 75 ml, transfer to a 500 ml I flask, and dil. to 100 ml mark. Acidify soln with 20 ml HCl, cool, and chill with 150 g crushed ice.

Run the $KBrO_3$ soln from a buret into the cold concentrate until a yellow color persists for at least 30 sec. Add 5 ml more, stopper, and let stand in an ice bath for 10 min. Add ca 2 g KI and titrate the liberated I with the $Na_2S_2O_3$ soln, using the starch indicator near the end point.

1 ml. of 0.05 *N* $KBrO_3$ soln = 3.29 mg subsidiary dye.

DISCUSSION

Although the analytical results indicate the method has merit, the recovery percentages are not truly quantitative. However, because the calculated figures are based on a 100 per cent reaction yield, a phenomenon

that is a rarity in organic synthesis, it is recommended that the proposed method be adopted as official, First Action.

It is very possible also, that a spectrophotometric assay, based on the chloroform removal of the bulk of the primary color, would yield results that are more in line with the calculated values. The Associate Referee therefore also recommends that such a study should be undertaken.

ACKNOWLEDGMENT

The Associate Referee wishes to thank the following collaborators for their cooperation and constructive efforts:

J. M. Remsen, E. I. du Pont de Nemours and Company, Wilmington, Delaware
F. Howard Hedger, Chas. Pfizer and Company, Brooklyn, New York
A. T. Schramm, National Aniline Division, Allied Chemical & Dye Corporation, Buffalo, New York
I. Hanig, H. Kohnstamm and Company, Inc., Brooklyn, New York

RECOMMENDATIONS

It is recommended* that the proposed method be adopted as official, First Action.

REFERENCE

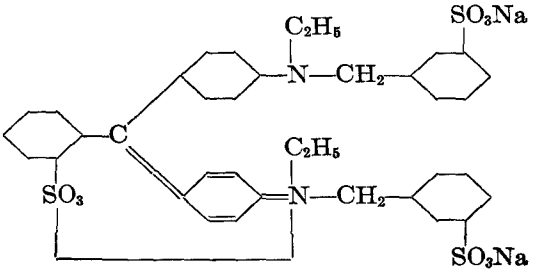
(1) Koch, L., *This Journal*, **33**, 405 (1950).

REPORT ON LOWER SULFONATED DYES IN FD&C BLUE NO. 1

By Meyer Dolinsky (Division of Cosmetics, Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C.), *Associate Referee*

At the 1950 A.O.A.C. meeting, several tentative methods for determining subsidiary dyes in FD&C colors were deleted (1), including a method for subsidiary dyes in FD&C Blue No. 1 (2). This method has been reinvestigated, and with some modification has been found to be suitable for determining lower sulfonated dyes in FD&C Blue No. 1.

FD&C Blue No. 1 (Brilliant Blue FCF) is a trisulfonated dye believed to have the following structure:



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

At least two disulfonated and two monosulfonated subsidiaries may be postulated. One of the disulfonated subsidiaries is certifiable as FD&C Green No. 1 (C.I. No. 666). One of the monosulfonated subsidiaries is readily obtained by condensing benzaldehyde *o*-sulfonic acid with two moles of ethylbenzylaniline and oxidizing the resulting leuco base. A sample of this monosulfonated subsidiary, plus a recrystallized sample of FD&C Green No. 1, were used to check the validity of the extraction procedure.

EXTRACTION DATA

In the original method, a solution of 100 mg of FD&C Blue No. 1 in 10 ml of water is diluted with 40 ml of salt-acetate solution and extracted with several portions of isoamyl alcohol. The isoamyl alcohol is then washed free of FD&C Blue No. 1 with several portions of salt-acetate solution, and the residual lower sulfonated dye is removed with water and determined colorimetrically.

When solutions of the lower sulfonated dyes containing no FD&C Blue No. 1 were treated as described in the original method, each of the lower sulfonated dyes tested was found to be extracted completely into the isoamyl alcohol (Table 1). They were not washed out of the alcohol to any appreciable extent by several washings with salt-acetate solution.

TABLE 1.—*Extraction of lower sulfonated subsidiaries*

	NOT EXTD INTO AMYL ALCOHOL	WASHED OUT OF AMYL ALCOHOL BY 4 WASHINGS WITH SALT-ACETATE SOLN
	per cent	*per cent*
Monosulfonated Subsidiary	0	0.2
FD&C Green No. 1 (Disulfonated Subsidiary)	0	0.5

From these data it appears reasonable to assume that any lower sulfonated subsidiary of FD&C Blue No. 1, when extracted alone, will be quantitatively recovered by the proposed method. Apparently, however, the extraction of some lower sulfonated subsidiaries may not be complete in the presence of a large amount of FD&C Blue No. 1. This was indicated by the following experiments:

(*a*) Two samples of FD&C Blue No. 1 were extracted as described in the original method. The salt-acetate solution from which the subsidiary had been presumably removed was then re-extracted a second time; an additional small amount of subsidiary was recovered. A third extraction produced practically no subsidiary. Data are shown in Table 2.

(*b*) The following solutions were extracted by the original method.

(1) A solution containing 100 mg of commercial FD&C Blue No. 1.

(2) A solution containing 5 mg of recrystallized FD&C Green No. 1.

TABLE 2.—*Subsidiary dye recovered by several extractions*

	1ST EXTRACTION	2ND EXTRACTION	3RD EXTRACTION	TOTAL
	per cent	*per cent*	*per cent*	*per cent*
Sample 1	1.2	0.3	trace	1.5
Sample 2	2.7	0.4	0.1	3.2

(3) A solution containing 100 mg of FD&C Blue No. 1 plus 5 mg. of recrystallized FD&C Green No. 1.

Recovery of subsidiary from the solution containing the mixture of dyes, as shown in Table 3, was less than the total subsidiary recovered when each of the components of the mixture was extracted separately.

TABLE 3.—*Extraction of FD&C blue No. 1 plus FD&C green No. 1*

COLOR EXTRACTED	COLOR RECOVERED (CALCULATED AS FD&C GREEN NO. 1
	mg
5.0 mg of FD&C Green No. 1 (approx. 90% pure dye)	4.3
100 mg FD&C Blue No. 1	1.8
	Total 6.1
5.0 mg FD&C Green No. 1 plus 100 mg FD&C Blue No. 1	5.2

A second extraction of the salt-acetate solution of the mixture recovered an additional 0.9 mg of subsidiary and a third extraction recovered less than 0.1% subsidiary. The color recovered in these latter two extractions was identified spectrophotometrically as FD&C Green No. 1.

It is apparent from these data that recoveries of subsidiary may not be complete when samples of FD&C Blue No. 1 are extracted as described in the original procedure. However, if the procedure is slightly modified by substituting a 10 mg sample of FD&C Blue No. 1 for the specified 100 mg sample, recoveries of subsidiary appear to be practically quantitative. A comparison of recoveries from two samples of FD&C Blue No. 1, extracted at a 10 mg and at a 100 mg level, are shown in Table 4.

SPECTROPHOTOMETRIC DATA

A large number of samples of FD&C Blue No. 1 were extracted, and the recovered subsidiary was examined spectrophotometrically. The subsidiary color appears to be identical in all cases (Fig. 1). A neutral aqueous solution of the subsidiary shows a major absorption peak at $617 \pm 2 m\mu$, with a secondary peak at 402 ± 2 mμ. In 0.1 *N* acid solution, the color changes from blue to green and in 0.1 *N* NaOH, fades rapidly.

TABLE 4.—*Extractions at different levels*

	SUBSIDIARY RECOVERED		
	1ST EXTRACTION	2ND EXTRACTION	3RD EXTRACTION
Sample A			
100 mg extracted	1.2	0.3	<0.1
10 mg extracted	1.6	<0.1	—
Sample B			
100 mg extracted	2.7	0.4	0.1
10 mg extracted	3.3	<0.1	—

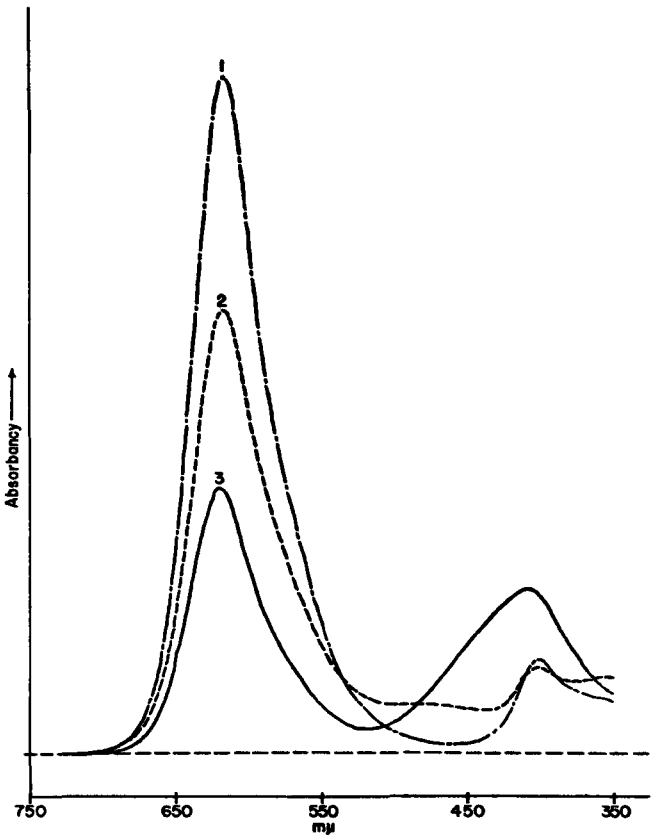


FIG. 1.—Lower sulfonated subsidiary of FD&C Blue No. 1. Curve 1.—in H_2O (*p*H 7.0). Curve 2.—in 0.1 *N* NaOH. Curve 3.—in 0.1 *N* HCl.

DISCUSSION

A number of samples of FD&C Blue No. 1, including at least one sample from each manufacturer of this color, were analyzed for lower sulfonated dyes. The subsidiary found ranged from 1.4 to 3.3 per cent. Thus, in no case did the amount of subsidiary exceed the 5 per cent tolerance allowed in the specification for certifiable FD&C Blue No. 1 (3).

On the basis of the data presented in this paper, it is believed that the original A.O.A.C. procedure for determining lower sulfonated dyes in FD&C Blue No. 1 is satisfactory, provided the following modifications are made:

(1) The sample size is reduced from 100 mg to 10 mg.
(2) The extracted subsidiary is determined spectrophotometrically, using the absorbance/mg/l of FD&C Green No. 1 as a standard.

ACKNOWLEDGMENT

The author wishes to thank Mr. Lee S. Harrow for preparing the leuco base of the monosulfonated subsidiary.

RECOMMENDATIONS

It is recommended*—

(1) That the extraction procedure for lower sulfonated dyes in FD&C Blue No. 1 be submitted to collaborative study.
(2) That the topic be continued.

REFERENCES

(1) FREEMAN, K. A., *This Journal*, **33**, 381 (1950).
(2) *Methods of Analysis*, 6th Ed., Association of Official Agricultural Chemists, Washington, D. C., 1945.
(3) S.R.A. F.D.C. 3, Coal-Tar Color Regulations.

REPORT ON PAPER CHROMATOGRAPHY OF COAL-TAR COLORS

By DORIS H. TILDEN (Food and Drug Administration, Department of Health, Education, and Welfare, San Francisco, Calif.), *Associate Referee*

Continuation of work reported last year (1) on the separation of coal tar colors by means of paper partition chromatography has resulted in recording some additional reactions for dyes or combinations of dyes already considered and several solvent systems found for the satisfactory separation and migration of some dyes not included in the former report.

Jaschik and Kramer (2) have reported the separation on paper of

* For report of Subcommittee B and action of the Association, see *This Journal*, **36**, 53 (1953).

FD&C Orange 1 and D&C Orange 2 by means of an ammonia solution. The author found that a very satisfactory separation is effected using this solvent system but much more compact spots are obtained using 1 per cent ammonium hydroxide-water solution saturated with amyl alcohol (solvent system #18) (1).

Another solvent system was added to the list previously reported: *n*-butyl alcohol saturated with water, 100 ml, and succinic anhydride, 3 grams, is excellent for separating FD&C Red 2 from Brilliant Scarlet (C.I.185). There is a clear-cut separation of colors, although the actual R_F values are close (Solvent system No. 11 is also very effective in separating these two dyes). It has given very good results in separating triphenyl methane dyes in sodium carbonate solution from many other dyes.

Table 1 below is a continuation of Table 3 in last year's report and Table 2 is in addition to Table 2 of that report.

Collaborative material consisting of three samples of authentic FD&C dyes in solution was sent out but only two replies have been received to date. The solutions were made up as follows:

(1) "Leaf green"—FD&C Yellow 5; FD&C Green 2; FD&C Orange 1.
(2) "Chocolate"—FD&C Red 2; FD&C Yellow 5; FD&C Orange 1 and FD&C Blue 1.
(3) "Raspberry"—FD&C Reds 1 and 2; FD&C Yellows 1 and 6 and FD&C Orange 1.

INSTRUCTIONS TO COLLABORATORS

APPARATUS

(a) *Glass tanks.*—Wooden or stainless steel cabinets air tight with racks; not less than 16″ high, suitable for ascending paper chromatography and capable of accommodating at least four paper strips about 2″ wide. For the present work, three tanks are desirable as there will be three different solvent systems used.

(b) *Whatman #1 filter paper.*—Sheets $18\frac{1}{4} \times 22\frac{1}{2}$ may be cut into strips the required size.

REAGENTS

(a) *Phenol.*—U.S.P.

(b) *Acetic acid.*—Glacial.

(c) *Hydrochloric acid.*—Reagent strength and grade to be diluted as required.

(d) *Ammonium hydroxide.*—Reagent strength and grade to be diluted as required.

(e) *Sodium carbonate.*—C.P. anhydrous.

(f) *Ethyl alcohol.*—70% v/v.

(g) *Butyl alcohol.*—Normal.

(h) *Solvent System.*—#1—Phenol, 150 g; H_2O, 48 g; glacial acetic acid, 2 g;
#2—Phenol, 80 g; H_2O, 20 g.
#11—*n*-Butyl alcohol, 200 ml; concd NH_4OH, 2 ml; H_2O, 88 ml; ethyl alcohol, 40 ml.

DETERMINATION

For this work, these solns may be used over a period of about two weeks without renewing, even though they may show some deterioration.

TABLE 1.—*Color reactions produced by various reagents on paper swatches from chromatograms*

DYE	CONCD. HCl	CONCD. H_2SO_4	10% NaOH	CONCD. NaOH
		*Group 1**		
Auramine	Decolorized	Almost decolorized	Decolorized	Paler
		Group 7		
D&C Red 8	Red brown	Deep red	Orange-yellow	Orange yellow
D&C Red 10	Lavender to violet	Same	Pink	Pink
D&C Red 14	Lilac	Lilac	Orange	Orange
D&C Red 31	Red brown	Deeper red brown	Brown orange	Orange
D&C Red 34	Lavender pink	Same	Pink	Pink
Ext D&C R12	Pink or red	Deeper red	Orange	Little change
		Group 8		
FD&C Orange 2	Pink	Bright pink	Orange pink	Pink
FD&C Red 32	Pink	Deep pink	Orange pink	Orange pink
FD&C Yellow 3	Deep pink	Lilac	Bright yellow	Bright yellow
FD&C Yellow 4	Deep pink	Lilac	Bright yellow	Bright yellow
D&C Green 6	Blue green	Dark green	Blue green	Little change
D&C O 17	Little change	Lavender	Little change	Little change
D&C Red 17	Pinker	Lavender	Blue, then fades	Fades
D&C Red 18	Lavender blue to blue	Blue, then green	Magenta	Magenta
D&C Red 35	Violet pink	Violet	Yellow orange	Orange yellow
D&C Red 37	Orange	Orange-red	Brilliant pink	Lighter but strong pink
D&C Violet 2	Fades	Green blue	Bright blue	Bright blue
D&C Yellow 11	Slightly deeper	Yellow	Decolorized	Fades
Ext. Orange 2	Yellow	Yellow	Brown	Orange brown
		Group 2		
D&C Y 7 & 8	Yellow	Yellow	Yellow	Yellow-green fluorescence
Ext. D&C R5	Fades, almost colorless	Red orange	Bright pink	Bright pink
D&C Orange 15	Yellow	Orange	Blue violet	Blue violet

* For groupings, see Koch, L., *This Journal*, 26, 245 (1943).

TABLE 2.—*Certifiable coal-tar dyes arranged according to Freeman's solubility sub-grouping, giving some chromatographic solvent systems for migration and/or separations*

KOCH'S[a] GROUP	COLOR	COLOR INDEX NO.	SPOTTED FROM	DEVELOPING SOLVENT SYSTEM	SOLVENT SYSTEM (1)	R_f VALUE	
	Auramine	655	70% Alcohol	MeOH-Formic Acid	12	0.87	
			70% Alcohol	2–4 Lutidine	24	0.81	
1 and 8	D&C Red 37	749	70% Alcohol	BuOH-Acetic Acid	9	0.90	4 hrs
			70% Alcohol	2–6 Lutidine (no water)		0.86	
2	FD&C Red 3	773	70% Alcohol	80% Phenol	2	0.64	
2	D&C Yellow 7	766	Sl. HCl	1% NH_4OH	6	0.76	
			NaOH			0.67	
			70% Alcohol		11	0.20	Compact spot
2	D&C Red 39	—	Sl. HCl	1% NH_4OH	6	0.86	
			Sl. HCl	Phenol-Acetic Acid-H_2O	1	0.93	
2 and 7	D&C Red 31	35	Sl. HCl	Phenol-Acetic Acid-H_2O	1	0.93	
2	Ext. D&C Red 4	777	Sl. HCl	BuOH-Acetic Acid	9	0.30	
2 and 7	D&C Red 14	214	Sl. HCl	BuOH-Acetic Acid	9	0.81	Spreads
2 and 7	D&C Red 15		Sl. HCl	80% Phenol	2	0.81	Compact spot
		214	Sl. HCl	Phenol-Acetic Acid-H_2O	1	0.83	Compact spot
2	D&C Yellow 8	766	Acetic	1% NH_4OH	6	0.90	Spreads
			NaOH			0.70	
2	D&C Orange 8	—	70% Alcohol		11	0.27	Compact spot
			70% Alcohol		11	0.30	Compact spot
7	D&C Red 10	189	HCl	Phenol-Acetic Acid-H_2O	1	0.79	
7	D&C Red 8	165	HCl	Phenol-Acetic Acid-H_2O	1	0.85	
3	D&C Red 7	163	70% Alcohol	MeOH-Formic Acid	12	0.82	Only fair
7 and 2	D&C Orange 15	1027	Na_2CO_3	Phenol-Acetic Acid-H_2O	1	0.92	
8	FD&C Orange 2		70% Alcohol	MeOH-Formic Acid	12	0.75	
8	FD&C Red 32		70% Alcohol	MeOH-Formic Acid	12	0.8 ±	best so far but shows 2 color zones
8	FD&C Yellow 3	22	70% Alcohol	MeOH-Formic Acid	12	0.88	
8	FD&C Yellow 4	61	70% Alcohol	MeOH-Formic Acid	12	0.81	
8	D&C Green 6		70% Alcohol	2–6 Lutidine (no H_2O)		0.88	
8	D&C Orange 17		70% Alcohol	80% Phenol	2	0.85	
8	D&C Red 18		70% Alcohol	2–6 Lutidine (no H_2O)		0.86	
8	D&C Red 35	69	70% Alcohol	Phenol-Acetic Acid-H_2O	1	0.9	Only fair
8	D&C Violet 2		70% Alcohol	Phenol-Acetic Acid-H_2O	1	0.90	
8	Ext. D&C Orange 2		70% Alcohol	0% NH_4OH-Amyl Alcohol	18		Seps. components
			70% Alcohol	2–6 Lutidine (no H_2O)		0.86	Best so far

[a] Koch, L., *This Journal*, **26**, 245 (1943).

In the largest tank, put Solvent System #1 to a depth of about 3/8″ and on the center of the tank bottom place a 50 ml beaker about one-fourth full of glacial acetic acid. Cover tank and allow reagents to equilibrate for about an hour.

In another tank put Solvent System #2 to a depth of about 3/8″ and cover.

In the third tank, preferably taller than the others (such as a one l cylinder) put Solvent System #11 and close tightly.

Authentic Dyes.—FD&C Reds 1 and 2; FD&C Blue 2; FD&C Green 2; FD&C Yellow 1, 5, 6; FD&C Orange 1.

In order to obtain chromatograms of authentic dyes for later reference work and also to familiarize collaborators with the chromatographic behavior of certain dyes under different conditions of *p*H and in some of the more widely applicable solvent systems, eight certified food colors are included with the collaborative samples.

PROCEDURE FOR PREPARING CHROMATOGRAMS OF AUTHENTIC DYES

Make a soln of each one of the dyes using about 10 mg of dry dye in 10 ml of H_2O. Take two 2 ml portions from each dye soln. To one portion add 4–5 drops of concd HCl; to the other portion, add dry Na_2CO_3 to distinct alkalinity.

Mark with pencil a point or circle about 1.5″ from the end of a strip of Whatman No. 1 filter paper, 1.5–2″ wide, cut to length required for the tank in which it will be used. If tank permits, several spots may be deposited separately along the end of a wider strip of paper. Spot the dye soln on the marked point, using a slender glass rod (about 3–4 mm diam.). Keep the color spot compact and about 3/8″ in diam. By repeated application, use sufficient soln to give a well-colored area (3–4 "dips" are usually enough). Allow spot to become almost dry between applications. After the final drop, hang the paper strip in the developing tank while the dye is still damp. Take care that the paper hangs free and straight and that it is fastened securely to a support at the top. It should dip into the solvent about ½″.

Spot all dyes from acid and from Na_2CO_3 solns and develop in solvent system #1. In addn, using Yellow 5, Blue 1, and Green 2, develop chromatograms spotted from Na_2CO_3 in solvent systems #2 and #11.

It is desirable to run at least triplicate strips of each dye in order that one may be used for spot testing, one for spectrophotometric examination, and one held for reference.

Solvent systems Nos. 1 and 2 travel relatively slowly and if the desired separation is not effected in 5–6 hrs, it may be continued overnight. A longer run will in most instances increase the distance between resolved spots. Solvent system No. 11 moves fairly rapidly and will probably serve the purpose in 4–6 hrs. If run overnight, the containing vessel should be air-tight or the advancing liquid will dry.

When a completed chromatogram is removed from the developing tank, mark the solvent front with pencil. The R_F (rate of flow) value is the distance traveled by the sample divided by the distance traveled by the solvent, measured from the point of deposit of the sample to the solvent front.

The dye solns furnished are prepared to represent some hues and combinations of dyes which might be met in routine analysis of food products for color. The colors of these solns are in themselves much more intense than those usually found in food samples. The aliquot taken for chromatographing, however, approximates the depth of colored soln that might be obtained after the dye material has been to some degree separated from the product in which it appears, and concd to a small volume.

PROCEDURE FOR CHROMATOGRAPHING UNKNOWN SOLUTIONS

I. "Leaf Green"

To ca 2 ml of the soln add Na_2CO_3 until alk. Spot sufficient to give a sub-

stantial amount of color on a paper strip and develop in solvent system #11. Run three strips.

II. "Chocolate"

Take two 2 ml portions of the soln. Make one portion acid by adding 4–5 drops of concd HCl; make the other portion distinctly alk. with anhyd. Na_2CO_3. Spot on separate paper strips and develop the acid spot in solvent No. 2; the alk. spot in solvent No. 11. Run three strips in each solvent system.

III. "Raspberry"

To 2 ml of the soln add 4–5 drops of concd HCl ; spot a substantial amount on a paper strip and develop in Solvent System #1. Run three strips.

Verification of coloring matter may be made by direct visual comparison with chromatograms of known dyes, although R_F values in cases of mixtures will probably be only relatively the same as for the dyes alone. Following this, the appropriate portion of the chromatogram may be cut out and compared with a swatch from the chromatogram of known dye, using the usual spotting reagents (*Methods of Analysis*, 7th Ed., Chap. 34, Table 1). Finally, using the Beckman spectrophotometer, compare the spectrophotometric curve prepared from a chromatogram of the unknown dye with that of the known dye which it is thought to be. For this procedure, cut out the spot of color and remove the dye from the paper by means of a small amount of 70% alcohol. Warm if necessary and filter into a 2″ cell. Read against a 70% alcohol blank.

DISCUSSION

At present, it does not seem advisable to be too concerned with R_F values, except as relative figures, for many conditions affect this ratio as applied to dyes and their combinations. For uniformity of results, however, the spot of material to be resolved should not be placed too far up the paper strip since there is a possibility of partial analysis of a mixed solvent system as the liquid travels away from the original surface. It will be observed that in general when using solvent system #1, the so-called high acid dyes do not migrate as far as the less acid ones, and the unsulfonated dyes travel farthest.

It has been estimated that over 90 per cent of the total volume of certifiable colors used are of the azo type. The occurrence also of dyes of the xanthene, indigoid, triphenylmethane, and nitro classifications make it necessary, in considering a possible over-all method for the separation of dyestuffs by means of paper chromatography, to employ more than one solvent system to achieve a satisfactory resolution of the individual colors in a mixture. Chromatograms produced in the work outlined above will illustrate the behavior of some known dyes under different conditions of *p*H and in different solvent systems. They will show where and how separations may be expected and also the appearance in some of the dyes of subsidiary material which might otherwise be confusing in the interpretation of a chromatogram of an unknown.

Tables 1 and 2 indicate the information solicited and reported results. Collaborator No. 1 developed the green sample solution in solvent system No. 1 (1); those results are thus not included here. The findings with that

TABLE 3.—*Results of collaborative work on three sample solutions*

SAMPLE SOLUTION	RASPBERRY			LEAF GREEN		CHOCOLATE		
COLLABORATOR	1	2	DHT	2	DHT	1	2	DHT
SOLVENT SYSTEM	1 (HCl SPOT)	1 (HCl SPOT)	1 (HCl SPOT)	11 (Na_2CO_3 SPOT)	11 (Na_2CO_3 SPOT)	2 (HCl SPOT) & 11 (Na_2CO_3 SPOT)	2 (HCl SPOT) & 11 (Na_2CO_3 SPOT)	2 (HCl SPOT) & 11 (Na_2CO_3 SPOT)
APPROXIMATE LENGTH OF RUN	10 MIN	23 MIN	12 MIN	15 MIN	12 MIN	10 MIN	18 & 6.5 MIN	12 MIN
NO. OF COLORS RESOLVED	4	5	5	3	3	5	4	4
Colors and R_F Values	Red 2 Yellow 6 or Red 1 Yellow 5 Orange 1	Red 2 0.36 Red 1 0.43 Yellow 6 0.45 Yellow 1 0.53 Orange 1 0.68	Red 2 0.15 (.35) Red 1 0.31 (.37) Yellow 6 0.41 (.37) Yellow 1 0.59 Orange 1 0.70 (.78)	Yellow 5 1.008 Green 2 0.12 Orange 1 0.46	Yellow 5 nil Green 2 0.25 Orange 1 0.60	Red 2 Yellow 5 Blue 1 or Green 2 Possibly Yellow 6 or Orange 1	Red 2 0.28 Yellow 5 0.35 Orange 1 0.44 Blue 1 0.11	Red 2 0.27 Yellow 5 0.31 Orange 1 0.56 Blue 1 0.25
Degree of Separation of Colors (1) Poorly defined						Poor in Sol. #2		Yes. Orange 1 & blue 1 in S.S. #2 Red 2 & Yellow 5 in #11
(2) Close but satisfactory for identification		Red 1 & Yellow 6	Red 1 & Yellow 6			Fair in Sol. #11		
(3) Widely spread	Yes	Red 2 Yellow 1 Orange 1	Red 2 ½ Yellow 1 Orange 1	Yes	Yes	Yes, except for Red 2 & Yellow 5 in Sol. #11	Yes	Blue & Orange in Sol. #11. Red & Yellow in Sol. #2
Identification of dye material A. Is the R_F value of the authentic dye and the corresponding one in the sample solution (1) The same?	Yes.	No	No	No	No	No, except in case of Yellow 5	No	
(2) Proportional?		Yes		Somewhat	Somewhat		In Sol. #2 No In Sol. #11 Yes	Fairly
(3) Helpful for use in identification?		Yes	Yes	Yes	Yes		Yes	Yes

SAMPLE SOLUTION	RASPBERRY			LEAF GREEN		CHOCOLATE		
COLLABORATOR	1	2	DHT	2	DHT	1	2	DHT
SOLVENT SYSTEM	1 (HCl SPOT)	1 (HCl SPOT)	1 (HCl SPOT)	11 (Na_2CO_3 SPOT)	11 (Na_2CO_3 SPOT)	2 (HCl SPOT) & 11 (Na_2CO_3 SPOT)	2 (HCl SPOT) & 11 (Na_2CO_3 SPOT)	2 (HCl SPOT) & 11 (Na_2CO_3 SPOT)
APPROXIMATE LENGTH OF RUN	10 MIN	23 MIN	12 MIN	15 MIN	12 MIN	10 MIN	18 & 6.5 MIN	12 MIN
NO. OF COLORS RESOLVED	4	5	5	3	3	5	4	4
B. Are Spot Tests (1) Unsatisfactory?	Yes					Yes		
(2) Characteristic?								
(3) Clear cut?								
(4) Helpful for use in identification?		Yes	Yes	Yes	Yes		Yes	Yes
Do the spectrophotometric curves from chromatographic spots from sample solutions show definite points directly comparable to curve obtained from spots of known dyes?		Yes	Yes	Yes	Yes	Sample too small to get good curves	Yes	Yes
Remarks	Yellow 1 trailed considerably. Unknown yellow spot between Y1 & Y5 but closer to Y5. Small amount of sample did not allow differentiation between R1 & Y6. Looked more like Y6.	Very satisfactory		A pale red spot was resolved above Orange 1 spot which believed to be a subsidiary of Orange 1. No attempt was made to identify it.			See Remarks *re* Orange 1 in Leaf Green. Subsidiary spot not very apparent when known Orange 1 was developed in Sol. 1.	

solvent system, however, agree very well with those of the Associate Referee when the same solvent system and sample solution were used.

No discussion of collaborative samples seems justified at this time.

Table 3 is a tabulation of results of collaborative work on three sample solutions received to date.

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 collaborative work be undertaken.

REFERENCES

(1) TILDEN, D. H., *This Journal*, **35**, 423 (1952).

(2) JASCHIK, S., and KRAMER, M., *Magyar Kem. Folyoirat*, **56**, 306 (1950).

No reports were received on intermediates in triphenyl-methane dyes, ether extract, halogens in halogenated fluoresceins, identification of coal-tar colors, volatile amine intermediates, non-volatile unsulfonated amine intermediates, sulfonated amine intermediates, unsulfonated phenolic intermediates, sulfonated phenolic intermediates, intermediates derived from phthalic acid, lakes and pigments, spectrophotometric testing, heavy metals, arsenic and antimony, and inorganic salts in coal-tar colors.

REPORT ON VITAMINS

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

Work by the Associate Referees this past year has been very gratifying, and from the reports given it is obvious that in most instances they have obtained excellent cooperation from collaborators. The introduction of a new type of Vitamin A supplement has made it necessary for the Referee on Vitamin A in mixed feeds to propose a new procedure for the extraction and separation of this vitamin before it can be measured spectrophotometrically. The Referee concurs in the recommendation of the Associate Referee on Vitamin A in mixed feeds.

Last year a meeting was held at which a method for the determination of Vitamin A in oleomargarine was discussed, and many people appeared to be interested in the subject. As a result of that meeting, the Referee on this subject arranged for a collaborative study. A large number of labora-

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

tories which indicated a desire to collaborate were sent samples. However, as indicated in the report, very few turned in results. This response was quite disappointing, since there is an important need for an officially recognized procedure, with the new standard for oleomargarine going into effect this year. Nevertheless I concur with the recommendations of the Referee on Vitamin A in Oleomargarine to continue collaborative study during the coming year. I also concur with the recommendation of the Referee on Carotene.

The official method for the determination of thiamine in flour has involved the enzyme conversion of cocarboxylase and the adsorption technique that are generally included in the determination of thiamine in natural materials. Because of the insignificant amounts of the bound forms of thiamine in enriched cereal products, it is possible to circumvent these steps. The results of the collaborative study carried out this year do not give a basis for adopting a short method for enriched cereal products at this time. I concur in the recommendations of the Referee on Thiamine in enriched cereal products.

Work on the pyridoxine method did not progress as rapidly as hoped and the Referee was unable to conduct a collaborative study this year but I concur in his recommendation that further work be undertaken. I also concur in the recommendation of the Referee on nicotinic acid, that based on further experience, the method now be made official.

The progress that has been made in the application of the microbiological method for the determination of Vitamin B_{12} to low potency materials now permits the Associate Referee's recommendation for adoption of a First Action method. This has been a difficult problem that involved not only methods of extraction but ways of stabilizing natural forms of the vitamin as well. It is encouraging that the results of this year's study support the conclusion that the method is applicable to feed supplements and I concur in the recommendations of the Associate Referee.

RECOMMENDATIONS

It is recommended*—

(1) That a new procedure be found for the extraction and separation of Vitamin A.

(2) That collaborative study on Vitamin A in oleomargarine be continued during the coming year.

(3) That work be continued on carotene analysis.

(4) That study be continued on the determination of thiamine in enriched flour.

(5) That the method for nicotinic acid be made official.

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* For report of Subcommittee A and action of the Association, see *This Journal*, 36, 52 (1953).

(6) That the microbiological assay for Vitamin B_{12} be continued.

(7) That the method for assay of Vitamin B_{12} feed supplements ranging from approximately 1.0 to 10 mg. of Vitamin B_{12} per pound be adopted, First Action.

REPORT ON VITAMIN A IN MIXED FEEDS

By MAXWELL L. COOLEY (General Mills, Inc., Minneapolis, Minnesota), *Associate Referee*

According to the 1951 report (1) on the collaborative study of Vitamin A in feeds, the status of the method was continued, First Action. Recommendations as to additions and changes in the procedure also were adopted (2). The collaborative study reported in the present article utilized these changes.

The number of collaborators who participated in this study was almost the same as last year—twenty-seven participants in 1952 and twenty-eight in 1951. Twenty-three laboratories collaborated both years.

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 all the feeds was derived from fish liver oil.

The composition of the experimental feed mixtures is given in Table 1. These data show that samples 1 through 5 were poultry mashes. Sample #6 is a dried milk product calf feed obtained from Western Condensing Company, Appleton, Wisconsin.

Examination of Table 1 shows that samples 4 and 5 were identical.

TABLE 1.—*Composition of experimental feed mixtures*

	SAMPLE					
	#1	#2	#3	#4	#5	#6
Wheat bran	10%	15%	5%	10%	10%	Peeble's Calf-Kit
Wheat middlings	15	10	15	10	10	
Corn gluten meal	—	—	5	5	5	
Ground yellow corn	20	20	20	20	20	
Soybean oil meal	40	35	30	40	40	
Dehydrated alfalfa meal	5	10	10	5	5	
Meat scraps	—	10	10	5	5	
Fish meal (Vitamin A-free)	10	—	5	5	5	
Vitamin A (calculated units of A per g)	2.5	4.0	6.0	8.5	8.5	45.0[a]

[a] Average results from shipper and Associate Referee's laboratory show approximately 45 units of A per g.

DISCUSSION OF PROCEDURE AND COMMENTS BY COLLABORATORS

Extraction.—The directions for extraction of the Vitamin A from a sample of feed by refluxing with hexane remains nearly the same as those given in the original method (3). Regarding the use of a Goldfisch extractor for refluxing, 3 collaborators commented that the regular extraction flask used with this apparatus is slightly small to accommodate the specified 100 ml volume. Correspondence with the manufacturer of the Goldfisch extractor indicates that a larger and more suitable flask soon may be available for this purpose.

Tracer for Vitamin A—The present procedure permits the use of a solution of pure β-carotene as an alternate source of tracer carotene. Of the 27 collaborators who participated this year, 13 continued to use dehydrated alfalfa meal to supply sufficient carotene to follow the band of this pigment in the chromatogram. Except for two laboratories which employed an ultraviolet light for tracing the progress of the Vitamin A band down the adsorption column, the other laboratories used a solution of β-carotene for this purpose.

Sample Weight—Although the prescribed sample weight was 10 g, approximately 25 per cent of the participants found it necessary to increase the weight for the lower potency sample and decrease it for the high potency sample. Except for one collaborator who used less than 1 g for sample 6, the use of 2–5 g was preferred by those who made any reduction in sample weight for this feed. The largest weight of material taken for assay on the low potency feed was 20 g. Experience indicates that a 20 g sample is about the maximum permissible for efficient extraction with the volume of solvent specified.

Adsorbent.—The procedure specifies equal parts by weight of diatomaceous earth (Johns-Manville Hyflo Super Cel) and Magnesia (Microbrand #2641, Westvaco Chlorine Products Corporation, Newark, California). The question has arisen as to the difference between magnesia #2641 and magnesia #2642; the latter is recommended for the chromatographic determination of carotene (4). As far as we have been able to ascertain, the only difference between the two products is that #2641 is heated at a higher temperature during the final stages of processing than is #2642.

The Westvaco Company has been contacted in an effort to obtain a magnesia, specifically for the determination of Vitamin A, which would be controlled by the manufacturer to give 90–100% recovery of Vitamin A. At this writing, no satisfactory arrangement has been made for providing such an adsorbent. However, the availability of a specific magnesia for the purpose seems feasible. This would mitigate to a considerable extent the erratic results obtained for Vitamin A in feeds due to nonuniformity of adsorption of various shipments of the powder.

As for using magnesias #2641 and #2642 interchangeably for the procedure in question, experiments at this laboratory indicate that either type may be used; if both absorbents are conditioned properly, recovery of Vitamin A is high. On the other hand, two collaborators stated that they have found #2641 to be more suitable than #2642. Apparently further investigation of adsorbents is necessary.

DISCUSSION OF COLLABORATIVE DATA

The average values of all results received for the six feed samples as compared with the calculated value for each sample are presented in Table 2. Examination of the results reported on samples 4 and 5 (dupli-

TABLE 2.—*Average values found for vitamin A in collaborative samples*

COLL. NO.	SAMPLE #1	SAMPLE #2	SAMPLE #3	SAMPLE #4	SAMPLE #5	SAMPLE #6
1	3.2	4.4	5.7	7.6	8.8	47.2
2	2.2	3.6	5.2	7.6	7.1	50.6
3	4.1	7.4	8.2	10.4	10.2	58.3
4	3.2	3.7	5.1	5.7	7.2	39.6
5	1.7	3.2	4.6	7.1	7.0	42.8
6	3.0	5.0	6.8	8.5	8.5	46.3
7	4.1	5.8	8.1	9.1	8.2	36.0
8	3.4	5.1	3.7	6.8	10.0	56.2
9	1.8	4.0	5.5	7.5	7.6	42.0
10	2.7	2.7	4.8	5.6	5.4	32.6
11	3.0	4.5	5.1	9.5	9.1	56.2
12	2.0	3.0	4.4	8.0	8.2	48.5
13	2.7	5.3	4.6	7.0	8.8	34.4
14	2.4	4.4	6.1	9.0	9.3	51.8
15	2.0	3.5	5.6	8.1	7.6	44.8
16	4.1	5.5	7.3	10.3	10.1	48.3
17	2.3	4.1	6.0	8.5	8.4	49.5
18	2.6	4.0	5.3	9.7	8.9	60.4
19	4.3	7.7	8.9	10.2	12.1	43.8
20	3.9	5.3	6.5	7.6	9.4	52.6
21	1.5	3.9	16.0[a]	6.6	7.5	48.7
22	5.5	3.4	3.5	3.6	6.5	40.7
23	3.2	4.7	6.3	9.6	10.0	44.4
24	2.1	3.6	5.5	7.6	7.3	39.5
25	2.2	3.8	5.5	7.7	7.8	43.3
26	2.4	4.0	4.2	6.8	7.4	37.7
27	3.1	5.3	7.5	10.0	9.3	43.4
Calcd. Value	2.5	4.0	6.0	8.5	8.5	45.0[b]
Mean	2.91	4.48	5.77	7.99	8.43	45.91
Std. Deviation	0.94	1.17	1.35	1.58	1.39	7.06
Coef. of Variation	32.3%	26.1%	23.4%	19.7%	16.5%	15.4%

[a] Omitted from average.
[b] Average results in Referee's laboratory indicate approximately 45.0 units of A per g.

cates) indicates a generally fair agreement within each laboratory on identical samples. The previous year's collaboration (2) included two sets of duplicate samples; there seems to be very similar accuracy achieved on identical samples in both studies.

Sample 6 is a milk replacement feed for calves. Because of its high dried milk products content, investigation of the applicability of the Vitamin A method to this type of material was desirable. The individual results, as well as the mean and coefficient of variation, indicate about the same agreement on this type of feed as on a poultry mash.

Examination was made of the data in Table 2, depending upon which of the above three tracers was used for following the Vitamin A down the column. As was previously stated, except for two laboratories which employed an ultraviolet light for this purpose, about half of the twenty-five used dehydrated alfalfa meal and the other half used a solution of β-carotene. No definite conclusions could be drawn as to which tracer—alfalfa meal or β-carotene—is the more desirable. Collaborators 11 and 14, who traced with ultraviolet light, reported results which were in better agreement with the means than those of the average participant. However, the accuracy of the values obtained by these two collaborators could not be considered superior to those obtained by a large number of other laboratories using either of the carotene sources as tracers.

Table 3 presents a comparison of the average values for the 1951 and 1952 collaborative studies. Of the 28 collaborators who participated in the 1951 study, 23 also participated in 1952. This table indicates no great change in agreement between laboratories, especially when results on samples of similar Vitamin A potencies are compared. Over-all examina-

TABLE 3.—*Comparison of average values found for Vitamin A in two collaborative studies*

	SAMPLES (UNITS OF VITAMIN A PER GRAM)					
	#1	#2	#3	#4	#5	#6
A.O.A.C. Study for 1952						
Calcd. Value	2.5	4.0	6.0	8.5	8.5	45.0
Mean (27 Collaborators)	2.91	4.48	5.77	7.99	8.43	45.91
Std. Deviation	0.94	1.17	1.35	1.58	1.39	7.06
Coef. of Variation	32.3%	26.1%	23.4%	19.7%	16.5%	15.4%
A.O.A.C. Study for 1951						
Calcd. Value	4.0	6.0	9.0	10.5	6.0	10.5
Mean (28 Collaborators)	4.10	5.31	7.90	9.70	5.46	9.49
Std. Deviation	1.02	1.13	1.48	1.80	1.00	1.41
Coef. of Variation	24.9%	21.3%	18.7%	18.6%	18.3%	14.8%

TABLE 4.—*Essential results of an analysis of variance*

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE
Collaborators	26	62.14[a]
Samples	5	21,132[a]
Interaction (C×S)	130	26.93[a]
Residual	324	6.19
Total	485	
Standard Error of a Single Determination	2.49	

[a] Exceeds 1% level of significance.

tion of these data shows a slightly better agreement in 1951.

The essential results of an analysis of variance are given in Table 4. Examination of this analysis demonstrates:

(1) There is a a highly significant variation between laboratories; (2) the samples vary as they would be expected to, inasmuch as they were made to have different individual potencies; (3) the highly significant interaction mean square indicates a definite absence of a tendency for each laboratory to obtain consistently high or consistently low results; (4) the standard error of a single determination (square root of residual mean square) is 2.49.

STABILIZED VITAMIN A PRODUCTS

The number of stabilized Vitamin A products available for use in feeds seems to be increasing. These are frequently referred to as dry Vitamin A preparations. One method commonly used in these products to inhibit oxidation of the Vitamin A is to embed the minute globules of Vitamin A oil in a matrix of digestible wax. These waxes are soluble in the extracting solvent (hexane) used in the method for Vitamin A in feeds. Correspondence with various laboratories as well as the 1950 A.O.A.C. report (5) indicate that the procedure is satisfactorily applied to assay of feeds containing stabilized Vitamin A products of this nature.

Two other types of dry A preparations have come to the attention of the Associate Referee. The stabilizing principle employed in one product is to surround particles of crystalline Vitamin A ester with a matrix of gelatin and in the other to envelop the Vitamin A with pectin. In either case the enveloping material is not soluble in hot hexane, and therefore the Vitamin A extraction procedure is not applicable.

Preliminary investigations indicate that saponification is necessary to dissolve the gelatin or the pectin, thus exposing the Vitamin A to extracting solvents. In this case, the vitamin ester is changed to Vitamin A alcohol.

In the regular collaborative procedure, the Vitamin A is extracted

and chromatographed in the ester form. The chromatography is based on the fact that Vitamin A ester is less strongly adsorbed than β-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.

Because saponification is necessary in a method for determining Vitamin A in feeds containing the stabilized A products in question, the following procedure is being investigated:

PROPOSED PROCEDURE

Extraction.—Weigh 5–10 g feed into a Goldfisch extraction flask or any suitable ground glass-joint refluxing flask. Add 60 ml of ethanol and 15 ml 50% KOH. Reflux for 30 min. Cool and transfer contents of flask to a 250 ml separatory funnel. (This separatory funnel must have a large opening, preferably using a pinch-cock rather than a ground-glass stop-cock, to facilitate passage of suspended feed particles.) Wash flask into separatory funnel with 60 ml H_2O. Extract the Vitamin A and carotenoids by shaking the suspension with 3 separate 30 ml portions of hexane. Break up emulsions with 2–5 ml ethanol. Collect extracts (ca 90 ml) and wash at least 3 times with 25 ml H_2O each. Make up to 100 ml with acetone, and shake thoroly with ca 5 g anhyd. Na_2SO_4.

Chromatography.—Pass a 50 ml aliquot through a magnesia-Hyflo Super-Cel column prepared according to the procedure (1). Elute with at least 100 ml 10% acetone in hexane. The eluate then contains the Vitamin A alcohol and the carotene from the feed. Make up to suitable vol. and mix.

Colorimetry.—Read the carotene present in the soln at 440 mμ. Evap. with mild heat and reduced pressure a suitable aliquot of this soln and dissolve the residue in sufficient $CHCl_3$ so that 1 ml after the addn of $SbCl_3$ reagent will give transmittance readings which are within the range of 30–65%. Set colorimeter at 100% transmission, using a blank comprised of 1 ml $CHCl_3$ and 10 ml of Carr-Price reagent. Place the assay tube in the colorimeter and add rapidly 10 ml of Carr-Price reagent. Using 620 mμ wavelength light, take max. colorimetric reading (color begins to fade within 3–5 sec.). Apply to a standard Vitamin A curve. Correct the results for the amount of carotene present in the soln. Use for this purpose a curve prepared from the reaction of $SbCl_3$ reagent with aliquots from a standard carotene (90% *beta*-10% *alpha*) soln under the same conditions as given above. Calc. the units of Vitamin A and the micrograms of carotene per g of feed.

The above procedure should be applicable to the detn 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.

The correction of the Vitamin A results because of the spurious color produced by the carotene present in the final color reaction has been reported in an earlier paper by Cooley, *et al.* (6).

Pending further developments, the proposed method may be desirable for the simultaneous detns of carotene and Vitamin A in feeds.

SUMMARY

Collaborative study of the procedure for the determination of Vitamin A in mixed feed, First Action, was continued in 1952. Results of this study indicated slightly less accuracy in applying the method than in the

previous collaboration. In part, this may be due to the use of generally less potent samples in the present work. One sample approached the lower sensitivity limit of the method, containing between 2 and 3 units of A per gram. Considering that 1 unit of Vitamin A is equivalent to 0.344 mmg of Vitamin A acetate, the method appears to be reasonably satisfactory for determining one or less p.p.m. of Vitamin A. As was pointed out in a previous report (1), because saponification is necessarily omitted, the interference of lipids and other extracted materials reduces the accuracy, especially when the procedure is applied to feeds containing low amounts of Vitamin A.

One collaborative sample in the present study was a feed having dried milk products as one of its main components. Results indicate the method applies about as well to this type of material as it does to regular poultry mashes.

Because of the availability of dry Vitamin A products for use in feeds which are stabilized by embedding the Vitamin A in a matrix of either gelatin or pectin, a modification of the present method is necessary. The proposed procedure employing saponification, as given in this report, is being investigated for applicability to feeds containing the above stabilized Vitamin A products. Saponification eliminates certain lipid interference, but because of the change in the chromatography, probably all of the carotene derived from the feed is present in the final Vitamin A solution. Influence of this carotene in the blue color reaction must therefore be subtracted. Further study of the modified method may indicate that it could be applied to the determination of Vitamin A in feeds without regard to source of the A. Possibly simultaneous determination of the carotene in feeds also could be accomplished.

ACKNOWLEDGMENT

Appreciation is extended to Mr. Ara O. Call, Western Condensing Company, Appleton, Wisconsin, for furnishing the dried milk product feed as well as the Vitamin A oil used in this study.

RECOMMENDATIONS

The Referee recommends* the status of the method be continued, First Action, subject to further collaborative study. Such a study also should include collaboration on samples of feed containing the stabilized Vitamin A products mentioned in this report. The prescribed modification of the method, employing saponification, should therefore be investigated.

LIST OF COLLABORATORS

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* For report of Subcommittee A and action of the Association, see *This Journal*, 36, 52 (1953).

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REFERENCES

(1) *This Journal*, **35**, 101 (1952).
(2) COOLEY, M. L., *ibid.*, **35**, 706 (1952).
(3) COOLEY, M. L., CHRISTIANSEN, J. B., and KOEHN, R. C., *Anal. Chem.*, **21**, 593 (1949).
(4) *Official Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Washington, D. C., 1950, p. 769.
(5) COOLEY, M. L., *This Journal*, **34**, 370 (1951).
(6) COOLEY, M. L., CHRISTIANSEN, J. B., and SCHROEDER, C. H., *Ind. Eng. Chem., Anal. Ed.*, **17**, 689 (1945).

REPORT ON VITAMIN A IN MARGARINE

BLANK OIL METHOD COMPARED WITH THE CHROMATOGRAPHIC PROCEDURE

By J. B. WILKIE (Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C.),
Associate Referee

This report on Vitamin A will be divided into three sections: (1) Some new developments in the Vitamin A field that are pertinent to problems of measuring Vitamin A; (2) A discussion of the results of a preliminary study in which the commonly used method (the blank oil method) has been compared with chromatographic procedures; (3) Detailed results of the collaborative study on margarine that has been conducted during the past few months.

COLLABORATIVE METHOD*

I. SPECIAL INSTRUCTIONS*

The same 10 g sample of margarine after saponification and extraction is used for the straight spectrophotometric and the optical neutralization evaluations, and the recommended $SbCl_3$ method check.

Before reporting results, preliminary trials should be made to assure checks within 5% of each other. If checks within 10% of each other cannot be obtained, the Associate Referee should be consulted.

After the method has been mastered, data and the calculated results should be submitted in triplicate from the direct spectrophotometric method, the optical neutralization modification, and the $SbCl_3$ method, both before and after chromatography—a minimum of 15–18 detns for each sample. A minimum of six saponifications and extractions must be completed.

Carotene spectrophotometric absorptions and corresponding Vitamin A potencies should also be reported in triplicate in addition to the corresponding Vitamin A potencies by the direct spectrophotometric, optical neutralization spectrophotometric, and $SbCl_3$ methods. The results of this study should be reported promptly, within one month if possible.

Please include comments on any phase of the methods used in this study or in the manner of conducting the work.

II. EQUIPMENT

General Precautions.—Thruout the procedure, protect the Vitamin A from strong illumination, either by working in subdued light or by using non-actinic glassware and avoiding undue exposure to air. Complete all steps in the procedure as promptly as consistent with the detailed instructions.

(a) *Spectrophotometer and Cells.*—Use any reliable ultraviolet spectrophotometer with any suitable ultraviolet source (the incandescent lamp is not a suitable source under 320 mμ). A spectrophotometer equipped with a continuous spectrum source reading to 200 mμ is recommended. Matched quartz cells with internal light path equal to 1 cm are preferable but sufficiently transparent cells of other materials

* Instructions and procedure have been revised primarily with editorial changes, in keeping with comments and criticisms of collaborators and with points raised during discussion at the time the report was presented at the A.O.A.C. meeting. The original instructions included the details for the antimony trichloride procedure, *Methods of Analysis*, A.O.A.C., 7th Ed., p. 767.

may be used. The cells should be matched or, if not, suitable corrections should be made.

(b) *Direct Reading Filter Photometer or Direct Reading Photoelectric Spectrophotometer for the Antimony Trichloride Method.*—Any direct reading photoelectric filter photometer or direct reading spectrophotometer may be used. The optical mechanism or filters should provide light at 620 mμ with transmission band narrowed sufficiently to give as nearly a straight line absorbance curve as possible. Test tubes should be matched for the particular photometer used.

(c) *Chromatographic Tubes.*—12 mm o. d. × 2½″ long with funnel on the upper end of tube; the lower end with a stem 8 mm in diam. and 1½″ long, provided with sealed-in disk of medium porosity which does not offer excessive resistance to flow of eluate.*

(d) *Standard Micro Bell Jar.*

(e) *Glass-Stoppered Volumetric Flasks.*—5 and 10 ml.

(f) *Long Wavelength Ultraviolet Lamp.*—This lamp should be just sufficiently intense to reveal the Vitamin A fluorescent band because Vitamin A may be easily destroyed by too much ultraviolet light. Such a lamp may be self-constructed, or a suitable lamp may be purchased complete from a scientific supply house.†

(g) *Vacuum gauge.*—Provided with a bleeder for controlling the vacuum applied to the column through the micro bell jar.

III. REAGENTS AND STANDARDS

(a) *KOH Solution.*—50%.

(b) *Ethanol,* 95%.—Commercial grade but with spectrophotometric characteristics as for absolute alcohol.

(c) *Absolute Alcohol.*—Must meet the following requirements for spectral purity: when measured in a 1 cm quartz cell against distd H_2O, it shall show an absorbance no greater than 0.01 between 350 and 320 mμ and no greater than 0.05 at 300 mμ.

(d) *Sodium Hydrosulfite.*—$Na_2S_2O_4$, J. T. Baker Purified Grade.

(e) *Sodium Sulfate, Anhydrous, Granular.*—A 10% soln not acid (red) to methyl red T. S. Must not absorb Vitamin A under conditions described.

(f) *Ether.*—Must be free from peroxides; use U.S.P. freshly distd, discarding first and last 10% portions; or U.S.P. anesthesia grade in ½ lb. cans. All solvents must be free from interfering absorption.

(g) *Phenolphthalein.*—1 g phenolphthalein in 100 ml alcohol.

(h) *Chloroform.*—Freshly redistd, discarding the first and last 10% portions or treated with 30–50 g anhyd. silica gel desiccant Grade A, Type 4 of the Davison Chemical Corporation, Baltimore 3, Md., before decanting or filtering.

(i) *Antimony Trichloride.*—Colorless, translucent crystalline mass or lumps. Keep in tight glass enclosed bottles in cool place. Dissolve contents of ¼ lb bottle (113.4 g) in 300–400 ml of hot $CHCl_3$. Add ca 5 g of silica gel desiccant described above, (h). Filter rapidly through sufficient filtering material to eliminate any possible turbidity. Finally dil. to ca 500 ml.

(j) *Reference Standard Vitamin A.*—Obtained from the U. S. Pharmacopeia, 46 Park Avenue, New York City.

(k) *$SbCl_3$ Stock Standard Vitamin A Solution.*—Prepare according to directions under Antimony Trichloride Procedure, A.O.A.C., *Methods of Analysis,* 7th Ed., p. 767.

(l) *Optical Neutralization Stock Standard Vitamin A Solution.*—Optical neutralization also requires a reference standard in alcohol contg an equivalent concn of

* No. G 9855, Scientific Glass Products Co., 49 Ackerman Street, Bloomfield, N. J.
† Fisher Scientific Co. No. 11-984-30, Model L, or *This Journal,* **28,** 76 (1945).

Vitamin A to match a 50% diln of 15,000 units/lb margarine or 33/2 = 16.5 units/g of Vitamin A. This in turn initially requires a stock soln of Vitamin A prepd by saponifying the contents of two U.S.P. Standard Vitamin A Acetate Capsules, according to U.S.P. or A.O.A.C. fish liver oil methods, and making to 50 ml vol. with absolute ethanol. To correct for possible loss of Vitamin A in the preceding operation, a 10% diln is measured on the spectrophotometer at 325 mμ. 10 × absorbance × 19 = units per ml of the stock soln. From this calcd strength the vol. to make to 10 ml to give the required 16.5 units/ml concn may be calcd.

(m) *Magnesium Oxide.*—Westvaco Chlorine Products Corp., Newark, California, #2641.

(n) *Analytical Filter Aid.*—Celite (Johns Mansville).

(o) *Adsorbent Mixtures.*—(1) *1 MgO:1 Celite (Body Mixture).*—Weigh equal parts of the components. Transfer to a ½-gallon screw lid Mason jar or other wide-mouth bottle for mixing. Mix thoroly by tumbling and/or shaking vigorously for 15 min. The jar should not be more than ⅔ full. After mixing, transfer to a wide-mouthed storage container with a tight fitting screw cap which should be kept tightly closed except when loading columns.

(2) *3 MgO:1 Celite:6 Anhyd.* Na_2SO_4 *(Header Mixture).*—Mix and keep in closed container as above.

The strength range of the above absorption mixtures is not very critical, although they may lose their activity by undue exposure to air. To determine the adsorption index in terms of g of FD&C Yellow #4 adsorbed per g of adsorption mixt., see *Anal. Chem.*, **24**, 1409–1411 (1952).

(p) *Petroleum Ether.*—The petr. ether used in this method should be substantially free from fluorescence and should have a transmission of greater than 85% at 300 mμ when measured in a 1 cm quartz cell against a no cell blank set at 100% transmission.*

(q) *Eluting Solutions.*—Four, consisting of the petr. ether contg 0.2, 0.5, 1, and 5% respectively, of absolute ethanol. It is convenient to have 200–500 ml of each of the above solns on hand.

IV. PROCEDURE

(a) *Saponification.*—Weigh 10 g margarine into a 300 ml beaker, add 75 ml 95% ethanol, then 25 ml 50% (w/w) KOH. Heat on elec. hot plate, stirring lumps to disperse sample completely. Bring to boil quickly and boil vigorously for 5 min. Remove from heat and allow to stand at room temp. for 20 min. with occasional stirring. Avoid rapid cooling.

(b) *Extraction.*—Transfer soln to 500 ml separatory funnel. Rinse saponification beaker with 100 ml of distd H_2O in several portions, adding these rinsings to the separatory funnel. Add 100 ml ether. Shake vigorously, and allow to stand ca 2 min. Separate aq. portion into another 500 ml separatory funnel. Likewise ext. this aq. fraction successively 4 times with 50 ml portions of ether, adding each ext. in turn to the original ext. In any case of slow separation, add 2–5 ml of 95% ethanol and swirl gently. Pour two 100 ml portions of distd H_2O thru the combined ether exts, swirling gently, and separate. Ext. these two rinses with two 50 ml portions of ether, adding them to the original ether exts. Pour two 100 ml portions of distd H_2O through the combined ether ext. and discard each washing without shaking. Add ca 10 ml of dil. KOH (0.02 *N*), shake vigorously, remove after separation occurs, then add successive portions of distd H_2O, with gentle agitation, removing each until the rinse H_2O is free of alkali as shown by phenolphthalein test. Allow

* Can be obtained in the boiling range 30–65°C. from the J. T. Baker Co. of Phillipsburg, N. J.

ether soln to stand 5 min., discard the separated H_2O, then transfer to a 400–500 ml tall beaker, add 3–5 g anhyd. Na_2SO_4, and stir moderately to remove traces of H_2O. Decant the ether ext. into another clean 400–500 ml beaker, rinsing the Na_2SO_4 thoroly (ca 6 times) with small portions of ether.

(c) *Evaporation of Extract.*—After adding ca 5 g of Na hydrosulfite to the ether soln, evap. on a steam bath to a vol. of ca 25 ml. Transfer the soln by decanting into a 50 ml beaker with 6 vigorous washings of the Na hydrosulfite using 2–5 ml portions of ether to remove Vitamin A completely from residue. Evap. this soln on steam bath to cessation of bubbling when stirred with a small glass rod. Heat for *precisely* 2 min. more, remove from the steam bath, and immediately subject to a vigorous stream of N for 1 min., then add 5 ml of petr. ether and make to vol. in a 10 ml glass-stoppered volumetric flask with petr. ether, for chromatographic separations.

(d) *Chromatography.*—Prepare a column by using the chromatographic tube specified under *Equipment*, fitted into a micro bell jar with a 50 ml beaker. Add sufficient 1:1 mixt. of MgO and Celite into tube, packing firmly but not tightly with a blunt rod under a 20–30″ vacuum to form a ¾″ column. This is called the body adsorbent segment. Next add header mixt. (MgO-Celite-Na_2SO_4), packing as for the previous segment, until a layer ½″ thick is in place.

Pipet 5 ml of prepared ext. into 10 ml beaker and reduce to ca 2 ml on a steam bath or with a stream of N. The remaining 5 ml of this soln may be evapd to dryness, dissolved in $CHCl_3$, and used for the $SbCl_3$ test.

After wetting the column with petr. ether and just before the surface is dry, pour the 2 ml of sample soln rapidly onto the column. A vacuum of 20″ on the column is preferred, although a full vacuum of 30″ will serve during the chromatographic separation.

The surface of the column should be completely covered with solvent at all times. The Vitamin A band is detected as a green fluorescent area with the weak ultraviolet lamp. The chromatography is carried out in a semi-darkened room. A completely darkened room is of advantage until the analyst is thoroly familiar with the bands to be observed.

The Vitamin A or carotene and certain extraneous materials form a compact series of bands in the header segment of the column which proceed into the body adsorbent segment where the initial separations become greater, so that the successive fractions may be collected as separate eluates. The bands to be saved are carotene, an orange band which appears first, and Vitamin A, a greenish fluorescent band that follows. The eluate preceding the first fraction to be saved is discarded. Each eluate fraction must be collected in a separate clean beaker and care should be taken to rinse the bottom of the filter disk and stem of tube, inside and out, at each change. An attempt should be made to elute the carotene with petr. ether alone. If the carotene band appears stalled for several (5) min., add 0.5 ml of 1% alc. eluting soln followed by 2–5 ml portions of petr. ether. Repeat this sequence as many times as necessary for slow, continuous movement on the column. When the carotene band reaches the filter disk, removal of the band is completed with petr. ether alone. Evap. the carotene eluate on a steam bath to cessation of bubbling when stirred with a glass rod, then to dryness at room temp. with a stream of N for 1 min. Take up residue in petr. ether and make to vol. in a 10 ml volumetric flask for spectrophotometric detn.

The Vitamin A band is then eluted by using 5% alc. eluting soln. This speeds up movement on the column and great care must be taken to collect the Vitamin A fraction selectively. When the initial point of the Vitamin A band reaches the filter disk a clean receiving beaker is put in place. Elution of the band is continued until the last tip of it disappears into the filter disk. Mark the meniscus of the eluant above the column and permit it to lower just ¼″ to obtain all the Vitamin A. Wash the

bottom of the disk and the stem inside and out with petr. ether from a wash bottle fitted with an upright nozzle, collecting the washings with the eluate. Evap. eluate on steam bath to cessation of bubbling when stirred with a glass rod, then to dryness at room temp. with a stream of N for 1 min. Promptly take up residue with absolute ethanol and make to vol. in a 10 ml volumetric flask for spectrophotometric and optical neutralization evaluations.

If an orange carotene band preceding the fluorescent Vitamin A band is not present, then elution of the Vitamin A band is accomplished by addn of 0.5 ml of 1% alc. eluting soln, followed by 2–5 ml petr. ether, repeating this sequence as many times as necessary for slow movement of the Vitamin A band to the filter disk. Final elution is then accomplished by adding 5% alc. eluting soln with collection of the eluate carried out as described above. Take up the final residue in absolute ethanol and make to vol. in a 10 ml volumetric flask for spectrophotometric and optical neutralization evaluations.

(e) *Spectrophotometric Determination.*—(1) *Carotene:* Obtain spectrophotometric transmittance of the carotene soln at 450 mμ and calc. the carotene content of the sample and its related Vitamin A value as follows:

A = Absorbance in petr. ether at 450 mμ.

l = Cell length.

W = g sample per ml of final measured soln.

$\frac{A \times 4.17^*}{lW}$ = mmg carotene per g of sample.

$\frac{A \times 4.17^* \times 455}{lW}$ = mmg carotene per pound sample.

$\frac{A \times 6.95^* \times 455}{lW}$ = units Vitamin A per pound.

Transmittance at 436 mμ may also be read as a check with the formula in use in current A.O.A.C. collaborative studies on carotene.

(2) *Vitamin A.*—*A. Use of Instrument.*—The directions are written specifically for the Beckman Model DU spectrophotometer. For all the measurements required, use the 0.1 sensitivity position exclusively. The sensitivity knob should be turned completely or nearly completely clockwise.

The directions lead to two final values for the Vitamin A potency of the sample: the purity index corrected value, and the optical neutralization value which may be regarded as checks of each other and should correspond closely. Further study will determine which is to be preferred.

B. Spectrophotometric Readings.—Place the following in the quartz cuvettes: the solvent in the first position, the sample soln in the second, and the standard soln in the third. With the solvent set at 100% transmission, record transmittancies of the sample soln in the usual manner at the following wavelengths: 310, 325, 334, 340, 345, 350, 436, and 450 mμ.

To calc. optical neutralization values, the following readings are taken at the above wavelengths. The sample soln is used as the reference, and the reference point is the original transmittancy reading of the sample soln at 325 mμ. For the first reading, set the wave length dial at 310 mμ. Balance the dark current in the usual manner, then with the sample soln in the light path and the transmission dial set at the reference point, open the photo cell shutter to "on" and adjust the meter reading to

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* The formulas are based upon an $E^{1\%}_{1\text{ cm }450\text{ m}\mu} = 2400$ and the definition that one unit of vitamin A = 0.6 mmg of *beta* carotene.

0 with the slit knob of the instrument. With photo cell shutter closed, shift the standard soln into the light path. Turn the photo cell shutter-switch to "on," balance the meter reading to 0 with the transmission dial, and record this dial reading. Repeat this sequence of operations for each of the other listed wavelengths.

C. Calculations.—1. The uncorrected Vitamin A value, based on absorbance at 325 mμ: convert the absorbance value read to $E^{1\%}_{1\text{ cm. }325\text{ m}\mu}$ by dividing by the per cent of the sample in the measured soln. Then $E^{1\%}_{1\text{ cm. }325\text{ m}\mu} \times 1900 \times 455 =$ units Vitamin A per lb.

2. The corrected value: Corrected value = uncorrected value × purity index.

Purity index is detd as follows, where A is absorbance value of the sample at the indicated wave length:

$$\text{Purity Index} = \text{P.I.} = \frac{\frac{A_{325}}{A_{340}}}{1.34} \quad \text{if} \quad \frac{A_{325}}{A_{340}} < 1.34$$

$$= \frac{2.68 - \frac{A_{325}}{A_{340}}}{1.34} \quad \text{if} \quad \frac{A_{325}}{A_{340}} > 1.34.$$

3. Optical neutralization value. Per cent of standard neutralized:

$$\frac{\frac{A'_{340}}{A'_{325}} \text{ for sample} - k}{1 - k} \times \frac{1}{\text{P.I.}} \times 100, \text{ if } \frac{A_{325}}{A_{340}} < 1.34$$

Per cent of standard neutralized, if

$$\frac{\frac{A'_{340}}{A'_{325}} \text{ for sample} - k}{1 - k} \times \text{P.I.} \times 100, \text{ if } \frac{A_{325}}{A_{340}} > 1.34$$

where A' is the absorption value corresponding to the optical neutralization transmission reading (transmission-absorption table) for the wavelength indicated, and k is $\frac{A_{340}}{A_{325}}$ for standard. This approximates 0.73. Then:

% Standard neutralized × units/ml of standard = units/ml of sample

and

$$\frac{\text{units/ml sample}}{\text{wt sample/ml}} \times 455 = \text{units/lb}$$

The Vitamin A potency of the margarine, where carotene is present, is the sum of the units of Vitamin A found in the two forms.

NEW DEVELOPMENTS

Recent studies, primarily from European laboratories (1, 2) indicate that the stereoisomers of Vitamin A and their esters have slight differences in their spectral absorptions. These are small but definite and must be taken into consideration by those working with pure or relatively pure Vitamin A preparations. The solvents used in making the spectral absorption measurements also cause slight differences in absorption and must be taken into consideration for ultimate accuracy.

Cama, Collins, and Morton (2) have published methods for determining

neovitamin A and Vitamin A_2 associated with Vitamin A_1 in natural products. There appears to be some difference in the biological value of these forms. As methods are perfected for Vitamin A measurement, the relationships will come to have a more important bearing.

Morton himself (2) urged caution in the use of the Morton-Stubbs correction and recommended that it be employed only where applicable. As the nonlinearity of background absorption increases, the applicability of this correction procedure decreases.

It has recently become apparent that the U.S.P. saponification procedure in the method for Vitamin A determination, which calls for 0.5 hour of boiling, generally suitable for fish liver oils, may lead to important losses of Vitamin A when applied to concentrates, to certain commercial products, and to margarines which lack sufficient natural antioxidant for Vitamin A protection. This loss may be prevented during the saponification step by the addition of fresh cotton seed oil or pyrogallic acid or both.

The method for determining Vitamin A in margarine suggested in the collaborative study this year involves chromatography. The Associate Referee believes that chromatographic separation is essential for this purpose to remove irrelevant absorption that invariably is an important source of error. In both England (3) and in Holland (4), chromatography has been used which is applicable to margarine Vitamin A. The adsorbent used was alumina, and required a relatively long column or columns and collection of multiple fractions in which the Vitamin A was determined by antimony trichloride technique. This procedure is more time-consuming than the present technique being tried by A.O.A.C. collaborators.

In evaluating the data accumulated in studies on margarine during the past year, the concept of purity index has been of help. The purity index used in this work is the ratio of A_{325}/A_{340} of the sample to the same ratio for pure transvitamin A which is 1.34, providing the sample A_{325}/A_{340} ratio is less than 1.34. If the sample ratio A_{325}/A_{340} is greater than 1.34, then the purity index formula is:

$$\frac{2.68 - \frac{A_{325}}{A_{340}}\text{ (sample)}}{1.34}.$$

Its significance lies in its deviation from the value 1.34. The purity index is partly empirical and not applicable without adequate purification of the sample. It can be applied to samples that have been chromatographed; although the correction is small, it is desirable, and it has a special significance when applied as a correction in the optical neutralization formulas.

"Optical neutralization" is a new concept used in the collaborative studies

this year. This has been developed in another field of spectrophotometry under the name of "variable reference" technique, in conjunction with the use of recording spectrophotometry (5). It may be applied to our problem with the ordinary Beckman spectrophotometer in the following manner: place a sample solution containing Vitamin A in a spectrophotometer cell in the blank position and a solution of Vitamin A standard in a cell in the sample position, and use as a reference point a fixed position in the middle of the transmission scale; then the resultant absorbance values indicate the degree of neutralization of the standard. If the neutralization is complete, the Vitamin A content of the standard solution is equivalent to that of the sample solution. At complete neutralization, the absorbance values at all wavelengths of the Vitamin A curve fall in a straight line. This concept and practice were developed with a recording spectrophotometer by varying the strength of the standard solution until a disappearance of the absorption peak was obtained, but by using a fixed reference standard, the Beckman Model DU spectrophotometer is applicable. The method is not generally applicable if the irrelevant absorbance is great, and thus chromatographing of the sample solution is essential. If the situation is represented fractionally relative to the absorption maxima by the use of the relative absorption, the numerical value of the neutralization axis becomes 1.0 and the length of each wavelength axis becomes 0.0 at complete neutralization; the length of any neutralization axis at a partial neutralization quantitatively represents the amount of the standard not yet neutralized. Algebraically, the neutralized ordinate is represented by the ratio A_{340}/A_{325} for the unneutralized absorbancies minus A_{340}/A_{325} of the neutralizing standard used. This difference divided by the ordinate represented by $(1-A_{340}/A_{325})$ for the neutralizing standard) represents the fraction of the standard neutralized.

The resulting complete formulas with corrections follow:

$$\frac{\frac{A_{340}}{A_{325}}\text{ unneutralized absorbancies} - \frac{A_{340}}{A_{325}}\text{ for standard } A}{1 - \frac{A_{340}}{A_{325}}\text{ for standard } A} = \frac{\frac{A_{340}}{A_{325}} - 0.73}{1 - 0.73} =$$

theoretical uncorrected fraction of std. neutralized

and if the original sample curve is of itself flatter than for the standard A, as indicated by the purity index it may be treated as an internal neutralization. To make the necessary compensation, the fraction of the standard neutralized, as above designated, is multiplied by 1/PI as previously developed. The complete formula then is:

$$\text{If } \frac{A_{325}}{A_{340}} < 1.34, \quad \frac{\frac{A_{340}}{A_{325}} - 0.73}{1 - 0.73} \times \frac{1}{\text{P.I.}} = \text{fraction of std. neutralized,}$$

$$\text{or if } \frac{A_{325}}{A_{340}} > 1.34, \quad \frac{\frac{A_{340}}{A_{325}} - 0.73}{1 - 0.73} \times \text{P.I.} = \text{fraction of std. neutralized}$$

This fraction times units per ml in the standard gives the units per ml in the sample.

The method serves as an additional check on the nature of the residual absorbance and is indicative of the thoroughness of the purification steps in the procedure. The residual curve may best be discerned at exact neutralization. If this is desired, one calculated dilution after the original evaluation will accomplish this purpose. The method also is very useful for comparison with the other methods such as the $SbCl_3$ method and the Morton-Stubbs correction evaluation.

THE "BLANK OIL" AND CHROMATOGRAPHIC PROCEDURES

The "blank oil" procedure is used in the laboratories of some of the magarine manufacturers for measuring Vitamin A in margarine. The basis for this procedure is given by Melnick, Luckmann, and Vahlteich (6–9). The Associate Referee has had an opportunity during the past year to carry out a preliminary study comparing the blank oil technique with the chromatographic procedure as previously applied. Samples of margarine and margarine constituents were kindly supplied by Dr. Luckmann of Best Foods Corporation. The blank oil procedure followed was essentially that described by these workers. The samples included various blank margarine oils, fortified margarine oils, fortified margarines, and the Vitamin A concentrates that were used in the fortification. The results are presented in Tables 1 and 2. Analyses were carried out by two analysts. Attention should be called to the discrepancies noted in column 1, Table 1, which gives the values for the unchromatographed and chromatographed samples read spectrophotometrically at 325 mμ. The discrepancies in the unchromatographed fractions can be explained, in part, by referring to column 6 which gives the apparent Vitamin A content of the column fractions that preceded and followed the Vitamin A fraction off the column. It appears that saponification increased the irrelevant absorption of this sample. That this is irrelevant absorption is shown further by the uniformity of the values for the chromatographed fractions measured in the several ways indicated in the table. One value for the concentrate A_1 given in column 5, Table 1, is considerably lower than the others. The value of 148,000 units per gram was obtained on the chromatographed material by optical neutralization. It appears possible that this A_1 concentrate may be relatively low in natural protective antioxidants and that loss in this case occurred on the chromatographic column. This could be demonstrated by the addition of cottonseed oil to the sample before saponification. In general, the values in Table 1 indicate a greater uniformity in the chromatographed fractions than in

TABLE 1.—*1952 A.O.A.C. vitamin A in margarine supplementary studies; comparative evaluation of A_1 and B_1 concentrates by various methods*[a]

(Values in units per gram when ×1000)

SAMPLE DESCRIPTION AND LABORATORY NO.	(1) SPEC. 325 Mμ		(2) PURITY INDEX		(6) PI COR. 325 Mμ		(4) $SbCl_3$		(5) OP. NEUT.		(6) SPEC. 325 Mμ CHROM.		(7) $SbCl_3$		(8) MORTON-STUBBS	
	UNCH.	CH.	UNCH.	CH.	UNCH.	CH.	UNCH.	CH.	UNCH.	CH.	PRE.	SUB.	PRE.	SUB.	UNCH.	CH.
	UNITS/G				UNITS/G		UNITS/G		UNITS/G		UNITS/G		UNITS/G		UNITS/G	
Whole Oil																
A_1 #25	222		0.90		204		218		148						183	
A_1 #24	211		0.91		192				139						155	
Sap., A_{1c} #25	210	183	0.965	0.955	203	183			175	175	7.7	16.2			165	186
Sap., A_1 #24 #25	216	164	0.81	1.0	175	164			171						144	
Sap., A_{1d} #25 (Pyrogallol)	240	200	0.935	0.95	223	190			190	178	6.9	17.4			210	167
Sap., A_{1a} #25	263	170	0.96	0.95	253	161	197	178	222	148	10.3	25.0	0.0	22.0	193	153
Sap., A_{1b} #25	256	193	0.935	0.95	240	183	210	204	180	177	9.7	16.9	14.5		160	163
Whole Oil																
B_1 #25	226		0.87		195				150						152	
Sap., B_{1a} #25	223	201	0.925	0.952	206	192	165	150	173	166	8.7	16.0	0.0	7.2	182	176
Sap., B_{1b} #25	223	186	0.955	0.955	213	177			169	166	8.7	20.4	0.0	14.5	182	176

[a] Unch.—Not chromatographed; Ch.—chromatographed. Pre—material before Vitamin A in column: Sub—material after Vitamin A in column. *a, b, c, d* refer to replicates.

TABLE 2.—*1952 A.O.A.C. vitamin A in margarine supplementary studies; vitamin A evaluations by various methods*

(Values in U.S.P. units/lb when ×1000)

SAMPLE DESCRIPTION AND LABORATORY NO.	(1) BLANK OIL		(2)	(3)		(4)		(5)		(6)		(7)	(8)		(8)	(9)
	NO LECITHIN + COLOR	LECITHIN + COLOR	SPEC. 325, CH.,	PURITY INDEX		μ/LB PI COR. 325 mμ		$SbCl_3$		$SbCl_3$		OP. NEUT. CH.	SPEC. 325		μ/LB MORTON-STUBBS	
				UNCH.	CH.	UNCH.	CH.	UNCH.	CH.	PRE.	SUB.		PRE.	SUB.	UNCH.	CH.
	UNITS/LB		UNITS/LB			BLANK OIL METHOD		UNITS/LB		UNITS/LB		UNITS/LB	UNITS/LB		BLANK OIL	
Wh. Oil Fort.																
A_1 #25		*17.9*		0.895		16.0									13.9	
Wh. Fort. Oil from Marg.																
A_1 #25	*18.1*			0.905		16.3									10.0	
Sap. Fort. Oil Alone																
A_1 #25		17.6	15.9	0.97	0.955	17.1	15.4	17.3	14.5	0.7	1.6	14.0	2.4	2.5	11.4	9.7
A_1 #24		*19.2*	19.3	0.97	1.0	18.6	19.4	19.1	16.7	1.2	1.2	18.5	0.6			
Sap. Fort. Oil from Marg.																
A_1 #25	16.7	17.3	20.0	0.98	0.98	16.4	19.5	17.1	15.5	0.6	1.3	18.2	2.7	7.6	16.7	−1.1
A_1 #24	*20.1*	20.1	18.4	0.98	1.0	19.7	18.4	19.6	16.3	0.38	2.0	15.0	0.9	5.3		
Wh. Oil Fort.																
B_1 #25		18.1		0.89		16.1									12.8	
Wh. Fort. Oil from Marg.																
B_1 #25	*17.5*	18.1		0.88		15.4									12.7	
				0.90											12.8	
Sap. Fort. Oil Alone																
B_1 #25		15.4	19.5	0.905	0.97	13.9	18.9	18.8	13.7	0.7	3.1	18.2	1.0	8.4		2.5
B_1 #24		*20.8*	18.1	0.88	0.99		18.0	21.1	16.7	0.7	3.7	15.1	1.1	11.5		
Sap. Fort. Oil from Marg.																
B_1 #25	16.9	16.2	17.2	0.995	0.96	16.8	16.5	20.4	13.7	0.9	1.5	17.5	1.3	7.9	10.9	6.9
B_1 #24	*17.1*	16.5	19.4	0.86	1.0	14.7	19.4	21.1	15.1	0.4	3.7	17.2	0.9	7.3		

the unchromatographed. It is of interest to note that the optical neutralization technique gave rather uniform results on both the chromatographed and unchromatographed fractions.

The italicized values in the first column of Table 2 represent the use of recommended blanks depending upon their lecithin content. Values in the first column not italicized represent the use of non-recommended blanks relative to the lecithin relationships. It has been stated by Melnick, *et al.*, that different blank oil compositions must be used for evaluating fortified oils and margarines. The explanation is that lecithin is separated from the oil in the oil separation step and thus should not appear in the blank used with a finished margarine. This study included in the blank oil technique fractions with lecithin and without lecithin. It was disturbing to observe results indicating an apparent absorption of less than zero for blanks containing lecithin compared with those without lecithin. Further study is needed to evaluate this point. Results obtained by two analysts are contained in Table 2. The manner in which the oil fraction was separated from the margarine was not standardized and this may have led to some minor but consistent differences in the results obtained. However, no wider discrepancies are observed in the results obtained by the antimony trichloride procedures either before or after chromatography, or the purity index corrected and the optical neutralization methods, than those noted with the blank oil method. Since all of the results are within the same order of magnitude, the methods appear to have equal validity. Closer scrutiny indicates that blank oil technique and antimony trichloride before chromatography gave consistently slightly higher values. This probably is the result of irrelevant absorption in the concentrate used in the case of the blank oil method and of the total irrelevant color developed in the case of the $SbCl_3$ method. This contention is supported by the apparent Vitamin A values of the fractions that preceded and followed the Vitamin A off the column indicated in columns 6 and 8, Table 2. That these fractions which show absorption do not contain Vitamin A is demonstrated by the lack of blue color upon treatment with antimony trichloride. This is a good demonstration of the importance of removing irrelevant absorption in margarine oils usually ignored in the commonly used antimony trichloride procedure.

The Morton-Stubbs corrected values from blank oil corrected curves are also given in column 9 of Table 2. These values are significantly lower than those from the other methods listed and show typical overcorrection. For the chromatographed fractions the Morton-Stubbs correction gives the values shown in column 9. The nonlinear irrelevant absorption at wavelengths lower than 325 is sufficient to disturb the correctness of this calculation. This irrelevant absorption has little influence on the results of the other procedures, as demonstrated by the small variation of the purity index values shown in column 3, Table 2,

which depend on changes in the longer wavelength side of the curve. It is quite apparent from the results of this study that the blank oil method offers no advantage from the standpoint of accuracy, and, in fact, is subject to important criticism because the irrelevant absorption effect is not overcome, no matter whether final evaluation is made colorimetrically or spectrophotometrically. There appears to be no alternative to the use of chromatography in this problem, from the standpoint of the official control chemist.

COLLABORATIVE STUDY

The A.O.A.C. collaborative study was based upon a method that involves chromatography. This method evolved from the original one proposed by Wilkie and De Witt (10). Continual study of this method has resulted in a number of modifications. The composition and size of the column have been changed and saponification, extraction, and drying procedures most suitable to attain sharpness of bands on the column have been found. Conditions under which control of absorption and elution of the Vitamin A and carotene bands are practical and feasible have been described.

Twenty-three laboratories indicated their intention to take part in this year's study. Samples and instructions were issued to all.* During the study, supplementary instructions were issued to increase the adequacy of the chromatography.

Two margarines were used for this study. Both were colored, one containing synthetic colors FD&C 3 and FD&C 4 and the other carotene. To date, seven laboratories have reported sufficient data for tabulation. Some of those that did not give reports had good intentions and expressed a desire to assist in these studies later as conditions would permit. Some of these reported difficulties in preliminary trials with the method, and suggestions for overcoming these difficulties were made. Several laboratories have made no response.

Results for Sample 1, the dye-colored sample, are given in Table 3, and for Sample 2, the carotene-colored sample, in Table 4. It will be observed that the reproducibility by each analyst as shown by the triplicates is generally excellent for all treatments except for the Morton-Stubbs procedure. There is one other exception. One of the results from Laboratory No. 1 of the optical neutralization is so relatively high that a possible explanation is that the chromatographic step may have been omitted.

Reproducibility of results between laboratories is not as good as reproducibility within each laboratory, particularly with the purity index and optical neutralization techniques. This is thought to be due to lack

* Instructions revised in accordance with comments and criticisms appear in the instructions to collaborators.

TABLE 3.—*1952 collaborators' reports on the chromatography method for vitamin A in margarine*

(Values are in U.S.P. units/lb when ×1000)

SAMPLE NO. 1—F&D COLORED

LABORATORY NO.	(1) SPEC. 325 UNCOR.	(2) SPEC. 340 UNCOR.	(3) PURITY INDEX	(4) PI COR. 325 mμ,	(5) PI COR. 340 MU,	(6) OPTICAL NEUT.,	(7) $SbCl_3$		(8) $SbCl_3$		(9) MORTON-STUBBS,	(10) SPEC. 325		(11) REL. AB.
							UNCH.	CH.	PRE.	SUB.		PRE.	SUB.	
	UNITS/LB	UNITS/LB		UNITS/LB	UNITS/LB	UNITS/LB	UNITS/LB		UNITS/LB		UNITS/LB	UNITS/LB		310 mμ
25	16.9	17.3	0.98	15.4	15.5	14.5	16.0	14.0	0.565	1.08	7.45	1.87	7.46	1.04
	15.6	16.3	0.965	15.0	15.7	13.75	15.0	12.0	0.0	2.64	6.9	1.67	6.00	1.02
	15.95	16.5	0.97	15.5	16.0	14.8	15.4	12.5	0.5	1.52	2.1	1.40	6.30	1.05
24	15.5	16.1	0.987	15.35	15.8	13.6	16.5	15.1			3.9			1.09
	15.4	15.6	1.0	15.4	15.6	13.4	17.8	13.1			4.8			1.04
	14.5	14.8	1.0	14.5	14.8	13.2	18.4	12.3			7.6			1.03
12	14.5	16.0	0.925	13.4	14.8						4.1			1.08
	14.7	16.5	0.91	13.4	15.0						5.2			1.08
	14.7	16.8	0.898	13.3	15.1						5.2			1.08
1	20.2	21.2	0.977	19.7	20.7	17.3	17.3				−26.4			1.68
	23.7	25.2	0.97	23.0	24.4	24.3	18.4				− 9.7			1.55
	18.5	18.9	0.985	18.2	18.6	15.6	17.9				−21.5			1.27
13	19.9	21.0	0.97	19.3	20.3	16.9	18.2	13.5			− 9.5			1.37
	19.7	20.7	0.97	19.1	20.1	16.8	16.2	12.5			− 8.5			1.38
	19.9	23.0	0.962	19.2	22.0	17.7	16.4	14.7			−12.3			1.42
2	21.6	22.7	0.977	21.1	22.2		20.5				−17.1			1.52
	20.9	21.7	0.985	20.6	21.4		20.6				− 7.2			1.36
	21.1	21.9	0.985	21.1	21.6		21.9				−0.864			1.23
3	12.9	12.7	0.955	12.3	12.0						−17.3			1.8
	12.8	12.8	0.985	12.8	12.9						−15.4			1.7
	12.9	12.5	0.948	12.9	12.5						−16.8			1.8

TABLE 4.—*1952 collaborators' reports on the chromatographic method for vitamin A in margarine*

(Values are in U.S.P. units/lb when ×1000)

SAMPLE NO. 2—CAROTENE COLORED

LABORATORY NO.	(1) SPEC. 325 UNCOR., UNITS/LB	(2) SPEC. 340 UNCOR., UNITS/LB	(3) PURITY INDEX	(4) PI COR., 325 mμ, UNITS/LB	(5) PI COR. 340 mμ, UNITS/LB	(6) OPTICAL NEUT., UNITS/LB	(7) $SbCl_3$ UNITS/LB		(8) $SbCl_3$ UNITS/LB		(9) MORTON-STUBBS, UNITS/LB	(10) CAROTENE UNITS/LB	(11) SPEC. 325 UNITS/LB		(12) REL. AB. 310 mμ
							UNCH.	CH.	PRE.	SUB.			PRE.	SUB.	
25	11.2	11.8	0.95	10.6	11.2	10.2	15.7	11.5	1.2	1.2	− 5.0	4.2	3.45	3.96	1.34
	11.0	11.4	0.95	10.45	10.6	10.2	14.6	12.7	0.6	1.2	8.3	4.2	3.02	4.50	1.10
	11.4	12.2	0.945	10.7	11.4	10.5	13.7	10.0	0.6	0.6	− 1.5	4.0	2.73	3.84	1.40
24	10.3	10.9	0.96	9.9	10.4	8.4	12.8	10.1			1.5	5.05			1.12
	11.4	11.0	0.98	11.2	11.5	9.7	13.1	9.1			5.8	4.8			1.05
	11.4	11.6	0.998	11.4	11.7	10.1	12.3	9.2			3.9	5.3			1.06
12	11.5	11.9	0.953	11.0	11.3						− 6.6	4.6			1.4
	14.5	11.7	0.965	14.0	11.3						− 7.4	5.2			1.2
	10.7	11.2	0.978	10.5	11.0						− 8.3	5.1			1.42
1	14.0	14.5	0.97	13.5	14.1	12.1	11.8				−46.7	6.2			2.49
	13.6	14.3	0.967	13.1	13.8	11.6	11.9				−42.0	5.9			2.38
	12.6	13.4	0.966	12.1	11.1	11.1	11.3				−31.2	5.7			2.19
13	11.3	12.4	0.944	10.7	11.7	10.3	13.3	12.8			−16.0	4.6			1.71
	12.1	13.0	0.955	11.6	12.4	10.6	11.7	9.9			−29.9	5.0			2.12
	12.6	13.7	0.948	11.9	13.0	10.6	13.6	11.0			−24.3	5.1			1.91
2	14.6	15.4	0.962	14.0	14.8		7.5				−31.0	3.1			2.0
	14.9	16.0	0.947	14.1	15.1		7.2				−27.0	3.0			1.86
	16.2	16.0	0.955	15.2	15.3		6.6				−27.0	3.0			1.30
3	8.9	9.3	0.963	8.5	9.0						−34.6	2.0			2.7
	8.6	8.6	0.985	8.5	8.5						−29.2	2.0			2.5
	8.4	8.2	0.955	8.0	8.2						−28.0	2.0			2.5

of precision in regulating the final cut-off of the Vitamin A fraction in the column. This is recognized as a weakness in the instructions and should be correctable by restatement, with perhaps a little more attention and practice by each analyst on this particular point. The antimony trichloride results on both the unchromatographed and chromatographed samples indicate the greatest uniformity between laboratories.

It is also interesting to observe that the unchromatographed sample gives a slightly higher antimony trichloride value than the chromatographed one. The antimony trichloride results on the pre- and subfractions, those preceding and following the Vitamin A off the column, account for the difference. The closeness of checks of the optical neutralization values and the antimony trichloride values is further evidence of the uniformity and reliability of the chromatography on the Vitamin A fraction.

The Morton-Stubbs values are out of line for reasons previously discussed. From data supplied by the manufacturer, it is known that 17,000 units of Vitamin A per pound were added to Sample No. 1. Four laboratories correctly reported the data required for optical neutralization. It is apparent from the other results reported that greater detail in describing the operation of the Beckman spectrophotometer for this purpose is needed.

It is of interest to note the agreement of values on the chromatographed sample measured either by optical neutralization, by antimony trichloride or at 325 mμ (P.I. corrected).

With respect to the Vitamin A value for Sample No. 2 (Table 4), agreement, both within laboratories and between laboratories, appears to be very good, even better than for Sample No. 1, and the same general considerations apply. Reproducibility in each laboratory is very good for the carotene but the results of Laboratory 25 appear to be somewhat low, while results from Laboratories 2 and 3 indicate the possibility of excessive losses of the carotene, or its incomplete separation. However, when it is realized that carotene may be somewhat less stable than Vitamin A, it is not too surprising to expect more variation in its evaluation, at least until the suggested chromatographic practice becomes more common than at present. The carotene may be the limiting factor in the final evolution of this procedure and may require some special precaution by those who obtain such abnormally low results. It was reported to the Associate Referee that the manufacturer of the carotene-fortified margarine added 12,000 units of Vitamin A as Vitamin A ester and 6000 units of Vitamin A as carotene. The data indicate that approximately 15,000–16,000 units of Vitamin A were recovered.

Tables 5 and 6 together with Tables 3 and 4 show some correlations of value to further collaborative work and illustrate how each collaborator may evaluate his own results.

TABLE 5.—*Criteria derived from sample No. 1*

LABORATORY	AV. 325 mμ PI COR. UNITS/LB WHEN×1000	AV. 340 mμ PI COR. UNITS/LB WHEN×1000	AV. $\frac{A_{325}}{A_{340}}$	AV. $\frac{A_{310}}{A_{325}}$
25	15.3	15.7	1.29	1.04
24	15.1	15.4	1.33	1.05
12	13.4	15.0	1.24	1.06
1	20.3	21.2	1.29	1.50
13	19.2	20.8	1.28	1.39
2	20.9	22.7	1.31	1.37
3	12.6	12.5	1.39	1.77

It will be observed that the first three laboratories obtained closer correlations between the results from different methods than did the other laboratories. Also it may be noted that the relative A_{310} is lower for the first three laboratories than for the others. Laboratory No. 1 which had higher average relative A_{310} has a greater discrepancy between the spectrophotometer 325 values and the more closely checking optical neutralization and $SbCl_3$ values.

Laboratory No. 3 having the excessively low results is the only one to have A_{325}/A_{340} ratios greater than 1.34.

The above results then give rise to the following tentative conclusions which may be used as criteria of operation by the method:

(1) A relatively low relative A_{310} value indicates effective chromatography. At this time a relative A_{310} value less than 1.1 indicates that the cut-off behind the Vitamin A band is satisfactory while a relative A_{310} greater than 1.40 with an A_{325}/A_{340} ratio less than 1.34 indicates that the cut-off behind the Vitamin A is allowing objectionable interference to pass and the results will be high.

(2) If the straight spectrophotometric results are too high because of an insufficiently sharp cut-off behind the Vitamin A band, an accurate evaluation is still possible through the optical neutralization or antimony

TABLE 6.—*Criteria derived from sample No. 2*

LABORATORY	AV. 325 mμ PI COR. UNITS/LB WHEN×1000	AV. 340 mμ PI COR. UNITS/LB WHEN×1000	AV. $\frac{A_{325}}{A_{340}}$	AV. $\frac{A_{310}}{A_{325}}$
25	10.6	11.0	1.27	1.28
24	10.8	11.2	1.11	1.08
12	11.8	11.2	1.29	1.34
1	12.9	13.6	1.30	2.35
13	11.4	12.36	1.27	1.91
2	14.4	15.0	1.23	1.72
3	8.33	8.56	1.35	2.56

trichloride methods. In a beginner's use of the chromatographic method, the antimony trichloride method would probably be the better means of evaluation because of the possible greater specificity under the developed conditions.

(3) An A_{325}/A_{340} ratio of greater than 1.34 with a corresponding relative A_{310} greater than 1.4 indicates Vitamin A loss.

With these criteria, the collaborators and the Associate Referee should be better guided in further studies. In view of the difficulties experienced by some of the collaborators, and the apparent need for further clarifications in the instructions, it is deemed advisable to continue this study along present lines.

RECOMMENDATIONS*

It is recommended that the method for Vitamin A in margarine be subjected to further collaborative study during the coming year.

REFERENCES

(1) BOLDINGH, J., *et al.*, *Nature*, **168**, 598 (1951).
(2) CAMA, H. R., COLLINS, F. D., and MORTON, R. A., *Biochem. J.*, **50**, 48 (1951).
(3) GRIDGEMAN, N. T., GIBSON, G. P., and SAVAGE, J. P., *The Analyst*, **73**, 662 (1948).
(4) BOLDINGH, J., and DROST, J. R., *J. Am. Oil Chem. Soc.*, **28**, 480 (1951).
(5) JONES, J. H., CLARK, G. R., and HARROW, L. S., *This Journal*, **34**, 135 (1951).
(6) MELNICK, D., LUCKMANN, F. H., and VAHLTEICH, H. W., *J. Am. Oil Chem. Soc.*, **29**, 104 (1952).
(7) LUCKMANN, F. H., MELNICK, D., and VAHLTEICH, H. W., *ibid.*, **29**, 121 (1952).
(8) LUCKMANN, F. H., GOODING, C. M., and MELNICK, D., *ibid.*, **29**, 174 (1952).
(9) LUCKMANN, F. H., MELNICK, D., and VAHLTEICH, H. W., *ibid.*, **29**, 321 (1952).
(10) WILKIE, J. B., and DEWITT, J. B., *This Journal*, **29**, 174 (1945).

REPORT ON THE DETERMINATION OF THIAMINE IN ENRICHED FLOUR

COMPARISON OF ACID HYDROLYSIS AND FLUOROMETRIC METHODS

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This report covers a collaborative study of rapid procedures for the determination of thiamine in enriched flour as compared with the official method (1).

Several investigators (3–5) have indicated that the natural thiamine of wheat flour is made available for determination by thiochrome procedure when the sample solution preparation is limited to acid hydrolysis

* For report of Subcommittee A and action of the Association, see *This Journal*, 36, 52 (1953).

because wheat flour contains little or no phosphorylated or protein bound thiamine. Various forms of rapid procedure have been employed, differing mainly in the means of effecting separation of the sample solution from the residual solids after acid digestion at 95–100°C.; either centrifuging or filtration is used for this purpose.

The official method provides alternatives of acid hydrolysis at 95–100°C. or at 121–123°C. by autoclaving and prescribes centrifuging and further treatment by enzyme digestion and base exchange. These variations in means of separation and temperatures of digestion have been included in the study. The rapid procedures are described in the instructions to collaborators.

INSTRUCTIONS TO COLLABORATORS

Sample No. (1) Enriched Flour (1 pt. jar—ca 200 g).
Sample No. (2) Enriched Flour (1 pt. jar—ca 200 g).
Sample No. (3) Enriched Flour (1 pt. jar—ca 200 g).

The jars are not completely filled to allow for mixing the samples in the closed jars. (Roll the jars for ca one min. just previous to opening.)

The present purpose is to compare results where the sample soln is obtained with plain acid hydrolysis as against the official fluorometric method (1). Some variations in the means of obtaining the sample solution should also be compared.

Analyze each sample by all of the following described procedures:

I. Fluorometric Method **40.18** thru **40.22.** (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 METHOD (ACID HYDROLYSIS)

REAGENTS AND APPARATUS

(a) Reagents described in Method I and additional description of preparation.

(b) *Isobutyl Alcohol.*—Use alcohol boiling between 106.5–108°C. with a fluorometer reading not exceeding 3 in the standard cuvette. Isobutyl alcohol may be recovered by acidifying waste solns and redistilling, retaining portion distilling between 106.5 and 108°C.

(c) *Starch.*—Reagent grade (thiamine free).

(d) *Sulfuric Acid.*—Ca 0.15 *N* (8.5 ml H_2SO_4 per 2000 ml H_2O).

(e) *Sulfuric Acid.*—Ca 0.10 *N* (5.7 ml H_2SO_4 per 2000 ml H_2O).

(f) *Potassium Chloride.*—Reagent grade.

(g) *Sodium Chloride.*—Reagent grade.

(h) *Filter Cel.*—(Diatomaceous Earth—"Hyflo-Super Cel" is suggested).

(i) *Thiamine Hydrochloride Stock Solution.*—Weigh accurately 20–25 mg of U.S.P. Thiamine Hydrochloride Reference Standard (2) which has been kept in a desiccator over P_2O_5 for at least 16 hrs. Since the reference standard is hygroscopic, take precautions to avoid absorption of moisture. Dissolve in 20% alcohol adjusted to *p*H 3.5 to 4.3 with HCl and make up to one l with the acidified 20% alcohol. Add additional acidified 20% alcohol to bring the concn to exactly 20 mmg per ml. Store in a cool place in a well-closed, light-resistant container.

(a) SAMPLE SOLUTION BY CENTRIFUGING (6)

Preparation of Assay Solution.—Weigh a 4.54 g sample (or an amount calcd to

contain 20 mmg of thiamine HCl) and transfer to a 100 ml centrifuge tube contg a glass rod. Mix thoroly with ca 35 ml of 0.15 N H_2SO_4 so as to avoid lumping. Wash down sides of the tube with ca 30 ml of acid and mix thoroly. Heat in a boiling H_2O bath for 30 min. Stir constantly for the first 5–7 min. and occasionally for the remainder of the 30 min. period. Place a drop of the soln on a spot plate and test with thymol blue. Soln should be distinctly red (*p*H 1 to 1.2). Cool, transfer quantitatively to a 100 ml volumetric flask and dil. to vol., using 0.1 N H_2SO_4 for transfer and diln. Mix and centrifuge. If centrifuging is done in original centrifuge tubes, use a speed of 2000 r.p.m. for ca 10 min. If 15 ml test tubes are used in small centrifuge, use speed of ca 3500 r.p.m. for 5 min. Decant supernatant liquid (assay soln).

Oxidation of Thiamine to Thiochrome.—Prepare standards by adding two 5.00 ml and two 2.50 ml aliquots of the standard thiamine soln to 50 ml glass-stoppered centrifuge tubes contg 2.5 g NaCl. Add 5.00 ml and 2.50 ml aliquots of the assay soln to addnl centrifuge tubes contg 2.5 g NaCl. Adjust the total vol. of all tubes to 5.0 ml with 0.1 N H_2SO_4. Swirl tubes gently several times for 5 sec. each time to dissolve part of the NaCl. (*The precision and accuracy of the results depend upon a uniform technique in carrying out the oxidation.*) For addn of oxidizing agent, use a pipet which will deliver 3 ml in 1 sec. Place the tip of the pipet holding the alk. ferricyanide soln in the neck of the tube and hold it so that the stream of soln will not hit the side of the tube. Give the tube a gentle swirl to impart a slight rotational motion in the liquid at the moment of oxidation. Add 3.0 ml of alk. ferricyanide soln, and then give the tube a gentle swirl through about one-half turn.

Within 30 min. following the oxidation, add 13 ml of isobutyl alcohol from a 100 ml buret, stopper the tube, and immediately shake vigorously for 10–15 sec. After the alcohol has been added to all tubes, shake tubes vigorously for two min. Centrifuge for 2–3 min. Pipet ca 10 ml of the alcohol layer into the photofluorometer cuvette.

Blanks.—Run blanks on 5 ml aliquot of standard and 5 ml and 2.5 ml aliquot of samples by adding 3 ml of the NaOH soln in place of the oxidizing reagent. Avoid contaminating the blanks with minute amounts of the oxidizing soln which might be on the stem of the isobutyl alcohol delivery buret. (There is no significant difference between readings on 5 ml and 2.5 ml *standard blanks.*)

Thiamine Hydrochloride Standard Solutions.—From a portion of the stock soln that has been warmed to the proper temp. pipet 5 ml into a 100 ml centrifuge tube, and add 60 ml 0.1 N H_2SO_4. Treat this soln exactly like the sample as regards acid digestion. Cool to room temp., transfer quantitatively to a 500 ml volumetric flask with 0.1 N H_2SO_4, and dil. to vol. with the acid. Do not use this standard more than one day.

Determination Standard.—Pipet an addnl 5 ml of the warmed stock soln into a 500 ml volumetric flask and dil. to vol. with 0.1 N H_2SO_4 (direct standard).

Measurement of Thiochrome Fluorescence.—Measure the fluorescence with a photofluorometer. Make a standard curve by plotting mmg of thiamine against readings corrected for blank. By means of this curve, convert sample readings corrected for blanks to mmg of thiamine and calc. to mg/lb.

(b) Repeat the procedure II **(a)** except autoclave at 121–123°C.

(c) SAMPLE SOLUTION BY FILTRATION

Transfer 9.07 g sample to a 200 ml centrifuge bottle, add 10 g of KCl or NaCl, and mix thoroly with a stirring rod. Add ca 70 ml of the 0.15 N H_2SO_4 and stir to obtain a finely divided suspension. Wash down the sides of the bottle with an addl 60 ml of the 0.15 N H_2SO_4. Digest in a H_2O bath at 95–100°C., stirring at frequent intervals until starch hydrolysis is complete—usually 10 min. A spot plate test is

then made to det. if acid to thymol blue (*p*H 1.0 to 1.2) and heating is continued for a total of 30 min. Cool, transfer the residual solids and soln to a 200 ml volumetric flask, and dil. to vol., using 0.10 *N* H_2SO_4 for transfer and diln. After mixing in a 400 ml beaker with ca 5 g of filter cel, filter through paper (S&S No. 597 or Whatman No. 41 H are recommended). Discard ca 25 ml of the first part of the filtrate. Continue as directed in II **(a)** beginning "Oxidation of Thiamine to Thiochrome."

Make each 2.5 ml aliquot to 5 ml by the addn of 2.5 ml of a soln prepared from 130 ml 0.15 *N* H_2SO_4, 70 ml 0.10 *N* H_2SO_4, and 10 g of KCl or NaCl.

(d) Repeat the procedure II **(c)** except autoclave at 121–123°C.

Thiamine standard solutions for II **(c)** *and* **(d).**—From a portion of the stock solution that has been warmed to the proper temp., pipet 10 ml into a 200 ml volumetric flask and dil. to vol. with the 0.15 *N* H_2SO_4 (1.0 mmg/ml). Pipet 40 ml of this soln into a 200 ml centrifuge bottle, add 10 g of KCl or NaCl and ca 1 g of starch, dil. to ca 130 ml with 0.15 *N* H_2SO_4, and treat exactly like the sample as regards acid digestion and filtration. Finally dil. to 200 ml in a volumetric flask with 0.1 *N* H_2SO_4 (0.20 mmg/ml) (Determination Standard).

Pipet an addnl 40 ml of the dild stock soln into a 200 ml volumetric flask contg 10 g of KCl or NaCl, dil. to ca 130 ml with the 0.15 *N* H_2SO_4 and make to vol. with the 0.1 *N* H_2SO_4 (0.20 mmg/ml) (Direct Standard).

NOTES

1. *Chlorine-free distilled water* should be used in the preparation of all reagents and standards. Test for chlorine with *o*-tolidine reagent (dissolve 1 g *o*-tolidine in one l of dil. HCl 1+9) and test according to A.P.H.A. methods for water analysis. If a yellow color is obtained, denoting presence of free chlorine, boil the distilled water down to two-thirds of original volume.

2. Filter paper and filter cell should be tested for thiamine absorption. Compare thiochrome readings on filtered and original standard solns.

3. The calculation to mg/lb may be made directly on a percentage basis. With a thiamine content of 2 mg/lb the sample weight of 9.07 g provides 40 mmg in the final vol. of 200 ml (0.20 mmg/ml). If 5 ml is found to contain 1.20 mmg, then the value is 20% high, a result of 2.40 mg/lb.

4. *Oxidation*: Small 35 ml glass-stoppered Pyrex bottles may be used instead of the specified 50 ml centrifuge tubes. The shoulder of the bottle aids in separating the two layers so that the isobutyl alcohol can be poured into the photofluorometer cuvettes.

5. *Reagent Blank*: To check any possible addn of thiamine as from added starch in the "determination standard" or other source, conduct one determination by method I **(a)** and one by method II **(c)**. These should be equivalent to the plain NaOH blank in the final oxidation.

6. The chart provided for reporting the results is an outline of the procedures described.

7. A total of six procedure variations have been described. Six separate weights per sample should be employed. Duplicate sample weights for each variation are not requested but may be used if the analyst desires. However, just previous to oxidation to thiochrome, two aliquots (5 ml and 2.5 ml) should be taken from each final assay solution and results reported in duplicate as mg/lb.

The results of the collaborators are tabulated in Tables 1, 2, and 3.

DISCUSSION

The three samples Nos. 1, 2, and 3 were submitted as providing the usual range of thiamine content found in enriched flour samples: No. 1

TABLE 1.—*Enriched flour sample No. 1 (Thiamin mg/lb)*

COLLABORATOR NO.	ALIQUOT, ML	I. OFFICIAL METHOD, A.O.A.C.		II. RAPID PROCEDURES								AUTOCLAVING LOSSES, PER CENT		
				CENTRIFUGED SEPARATION				FILTRATION SEPARATION						
		(a) ACID DIGEST, (95–100°C.)	(b) ACID DIGEST, AUTOCLAVE (121–123°C.)	ACID DIGEST (95–100°C.)		ACID DIGEST AUTOCLAVE (121–123°C.)		ACID DIGEST (95–100°C.)		ACID DIGEST AUTOCLAVE (121–123°C.)		METHOD I(b)	METHOD II(b)	METHOD II(d)
				(A)[a]	(B)[a]	(A)	(B)	(A)	(B)	(A)	(B)			
(1)	5.0	2.12	2.04	2.02	2.02	2.04	2.02	1.96	1.96	2.00	2.00			
	2.5	2.08	2.08	2.00	2.00	2.00	2.00	1.96	1.96	2.00	2.00			
	Av.	2.10	2.06	2.01	2.01	2.02	2.01	1.96	1.96	2.00	2.00	None	None	None
(2)	5.0	2.04	2.08	2.16	2.14	1.96	1.98	1.89	1.79	1.89	1.65			
	5.0	—	—	2.01	1.98	—	—	—	—	—	—			
	2.5	2.02	2.12	2.05	2.04	1.98	1.99	1.85	1.79	1.87	1.64			
	2.5	—	—	2.03	2.02	—	—	—	—	—	—			
	Av.	2.03	2.10	2.06	2.09	1.97	1.99	1.87	1.79	1.88	1.65	None	4.4	None
(3)	5.0	2.13	2.17	2.08	2.06	1.52	1.52	1.98	2.01	1.49	1.49			
	2.5	2.13	2.15	2.16	2.16	1.62	1.58	2.04	2.06	1.52	1.54			
	Av.	2.13	2.16	2.12	2.11	1.57	1.55	2.01	2.04	1.51	1.52	None	26.0	25.0
(4)	5.0	1.57	1.52	1.88	1.78	1.84	1.84	1.98	1.91	1.93	1.78			
	5.0	1.65	1.42	1.89	1.79	1.85	1.85	2.02	1.98	1.95	1.81			
	2.5	1.44	1.25	1.92	1.88	1.86	1.86	2.04	2.08	1.86	1.96			
	2.5	1.40	1.25	1.90	1.90	1.90	1.90	1.98	2.04	1.92	2.02			
	Av.	1.52	1.36	1.90	1.84	1.86	1.86	2.01	2.00	1.92	1.89	10.5	None	4.5
(5)	5.0	2.00	2.02	2.12	2.06	1.78	1.66	2.00	2.02	1.80	1.70			
	5.0	2.06	2.08	2.12	2.06	1.84	1.72	2.02	2.04	1.72	1.62			
	2.5	2.08	2.04	2.04	2.00	1.92	1.80	2.08	2.08	1.92	1.80			
	2.5	2.04	2.04	2.08	2.00	1.92	1.76	2.04	2.08	1.84	1.72			
	Av.	2.05	2.05	2.09	2.03	1.87	1.73	2.04	2.06	1.82	1.71	None	10.5	10.8
(6)	5.0	1.97	1.95	2.05	1.96	1.03	0.99	1.89	1.83	1.09	1.09			
	2.5	2.06	2.08	2.40	2.26	1.39	1.35	1.83	1.82	—	—			
	Av.	2.01	2.02	2.23[b]	2.11[b]	1.21[b]	1.14[b]	1.86[b]	1.82[b]	1.09[b]	1.09[b]	None	45.7[b]	41.4[b]
(7)	5.0	1.99	1.85	2.07	2.12	1.70	1.66	2.09	2.04	1.84	1.75			
	5.0	—	2.02	1.99	2.08	—	—	—	—	—	—			
	Av.	1.99	1.94	2.03	2.10	1.70	1.66	2.09	2.04	1.84	1.75	None	16.2	12.0
Averages		1.93	1.90	2.03	2.01	1.85	1.81	2.00	1.99	1.84	1.77	None	8.9	8.00

[a] Notes: (A) Calcd. basis "determination standard"; (B) Calcd. basis "direct standard."
[b] Results not included in averages.

TABLE 2.—*Enriched flour sample No. 2 (Thiamin mg/lb)*

COLLABORATOR NO.	ALIQUOT, ML	I. OFFICIAL METHOD, A.O.A.C.		II. RAPID PROCEDURES								AUTOCLAVING LOSSES, PER CENT		
				CENTRIFUGED SEPARATION				FILTRATION SEPARATION						
		(a) ACID DIGEST, (95–100°C.)	(b) ACID DIGEST, AUTOCLAVE (121–123°C.)	ACID DIGEST (95–100°C.)		ACID DIGEST AUTOCLAVE (121–123°C.)		ACID DIGEST (95–100°C.)		ACID DIGEST AUTOCLAVE (121–123°C.)		METHOD I(b)	METHOD II(b)	METHOD II(d)
				(A)[a]	(B)[a]	(A)	(B)	(A)	(B)	(A)	(B)			
(1)	5.0	1.66	1.66	1.58	1.58	1.62	1.58	1.50	1.50	1.58	1.58			
	2.5	1.68	1.68	1.56	1.56	1.56	1.52	1.52	1.52	1.60	1.60			
	Av.	1.67	1.67	1.57	1.57	1.59	1.55	1.51	1.51	1.59	1.59	None	None	None
(2)	5.0	1.67	1.68	1.63	1.62	1.56	1.58	1.53	1.45	1.55	1.37			
	5.0	—	—	1.58	1.57	—	—	—	—	—	—			
	2.5	1.69	1.77	1.64	1.62	1.52	1.53	1.49	1.41	1.57	1.38			
	2.5	—	—	1.60	1.58	—	—	—	—	—	—			
	Av.	1.68	1.73	1.61	1.60	1.54	1.56	1.51	1.43	1.56	1.38	None	4.3	None
(3)	5.0	1.65	1.69	1.74	1.74	1.23	1.22	1.66	1.70	1.21	1.22			
	2.5	1.63	1.69	1.80	1.76	1.28	1.28	1.66	1.68	1.28	1.30			
	Av.	1.64	1.69	1.77	1.75	1.26	1.25	1.66	1.69	1.25	1.26	None	28.8	24.7
(4)	5.0	1.49	1.23	1.50	1.44	1.50	1.50	1.54	1.49	1.54	1.47			
	5.0	1.40	1.30	1.60	1.54	1.49	1.49	1.61	1.56	1.62	1.54			
	2.5	1.40	1.24	1.46	1.52	1.56	1.56	1.56	1.66	1.46	1.62			
	2.5	1.36	1.20	1.50	1.58	1.58	1.58	1.58	1.68	1.52	1.68			
	Av.	1.41	1.24	1.52	1.52	1.53	1.53	1.57	1.60	1.54	1.58	12.0	None	None
(5)	5.0	1.66	1.62	1.72	1.68	1.36	1.28	1.62	1.64	1.38	1.30			
	5.0	1.68	1.60	1.68	1.64	1.38	1.30	1.64	1.66	1.38	1.30			
	2.5	1.68	1.64	1.68	1.64	1.44	1.36	1.60	1.60	1.44	1.36			
	2.5	1.64	1.62	1.64	1.60	1.48	1.36	1.64	1.68	1.48	1.40			
	Av.	1.67	1.62	1.68	1.64	1.41	1.33	1.63	1.65	1.42	1.34	None	16.1	12.9
(6)	5.0	1.65	1.62	1.64	1.57	0.83	0.79	1.48	1.43	0.86	0.86			
	2.5	1.68	1.69	1.89	1.77	1.09	1.01	1.45	1.44	—	—			
	Av.	1.67	1.66	1.77[b]	1.67[b]	0.96[b]	0.80[b]	1.46[b]	1.43[b]	0.86[b]	0.86[b]	None	45.8[b]	41.1[b]
(7)	5.0	1.55	1.58	1.62	1.70	1.32	1.29	1.67	1.63	1.49	1.43			
	5.0	—	1.60	1.64	1.72	—	—	—	—	—	—			
	Av.	1.55	1.59	1.63	1.71	1.32	1.29	1.67	1.63	1.49	1.43	None	19.0	10.8
Averages		1.60	1.56	1.62	1.62	1.46	1.43	1.59	1.59	1.47	1.44	None	9.9	7.6

[a] Notes: (A) Calcd. basis "determination standard"; (B) Calcd. basis "direct standard."
[b] Results not included in averages.

TABLE 3.—*Enriched flour sample No. 3 (Thiamin mg/lb)*

COLLABORATOR NO.	ALIQUOT, ML	I. OFFICIAL METHOD, A.O.A.C.		II. RAPID PROCEDURES								AUTOCLAVING LOSSES, PER CENT		
				CENTRIFUGED SEPARATION				FILTRATION SEPARATION						
		(a) ACID DIGEST, (95–100°C.)	(b) ACID DIGEST, AUTOCLAVE (121–123°C.)	ACID DIGEST (95–100°C.)		ACID DIGEST AUTOCLAVE (121–123°C.)		ACID DIGEST (95–100°C.)		ACID DIGEST AUTOCTAVE (121–123°C.)		METHOD I(b)	METHOD II(b)	METHOD II(d)
				(A)[a]	(B)[a]	(A)	(B)	(A)	(B)	(A)	(B)			
(1)	5.0	1.42	1.38	1.32	1.32	1.32	1.32	1.32	1.32	1.36	1.36			
	2.5	1.40	1.36	1.32	1.32	1.24	1.24	1.32	1.32	1.32	1.32			
	Av.	1.41	1.37	1.32	1.32	1.28	1.28	1.32	1.32	1.34	1.34	None	None	None
(2)	5.0	1.36	1.41	1.36	1.35	1.27	1.28	1.25	1.18	1.23	1.08			
	5.0	—	—	1.34	1.33	—	—	—	—	—	—			
	2.5	1.37	1.40	1.41	1.40	1.29	1.29	1.27	1.20	1.22	1.07			
	2.5	—	—	1.35	1.35	—	—	—	—	—	—			
	Av.	1.37	1.41	1.37	1.36	1.28	1.29	1.26	1.19	1.23	1.08	None	6.6	None
(3)	5.0	1.39	1.44	1.48	1.48	0.96	0.96	1.39	1.40	0.91	0.93			
	2.5	1.38	1.43	1.44	1.44	1.00	1.00	1.44	1.44	0.98	0.96			
	Av.	1.39	1.44	1.46	1.46	0.98	0.98	1.42	1.42	0.95	0.95	None	32.9	33.1
(4)	5.0	1.22	1.07	1.12	1.24	1.12	1.12	1.27	1.27	1.35	1.31			
	5.0	1.24	1.09	1.27	1.11	1.19	1.19	1.30	1.28	1.37	1.33			
	2.5	1.10	1.10	0.96	1.08	1.12	1.12	1.24	1.40	1.20	1.42			
	2.5	0.98	0.98	0.98	1.12	1.12	1.12	1.28	1.42	1.20	1.42			
	Av.	1.14	1.06	1.08	1.14	1.14	1.14	1.27	1.34	1.28	1.37	7.0	None	None
(5)	5.0	1.36	1.40	1.42	1.38	1.12	1.06	1.36	1.38	1.22	1.16			
	5.0	1.36	1.34	1.40	1.36	1.12	1.04	1.36	1.38	1.24	1.18			
	2.5	1.36	1.40	1.32	1.28	1.12	1.08	1.32	1.32	1.24	1.20			
	2.5	1.36	1.40	1.40	1.36	1.20	1.16	1.32	1.32	1.24	1.16			
	Av.	1.36	1.39	1.39	1.35	1.14	1.09	1.34	1.35	1.24	1.18	None	14.3	6.1
(6)	5.0	1.41	1.37	1.39	1.33	0.69	0.65	1.22	1.18	0.71	0.72			
	2.5	1.47	1.47	1.57	1.48	0.93	0.87	1.19	1.18	—	—			
	Av.	1.44	1.42	1.48[b]	1.40[b]	0.78[b]	0.76[b]	1.20[b]	1.18[b]	0.71[b]	0.72[b]	None	47.3[b]	40.8[b]
(7)	5.0	1.20	1.30	1.38	1.45	1.14	1.11	1.40	1.37	1.18	1.12			
	5.0	—	—	1.30	1.35	—	—	—	—	—	—			
	Av.	1.20	1.30	1.34	1.40	1.14	1.11	1.40	1.37	1.18	1.12	None	15.0	16.0
Averages		1.32	1.31	1.31	1.32	1.16	1.14	1.32	1.33	1.22	1.20	None	11.5	7.6

[a] Notes: (A) Calcd. basis "determination standard"; (B) Calcd. basis "direct standard."
[b] Results not included in averages.

with about 2.0 mg per lb (minimum standard) and Nos. 2 and 3 with shortages of 15 and 30 per cent. In the interpretation of the results, the following comments are applicable to all three samples.

OFFICIAL METHOD

With one exception, the results of the seven chemists are considered to be in excellent agreement. Comparable results were obtained by the alternatives of acid digestion at 95–100°C. for one-half hour or by autoclaving at 121–123°C. for the same length of time. The method specifies that the standard be carried through the complete procedure.

RAPID PROCEDURES

(1) *Standards comparison.*—The collaborators were asked to calculate two sets of results making use of (A) "determination standard" treated the same as the sample and (B) "direct standard" containing the same amounts of acids or KCl that were used in the procedure. In general the results are slightly higher by standard (A) indicating some loss in the determination standard. However, in general either standard is considered applicable.

(2) *Comparison of centrifuging and filtration.*—The results of this study indicate that centrifuging or filtration may be considered as alternatives to be used in the preparation of the sample solution. One collaborator reported difficulty with filtration in Method II (c). The Associate Referee noticed that filtration was slower than experienced in previous determinations where 0.1 *N* HCl was used, rather than the 0.10 *N* and 0.15 *N* H_2SO_4 that were specified for the rapid procedures.

(3) *Digestion temperature comparison.*—The results reported are difficult to interpret in view of the differences between collaborators and the small amounts of information supplied. Three of the collaborators have no appreciable differences resulting from use of the two temperatures by the rapid procedures, while four have differences of from 10 to 47 per cent. Collaborator #6 reported excessively high sample blanks throughout all of the rapid determinations and therefore these results have been omitted from the averages. Several have reported that sample blanks tended to go up with the autoclaving procedure, which may account in some measure for low results. This was also the experience of the Associate Referee where the sample blank increased from 3 to 6 or from 5 to 7, comparing 95–100°C. and 121–123°C. respectively, with a reading of about 80 for 1 microgram of thiamine. However, check results were obtained with the use of the two temperatures.

RECOMMENDATIONS

It is recommended* that this study be continued in the further development and investigation of the rapid thiochrome procedures here de-

* For report of Subcommittee A and action of the Association, see *This Journal*, **36**, 52 (1953).

scribed and that other types of enriched flour be analyzed in order to compare the rapid and official procedures.

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REFERENCES

(1) *Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Washington, D. C., 1950 **(40.18–40.22).**
(2) United States Pharmacopeia, Fourteenth Revision, Mack Publishing Co., Easton, Pa., 1950, p. 770.
(3) BOOTH, R. G., *J. Soc. Chem. Ind.*, **59,** 181 (1940).
(4) ANDREWS, J. S., and NORDGREN, R., *Cereal Chem.*, **18,** 686 (1941).
(5) KLINE, O. L., *This Journal*, **31,** 455 (1948).
(6) TOLLE, C. D., and HALL, W. L., personal communication; JOINER, C. R., personal communication.

REPORT ON VITAMIN B_6 (CHEMICAL METHODS)

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

During the past year the greater part of the study on Vitamin B_6 was directed towards the use of the reagent 2,6-dichloroquinonechloroimide for Vitamin B_6 analysis. The procedure of Scudi* as modified by Oser, *et al.*† was followed essentially. Excellent results were obtained on most pharmaceutical products; however, injection solutions containing phenol, yeast, and low potency feed products caused difficulty.

Recourse was obtained by use of the new chemical method for Vitamin B_6 as presented before the Association last year. Yet on low potency feedstuffs or similar materials wherein highly colored hydrolysates are found, difficulty was encountered. Aid was sought in the use of various ion exchangers either selectively to adsorb troublesome interfering colors or to

* *J. Biol. Chem.*, **139,** 707 (1941).
† *Ibid.*, **155,** 119 (1944).

concentrate the vitamin. No definite progress can be reported at this time on this phase of the study, except that while carrying on the work, it was noted certain phenomena occurred when small amounts of Vitamin B_6 were present with large amounts of nicotinic acid. Separation of these compounds on a synthetic ion exchanger was investigated and reported in another paper describing one part of the work (*This Journal*, **36**, 1018 (1953)).

RECOMMENDATION*

It is recommended that further work be undertaken to study a suitable chemical method for Vitamin B_6.

REPORT ON VITAMIN B_{12} (MICROBIOLOGICAL METHODS)

By CARL H. KRIEGER (Wisconsin Alumni Research Foundation, Madison, Wisconsin), *Associate Referee*

The 1951 A.O.A.C. Vitamin B_{12} collaborative study (1) revealed that, in the assay of crude materials, the method of sample treatment has a significant bearing on the subsequent assay value. Apparently the necessity of stabilizing the Vitamin B_{12b} is an important factor in preparing samples for assay. Of the four methods of sample preparation employed, the one consisting of a buffered bisulfite treatment appeared to show the most promise. The use of bisulfite in stabilizing Vitamin B_{12b} had its inception with the work of Fricke, *et al.* (2), and Frost, *et al.* (3). Subsequently Loy, Haggerty, and Kline (4) and Prier, Derse, and Krieger (5) have reported additional observations on the stabilizing phenomenon. In view of the success obtained by this method of sample treatment, it was deemed desirable to employ it in the 1952 collaborative study.

The 1951 A.O.A.C. study also indicated that a turbidimetric evaluation of the growth of the microorganisms after eighteen to twenty-four hours of incubation appeared practicable. Such a procedure has a distinct advantage over the titrimetric evaluation which requires an incubation period of seventy-two hours. Because of this, the turbidimetric evaluation was again employed in the 1952 study.

Three crude test materials, affording a wide range in potency, and a solution of U.S.P. standard cyanocobalamin served as the collaborative study samples. The method of assay employed was basically the U.S.P. XIV method (6). This method was modified in the 1951 A.O.A.C. study (1) to allow for a turbidimetric evaluation. In this study, the latter was modified further to include more specific instructions for sample treatment, basal medium preparation, and procedure. Since the desirability of including tomato juice in the basal medium has been strongly questioned, this ingredient was omitted from the medium.

* For report of Subcommittee A and action of the Association, see *This Journal*, 36, 52 (1953).

In an attempt to determine what intra- as well as interlaboratory agreement could be obtained in this study, each sample was assayed on three separate assay days.

The samples subjected to assay were as follows. Sample A was a water-phenol solution of Cyanocobalamin Reference Standard U.S.P., prepared to contain 15 mmg of Vitamin B_{12} per ml. The solution was colored yellow by means of a food dye. Sample B was a commercial grade 50 per cent condensed fish solubles. Sample C was a commercial Vitamin B_{12} antibiotic feed supplement containing adsorbed Vitamin B_{12}. Sample D was evaporated milk purchased on the open market. All cans bore the same code number and three cans were sent to each collaborator to permit the use of an unopened can on each of the three assay days.

VITAMIN B_{12} ASSAY PROCEDURE

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 (4.54 ml 0.2 *M* Na_2HPO_4 plus 5.46 ml 0.1 *M* citric acid) contg 1.0% freshly prepd $NaHSO_3$. Allow any undissolved particles to settle, or centrifuge if necessary. Dil. an aliquot of the clear soln with H_2O so that the final test soln contains a vitamin B_{12} activity equivalent to ca 0.025 millimicrogram of cyanocobalamin.

The amount of bisulfite in the assay soln should not exceed 0.025 mg/ml or the assay tube should not contain more than 0.125 mg.

REAGENTS

(a) *Standard cyanocobalamin stock solution.*—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 1.25 mmg of cyanocobalamin. Store in a cool place and use no longer than 60 days.

(b) *Standard cyanocobalamin solution.*—To 10 ml of standard cyanocobalamin stock soln **(a)** add 25 ml 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 0.025 millimicrogram of cyanocobalamin. Prepare a fresh standard soln for each assay.

(c) *Basal medium stock solution.*—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 additions should be followed:

100 mg *l*-cystine and 100 mg *d,l*-tryptophane dissolved in ca 10 ml 1 *N* HCl.	
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 sodium acetate, anhyd. and 1 g ascorbic acid dissolved in 50 ml 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 solution.*—Mix 100 g vitamin-free casein with 500 ml dil. HCl (1 in 2) and reflux the mixture 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 ($\pm$0.1) with NaOH soln and add H_2O to 1000 ml. Add 20 g activated charcoal, stir 1 hr and filter. Repeat twice this treatment with activated charcoal. Store under toluene in refrigerator at a temp. not below 10°C. Filter soln if a ppt forms upon storage.

(e) *Asparagine solution.*—Dissolve 2.0 g *l*-asparagine in H_2O to make 200 ml. Store under toluene in a refrigerator.

(f) *Adenine-guanine-uracil solution.*—Dissolve 0.2 g each 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 a refrigerator.

(g) *Xanthine solution.*—Suspend 0.2 g xanthine in 30–40 ml 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 a refrigerator.

(h) *Vitamin solution I, riboflavin-thiamine-biotin-nicotinic acid solution.*—Prep. a soln in 0.02 *N* acetic acid, each ml to contain 25 mmg riboflavin, 25 mmg thiamine hydrochloride, 0.2 mmg biotin, and 50 mmg nicotinic acid. Store, protected from light under toluene in refrigerator.

(i) *Vitamin solution II, p-aminobenzoic acid-calcium pantothenate-pyridoxine-pyridoxal-pyridoxamine-folic acid solution.*—Prep. a soln in 25% neutralized alcohol, each ml to contain 50 mmg *p*-aminobenzoic acid, 25 mmg Ca pantothenate, 100 mmg pyridoxine HCl, 100 mmg pyridoxal HCl, 20 mmg pyridoxamine dihydrochloride, and 5 mmg folic acid. Store in refrigerator.

(j) *Salt solution A.*—Dissolve 10 g KH_2PO_4 and 10 g K_2HPO_4 in H_2O to make 200 ml. Add 2 drops HCl and store under toluene.

(k) *Salt Solution B.*—Dissolve 4.0 g $MgSO_4 \cdot 7H_2O$, 0.2 g NaCl, 0.2 g $FeSO_4 \cdot 7H_2O$, and 0.2 g $MnSO_4 \cdot H_2O$ in H_2O to make 200 ml. Add 2 drops HCl, and store under toluene.

(l) *Polysorbate 80 solution.*—Dissolve 20 mg of polysorbate 80 in sufficient alcohol to make 200 ml. Store in refrigerator.

(m) *Culture medium.*—Dissolve 0.75 g water-soluble yeast extract, 0.75 g peptone, 1 g anhyd. dextrose, and 0.2 g KH_2PO_4 in 60–70 ml H_2O. Add 10 ml tomato juice prepn (n), and 1 ml polysorbate 80 soln. 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 an autoclave for 15 min. at 121–123°C. (exhaust line temp.). Cool as rapidly as possible to avoid color formation from overheating.

(n) *Tomato juice preparation.*—Centrifuge 3000 ml commercially canned tomato juice. Suspend 10–20 g analytical filter-aid in the supernatant liquid and filter with suction thru a layer of analytical filter-aid of sufficient thickness so that a clear, straw-colored filtrate is obtained.

(o) *Suspension medium.*—Dil. measured vol. of basal medium stock soln with an equal vol. H_2O. Place 10 ml portions of the dild medium in test tubes. Sterilize, and cool as directed for the culture medium (m).

(p) *Stock culture of the Lactobacillus leichmannii.*—To 100 ml culture medium add 1.0–1.5 g agar, and heat with stirring on steam bath until the 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.** (Before using a fresh culture in this assay, make at least 10 successive transfers of the culture in a two-week period.) Incubate 16–24 hrs at

* 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.

any selected temp. between 30° and 37° but held constant to within ±0.5°, and finally store in refrigerator.

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 culture medium. Incubate for 16–24 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 sterile suspension medium, centrifuge, and decant supernatant liquid. Again suspend cells in 10 ml sterile suspension medium, centrifuge, and decant supernatant liquid. Repeat this process a third time. Finally resuspend cells in 10 ml sterile suspension medium, add 1 ml of this suspension to 10 ml of sterile suspension medium, and mix. The cell suspension so obtained is the inoculum.

DETERMINATION

Because of the extremely high biological activity of the cobalamins the cleaning of glassware is *highly important.*

To 20×150 mm test tubes, add *in triplicate,* 0.0 ml, 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 basal medium stock soln and sufficient H_2O to make 10 ml.

To 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 throughout the assay.

Sterilization of the assay tubes is critical. During heating, cyanocobalamin, and perhaps other analogues, are converted to an analogue of the vitamin that is completely available to the organism. The 5 min. sterilization time is sufficient for this purpose, and to assure adequate sterilization. Longer heating periods cause excessive browning which may inhibit the growth of the organism. It is important to use a sterilizer that can be brought to temp. in a short time and which gives uniform heating. Too close packing of tubes in the autoclave, or over-loading, may cause variation in heating rate. Identical conditions for the cooling of tubes should be maintained.

Aseptically inoculate each tube with 1 drop of inoculum, and incubate for 16–24 hrs at any temp. between 30° and 37°C. but held constant to within ±0.5°C. Read turbidity of the tubes in a suitable instrument at a wavelength of 660 mμ after thoro shaking. Allow ca the same time interval to elapse prior to each tube reading.

Disregard results of an assay in which contamination with a foreign organism is evident or in which the control tubes of the standard series, to which no standard cyanocobalamin soln has been added, give a reading less than 85% transmission (preferably less than 90%), when read against an H_2O blank, which indicates interference due to vitamin B_{12} activity in the basal medium stock soln or inoculum.

CALCULATION

Prepare a standard concn response curve by plotting the galvanometer readings for each level of the standard cyanocobalamin soln used, against millimicrograms of

cyanocobalamin contained in the respective tubes. Draw the smooth curve which by visual inspection appears best to fit the plotted points. 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 average 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.

LIST OF COLLABORATORS

(1) T. F. Andrews, Western Condensing Co., Appleton, Wisconsin.

(2) C. Bergeron and J. R. Mallen, The Armour Laboratories, Chicago, Illinois.

(3) D. Billman, Jr. and J. R. McMahan, Chas. Pfizer & Co., Terre Haute, Indiana.

(4) F. Butzi and R. M. Stern, Pabst Laboratories, Milwaukee, Wisconsin.

(5) G. H. Craig and R. E. Bennett, Commercial Solvents Corporation, Terre Haute, Indiana.

(6) S. Derenuik, Fermentation Products Inc., Newaygo, Michigan.

(7) M. C. Firman and N. E. Rigler, Heyden Chemical Corp., Princeton, New Jersey.

(8) R. B. Grainger and L. M. Flynn, University of Missouri, Columbia, Missouri.

(9) M. L. Hobbs, R. P. Scherer Corp., Detroit, Michigan.

(10) R. G. Kluene and H. E. Wright, Schenley Laboratories, Inc., Lawrenceburg, Indiana.

(11) H. W. Loy and O. L. Kline, Division of Nutrition, Food & Drug Administration, Washington, D. C.

(12) R. F. Prier and P. H. Derse, Wisconsin Alumni Research Foundation Madison, Wisconsin.

(13) F. E. Randall, Cooperative G. L. F. Exchange, Inc., Buffalo, New York.

(14) L. Siegel and K. Morgareidge, Food Research Laboratory, Long Island City, New York.

(15) H. R. Skeggs, Sharp and Dohme, Inc., West Point, Pennsylvania.

(16) C. E. Smith and M. A. Douglas, Northern Regional Laboratory, Peoria, Illinois.

(17) J. T. Stephenson, Eli Lilly & Co., Indianapolis, Indiana.

(18) I. Terrill and R. Zorn, Grain Processing Corp., Muscatine, Iowa.

COMMENTS AND SUGGESTIONS BY COLLABORATORS

Collaborator No. 4: You requested that we do not report results which are more than 125 per cent of the estimated value. The results of our assay of Samples A and C were consistently out of this range; however, we are reporting the results of them for whatever worth they might have.

Collaborator No. 5: We found the procedure to be very satisfactory in most respects. However, the slope of the response curve is quite shallow, thus limiting the range of the assay and increasing the point-to-point error within a sample.

Collaborator No. 6: Uninoculated blanks gave 75–85% light transmission aginst water. Inoculated blanks gave 55–75% light transmission against water. Inoculated blanks gave 75–90% light transmission against uninoculated. The following were

tried, without success, in an attempt to cut down growth in blanks: (*a*) several different casein hydrolysates; (*b*) repeated charcoal treatment of casein hydrolysate; (*c*) reduction of amount of ascorbic acid in the medium; (*d*) washing of inoculum 5 times with suspension medium and with normal saline; (*e*) frequent checking for purity of culture. Sample D retained some turbidity although filtered after bisulfite treatment; consequently values were high, e.g., 2.6 mmg/ml, and 2.5 mmg/ml.

Collaborator No. 8: Some attempt should be made to standardize the inoculum. We have been adjusting the washed cells to a light transmittancy of 60%, using a 660 mμ filter and reading against a water blank. One drop of this is used in inoculating each tube. A few more points should be added to the standard curve. We have found that the steep straight line portion of most of our A.O.A.C. B_{12} curves extends to 0.175 millimicrograms. The curves tend to flatten beyond this point. The addn of 0.175 and 0.250 millimicrogram points will produce a more complete curve.

Collaborator No. 11: We found it necessary to wash the culture two additional times over the specified procedure in order to obtain a high reading in the zero tube of the standard curve.

Collaborator No. 14: Over a period of several months we ran parallel assays on several hundred samples evaluated on a turbidimetric basis, using 16 hour and 40 hour incubation. In our hands, the 40 hour test proved to be much more satisfactory than the 16 hour test. The range of the reference standard should be tenfold instead of five as it is now. In practice we believe you will find it difficult to maintain ten tubes per sample that will meet the requirements of this test with a standard of five fold range. We believe that six separate assays are a minimum upon which the B_{12} value of a sample should be calculated.

Collaborator No. 16: As usual we found considerable discrepancy in the samples which contained very little B_{12}, and good agreement for those two which were loaded with B_{12}. . . . Generally our checks for a single day's assay were good. Our curve was quite good. However, on the same day, we ran levels of B_{12} by our method of the same magnitude as the A.O.A.C. method and found a somewhat greater response for our method. As you know, we use cysteine in place of cystine, and also include fumaric acid in the medium. Our *p*H adjustment is 5.7 rather than 6.0.

Collaborator No. 17: The standard curve obtained was very flat when compared with the range of our regular U.S.P. acidimetric determinations as made electrometrically, using *p*H 7.0 as the end point.

DISCUSSION OF COLLABORATIVE DATA

A summary of the Vitamin B_{12} values obtained for samples A, B, C, and D are given in Tables 1, 2, 3, and 4 respectively. In addition to reporting the results obtained by the collaborative procedure, a number of laboratories also supplied data obtained by the method currently employed in their laboratory. These values are included in the tables.

The best results were obtained on sample A, which consisted of a solution of U.S.P. Cyanocobalamin Reference Standard, as is evidenced by the relatively low coefficient of variation, not only for each laboratory but also between laboratories. In comparing the results obtained by the collaborative procedure with those obtained by the current method, the former yielded values in closer agreement to the theoretical in most instances.

Considerably greater variations were experienced with sample B

TABLE 1.—*Vitamin B_{12} values for sample A (micrograms/ml)*

COLLABORATOR	COLLABORATIVE METHOD			CURRENT METHOD		
	MEAN[a]	STANDARD DEVIATION	COEFFICIENT OF VARIATION	MEAN[a]	STANDARD DEVIATION	COEFFICIENT OF VARIATION
			per cent			*per cent*
1	15.70	1.860	11.80	15.3	2.69	17.60
2	14.90	0.879	5.90			
3	15.33	0.321	2.09			
4	28.30[b]	2.040	7.29			
5	15.30	0.500	3.30			
6	16.45	3.180	19.30			
7	18.70	1.370	7.30			
8	16.02	1.301	8.12			
9	14.48	2.190	15.10			
10	18.10	1.852	10.23	22.90	2.760	12.05
11	11.58	0.500	4.32			
12	16.20	1.600	9.90			
13	14.20	0.819	5.77			
14	15.16	1.240	7.82	13.90 13.27	0.200 0.513	1.43 3.87
15	14.80	0.865	5.84	14.70	0.613	4.17
16	14.60	0.926	6.35	16.10	0.283	1.75
17	14.17	0.183	1.29			
18	13.60	0.300	2.20			
Mean	15.30					
Std. Deviation	1.65					
Coef. of Variation, %	10.8					

[a] Each value represents the mean of at least three separate assays.
[b] Value omitted from calculation.

(condensed fish solubles) not only between laboratories but in a number of instances within one laboratory. A similar condition prevailed with the the current methods. It is not surprising that this test material, being of relatively low potency, should reveal a greater variation.

A crude test material such as sample C (Vitamin B_{12} antibiotic feed supplement) of relatively high Vitamin B_{12} content lent itself to better within laboratory and between laboratory agreement than sample B. The variation, however, was greater than that experienced with sample A which had essentially the same potency. Although the difference in the coefficient of variation between samples A and C is not seriously large, it indicates that some difficulty is still extant in the elution and/or stabilization of Vitamin B_{12} or its analogs. However, the method appears readily applicable to Vitamin B_{12} feed supplements.

It is evident from the results obtained on sample D (evaporated milk) that such a test material possessing an extremely low Vitamin B_{12} content, *i.e.*, approximately 1.0 millimicrogram of Vitamin B_{12} per ml., markedly

TABLE 2.—*Vitamin B_{12} values for sample B (micrograms/g)*

COLLABORATOR	COLLABORATIVE METHOD			CURRENT METHOD		
	MEAN[a]	STANDARD DEVIATION	COEFFICIENT OF VARIATION	MEAN[a]	STANDARD DEVIATION	COEFFICIENT OF VARIATION
			per cent			*per cent*
1	0.177	0.0115	6.50	0.143	0.0153	10.70
2	0.098	0.0146	14.90			
3	0.161	0.0026	1.61			
4	0.267	0.037	13.20			
5	0.190	0.016	8.20			
6	0.193	0.0116	6.00			
7	0.184	0.032	16.90			
8	0.187	0.01749	9.35			
9	0.178	—	—	0.148	0.0202	13.65
10	0.161	0.03701	22.98	0.175	0.0366	20.91
11	0.123	0.0153	12.42			
12	0.220	0.01	4.50			
13	0.150	0.0224	14.93			
14	0.177	0.019	15.09	0.167 0.167	0.0058 0.0152	3.47 9.10
15	0.127	0.003	2.36	0.135	0.0119	8.81
16	0.211	0.908	43.00	0.201	0.0420	20.89
17	0.173	0.005774	3.33			
18	0.176	0.0015	0.85			
Mean		0.175				
Std. Deviation		0.0496				
Coef. of Variation, %		28.3				

[a] Each value represents the mean of at least three separate assays except collaborator #9 where only one assay value was reported.

increased the intra- and interlaboratory coefficient of variation. In most cases where both the collaborative and current procedures were applied, the variation obtained by the current method was definitely the lesser. Undoubtedly, greater familiarity with the current method aided in lowering the coefficient of variation.

In general, reasonably good inter- and intralaboratory agreement was obtained, particularly with the higher potency samples. The greater variation with the low potency materials indicates that further work must be done to afford more reliable results. As was to be expected, the within laboratory agreement was better than that observed between laboratories. Seemingly, the bisulfite treatment presented no particular problems. Although the 1951 study, as well as the present one, indicates that the bisulfite treatment yields reasonably satisfactory results, the variations observed on the lower potency materials may be suggestive of incomplete elution or stabilization of the Vitamin B_{12} activity. Consequently, further consideration should be given this phase of the assay in an effort to minimize these discrepancies. The within and between

TABLE 3.—*Vitamin B_{12} values for sample C (micrograms/g)*

COLLABORATOR	COLLABORATIVE METHOD			CURRENT METHOD		
	MEAN[a]	STANDARD DEVIATION	COEFFICIENT OF VARIATION	MEAN[a]	STANDARD DEVIATION	COEFFICIENT OF VARIATION
			per cent			*per cent*
1	12.50	2.380	19.05	14.10	1.960	13.90
2	13.40	0.510	3.80			
3	13.00	0.308	2.37			
4	27.70[b]	6.850	26.70			
5	13.70	1.600	11.80			
6	12.10	2.180	18.00			
7	14.50	1.170	7.90			
8	13.53	0.505	3.72			
9	12.27	1.532	12.48			
10	10.20	0.903	8.85	11.30	0.436	3.86
11	9.80	1.058	10.80			
12	12.60	1.600	12.70			
13	11.30	2.544	22.51			
14	15.33	1.360	8.89	13.50 14.37	0.693 0.710	5.13 4.94
15	10.90	0.200	1.83	11.30	0.630	5.58
16	13.04	1.130	8.66	13.40	0.720	5.37
17	17.05	2.758	16.17			
18	9.97	0.058	0.58			
Mean	12.10					
Std. Deviation	2.010					
Coef. of Variation, %	16.6					

[a] Each value represents the mean of at least three separate assays.
[b] Value omitted from calculation.

laboratory agreement obtained in this study also lends further support to the contention that a turbidimetric evaluation is feasible. One collaborator (No. 14) indicated that better results were obtained after 40 hours rather than 16 hours. Similar situations have been rectified by the use of a more active inoculum such as is obtained by frequent transfer of the microorganism within a given day.

Even at best, it is obvious from the data and the comments made by some of the collaborators that room for improvement remains. As indicated above, the elution and stabilization of the Vitamin B_{12} activity is an important factor. However, consideration must also be given to the actual execution of the assay itself. Contradictory comments relative to the extent of the usable part of the standard curve, the percentage of light transmission in the blanks, the activity of the microorganism, together with occasional very high or low values, seem to indicate a failure to perform the assay satisfactorily as now written. In general this was not the case; however, its occurrence was frequent enough to warrant con-

TABLE 4.—*Vitamin B_{12} values for sample D (millimicrograms/ml)*

COLLABORATOR	COLLABORATIVE METHOD			CURRENT METHOD		
	MEAN[a]	STANDARD DEVIATION	COEFFICIENT OF VARIATION	MEAN[a]	STANDARD DEVIATION	COEFFICIENT OF VARIATION
			per cent			*per cent*
1	1.07	0.186	17.40	0.91	0.086	9.50
2	0.41	0.128	31.00			
3	0.68	—	—			
4	1.23	0.230	18.70			
5	0.99	0.010	1.10			
6	—	—	—			
7	1.05	0.069	6.60			
8	0.54	0.052	9.62			
9	0.88	0.707	8.03	1.43	0.424	2.96
10	2.49	0.541	21.71	1.89	0.216	11.42
11	0.68	0.054	7.99			
12	0.87	0.150	17.10			
13	0.62	0.130	21.03			
14	1.20	0.012	8.33	1.16 1.23	0.012 0.058	10.00 4.72
15	0.50	0.090	18.00	0.53	0.067	12.70
16	0.65	0.174	26.60	1.11	0.205	18.46
17	1.09	0.064	5.89			
18	1.45	0.010	0.69			
Mean		0.96				
Std. Deviation		0.490				
Coef. of Variation,		% 50.6				

[a] Each value represents the mean of at least three separate assays except collaborator #3 which is one assay.

sideration. Particular attention to the use of scrupulously clean glassware, the length of time and manner of sterilization, and the maintenance of identical conditions for the cooling of the tubes often obviate many of these difficulties.

SUMMARY

A proposed procedure for the microbiological assay of vitamin B_{12} in crude materials was subjected to collaborative study in 1952 by eighteen laboratories. The method of assay was a modification of that employed in the 1951 study. A bisulfite treatment of the test material to stabilize vitamin B_{12b} and a turbidimetric evaluation of the growth of the microorganisms after sixteen to twenty-four hours were included. Three crude test materials, varying widely in potency, and a solution of Cyanocobalamin Reference Standard were subjected to assay on each of three assay days. In general reasonably good inter- and intralaboratory agreement was obtained. Far less variation was observed with the higher potency materials than with the low potency samples. The results ob-

tained on the Vitamin B_{12} feed supplement in both the 1951 and 1952 studies were of an order to indicate that this method is sufficiently satisfactory for general use on materials ranging from approximately 1–10 mg of Vitamin B_{12} per pound. The greater variation experienced with the low potency samples indicates the desirability of further work including continued consideration of sample treatment and assay execution.

RECOMMENDATIONS

It is recommended*—

(1) That the method be adopted First Action for assay of Vitamin B_{12} feed supplements ranging from approximately 1.0 to 10 mg of Vitamin B_{12} per pound.

(2) That the work on the microbiological assay for vitamin B_{12} be continued.

(3) That the test materials should continue to be crude materials of relatively low potency and that particular attention be given to milk.

REFERENCES

(1) KRIEGER, C. H., *This Journal*, **35**, 726 (1952).
(2) FRICKE, H. H., LANIUS, B., DEROSE, A. F., LAPIDUS, M., and FROST, D. V., *Federation Proc.*, **9**, 173 (1950).
(3) FROST, D. V., ARMSTRONG, K. L., LAPIDUS, M., and FRICKE, H. H., *Am. Chem. Soc.*, Abstract of Papers, 70 C, 118th Meeting, Chicago, Sept. 1950.
(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.*, **40**, 474 (1952).
(6) United States Pharmacopeia, Fourteenth Revision, Third Supplement, Mack Publishing Co., Easton, Pa., 1952, p. 15.

REPORT ON A CHEMICAL METHOD FOR NICOTINIC ACID

By J. P. SWEENEY (Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D.C.),
Associate Referee

A report on a collaborative study of methods for the chemical determination of nicotinic acid was given at the 1950 A.O.A.C. meeting. In this study sulfanilic acid and Tobias acid were used as aromatic amines. As the result of the study, the sulfanilic acid method was adopted as official, First Action.

The nicotinic acid must be extracted from food and feed products and any nicotinamide present converted to nicotinic acid. For this purpose,

* For report of Subcommittee A and action of the Association, see *This Journal*, **36**, 52 (1953).

the samples were autoclaved with 0.25 *N* sulfuric acid for 30 min. at 15 lb pressure.

However, further work revealed that 0.25 *N* sulfuric acid is not of sufficient strength for complete conversion of nicotinamide to nicotinic acid. Therefore, it was recommended at the 1951 meeting that the strength of the acid be increased from 0.25 *N* to 1.0 *N* sulfuric acid.

This modified method has been used during the past year by districts of the Food and Drug Administration for the determination of the nicotinic acid content of a series of check samples. The results obtained thus far are presented in Table 1.

TABLE 1.—*Nicotinic acid assay*

NO.	SAMPLE	NICOTINIC ACID (MG PER LB)				
		MICROBIOLOGICAL	CHEMICAL			
			DISTRICTS			
			1	2	3	4
91	Enriched flour	—	16.9	16.8 17.1	17.0	17.75
92	Enriched bread	16.2	16.0	15.7	16.4	15.5
93	Unenriched flour	—	6.5	5.9	6.3	6.7
94	Enriched corn meal	13.8	13.2	13.6 13.9	17.1	12.0 13.6
95	Enriched flour	—	20.3	22.1 21.0	19.4	21.0 18.8
96	Enriched flour	—	15.5	15.0 14.1	13.7	16.3
97	Enriched bread	—	13.9	15.8	15.7	15.7
98	Unenriched flour	6.2	6.2	—	—	6.0

RECOMMENDATION

It is recommended* that the sulfanilic acid method for nicotinic acid be made official.

REPORT ON CAROTENE

By F. W. QUACKENBUSH (Purdue University Agricultural Experiment Station, Lafayette, Indiana), *Associate Referee*

The monthly check sample for carotene in alfalfa meal was continued this year with a view toward the elimination of factors responsible for variations between laboratories. Since carotene in alfalfa meal is subject to deterioration during mailing and holding of samples prior to analysis,

* For report of Subcommittee A and action of the Association, see *This Journal*, 36, 52 (1953).

TABLE 1.—*Carotene values reported on alfalfa meal samples (mg/lb)*

COLLABORATOR NUMBER[a]	SAMPLE 32 REGULAR MAIL ROOM TEMPERATURE	SAMPLE 33 AIRMAIL ROOM TEMPERATURE	SAMPLE 34 AIRMAIL REFRIGERATED	SAMPLE 35 AIRMAIL REFRIGERATED
1	72.2	97.5	88.8	127.0
2	79.8	97.8	85.2	122.5
3	78.0	88.0	84.6	115.5
4	82.5	98.3[b]	90.1	105.9
5	78.0	76.2[b]	100.5	99.1[b]
6	82.6	92.9	84.3	108.2
7	78.0	87.8	89.3[b]	112.2
8	77.6	86.9	82.6	107.9
9	83.7	98.4	90.6	103.5
10	75.6	85.3	84.4	97.1
11	78.2	80.4	87.9	108.4
12	73.8	89.6	88.1	113.5
13	76.3	95.0	89.2	109.0
14	95.7	86.8	93.5	115.6
15	73.4	89.7	81.4	101.4
16	90.7	94.8	101.2	116.8
17	88.9	89.5	84.8	106.9
18	73.7	93.2	91.4	109.5
19	86.5	92.6	83.5	125.3
20	78.8	87.9	96.0	111.6
21	81.7	99.2	89.9	114.4
22	86.8	102.2	79.5	103.8
23	77.0	84.6	84.2	105.7
24	76.2	97.2	81.0	97.4
25	80.7	92.6	100.8	109.0
26	80.6	100.7	90.7	104.0[b]
27	87.5	97.2	86.7	105.6
28	77.2	82.5	83.6	101.3
29	75.9	99.1	90.0[b]	124.2
30	68.9	68.0[b]	87.9	102.3
31	80.5	96.7	88.5	103.0
32	82.2[b]	92.6	90.5	90.8
33	88.8	96.0	83.6	101.9
34	62.6[b]	104.4	85.5	111.6
35	76.2	91.6	92.5	110.7
36	82.6	88.5	71.2	107.1
37	80.5	92.4	90.4	112.0
38	81.7	96.1	96.3	112.6
39	86.2	86.3	92.8	100.6
40	95.6	103.6	91.1	119.8
41	90.7	108.9[b]	104.3	99.8
42	80.0	88.1	82.5	87.9
43	69.4	73.5[b]	98.0	123.0
44	85.2	87.0	85.2	94.2
45	78.9	91.8	78.6	108.0[b]
46	80.5	91.4	91.2	107.1
47	83.2	94.5	89.5	102.7
48	83.1	108.7	98.1	118.5[b]
Average	80.9	93.1	88.7	108.3
Std. Dev.	6.05	6.11	6.59	8.58
Coeff. of Var.	7.5%	6.6%	7.4%	7.9%

[a] Numbers do not correspond to those previously assigned.
[b] Omitted from average and statistical calculations.

collaborators have often pointed to this as an important source of variation, in some cases supporting their view with results of successive analysis on their sample at intervals of several days or a week. In an

attempt to evaluate this error samples were sent out as follows:

Sample No. 32 (mailed May 28), like previous samples, was delivered by regular mail with instructions to hold at room temperature and analyze within two weeks from mailing date. In some cases the samples were in the mail during nearly the entire two weeks' time.

Sample No. 33 (mailed June 28) was sent airmail with instructions to hold at room temperature and analyze within two weeks from mailing date. Most samples were delivered within two days.

Sample No. 34 (mailed July 28) was sent airmail with instructions to refrigerate immediately on arrival and until analysis could be made.

Sample No. 35 (mailed August 28) was sent airmail with the same instructions as No. 34. In addressing packaged samples before mailing, each tenth package was set aside for the purpose of checking on uniformity of sample. The ten samples so set aside were analyzed at Lamar, Col., then sent airmail to Lafayette, Ind., where they were analyzed again.

RESULTS

48 collaborators analysed all four samples by the official method and reported results within the period specified (Table 1). Airmailing and refrigeration did not reduce the variations between laboratories.

Statistical analysis of the regression of reported carotene values on number of days between mailing from Lamar and analysis in the laboratory seemed to show a significant advantage for airmailing, but not for refrigeration (Table 2). Evidently the differences were overshadowed by other uncontrolled variables.

TABLE 2.—*Relation of carotene values to time lapsed between mailing and analysis*

SAMPLE	MAILING	HOLDING	SLOPE OF REGRESSION LINE
32	Regular	Room Temp.	−.70[a]
33	Airmail	Room Temp.	+.22
34	Airmail	Refrigerated	−.16
35	Airmail	Refrigerated	+.36

[a] Significant at the 5% level.

Since the results on Samples 33 and 34 showed little reduction in variability as compared with previous samples, the ten samples of No. 35 were analyzed as a check on the uniformity of alfalfa meal sent out as the check samples. The results obtained at Lamar and at Lafayette are shown in Table 3. The data indicate fairly good uniformity of samples.

SUMMARY

Deterioration of samples between date of mailing to collaborators and date of analysis does not appear to be the chief source of variation in

TABLE 3.—*Carotene found in ten containers of sample No. 35 (mg/lb)*

SAMPLE	LAMAR (AUG 28)	LAFAYETTE (SEPT 3)	DIFFERENCE
10	117.5	106.5	11.0
20	117.5	103.5	14.0
30	120.0	102.6	17.4
40	117.0	102.1	14.9
50	117.0	101.1	16.0
60	113.3	106.1	7.2
70	117.5	104.5	13.0
80	113.3	101.7	11.6
90	116.5	101.4	15.1
100	116.0	102.1	13.9
Average	116.56	103.15	13.4

carotene analyses on alfalfa meal check samples. Evidence also indicates that the samples have been fairly uniform in composition when mailed.

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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. He also wishes to express his appreciation to all of the collaborators for their participation and to Professor S. R. Miles of the Purdue staff for statistical analysis of the results.

RECOMMENDATIONS

It is recommended* that work on carotene analysis be continued.

REPORT ON FILL OF CONTAINER STUDIES ON FROZEN FRUITS

By WILLIAM W. WALLACE, *Associate Referee*, and R. A. OSBORN, *Referee* (Food and Drug Administration, Department of Health, Education, and Welfare, Seattle, Wash. and Washington, D. C.)

This Association has as yet no official procedure for the determination of the fill of container for small packages of frozen fruits. A simple measurement of headspace is usually not practicable, since packages of fruits are frozen in various positions and the packages may not be rigid in construction.

A displacement method for the measurement of the volume of frozen

* For report of Subcommittee A and action of the Association, see *This Journal*, 36, 52 (1953).

fruit was proposed early in 1948 by Osborn.* Later, Nickerson and Evers (unpublished work) of the National Association of Frozen Food Packers, Washington, D. C., measured the per cent fill of many commercial packages of various frozen fruits, using the displacement procedure.

The work reported here was undertaken (*a*) to obtain collaborative results with the method; (*b*) to determine the per cent fill of containers of frozen fruits obtained by current commercial practice; (*c*) to determine the per cent fill of containers of frozen fruits that can be obtained by good commercial practice; and (*d*) to study the effect of slack filling on the measurements of per cent fill.

PROCEDURE FOR DETERMINING NET VOLUME IN CERTAIN CONSUMER-SIZE PACKAGES OF FROZEN FRUITS

APPARATUS

(a) *Overflow can*, with device for lowering frozen fruit into liquid and for removing it (Fig. 1). Can is ca 8 in. in diam. and ca 9 in. high with an overflow spout of $\frac{3}{16}$ in. I.D. copper tubing. The tubing is soldered to an opening on side of can ca $\frac{1}{2}$ in. from bottom and bent upward parallel to side of can to ca 2 in. below top of can where it is bent away and downward to form an inverted U. The spout is formed by cutting tubing on outer side of U where it makes ca 45° angle with can, making cut parallel to bottom of can. The opening of spout is ca $\frac{1}{8}$ in. below lower surface of U-bend. The end of spout is bent up or down until overflow, caused by adding an excess of liquid to can, will end abruptly. (Proper adjustment of tube and addn of sufficient liquid will secure this effect.)

(b) *Plastic bags*, pliable at 0°F.; capable of holding a vacuum; ca 8×10 in. when flat. (Cry-o-rap bags, Type L, Dewey & Almy Chemical Co., Locksport, N. Y., or equivalent.)

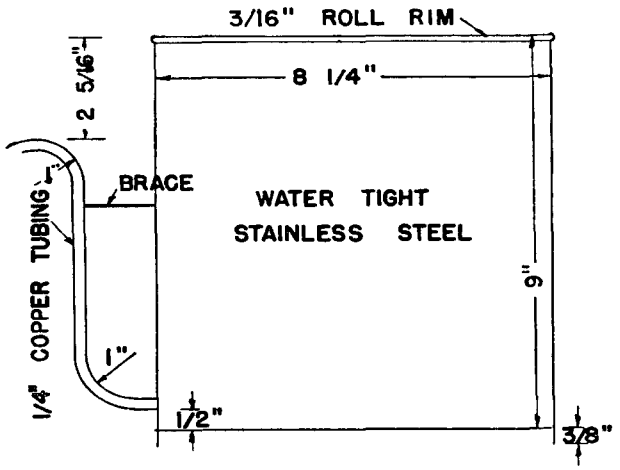

FIG. 1.—Apparatus for determining volume of frozen fruits by displacement.

* *This Journal*, 32, 174 (1949).

(c) *Deep freeze or cold room,* at or near 0°F.
(d) *Refined light mineral oil,* such as odorless kerosene.

DETERMINATION

Remove frozen sample from its container and inner wrapper, if any, and transfer it to plastic bag. Remove excess air from bag by inserting glass tube attached to vacuum line. Twist bag top to close, hold twist with pinch clamp, and trim off loose end. The bags should be pre-tested to be certain that they will not leak.

Place overflow can in freezing compartment and fill can, in which lifting device is inserted, with light mineral oil at temperature of freezing compartment (ca 0°F). Add sufficient excess of mineral oil (ca 300 ml) to produce siphon effect in overflow, collecting overflow in beaker. Place an empty, calibrated graduated cylinder under overflow tube and immerse frozen fruit sample completely in the mineral oil by means of lifting device. Record volume of overflow in cylinder to nearest ml. Correct this vol. for displacement of empty plastic bag and pinch clamp (ca 7 ml). Redetermine displacement of sample to check reproducibility of procedure.

Det. per cent fill of container by dividing net displacement of frozen fruit by H_2O capacity of outer container and multiply by 100.

For packages with square corners, calc. H_2O capacity of outer container by multiplying inside length, breadth, and height in centimeters. For packages with curved edges or irregular shape, det. H_2O capacity, using method for H_2O capacity of fiber containers described below.

The above procedure was subjected to collaborative study, using authentic and commercial packs of a number of fruits. The preparation of these packs is described later in this report.

The results obtained by the collaborators are shown in Table 1. The volumes reported are for the packs of commercial or experimental mixes of fruit and sugar (or syrup), which had been filled to exact declared net weight. Each figure reported is an average of individual determinations on 3 to 6 packages of each series.

The results show generally close agreement between collaborators, in most instances within 10 ml. Due to the physical nature of the products, some difference in net volume from package to package of certain fruits, such as blackberries and cherries packed with sugar, was expected. Other mixtures, such as sliced strawberries and sugar, or fruits and syrup, would be expected to have a more uniform fill, and results reported here bear this out.

In order to calculate the per cent fill of consumer size packages of frozen fruits, it is necessary to know the volume of the container. For rectangular packages, this can be calculated from the internal measurements. However, many of the packages now in use have tin ends and waxed paper sides with rounded corners. In addition, the sides of these containers bulge in varying degrees. Considerable variation in capacity was found, depending on whether the sides were held even with the ends by a rigid form, or were allowed to bulge with the weight of water added. It was felt that the capacity measurement should be made with the flexible sides of the package unchanged from their normal or empty position. The following method was devised.

TABLE 1.—*Comparison of results by collaborators for volume of frozen fruits determined by displacement method*

FRUIT	WEIGHT RATIO— FRUIT TO PACKING MEDIA	NO. PACKS EXAMINED	VOL. IN ML/LB FRUIT SAMPLE			
			COLLABORATORS			
			1	2	3	4
Sl. Strawberries and sugar	4 plus 1	8	438	436	438	—
Sl. Strawberries and syrup	3 plus 1	2	451	446	449	—
Whole Strawberries and sugar	3 plus 1	1	421	419	426	—
Whole Strawberries and syrup	3 plus 1	1	461	459	461	—
Red Raspberries and syrup	2 plus 1	6	436	440	435	—
Red Raspberries and syrup	7 plus 3	1	464	473	437	—
RSP Cherries and sugar	5 plus 1	1	436	433	433	—
RSP Cherries and sugar	3 plus 1	1	397	400	410	—
RSP Cherries and syrup	3 plus 1	1	441	445	435	—
Himalaya Blackberries and syrup	2 plus 1	1	450	471	464	—
Himalaya Blackberries and syrup	3 plus 1	1	457	480	467	—
Evergreen Blackberries and syrup	4 plus 1	1	497	507	491	—
Apricot halves and syrup	60 plus 40	1	431	445	—	444
Apricot halves and syrup	65 plus 35	1	440	447	—	451
Apricot halves and syrup	70 plus 30	1	440	459	—	453
Sl. Freestone Peaches and syrup	65 plus 35	2	449	449	—	—
Sl. Freestone Peaches and syrup	69 plus 31	3	447	454	—	451
Sl. Freestone Peaches and syrup	75 plus 25	3	460	464	—	465

PROCEDURE FOR DETERMINING WATER CAPACITY OF CONTAINERS

Place empty container in beaker or pan contg enough H_2O to reach to within 1 cm of top of container when it is resting on bottom of beaker or pan. Note that no air is trapped by bottom of container.

Add H_2O from a calibrated 500 ml buret to fill container to capacity, or to measured headspace if indented top has been removed. Read H_2O capacity directly from buret.

This method was used to determine the water capacity of the eleven different sizes or types of containers representing 25 authentic packs of frozen fruit put up for this investigation. Empty, unused containers were obtained when the fruit packs were prepared. In most instances, these empty containers were machine capped in the same manner as the filled containers. A $\frac{1}{4}''$ hole was drilled in the cap and the container was filled to capacity through the hole.

A description of the containers and their water capacities is given in Table 2.

When the water capacity of empty, *used* containers was determined by the same method, it was found that the capacity had generally increased.

TABLE 2.—*Water capacity of unused, consumer-size, frozen fruit containers*

NO.	MANUFACTURER	EXT. DIMENSIONS INCHES	DECLARED NET	DESCRIPTION	NO. EXAM.	WATER CAPACITY, ML	
						RANGE	AVERAGE
1	Sefton	$4\times3\frac{1}{2}\times1\frac{3}{4}$	12 oz.	Metal end, paperbd. sides, lithographed label	61	365–378	372
2	Sefton	$5\times3\frac{1}{2}\times1\frac{3}{4}$	1 lb.		16	467–490	477
3	Sefton	$5\frac{1}{2}\times3\frac{1}{2}\times1\frac{3}{4}$	1 lb.		9	512–522	517
4	Canco	$3\frac{3}{4}\times3\frac{1}{2}\times1\frac{3}{4}$	12 oz.	Metal end, waxed paperbd. sides	0		345[a]
5	Canco	$3\frac{7}{8}\times3\frac{1}{2}\times1\frac{3}{4}$	12 oz.		49	349–362	357
6	Canco	$4\frac{7}{8}\times3\frac{1}{2}\times1\frac{3}{4}$	1 lb.		2	456–457	457
7	Canco	$5\times3\frac{1}{2}\times1\frac{3}{4}$	1 lb.		12	478–495	486
8	Canco	$5\times3\frac{1}{2}\times1\frac{3}{4}$	1 lb.	Metal end, paperbd. sides, lithographed	7	447–455	450
9	Lilly Tulip or Cupples-Hesse	Round cups	1 lb.	Waxed paperbd., recessed top & bottom	16	457–471	464
10	Marathon	$4\times3\frac{9}{16}\times1\frac{3}{4}$	12 oz.	"Paxcel," thin paperbd. box	8	321–337	326
11	Marathon	$5\frac{3}{8}\times4\times1\frac{3}{4}$	1 lb.	Paperbd. box with paper inner-bag	6		602[b]

[a] Calcd from water capacity of Canco $3\frac{7}{8}$ in. container (No. 5) since no empty, unused containers were available for measurement. Used containers measured 355–358 ml capacity.

[b] Calcd from internal dimensions of rectangular box.

The increase was as much as 25 ml in some instances and averaged 12 ml for all containers measured.

FILL OF CONTAINER OF COMMERCIALLY FROZEN FRUITS (1951 SEASON) AND FILL OF CONTAINER OBTAINABLE BY GOOD COMMERCIAL PRACTICE

A total of 25 authentic packs representing 11 fruits were prepared during the 1951 packing season. These packs were made at 12 plants in Washington, Oregon, and California. Each authentic pack consisted of several series of 12 or more package units. The packs included:

(1) The regular commercial pack taken from the factory line. The gross weight of each container and the average tare weight was obtained. The fruits in these packs included sliced and whole strawberries, red raspberries, and sliced freestone peaches.

(2) Consumer-size packages of commercial mixtures of fruit and sugar (or syrup) filled to the exact net weight declared on the commercial packages. In addition to fruits listed in series 1, this series included apricot halves, black raspberries, red sour pitted cherries, evergreen blackberries, Himalaya blackberries, boysenberries, loganberries, and Pacific blackberries.

(3) When the ratio of fruit to packing medium in the commercial samples differed from the proposed standard for frozen fruit,* packages containing the proposed ratio were prepared and filled to the declared net weight. This series included red raspberries and sliced peaches.

Sufficient packages were prepared for each series to permit independent examination by two or more analysts. All weighings were made with calibrated weights on a torsion balance sensitive to 0.01 oz. The containers were sealed by the factory machines and frozen in the regular freezing room of the packer. After the fruit was frozen solid (twenty-four hours or more) the packages were moved, with protection from thawing, to laboratory cold storage at ca 0°F. and held there until examined.

The net volume was determined, using the overflow method given above. The water capacity of empty, unused containers, taken from the same lot as those used for each pack, was also determined as described above. The per cent fill is the net volume divided by the water capacity of the empty, unused container and multiplied by 100. The per cent fill was calculated to declared net weight as explained later in this report.

The results reported in Tables 3 and 4 are averages of all containers examined by all analysts.

With the exception of the paper box with inner bag, any container encountered could be commercially filled to 90 per cent or more of its water

* Federal Register Oct. 4 (1950), 6674–6686.

TABLE 3.—*Fill of frozen fruits by container types; commercial packs—1951 season*

FRUIT	CONTAINER	NO. EXAMINED		FILL FOUND, PER CENT	FILL CALCD TO DECL. NET WT, PER CENT
		PACKS	PKGS.		
Sl. strawberries	12 oz. Sefton	3	35	86.3	86.2
4 plus 1 sugar	12 oz. Canco, 3⅞″	1	14	94.8	95.5
	12 oz. Paxcel	1	16	101.2	98.7
	1 lb. Canco, 5″ lithogr.	1	16	97.7	97.9
	1 lb. Canco, 5″ waxed	1	6	88.8	88.9
	1 lb. Canco, 4⅞″ waxed	2	22	95.0	94.3
3 plus 1 syrup	12 oz. Sefton	1	15	91.3	91.9
Whole strawberries					
3 plus 1 sugar	1 lb. cup	1	15	96.1	90.8
11 plus 5 sugar	1 lb. cup	1	4	92.0	91.3
Red Raspberries	12 oz. Sefton	2	31	90.0	88.3
2 plus 1 syrup 50°	12 oz. Canco, 3⅞″	2	28	92.8	91.6
Sl. Freestone Peaches	12 oz. Sefton	1	15	94.1	91.3
7 plus 3 syrup 50°	12 oz. Canco, 3⅞″	1	15	98.9	94.8
	1 lb. Sefton	1	15	98.5	94.0
	1 lb. Marathon (with inner bag)	1	15	78.9	78.7

capacity. Packages with a fill below 90 per cent appeared to be slack-filled. With containers having an inner bag, it may not be practicable to obtain 90 per cent fill.

FACTORS AFFECTING THE VOLUME OF A GIVEN WEIGHT OF FROZEN FRUITS

Table 5 summarizes results from the examination of authentic packs of a number of consumer size packages of frozen fruits which are commonly packed with net weight declarations of 12 oz. and 1 lb. Other packages marked 10½ oz., 15 oz., 1 lb 4 oz., etc., may occur. It is a common practice for container manufacturers and packers to have the cubic capacity of the packages related to the net weight of the fruit and sugar, or fruit and syrup mixture. Data in Table 5 bears on this subject. Here will be found the volume (ml) required for one pound of various frozen fruits when packed in several ratios of fruit to sugar, or fruit to syrup. It will be observed that with the higher ratios of fruit to sugar or syrup a somewhat larger volume is required for one pound of the frozen fruit. This is to

TABLE 4.—*Fill of frozen fruits by container types; experimental packs—1951 season*

FRUIT	CONTAINER	NO. PACKS	NO. PKGS. EXAMINED	FILL FOUND, PER CENT	CALCD TO NET WT, 12 OZ. OR 16 OZ., PER CENT
Apricot halves & 47° syrup					
60 plus 40	12 oz. Canco, 3 7/8"	1	12	91.7	92.5
65 plus 35	12 oz. Canco, 3 7/8"	1	18	93.3	94.0
70 plus 30	12 oz. Canco, 3 7/8"	1	12	93.8	94.7
Black Raspberries & 51° syrup					
7 plus 3	12 oz. Canco, 3 7/8"	1	10	92.8	93.5
Boysenberries & 50° syrup					
2 plus 1	12 oz. Canco, 3 7/8"	1	9	92.5	93.0
3 plus 1	12 oz. Canco, 3 7/8"	1	7	95.6	96.4
Evergreen Blackberries & 50° syrup					
2 plus 1	1 lb. Sefton, 5 1/2"	1	6	86.2	89.2
3 plus 1	1 lb. Sefton, 5 1/2"	1	10	92.3	95.8
4 plus 1 sugar	1 lb. Sefton, 5 1/2"	1	11	92.5	96.4
Himalaya Blackberries & 50° syrup					
3 plus 2	1 lb. Sefton, 5"	1	8	89.4	91.6
2 plus 1	1 lb. Sefton, 5"	1	12	92.4	94.6
3 plus 1	1 lb. Sefton, 5"	1	15	94.4	96.4
Loganberries & 60° syrup					
2 plus 1	12 oz. Canco, 3 7/8"	1	6	93.3	94.1
Pacific Blackberries & 50° syrup					
3 plus 1	12 oz. Canco, 3 7/8"	1	10	93.0	93.5
RSP Cherries					
3 plus 1 sugar	12 oz. Canco, 3 7/8"	1	12	85.4	86.0
5 plus 1 sugar	12 oz. Canco, 3 7/8"	1	12	92.0	93.0
5 plus 1 sugar	12 oz. Canco, 3 3/4"	1	8	98.4	99.2
2 plus 1 syrup 61°	12 oz. Canco, 3 7/8"	1	8	88.1	88.8
4 plus 1 syrup	12 oz. Canco, 3 7/8"	1	9	93.4	94.1
2 plus 1 syrup	12 oz. Canco, 3 3/4"	1	5	91.3	91.9
3 plus 1 syrup	12 oz. Canco, 3 3/4"	1	11	95.6	95.8
4 plus 1 syrup	12 oz. Canco, 3 3/4"	1	9	99.0	99.7

TABLE 5.—*Volume occupied by frozen fruits, ml/lb*

FRUIT MIXTURE	RATIO FRUIT TO PACKING MEDIA	NO. PKGS EXAMINED	ML/LB	
			RANGE	AVERAGE
Sl. Strawberries & sugar	4 plus 1	178	419–466	438
Whole Strawberries & sugar	3 plus 1	15	419–426	422
Sl. Strawberries & 60° syrup	3 plus 1	63	439–455	449
Whole Strawberries & 60° syrup	3 plus 1	10	459–461	460
Sl. Peaches & 50° syrup	65 plus 35	32	437–455	445
	69 plus 31	58	440–459	451
	75 plus 25	28	457–470	463
Apricot halves & 47° syrup	60 plus 40	12	431–445	440
	65 plus 35	18	440–451	446
	70 plus 30	12	440–459	451
Red Raspberries & 50° syrup	2 plus 1	83	423–446	437
	7 plus 3	26	432–464	446
	3 plus 1	15	442–455	448
Black Raspberries & 50° syrup	7 plus 3	10	444–446	445
Blackberries & 50° syrup	3 plus 2	8	439–455	447
	2 plus 1	33	433–471	455
	3 plus 1	51	439–501	465
Blackberries & sugar	4 plus 1	11	491–507	498
RSP Cherries & 60° syrup	2 plus 1	13	413–423	417
	3 plus 1	11	435–445	440
	4 plus 1	18	435–466	449
RSP Cherries & sugar	5 plus 1	20	433–459	443

be expected since the specific gravity of the fruit ingredient is lower than that of the packing medium.

Some consideration was given to the effect of slack-filling on the fill of the container and on the volume occupied by a given weight of the frozen fruit. The results of this study are given in Table 6. It will be observed that a reduction of 10 to 25 per cent in put-in weights results in corresponding decreases in the per cent fill of container. The two blackberry packs differed to some extent from the other fruit packs by showing larger volumes per pound as the put-in weights were reduced; this affected the per cent fill.

CONCLUSIONS

From the examination of commercially prepared packages of frozen

TABLE 6.—*Correlation of slack-filling and per cent fill of container*

SAMPLE DESCRIPTION	PUT-IN WEIGHT, OZ.	NO. PKGS. EXAMINED	ML/LB	FILL, PER CENT
FS 78-381 K, Sliced Strawberries & Sugar, 4 plus 1	12.0	12	458	95.4
12.0 oz. Canco tin end paper box	10.8	12	458	85.7
$3\frac{7}{8}\times3\frac{1}{2}\times1\frac{3}{4}''$, capacity 357 ml	9.0	2	457	71.1
FS 78-383 K, Sliced Strawberries & Syrup, 3 plus 1	12.0	12	444	86.7
12.0 oz. Sefton tin end paper box	10.8	12	438	76.0
$4\times3\frac{1}{2}\times1\frac{3}{4}''$, capacity 372 ml				
FS 78-391/6 K, Red Raspberries & Syrup, 2 plus 1	12.0	24	435	90.6
12.0 oz. Canco tin end paper box	9.0	12	455	71.2
$3\frac{7}{8}\times3\frac{1}{2}\times1\frac{3}{4}''$, capacity 357 ml				
FS 78-389 K, Red Raspberries & Syrup, 7 plus 3	12.0	12	458	90.3
12.0 oz. Sefton tin end paper box	10.8	13	473	81.8
$4\frac{7}{8}\times3\frac{1}{2}\times1\frac{3}{4}''$, capacity 372 ml	9.0	4	459	68.8
FS 37-493 K, Himalaya Blackberries & Syrup, 3 plus 1	16.0	15	468	94.5
1 lb. Sefton tin end paper box	14.4	12	498	89.5
$5\times3\frac{1}{2}\times1\frac{3}{4}''$, capacity 477 ml	12.0	12	525	77.6
FS 78-399 K, Evergreen Blackberries & Sugar, 4 plus 1	16.0	11	498	92.7
1 lb. Sefton tin end paper box	14.4	8	520	87.9
$5\frac{1}{2}\times3\frac{1}{2}\times1\frac{3}{4}''$, capacity 517 ml	12.0	2	556	79.5
FS 34-995 K, Sliced Freestone Peaches & Syrup, 65+35	16.0	12	456	75.9
1 lb. Marathon paper box with inner bag	14.4	9	455	68.2
$5\frac{3}{8}\times4\times1\frac{3}{4}''$, capacity 602 ml				
FS 34-999 K, Sliced Freestone Peaches & Syrup, 65 plus 35	16.0	11	448	93.9
1 lb. Sefton tin end paper box	14.4	11	445	84.3
$5\times3\frac{1}{2}\times1\frac{3}{4}''$, capacity 477 ml				
FS 78-398 K, Red Sour Pitted Cherries & Sugar, 5 plus 1	12.0	8	457	98.4
12.0 oz. Canco tin end paper box	10.8	12	454	88.4
$3\frac{3}{4}\times3\frac{1}{2}\times1\frac{3}{4}''$, capacity 345 ml	9.0	7	440	71.4

fruits it appears that most of such packages are reasonably well filled. The average of all analysts' results on all fruits examined was 92.7 per cent fill for 262 commercial packages.

Data are presented on the volume occupied by various frozen fruits with several ratios of fruit to sugar or syrup. The volume increases as the ratio of fruit to packing medium increases.

Slack-filling has little effect on the volume per pound for fruit mixtures such as sliced strawberries and sugar (or syrup), sliced peaches and syrup, and the softer berries and syrup. It appears that blackberries occupy larger volumes per pound when put-in weight is reduced.

The displacement method for the determination of the volume of frozen fruits gives reproducible results in the hands of collaborators.

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Hudson House, Inc., Forest Grove, Oregon
Kelley-Farquhar & Co., Salem, Oregon, and Sumner, Washington
National Farmers Union Service Corp., Puyallup, Washington
National Fruit Canning Co., Seattle, Washington
Stokeley-Van Camp, Inc., Bellingham and Kent, Washington
Twin City Foods, Inc., Stanwood, Washington
United Growers, Inc., Liberty, Oregon
Willamette Cherry Growers, Salem, Oregon
Chapman Frozen Foods, Modesto, California
Glacier Packing Co., Inc., Sanger, California
Spiegl Food Co., Salinas, California
Driscoll Strawberries, Inc., San Martin, California

It is recommended*—

That the displacement method for determination of volume of frozen fruits be given further collaborative trial with view to adoption as an official method.

* This report was received too late to be included in its proper place in the program. For the previous recommendation of the Referee, see *This Journal*, **36**, 277 (1953) and for the report of Subcommittee D and action of the Association, see *This Journal*, **36**, 63 (1953).

ANNOUNCEMENTS

Referee Assignments, Changes, Appointments.

ECONOMIC POISONS:

Paul A. Giang, Bureau of Entomology and Plant Quarantine, Beltsville, Maryland, has been appointed Associate Referee on Systemic Poisons.

NUTRITIONAL ADJUNCTS:

Dr. C. Ray Thompson, Western Regional Research Laboratory, Albany, California, has been appointed Associate Referee on Xanthophylls in Mixed Feeds.

ALCOHOLIC BEVERAGES:

Dr. Dwight West, J. E. Siebel Sons' Co., Inc., Chicago, Illinois, has been appointed Associate Referee on Malt Beverages, Sirups, Extracts, and Brewing Materials to succeed Dr. Robert I. Tenny.

CONTRIBUTED PAPERS

DIRECT DETERMINATION OF AVAILABLE PHOSPHORIC ACID IN FERTILIZERS*

By H. R. ALLEN (Kentucky Agricultural Experiment Station, Lexington, Kentucky)†

In 1951, the writer reported to this Association (1) on a method for the direct determination of available phosphoric acid in fertilizers. In this method the water-soluble and citrate-soluble extracts from a 1 gram sample of fertilizer are combined in a 500 ml volume; the phosphorus is precipitated as ammonium phosphomolybdate and titrated in the usual manner. Precipitation is induced by shaking the solutions on a Ross-Kershaw shaker at approximately room temperature. It is necessary to reduce the sample aliquot to 0.05 gram (25 ml) in order to overcome the effect of citrate ion on precipitation.

In a later investigation, it was found that a 0.1 gram (50 ml) sample aliquot could be used if the precipitation were carried out in a water-bath equipped with a continuous agitation device. The flasks are shaken for 1 hour at 50°C.

The direct method is used as a screening procedure for most samples. Samples which analyze below or considerably above guarantee are rechecked by the official method. The citrate-insoluble residues obtained in the direct method are not discarded until it is determined which samples must be rechecked and can then be used for the insoluble phosphoric acid determination.

It appears that the use of ammonium nitrate as an aid to precipitation is unnecessary. It also appears that ammonium nitrate is not necessary in the official method when the Ross-Kershaw shaking machine is used. If ammonium nitrate is used, it must be practically phosphorus-free.

The direct method is applied to mixed fertilizers and superphosphate. Reagents are the same as in the official method (2), except that extra HNO_3 is not added to the molybdate soln.

REVISED METHOD

Weigh a 1 g sample into a 250 ml Erlenmeyer flask, add 50 ml of H_2O and shake for 15 min. on a Ross-Kershaw shaker (or stir by other method). Add 10 ml of 1+1 HNO_3 to a one l filter flask. Filter with suction through 9 cm S&S No. 597 or Whatman No. 1 paper into the filter flask. Wash with H_2O at room temperature to a vol. of about 125 ml. Proceed with the insoluble portion as in the official method (2) by digestion in neutral NH_4 citrate soln at 65°C. (In this laboratory, a H_2O bath with a continuous agitation device is used.) After digestion for 1 hr, filter the soln into the flask contg the water-soluble portion, using a 5.5 cm Whatman No. 7

* Presented at the annual meeting of the Association of Official Agricultural Chemists, September 29, 30, and Oct. 1, 1952, at Washington, D. C.
† This investigation is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

TABLE 1.—*Available phosphoric acid by official method and by direct method. Results in per cent P_2O_5*

GUARANTEE	OFFICIAL METHOD	DIRECT METHOD—0.1 G SAMPLE		
		1 HR. AT 50°C.[a]	½ HR. AT 50°C.[a]	1 HR. 50°C.[b] HAND-SHAKEN
8	8.11	8.15	8.05	7.45
12	12.83	12.80	12.55	11.05
16	15.84	15.80	15.40	14.10
20	21.10	21.15	20.75	20.40
20	20.26	20.15	20.00	19.65
46	46.15	45.80	42.15	40.35

[a] Flasks in continuous agitation bath for specified time.
[b] Flasks shaken by hand 6 times at 10 min. intervals, in place of continuous agitation.

filter paper. Wash with H_2O at 65°C. to a total vol. of 450 ml as indicated by a mark on the side of the flask. Transfer contents of filter flask to a 500 ml volumetric flask, cool, and adjust to 500 ml. Mix and transfer 50 ml to a 200 or 250 ml Erlenmeyer flask, add 35 ml of molybdate soln (45–50 ml for 45% P_2O_5) slowly with shaking, place flask in a constant temperature H_2O bath equipped with continuous agitation device, and shake for 1 hr at 50°C. Filter and titrate as in the official method.

If a continuous agitation bath is not available, use 25 ml sample, adjust soln to 30–35°C., and stir on Ross-Kershaw shaker for 30 min. When the soln is highly colored by organic matter the smaller aliquot is preferable.

DISCUSSION

Results in Table 1 indicate that when a 0.1 gram sample is used, continuous agitation for 30 minutes at 50°C. is not quite sufficient. These results also show that shaking by hand at 10 minute intervals cannot be substituted for continuous agitation.

Table 2 shows the results obtained with and without the addition of ammonium nitrate, and with and without the addition of calcium sulfate, to samples of triple superphosphate. It appears that the presence of sulfate does not give higher results.

TABLE 2.—*Available phosphoric acid by direct method. Effect of addition of calcium sulfate to triple superphosphate. Effect of ammonium nitrate on precipitation. Results in mg of P_2O_5*

GUARANTEE	OFFICIAL METHOD	DIRECT METHOD—1 HR. AT 50°C.[a]			
		NH_4NO_3	NO NH_4NO_3	SAMPLE, 0.8 G	SAMPLE, 0.8 G PLUS 0.2 G $CaSO_4$
46	46.15	45.65	45.80	37.05	36.90
48	48.44	48.05	47.85	38.50	38.40
48	47.74	47.35	47.55	38.20	38.05
48	48.35	48.10	48.15	38.80	38.40

[a] Flasks in continuous agitation bath for 1 hr.

Suction filtering, using filter paper in a Hirsh porcelain funnel, has been satisfactory in the three filtrations described. A funnel 9 cm in diameter with a 9 cm paper is used for filtering the water-soluble extract. This allows the filter paper to extend up the wall of the funnel about one-half inch, and facilitates the transfer of residue to the citrate solution. A paper 5.5 cm in diameter is used in the same funnel for filtering the citrate-soluble extract. This paper just covers the bottom of the funnel. For filtering the ammonium phosphomolybdate precipitates, a Hirsh funnel of 7.5 cm diameter is used, with an 8 cm (special size) Whatman No. 2 paper. (This funnel is the No. 00 size but was made on special order with more filter openings.) The filter paper extends up the wall of the funnel, and paper and precipitate can be easily transferred to the titrating flask.

SUMMARY

Available phosphoric acid in mixed fertilizers and superphosphate is determined directly from combined water-soluble and citrate-soluble extracts. Precipitation of phosphorus as ammonium phosphomolybdate is induced by shaking the flasks for 1 hour at 50°C. in a bath equipped with a continuous agitation device. An aliquot of 0.1 gram is used.

Use of ammonium nitrate as an aid to precipitation is unnecessary. The method is not applicable to calcium metaphosphate.

REFERENCES

(1) ALLEN, H. R., SWIFT, E., and HAYS, R., *This Journal*, **35**, 764 (1952).
(2) *Official Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Washington, D. C., 1950, pp. 9–11.

THE USE OF PERCHLORIC-NITRIC ACID DIGESTION IN THE DETERMINATION OF PHOSPHORIC ACID IN FERTILIZERS*

By L. J. HARDIN (The University of Tennessee Agricultural Experiment Station, Knoxville, Tenn.)

The official method (1) for the determination of total phosphoric acid in fertilizers gives four alternate procedures for digestion of the sample charge in preparation for the determination. Each of these is designed to meet the requirements of certain fertilizer components, especially with respect to the complete decomposition and ionization of the phosphorus content of the included organic materials.

The stipulated H_2SO_4-HNO_3 digestion, **2.9 (d)**, of fertilizer samples that contain large amounts of organic matter, such as cottonseed meal, ade-

* Presented at the annual meeting of the Association of Official Agricultural Chemists, September 29, 30, and Oct. 1, 1952, at Washington, D. C.

quately effects complete solution and ionization of the phosphorus content, but is somewhat tedious. Moreover, when the solution is prepared in this manner, precipitation of the ammonium phosphomolybdate from hot solution and subsequent gravimetric determination of the phosphoric acid content then becomes necessary. These undesirable steps would be unnecessary if the sulfuric acid digestion were eliminated, since the determination can be made volumetrically when sulfates are absent, or are present in only small amounts.

Allen and Gault (2) reported on the use of various solvents in the preparation of charges of organic fertilizers for phosphoric acid analysis. Some agreement was obtained between results obtained through the use of $HClO_4$-HNO_3 digestant and the official H_2SO_4-HNO_3 digestion, although results from the sulfuric acid procedure usually were higher. Decidedly lower results were obtained, however, when the aqua regia digestion was employed; the magnitude of such discrepancies depended on the type and amount of organic material present. When that procedure was used, the greatest losses of P_2O_5 occurred in the analysis of cotton seed meal, soybean meal, and castor pomace. Other investigators also have found that extended HNO_3-HCl digestions did not bring solutions of certain synthetic organic phosphates into ionized form suitable for the molybdate precipitation (4).

The rapid $HClO_4$-HNO_3 digestion of vegetation or other samples congaining organic matter was found to be adequate for the determination of calcium, phosphorus, etc., (6). This procedure, with precautions, has been used in this laboratory as a multi-purpose digestant and in many laboratories preparatory to the determination of the P_2O_5 content of fertilizers, but such use is unofficial. Brabson and Edwards (3) showed the effectiveness of perchloric acid in dissolving phosphates, with special emphasis on calcium metaphosphate.

At the 1950 meeting of the Association, the Associate Referee on phosphoric acid in fertilizers suggested that the $HClO_4$-HNO_3 digestion be considered in lieu of the four alternate treatments stipulated in the *Official Methods* for the P_2O_5 determination. A recommendation to that effect was included in his report (5) but has not been acted upon, however. The present study was prompted by that recommendation and is intended to call attention to the previous work, to supplement that work, and to give impetus to such collaborative work as may be necessary to make the HNO_3-$HClO_4$ procedure official.

METHOD

The procedure is as follows: weigh a 1 g charge of the fertilizer sample and transfer to 250 ml "fertilizer" flask. Add 10–15 ml of HNO_3, shake to wet the charge, and then add 10 ml of 70% $HClO_4$. Place flask on hot plate, bring to boiling, and continue boiling until copious white fumes are evolved. At this stage, the soln will be essentially colorless, and the HNO_3 will have been removed. Cool the digestate, dil.

to volume, stopper the flask, and shake well.* Filter, or allow to settle, transfer an appropriate aliquot of the clear soln to a pptn flask and proceed with the volumetric detn as directed in **2.13 (a)**.

DISCUSSION

Cottonseed meal is frequently used in fertilizer mixtures and its phosphorus content is of principal importance. In the present experiments cottonseed meal was used alone and in combination with a variety of fertilizer materials. The samples were subjected to the digestions prescribed in the *Official Methods*, and to the $HClO_4$-HNO_3 digestion. The values obtained by that method are shown in Table 1, and are in close agreement with actual values.

The results obtained by means of the aqua regia digestions are consistently low, even though that treatment was prolonged. Apparently, these low results are due to both incomplete solution and ionization of the dissolved organic phosphorus, shown by the slightly higher values obtained in some cases when the aliquot of the digestate was boiled with HNO_3 prior to the phosphomolybdate precipitation.

Urea was included in some mixtures, since a mixed fertilizer might include that material as a supplemental source of nitrogen. In previous unsuccessful attempts to effect $HClO_4$-HNO_3 digestion of urine in determinations of its phosphorus content, it was observed that an insoluble crystalline precipitate was formed in which the nitrogen content corresponded to that of urea. Urea was included in some of the present mixtures to see if any material, other than SiO_2, was left undissolved in $HClO_4$. Insoluble residues would cast doubt upon the efficacy of the $HClO_4$-HNO_3 digestion. These were not noted, and apparently urea can be handled by the $HClO_4$-HNO_3 digestion of fertilizer charges.

It is well known that the presence of sulfate causes high P_2O_5 results in the volumetric determination. Where sulfate is low, however, this effect can be diminished through the stipulated addition of HNO_3 to the molybdate precipitant, **2.11 (a)**. This was shown by the findings of Allen and Gault (1), and by results in Table 1 for mixtures that contained ammonium sulfate and potassium sulfate. Even though these materials were included at the rate of only 500 and 250 lb. per ton, respectively, the P_2O_5 values found exceeded the actual values, but the high values were essentially corrected when the acidified molybdate was used. Similar higher results are shown in Table 2, where somewhat excessive amounts of sulfuric acid were added to solutions of known phosphate content prior to the cold precipitation and volumetric determination. Neutralization of the added H_2SO_4 with NH_4OH did not eliminate the effect of the sulfate, nor did

* *Precaution.*—The potential explosion hazard for mixtures of $HClO_4$ and organic matter is recognized and has been stressed in publications and news items (7). The prescribed prior saturation of the charge of organic matter with HNO_3, and the low initial temperature of the digestion, have obviated explosions in several thousand such digestions made in the laboratory.

TABLE 1.—*Comparative recovery of the phosphoric acid content of fertilizer mixtures by the perchloric-nitric acid digestion*

NO.	COMPONENTS OF SAMPLE	TOTAL P_2O_5[a]				
			METHOD OF DIGESTION OF SAMPLE CHARGE			
		ACTUAL[e]	I[b]	II[c]		III[d]
				PRECIPITATION DIRECT	ALIQUOT DIGESTED[f]	
1	Cotton seed meal	*per cent* 2.78[g]	*per cent* 3.10	*per cent* 0.65	*per cent* 0.80	*per cent* 2.75
2	9 C.S.M. 1 Urea	2.50	2.95	0.70	0.78	2.60
3	2 C.S.M. 1 Urea 1 phos. rock	9.63	10.15	8.68	8.68	9.70
4	Phosphate rock	33.01[h]	—	—	—	32.90[i]
5	TVA Fused phos. rock	29.80[j]	—	—	—	29.75
6	TVA calcium Metaphosphate	—	—	—	—	58.80
7	2 C.S.M. 1 Metaphosphate 1 Urea	16.07	—	—	—	16.15
8	2 C.S.M. 1 Fused phos. rock 1 Urea	8.84	—	—	—	8.70
9	2 C.S.M. 1 Phos. rock 1 Sul. am.	9.63	—	8.65	—	9.82 9.65[k]
10	4 C.S.M. 2 Phos. rock 1 K_2SO_4 1 Urea	9.63	—	8.80	—	9.82[k]
11	Feces	1.58	—	1.10	—	1.55
12	Basic slag	—	—	11.05	—	11.07

[a] The detn of phosphoric acid in aliquots of the digested sample was made volumetrically, after a pptn of a NH_4 phosphomolybdate at room temperature and 30 min. agitation.

[b] Method I: Sample charge digested by boiling with 25 ml H_2SO_4 and 5 ml HNO_3. (*Official Methods of Analysis*, 2.9 (d)).

[c] Method II: Sample charge digested by boiling with 30 ml of HNO_3 and 5 ml of HCl (*Official Methods of Analysis*, 2.9 (a).

[d] Method III: Sample charge digested by boiling with 10 ml $HClO_4$ and 15 ml of HNO_3 until colorless soln and white fumes obtained.

[e] These values obtained from average of detns by official procedure or calcd from values obtained in that manner.

[f] Addl digestion of aliquot made by boiling with 5 ml of HNO_3.

[g] Average of triplicate values obtained when sample charge slurried with satd $Mg(NO_3)_2$ soln, dried, and incinerated; ash digested with 10 ml of HNO_3 and dild to volume. (*Official Methods of Analysis*, 2.9 (c)).

[h] Bureau of Standards analysis.

[i] Uncor. for moisture.

[j] Original analysis reported by Wilson Dam laboratory of T.V.A.

[k] Molybdate acidified with 5 ml HNO_3/100 ml molybdate.

TABLE 2.—*Effect of H_2SO_4[a] in phosphate solutions on the volumetric determination of phosphoric acid*[b]

NO.	SOLUTION VOLUME (ML)	1+1 H_2SO_4 ADDED (ML)	PER CENT P_2O_5		
			H_2SO_4 NEUTRALIZED WITH NH_4OH	ACIDIFIED MOLYBDATE[b,c]	H_2SO_4 NOT NEUTRALIZED WITH NH_4OH
1	61	1	0.02	—	0.00
2	60	None	35.30	35.20	35.25
3	61	1	36.40	36.40	36.20
4	62	2	36.40	36.80	36.20
5	63	3	36.20	36.40	36.20
6	64	4	36.20	36.40	36.20

[a] With and without NH_4OH neutralization prior to pptn of NH_4 phosphomolybdate.
[b] Each pptn used in the comparison was made from a 10 ml aliquot of aqua regia soln of Bureau of Standards Phosphate Rock No. 120; average analysis, 35.33% P_2O_5. Ten g of NH_4NO_3 in soln was added to each soln and 55 ml of NH_4 molybdate soln was used as the precipitant. After pptn by agitation at room temperature, the detns were made volumetrically.
[c] NH_4 molybdate precipitant acidified with 5 ml of HNO_3 per 100 ml molybdate.

use of the acidified molybdate serve as a reliable corrective in the presence of such excessive amounts.

Invariably, low values have been obtained by this investigator when the P_2O_5 determination was made volumetrically, following the removal of small amounts of sulfate by the barium chloride precipitation, **2.12 (b).** It is presumed that these low values have been caused by loss of P_2O_5 through precipitation of barium phosphate, or as a result of occlusion.

In practice, if a fertilizer sample has a low content of sulfate, the $HClO_4$-HNO_3 digestate may be analyzed accurately for P_2O_5 content by means of the official volumetric method. However, the amount of sulfate in the H_2SO_4 digestate is known to vitiate the accuracy of results obtained by means of the volumetric determination although the official method is easily interpreted to permit use of that digestion in conjunction with the volumetric method.

The results in Table 3 show that $HClO_4$, or perchlorates, present from the $HClO_4$-HNO_3 digestion, do not cause erroneous results in the volumetric determination of P_2O_5.

SUMMARY AND CONCLUSIONS

Table 1 shows that accurate results for the P_2O_5 content of mineral and organic materials are obtained by digestion with $HClO_4$-HNO_3 and volumetric determination.

When sulfates are present in moderate amounts, the determination of the P_2O_5 content may be made on the $HClO_4$-HNO_3 digestate by means of the prescribed volumetric procedure in which the molybdate precipitant is acidified.

The presence of $HClO_4$, or chlorate ions, causes no adverse effect upon the accuracy of the volumetric determination of phosphoric acid. The well-

TABLE 3 —*Effect of unneutralized $HClO_4$ in phosphate solutions on the volumetric determination of phosphoric acid*[a]

NO.	SOLUTION VOLUME, ML	70% $HClO_4$ ADDED, ML	PER CENT P_2O_5
1	60	None	35.30
2	60	None	35.40
3	61	1	35.40
4	61	1	35.50
5	62	2	35.40
6	62	2	35.40
7	63	3	35.40
8	63	3	35.40

[a] Each pptn in the comparison was made from a 10 ml aliquot of aqua regia soln of Bureau of Standards Phosphate Rock No. 120; average analysis, 35.33% P_2O_5. A constant of 10 g of NH_4NO_3 in soln was added to each soln and 55 ml of NH_4 molybdate soln was used as the precipitant. After pptn by agitation at room temperature, the detns were made volumetrically.

known effect of the presence of H_2SO_4, or sulfate ions, is substantiated as being a cause of high P_2O_5 values in the volumetric determination.

From the present results, and from related findings by other investigators, it is concluded that the $HClO_4$–HNO_3 digestion of fertilizer charges, with subsequent precipitation by acidified molybdate at room temperature and volumetric determination, is adequate for the wide variety of fertilizer materials that have been tried.

The aqua regia digestion does not effect complete recovery of organic phosphorus, such as that in cottonseed meal, and its use should be restricted to samples known to contain only mineral phosphates.

The magnesium nitrate incineration procedure is satisfactory, although its use introduces extra and tedious steps in the analysis.

The H_2SO_4–HNO_3 digestion is also tedious and results in a solution so heavily laden with sulfate that the usual volumetric procedure (room temperature precipitation with acidified molybdate) is not applicable. The alternative is the use of the longer gravimetric method.

ACKNOWLEDGMENT

Editorial suggestions by Doctor W. H. MacIntire of the Tennessee Experiment Station and by Mr. K. D. Jacob, Bureau of Plant Industry, U. S. Department of Agriculture, are greatly appreciated.

REFERENCES

(1) *Official Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Washington, D. C., 1950.
(2) ALLEN, H. R., and GAULT, L., *This Journal*, **30**, 135 (1947).
(3) BRABSON, J. A., and EDWARDS, D. W., *ibid.*, **34**, 771 (1951).
(4) HARDIN, L. J., and MACINTIRE, W. H., *ibid.*, **31**, 400 (1948).
(5) JACOB, K. D., *ibid.*, **34**, 624 (1951).
(6) STERGES, A. J., HARDIN, L. J., and MACINTIRE, W. H., *ibid.*, **33**, 114 (1950).
(7) ZAHN, V., *Ind. Eng. Chem., News Ed.*, **15**, 214 (1937).

A MODIFIED SPECTROPHOTOMETRIC PROCEDURE FOR THE DIRECT DETERMINATION OF AVAILABLE PHOSPHORIC ACID IN FERTILIZERS AS THE HETEROPOLY-PHOSPHOVANADOMOLYBDATE COMPLEX*

By ROBERT T. TEAGUE, JR. (Analytical Division, North Carolina Department of Agriculture, Raleigh, North Carolina)

The direct procedure under study makes use of the combined water- and citrate-soluble filtrates without deviating substantially from the official A.O.A.C. method (3) for obtaining these filtrates. The phosphoric acid present in the filtrates is then converted to the *ortho* form, and at the same time the natural colors of the fertilizer solutions are destroyed.

The *ortho*-phosphoric acid in the filtrates is subsequently determined by the photometric phosphovanadomolybdate method (6, 8, 10), thus eliminating the time-consuming precipitation, filtration, and titration steps in the official A.O.A.C. method and also in earlier direct methods described below.

MacIntire, *et al.* (9), proposed a direct method, involving titration of ammonium phosphomolybdate, based upon extraction of the sample with citrated ammonium nitrate aided by a steam generator. Allen, *et al.* (2), has worked with the combined filtrates, incorporating a reduction in aliquot in order to obtain complete ammonium phosphomolybdate precipitation. Epps (6) applied the photometric phosphovanadomolybdate method to the combined filtrates.

The studies by both Allen and Epps are not applicable to those samples containing organic and metaphosphates, since it is questionable whether all the phosphoric acid present is in the *ortho* form. It also appears that the Epps method is subject to error from the wide variations in the natural color of the extracts from different samples. The cumbersome precipitation, filtration, and titration steps, although reduced by one-half in comparison to the official method, are still present in Allen's and MacIntire's proposals.

METHOD

REAGENTS

Vanadomolybdate.—To 700 ml of H_2O, add 80 g 85% molybdic acid and dissolve with 65 ml concd NH_4OH. Dissolve 2 g of NH_4 metavanadate in 600 ml hot H_2O and add 200 ml concd HNO_3. Allow both solns to cool, and mix, with constant stirring, by pouring the molybdate soln into the vanadate soln. Cool and dil. to 2 l.

Neutral ammonium citrate.—Dissolve 370 g of crystalline citric acid in 1500 ml of H_2O and add 345 ml concd NH_4OH. Adjust to *p*H 7.0 by adding either NH_4OH or citric acid soln. Then adjust specific gravity to 1.09 at 20°C.

Standard phosphoric acid solution.—Dissolve 0.3836 g potassium dihydrogen

* Originally formulated by Mission, G., *Chem. Ztg.*, 32, 633 (1908).

phosphate in one l distd H_2O. For each % P_2O_5 desired, introduce 4 ml of this soln into a 200 ml volumetric flask contg a reagent blank consisting of 8 ml neutral NH_4 citrate digested in 10 ml concd H_2SO_4 with addn of $NaNO_3$ until all organic matter is destroyed. Make up to mark and shake. Use 25 ml.

Nitric acid.—C. P., concd.

Sulfuric acid.—C. P., concd.

Sodium nitrate.—C. P., pellets.

GENERAL PROCEDURE

Weigh a 1 g sample on a 9 cm Whatman No. 1 filter paper and leach 10 to 12 times with 10 ml portions of distd H_2O into a 500 ml volumetric flask. Add 25–30 ml concd HNO_3 to the filtrate. Transfer the filter paper and residue to a flask contg 100 ml neutral NH_4 citrate, previously heated to 65°C. in a H_2O bath. Stopper and shake until paper is reduced to pulp. Digest with intermittent shaking every 5 min. (or continuously) for 1 hr at 65°C. Remove from bath and filter thru a Büchner funnel using 7 cm Whatman No. 5 filter paper, with suction, into the 500 ml volumetric flask* contg the H_2O-soluble filtrate, and wash with distd H_2O at 65°C. Let cool, make up to mark, and shake. Pipet 40 ml into a 200 ml Pyrex volumetric flask. Add a few glass beads or phosphorus-free sand to prevent bumping and 10 ml concd H_2SO_4. Place on a hot plate† and boil. When vol. reaches about 12–15 ml, a slight foaming action will take place as the organic matter begins to char. Allow foaming action to cease and add small portions of $NaNO_3$ to the contents until all organic matter is destroyed. Retain on hot plate until all oxides of nitrogen are removed and a light yellow hue remains. Remove from hot plate and cool. The light yellow hue should disappear upon cooling. Fill nearly to mark with distd H_2O, allow to cool, make up to mark, and shake. Pipet a 25 ml aliquot into a 100 ml volumetric flask and add 25–35 ml distd H_2O and 25 ml vanadomolybdate reagent. Make up to mark, shake, and let stand for 10 min. for development of color.

Det absorbance at 400 mμ, using a Coleman Universal Spectrophotometer, Model 14, or similar instrument with a 19×150 mm or other cuvette. A reagent blank must be used because of the color of the reagent. Adjust galvanometer until blank reads zero absorbance, and check from time to time as measurements of samples continue. Select standard P_2O_5 solns which will cover the range of P_2O_5 present in samples, and develop color. Det. absorbance as indicated above. Plot a standard curve using standards and calc. per cent P_2O_5 of unknown from this graph. Calculation can be simplified for samples contg a max. of 12% P_2O_5 by using the relationship:

$$\frac{(\text{Absorbance Unknown})(\%\ P_2O_5\ \text{Std.})}{\text{Absorbance Standard}} = \%\ P_2O_5\ \text{of Unknown},$$

but necessitates the use of standards close to the unknown to obtain best accuracy.

DATA AND DISCUSSION

Kitson and Mellon (8) have found that sulfuric acid behaves like nitric acid in the development of color. The acid concentration range for sulfuric acid is 0.2 to 1.4 *N*, with 0.5 *N* optimum. The concentration of solutions studied is approximately 0.8 *N*.

* If facilities are not available for filtering directly into the 500 ml volumetric flask, the soln may first be filtered into a suction flask and then transferred into the 500 ml flask.

† Best heating results are obtained by using a 3-heat, 3600-watt hot plate. Use all three heats for initial boiling and then turn down to two heats, 2400 watts, for remainder of digestion.

TABLE 1.—*Analysis of organic phosphates*

MATERIAL	AVAILABLE PHOSPHORIC ACID, PER CENT (AVERAGE OF TRIPLICATES)	
	A.O.A.C.	SPECTROPHOTOMETER
Cotton Seed Meal, 3%	2.99	2.98
Cotton Seed Meal, 2.5%	2.44	2.59
Castor Pomace	2.23	2.06
Cocoa Tankage	1.58	1.72
Cocoa Meal	1.06	1.01

The preliminary investigation of materials represented in Tables 1, 2, and 3 indicates that the method is adapted to analysis of materials containing organic and metaphosphates.

The procedure was applied to a number of organic phosphates normally found in fertilizers (Table 1); metaphosphates and superphosphates (Table 2); an assorted mixture of organic phosphates, metaphosphates, superphosphates, and sand (Table 3); and one hundred official samples of the North Carolina Department of Agriculture (Table 4). All organic phosphates and metaphosphates and accompanying A.O.A.C. alkalimetric titrations were run in triplicate. The assorted mixtures and each separate determination of the official North Carolina Department of Agriculture samples were made in duplicate. Comparison is shown between the A.O.A.C. alkalimetric titrations and the spectrophotometric procedure in all tables.

Average results by the A.O.A.C. method in Tables 1 and 3 were obtained by digestion with 15 ml concentrated sulfuric acid and 10 ml of concentrated nitric acid. Samples in Table 2 were digested with 30 ml of concentrated nitric acid and 10 ml of concentrated hydrochloric acid. All insolubles were digested with the nitric-hydrochloric mixture.

Table 4 shows the relationship between samples digested with 15 ml of concentrated sulfuric acid and 10 ml of concentrated nitric acid

TABLE 2.—*Analysis of metaphosphates and superphosphates*

MATERIAL	AVAILABLE PHOSPHORIC ACID, PER CENT (AVERAGE OF TRIPLICATES)	
	A.O.A.C.	SPECTROPHOTOMETER
Ca Metaphosphate, 60%	60.47	60.25
Ca Metaphosphate, 20%	20.22	20.72
Ca Metaphosphate, 11%	11.55	11.45
Calgonite ($NaPO_3$)	15.19	14.92
Triple Superphosphate, 49%	49.27	49.42
Superphosphate, 19%	18.42	18.49

TABLE 3.—*Analysis of mixtures*

NO.	MATERIAL (APPROXIMATE COMPONENTS)	AVAILABLE PHOSPHORIC ACID, PER CENT (AVERAGE OF DUPLICATES)	
		A.O.A.C.	SPECTRO-PHOTOMETER
1	$Ca(PO_3)_2$ 2, 3% C.S.M. 1, Cocoa Tankage 1, Sand 12	8.34	8.28
2	$Ca(PO_3)_2$ 1, Triple Super 1, Castor Pomace 2, Sand 12	7.68	7.62
3	$Ca(PO_3)_2$ 1, 19% Super 6, Cocoa Meal 1, Sand 6	12.66	12.87
4	$Ca(PO_3)_2$ 1, Cocoa Tankage 1, Castor Pomace 1, Sand 16	4.03	3.95
5	$Ca(PO_3)_2$ 1, 19% Super 4, 3% C.S.M. 1	22.85	22.92
6	$Ca(PO_3)_2$ 1, 19% Super 4, Cocoa Tankage 4	16.24	16.32
7	Triple Super 1, 2½% C.S.M. 1, Castor Pomace 1, Sand 4	8.15	7.90
8	Triple Super 4, Cocoa Meal 1, Sand 16	9.84	9.60
9	19% Super 1, 2½% C.S.M. 1	10.14	10.37
10	19% Super 8, Castor Pomace 1, Sand 8	8.88	8.80

(method I) and the same samples digested with 10 ml of concentrated hydrochloric acid and 30 ml of concentrated nitric acid (method II), as against those run by the spectrophotometer (method III). The same results for insolubles were used to calculate the available phosphoric acid for the two separate total digestions.

Results of method I averaged 0.15 per cent higher than method II and 0.12 per cent higher than method III. The over-all average of method III

TABLE 4.—*Analysis of official fertilizer samples of N.C.D.A.*

NUMBER OF SAMPLES	GRADE	ORGANIC MATTER CONTENT (AVERAGE PER CENT INSOLUBLE NITROGEN)	AVAILABLE PHOSPHORIC ACID, PER CENT (AVERAGE OF NUMBER OF SAMPLES ANALYSED)		
			H_2SO_4–HNO_3 (METHOD I)	HNO_3—HCl (METHOD II)	SPECTROPHOTOMETER (METHOD III)
41	3-9-6	0.85	9.24	9.04	9.11
26	4-10-6	0.11	10.00	9.91	9.88
8	3-9-9	0.32	9.33	9.15	9.15
5	6-8-6	0.24	8.22	8.10	8.20
4	3-12-6	0.30	11.77	11.62	11.78
4	5-10-5	0.11	10.12	10.06	9.96
4	2-12-12	0.00	12.15	12.06	11.99
3	5-10-10	0.00	10.36	10.26	10.16
2	0-14-14	0.00	13.70	13.67	13.96
2	Superphosphate	0.00	20.35	20.08	20.33
1	4-8-3	0.00	8.28	8.10	7.98

was, therefore, 0.03 per cent higher than method II, and agreement throughout was good. It was anticipated that method I would give high results because of sulfate interference, as shown by Allen and Gault (1), Brabson and Edwards (5), and Jacob (7), even when precipitated in the recommended temperature range of 25–30°C.

The average deviation between methods II and III was 0.11 per cent and the maximum 0.33 per cent.

The average deviation between duplicates of a single sample and of a single method was 0.11 per cent for method I, 0.10 per cent for method II, and 0.13 per cent for method III. Maximum deviation was 0.35 per cent for methods I and II and 0.33 per cent for method III.

SUMMARY

The method comprises three steps:

(1) Separation of available phosphoric acid, using combined water- and citrate-soluble filtrates.

(2) Oxidation or hydration to the *ortho-* form.

(3) Development of color.

The comparative accuracy of the A.O.A.C. alkalimetric titration (using the HNO_3-HCl mixture) and the spectrophotometric method is as good as duplicates by the same method. Results using sulfuric acid digestions for totals were consistently higher than the results by the spectrophotometer or digestions with nitric-hydrochloric acid mixture; this is attributed to sulfate interference. There is no sulfate interference in development of color with the spectrophotometric procedure. The spectrophotometric method requires about half the time and equipment of the alkalimetric titration of the A.O.A.C. method, and should prove valuable to laboratories where many determinations are made. The method is especially adapted to the determination of fertilizers containing organic phosphates and metaphosphates.

ACKNOWLEDGMENT

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REFERENCES

(1) Allen, H. R., and Gault, L., *This Journal*, **30**, 135 (1947).
(2) Allen, H. R., *et al.*, *ibid.*, **35**, 764 (1952).
(3) *Official Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Washington, D. C., (1950).
(4) Barton, C. J., *Anal. Chem.*, **20**, 1068 (1948).
(5) Brabson, J. A., and Edwards, O. W., *This Journal*, **34**, 771 (1951).
(6) Epps, E. A., Jr., *Anal. Chem.*, **22**, 1062 (1950).
(7) Jacob, K. D., *et al.*, *This Journal*, **31**, 225 (1948).

(8) KITSON, R. E., and MELLON, M. G., *Ind. Eng. Chem., Anal. Ed.*, **16**, 379 (1944).
(9) MACINTIRE, W. H., *et al., ibid.*, **10**, 143 (1938).
(10) MISSION, G., *Chem. Ztg.*, **32**, 633 (1908).

INSTRUMENTAL METHODS FOR THE DETERMINATION OF AVAILABLE PHOSPHORIC ACID AND POTASH IN FERTILIZERS*

By H. C. AUSTIN, JR., W. P. DENSON, and E. A. EPPS, JR. (Louisiana Agricultural Experiment Station, University Station, Baton Rouge 3, La.)

The continued increase in the use of commercial fertilizers has greatly increased the work of control chemists and has made it desirable to search for analytical methods requiring less time. This is particularly true for available phosphoric acid and for potash, as the official methods (1) are lengthy. If more rapid procedures or time-saving innovations are found to give reasonable accuracy they should be satisfactory at least for rapid screening; the questionable samples could then be subjected to analysis by official methods. The photometric method of Epps (2) for available phosphoric acid and the flame photometric method for potash are potentially capable of providing real savings of time in routine analyses. The photometric method gives a fifty per cent reduction in time required for determination of available phosphoric acid, and the flame photometric method permits determination of potash in about one-tenth the time required by the official procedure. Neither of these methods has been generally adopted by control chemists, but they have found satisfactory application in a number of laboratories. This work was undertaken to establish further the reliability of the methods and to investigate the possibility of additional saving in time by using the same solution for determination of both available phosphorus pentoxide and potash.

METHOD

REAGENTS

Standard sodium hydroxide solution.—0.3240 *N*.

Standard nitric acid solution.—0.1620 *N*.

Phenolphthalein indicator.—1 g in 100 ml ethanol.

Molybdate solution.—Dissolve 100 g of MoO_3 in 144 ml of NH_4OH and 271 ml of H_2O. Cool and pour slowly and with constant stirring into cool mixt. of 489 ml of HNO_3 and 1148 ml of H_2O.

Ammonium citrate solution.—Dissolve 370 g of citric acid in 1500 ml H_2O and nearly neutralize with 345 ml of NH_4OH. Cool and adjust to *p*H 7 and sp. gr. of 1.09 at 20° C.

Vanadomolybdate reagent.—Dissolve 40 g of molybdic acid, 85%, in 400 ml H_2O. Dissolve 1.0 g of NH_4 metavanadate in 300 ml of H_2O and add 200 ml HNO_3. Allow

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the two solns to cool and mix by pouring the molybdate soln into the vanadate soln. Dil. to one liter.

Standard phosphorus pentoxide soln.—Dissolve 0.4792 g of potassium dihydrogen phosphate in one l of H_2O. This soln contains 0.25 mg P_2O_5 per ml.

Ammonium oxalate soln.—Satd.

Ammonium chloride soln.—Dissolve 100 g of NH_4Cl in 500 ml H_2O, add 5–10 g powd K_2PtCl_6 and shake at intervals for 6–8 hrs. Allow to settle overnight and filter.

Platinum soln.—Contains equivalent of 10% Pt.

Acid-alcohol.—Mix 200 ml of ethanol with 20 ml of HCl and cool to room temperature.

PROCEDURES

Available Phosphorus Pentoxide

A. Official method (1).—Dissolve 2 g of sample in 30 ml HNO_3 and 5 ml HCl, heat to destroy organic matter, cool, and dil. to 250 ml. Pipet 25 ml aliquot into 250 ml Phillips beaker, add 3 g NH_4NO_3, and adjust to neutrality with NH_4OH and HNO_3. Add 35 ml molybdate soln and shake for 30 min at room temperature. Decant thru a Shimer filter with an asbestos mat. Transfer ppt to the mat and wash 6 times with H_2O. Extrude the mat and ppt into the precipitating vessel, and add a small vol. of H_2O. Now add standard alk. until the ppt dissolves. Add a drop of phenolphthalein indicator and back titrate with standard acid.

Citrate-insoluble phosphorus pentoxide.—Place 1 g sample on a 9 cm filter paper and wash with successive small portions of H_2O until the filtrate is ca 250 ml. Transfer the filter and residue within 1 hr to a 200 ml flask contg 100 ml NH_4 citrate soln previously heated to 65°C. Close flask with rubber stopper and shake vigorously until filter paper is distintegrated. Relieve pressure by removing stopper. Loosely stopper flask and place in H_2O bath at 65°C. for 1 hr. Level of H_2O in bath must be above level of soln in flask. Shake flask every 5 min. At end of 1 hr filter by suction on a Shimer filter with a paper pulp mat. Wash with 250 ml of H_2O at 65°C. Allow thoro draining before adding each fresh portion of wash H_2O. Transfer mat and residue to a 250 ml flask and dissolve residue by heating with 35 ml HNO_3 and 10 ml HCl. Let cool and dil. to 250 ml. Det. P_2O_5 in a 50 ml aliquot as in pptn and titration of total P_2O_5.

The difference between total P_2O_5 and citrate-insoluble P_2O_5 is the available P_2O_5.

B. Photometric method (1).—Proceed as in detn of citrate-insoluble P_2O_5 except the H_2O washings (wash to vol. of 100 ml instead of 250 ml) and filtrate and washings from the NH_4 citrate digestion are caught in a 500 ml Kohlrausch flask contg 25 ml HNO_3. Let cool and dil. to 500 ml. Transfer 5 ml aliquot to 100 ml volumetric flask and add 25 ml H_2O and 25 ml of vanadomolybdate reagent. Make to the mark, mix thoroly, and let stand 10 min. for development of color.

Det. absorbance at 400 mμ, using a Beckman spectrophotometer with a 1 cm cell. Per cent available P_2O_5 may be read directly from a curve prepared by carrying suitable aliquots of the standard P_2O_5 soln thru the same procedure. A reagent blank must be placed in the reference cell and it is desirable to check the standard curve by running two or more known standards with each set of detns.

Potash

A. Official method (1).—Place 2.5 g of the sample in a 250 ml volumetric flask and add 125 ml of H_2O and 50 ml of satd NH_4 oxalate soln. Boil 30 min., add a slight excess of NH_4OH, and after cooling, dil. to 250 ml. Mix thoroly and pass thru a dry filter. Evap. a 25 ml aliquot to dryness in a 100 ml Pt dish. Add 1 ml H_2SO_4 (1+1) and a few granules of sugar, and ignite to whiteness. Dissolve residue

in hot H_2O, using at least 20 ml for each decigram of potash present; add a few drops of HCl and an excess of Pt soln. Evap. to a thick paste on a steam bath, avoiding contamination by NH_3 fumes. Treat residue with 6 ml of acid-alcohol. After 15 min., filter on Gooch crucible. Wash with 75 ml of alcohol and then wash 5 or 6 times with 10 ml portions of NH_4Cl soln. Wash thoroly again with alcohol, dry 30 min. at 100°C., and weigh. Dissolve K_2PtCl_6 with hot H_2O, dry, and reweigh. Calc. per cent K_2O from the difference in the two wts.

B. Flame photometric method.—Dil. the soln as prepared for analysis by the official method in such a manner that the final soln contains the equivalent of 250 p.p.m. of original sample. In the case of muriates, dil. to 100 p.p.m. of original sample. Introduce the dild solns into the flame of a Beckman flame photometer. Take at least three readings for each sample. Operating conditions for the flame photometer are: slit width, 0.14 mm; wavelength, 767 mμ; oxygen pressure, 14 p.s.i.; acetylene pressure, 2 p.s.i. Prepare a standard curve by plotting % transmission against % K_2O, using solns contg 60, 50, 40, 30, 20, and 10 p.p.m. K_2O in the form of KCl dissolved in distd H_2O. Set the instrument at 100% transmission for the 60 p.p.m. K_2O soln.

C. Flame photometric method using phosphoric acid solution.—Instead of using the sample soln as prepared for analysis of potash by the official method, make suitable dilns of the soln used for detn of available P_2O_5 by the photometric method. Det. the per cent transmission as in the flame photometric method. Set the instrument at 100% transmission using a soln contg 60 p.p.m. K_2O and proportionate amounts of NH_4 citrate soln and HNO_3.

DISCUSSION AND DATA

Analyses of a number of samples were made in triplicate by the methods described. The commercial fertilizers were official samples submitted by the State Department of Agriculture. The Magruder samples were taken from a series of check samples issued by the Royster Guano Company and analyzed, using A.O.A.C. methods, by more than 100 state and commercial analysts. The average of the analyses so obtained should closely represent the true values for nitrogen, available phosphoric acid, and potash. Because of the difficulty of preparing fertilizers of known analysis similar to commercial ones, it is convenient to use the Magruder samples to test the accuracy of analytical methods.

Table 1 gives a comparison of the A.O.A.C. and photometric methods for determination of available phosphoric acid in commercial fertilizers. The average difference between the two methods of analysis is 0.24 per cent. Where the two methods are compared with average analysis of Magruder samples (Table 2) it may be seen that the average difference between the photometric method and the Magruder results is 0.14 per cent (omitting the results on triple superphosphate which are obviously in error).

Analyses of the samples by the A.O.A.C. method show an average difference of 0.17 per cent from the Magruder averages. It appears that under routine conditions, the results obtained by the photometric method on mixed fertilizers are as accurate and reproducible as results obtained by the A.O.A.C. method. The differences in analyses between the photo-

TABLE 1.—*Comparison of A.O.A.C. and photometric methods for determination of available phosphorus pentoxide in commercial fertilizers*

LABORATORY NUMBER	SAMPLE	A.O.A.C.	PHOTOMETRIC
245	0-14-7	13.99	14.16
246	3-12-12	12.37	12.37
265	3-12-12	11.03	11.26
222	4-8-8	8.12	8.40
224	4-12-4	12.09	12.29
548	5-10-5	10.80	10.60
345	5-10-10	10.50	10.62
255	5-20-20	20.73	20.54
571	6-8-8	8.12	8.03
451	6-8-12	8.52	8.74
229	6-10-4	10.01	10.22
565	6-10-4	10.09	9.80
583	6-12-6	12.06	12.27
232	6-24-24	24.81	24.49
235	8-8-8	8.27	8.53
433	10-8-4	8.94	9.37
429	10-10-5	10.22	10.37
326	10-30-10	30.19	29.80
370	12-8-0	9.45	9.59
282	12-12-12	12.15	12.26
236	Superphosphate	21.41	21.00
346	Triple Superphosphate	49.98	49.20

metric and A.O.A.C. methods are sometimes positive and sometimes negative, indicating random variations with no tendency to give consist-

TABLE 2.—*Comparison of A.O.A.C. and photometric methods of analysis of available phosphorus pentoxide in Magruder check samples*

SAMPLE	A.O.A.C.	A.O.A.C. AVERAGE	PHOTOMETRIC
Nov. 1950, 5-10-5	10.50	10.26	10.48
Dec. 1950, Triple Superphosphate	45.69	45.31	46.50
Jan. 1951, 7-7-7	7.03	7.19	7.03
Feb. 1951, 3-9-6	9.25	9.07	9.08
Apr. 1951, 4-12-4	12.51	12.42	12.78
May 1951, 4-10-6	9.82	9.75	9.77
July 1951, 3-9-9	8.98	9.01	9.13
Nov. 1951, 5-10-5	10.05	10.10	10.22
Dec. 1951, 8-16-16	16.07	16.56	16.73
Mar. 1952, 15-15-6	15.58	15.56	15.60
Apr. 1952, 3-12-6	12.00	12.32	12.08
May 1952, 5-10-10	10.59	10.55	10.65
June 1952, 0-14-14	13.66	14.06	14.13

ently higher or lower results. Analysis of triple superphosphate has not been satisfactory but it may be due to inherent limitations in instrumentation as the percentage error is of the same order as that on samples of lower available phosphoric content.

TABLE 3.—*Comparison of A.O.A.C. and flame photometric methods for determination of potash in commercial fertilizers*

LABORATORY NUMBER	SAMPLE	A.O.A.C.	FLAME PHOTOMETER	
			I	II
222	4-8-8	8.75	8.86	8.72
224	4-12-4	4.47	4.41	4.42
229	6-10-4	4.25	4.17	4.27
232	6-24-24	19.80	19.92	19.52
235	8-8-8	7.78	7.73	7.67
245	0-14-7	7.48	7.35	7.62
246	3-12-12	12.25	12.44	12.58
255	5-20-20	19.52	19.35	19.43
265	3-12-12	13.80	13.87	13.98
282	12-12-12	11.62	11.59	11.87
326	10-30-10	9.59	9.49	9.87
345	5-10-10	9.87	9.87	9.93
429	10-10-5	5.10	5.00	5.27
433	10-8-4	4.24	4.15	4.35
451	6-8-12	11.21	11.36	11.33
548	5-10-5	5.23	5.25	5.45
565	6-10-4	4.09	4.16	4.53
571	6-8-8	8.47	8.47	8.63
583	6-12-6	6.39	6.41	6.78
231	50% Muriate	48.11	49.00	48.17
233	60% Muriate	61.15	61.27	61.87
242	60% Muriate	59.51	59.70	60.20
676	60% Muriate	59.46	59.27	59.60

Tables 3 and 4 show comparison of the flame photometric (Column I), the modified flame photometric (Column II), and the A.O.A.C. methods. The average difference between the A.O.A.C. and the flame photometric methods is 0.08 per cent and that between the A.O.A.C. and modified flame photometric methods is 0.18 per cent. These values apply only to the mixed fertilizers, as the muriates do not show such good agreement. The reason for poor checks on muriates may be due to limitations in the instrument. Even with the wide variation it still might be permissible to use the flame photometer for screening, as higher tolerances are usually provided for high analysis goods. Examination of data in Table 4 shows the flame photometric method to give results closely comparable to the A.O.A.C. method. The average difference between the A.O.A.C. and

TABLE 4.—*Comparison of A.O.A.C. and flame photometric methods for determination of potash in Magruder check samples*

SAMPLE	A.O.A.C.	A.O.A.C. AVERAGE	FLAME PHOTOMETER	
			I	II
Nov. 1950, 5-10-5	4.99	5.14	5.07	5.17
Feb. 1951, 3-9-6	6.36	6.25	6.56	6.62
July 1951, 3-9-9	8.93	8.92	8.97	9.15
Mar. 1952, 15-15-6	5.67	5.95	5.73	5.93
June 1952, 0-14-14	14.78	14.61	14.71	15.12

Magruder results is 0.14 per cent, between the flame photometric and the Magruder results, 0.15 per cent, and between the modified flame photometric and the Magruder results, 0.23 per cent. Both flame photometric methods give good agreement, although those obtained by the modified method do not check as closely. This modification, however, gives sufficiently accurate results to justify its use for screening purposes.

CONCLUSIONS

(1) The photometric method for determination of available phosphoric acid yields results comparable in accuracy to the official A.O.A.C. method. The former method is much more rapid and has the additional advantages that consumption of reagents is reduced and there is no necessity for preparing and standardizing solutions.

(2) The flame photometric method for determination of potash in fertilizers is very rapid and gives results as accurate as the official A.O.A.C. method, except for muriate of potash. This method also permits considerable saving of chemicals and equipment.

(3) The flame photometric method for potash and the photometric method for phosphoric acid are recommended for analysis of commercial fertilizers. Large numbers of samples may be screened rapidly, and questionable results then checked, using official A.O.A.C. methods.

(4) Additional saving in the time required for analysis may be obtained by the flame photometric determination of potash, using the solution prepared for photometric phosphoric acid analysis.

REFERENCES

(1) *Official Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Washington, D. C., 1950.

(2) EPPS, E. A., JR., *Anal. Chem.*, **22**, 1062 (1950).

DETERMINATION OF POTASSIUM IN MIXED FERTILIZERS*

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A short, inexpensive method for the determination of potassium in mixed fertilizers has been needed in the industry for some time. Work with the Beckman Model DU flame photometer has shown that the flame method could be used as a screening procedure. If sufficient digesting and cooling space were available, two people could easily make 100 potassium analyses per day.

The bulletins that accompany the Beckman instrument give thorough instructions for setting up the instrument and for detecting and correcting any trouble. However, the actual calibration of the flame photometer is a lengthy, trial and error operation. It is the purpose of this report to assist operators in the calibration of their equipment without having to duplicate some of the steps which have been found unnecessary.

All of the fertilizer samples, containing up to and including 16 per cent potash, are being screened with the flame photometer; only those that do not agree within certain specified limits with the manufacturers' guarantee, and those with a guarantee of over 16 per cent potash, are being analyzed by the official method. Due to the saving in man-hours and materials, principally platinum, this procedure has reduced the cost of each potash determination by about two-thirds. On this basis the flame photometer will pay for itself after approximately 3000 analyses.

EXPERIMENTAL

In the newer Beckman instrument, the solution to be analyzed is aspirated into the oxy-acetylene flame by the oxygen. A narrow region of the spectrum, characteristic of the element to be measured, is focused through a slit and projected onto a phototube, from which a direct reading of the intensity is obtained. The reading is a measure of the total intensity of the flame background plus the light emitted by the element or elements whose spectra fall within the region covered by the slit. To derive the latter, the flame background is measured before introduction of the sample or standard solution and is subtracted from the total intensity reading. Spectrophotometric readings of the intensity of the element under study are converted into measurements of its concentration by means of standard curves.

Each flame photometer has certain individual characteristics with which the operator must become familiar. The bore of the capillary tube in the aspirator may vary in different burners. Therefore, the oxygen and fuel pressures must be adjusted to the best condition for each instrument, which is accomplished by measuring transmittance at different oxygen

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pressures while the fuel pressure is kept constant. With progressive increase in oxygen pressure, transmittance values rise to a maximum and then decline, as shown in Fig. 1. In the vicinity of the peak, relatively large variations in oxygen pressures cause relatively small changes in transmittance readings. It is in this less sensitive area of oxygen and gas pressure that transmittance readings are most accurate.

The optimum pressures for the potassium determination for the instrument tested were: oxygen, 10 lb per sq. in.; acetylene, 5 lb per sq. in. These values may shift from time to time and require rechecking at intervals.

For the initial dark current check, the fixed switch is set at 0.1, the shutter is closed, and the spectrophotometer circuit is balanced to the zero point with the dark current rheostat. The fixed switch is then moved to the "check" position, and if the circuit is balanced, the needle should remain at the zero position.

To adjust the instrument for sensitivity, the fixed switch is turned to

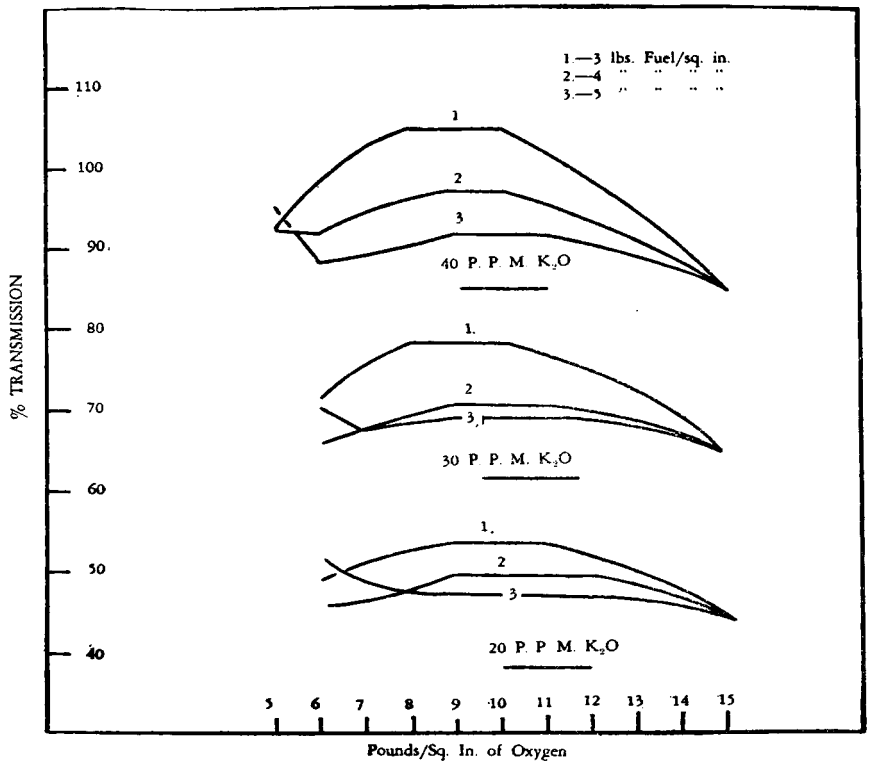

FIG. 1.—Effect of variation of oxygen pressure on transmittance values for K_2O.

the 0.1 position. The variable rheostat is set at a point which provides optimum transmittance spread with minimum fluctuation of the galvanometer, and is fixed at this point throughout the succeeding determination. During this time, all of the settings used for the standards are kept constant, except that of the transmission dial. Pressure gauges should be kept as close to the original setting as possible.

In calibrating the flame photometer, two solutions were used at several concentrations. The first attempts were made using solutions of C. P. KCl and K_2SO_4, both separately and mixed, each containing an equivalent of 1 g/liter of K_2O or 1000 p.p.m. Aliquots of these solutions were diluted to give a series of solutions containing from 100 to 1000 p.p.m. of K_2O in 100 p.p.m. increments. The solution containing 1000 p.p.m. was used to zero the instrument (100% on the transmission scale). The transmission reading for each of the other solutions was taken, and these values were plotted against the known K_2O content. The curves were irregular, and, as most of the potash results of actual samples fell in the lower portion of the curve, more dilute solutions seemed advisable.

A series of ten solutions containing from 50 to 500 p.p.m. of K_2O in 50 p.p.m. increments was used for the next test. Curves were constructed in the same manner as above, and were found to be more regular than those obtained with the more concentrated solutions. Using these curves as a basis for determining potash content, more than 100 samples of mixed fertilizers were then tested with the flame photometer. In comparing these results with those obtained on the same solutions by the official method, very close agreement was found in about 80 per cent of the cases. The remaining 20 per cent differed by over 20 points or 0.2 per cent (some higher and some lower); there was no definite trend. There has not been sufficient time in which to determine the cause of this variation of potash results; however, it has been noticed that fertilizers with excessive amounts of cottonseed tend to run high. By first burning off the organic material and then digesting in the usual way, the results were in closer agreement with the guarantee. It is quite possible that other sources of organics such as tankage, tobacco stems, tobacco dust, sludges, and dried blood would have the same enhancing effect.

Another series of tests was made using solutions containing 0–50 p.p.m. K_2O in 5 p.p.m. increments. The 50 p.p.m. solution represented 10 per cent potash (100 per cent on the transmission scale). This dilution was made to expand the section of the curve in which most of the samples fell and also to reduce the possibility of error due to interference from secondary elements which are present in nearly all of the fertilizers used in this state. One-half of the normal aliquot was used on materials containing over 8 per cent, and up to and including 16 per cent K_2O. (Samples containing over 16 per cent were not analyzed by this procedure, as stated before.) Too great a dilution was found to multiply small errors due to

interference, pipetting, or weighing to such a degree that results were not reliable.

With the settings of 10 p.s.i. for oxygen, 5 p.s.i. for fuel, and a slit opening of 0.2 mm, and using the solutions containing 0–50 p.p.m. of K_2O, a straight line curve was found between 5 p.p.m. (1 per cent K_2O) and 40 p.p.m. (8 per cent K_2O). Below 5 and above 40 p.p.m., up to 50 p.p.m. (top of scale), the curve had a slight slope. As most of the potash guarantees in this state fall between 20 p.p.m. (4 per cent) and 40 p.p.m. (8 per cent), this slight deviation from the straight line does not affect the results appreciably. This curve is shown in Fig. 2.

Although potassium is present in fertilizers as both the chloride and the sulfate, results using K_2SO_4 as the standard seem to be in closest agree-

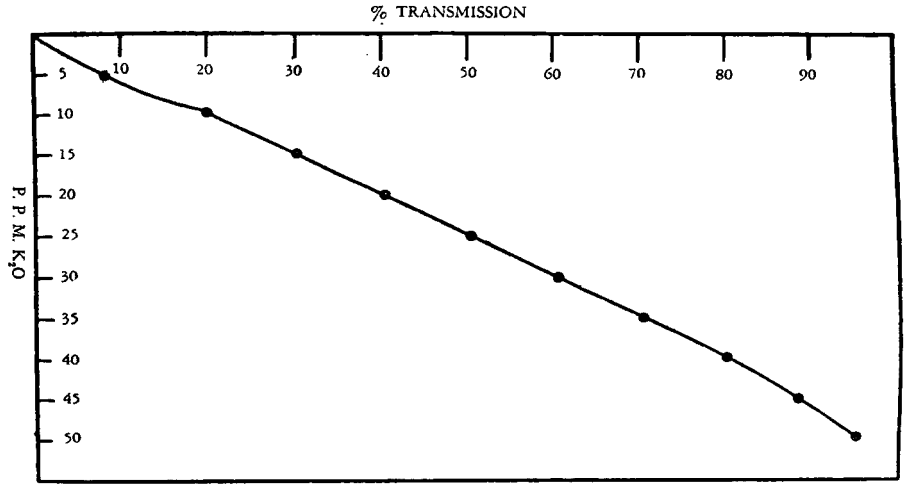

FIG. 2.—Relation between transmission values and concentration of standards.

ment with values by the official method. All of the routine work now being done is based on the sulfate standards in the 0–50 p.p.m. range.

Several variations in the preparation of solutions of the fertilizer were tried; the most satisfactory procedure was found to be the A.O.A.C. method for mixed fertilizers.

Other methods of preparing the solutions included a straight water digestion and a water digestion followed by acidification with HCl. Tests were made with these solutions diluted to the 0–50 p.p.m. range and also with these solutions to which a buffer had been added. This buffer contained known amounts of Na, Ca, and Mg and was prepared according to

TABLE 1.—*Comparison of solution methods. Results as per cent K_2O*

OFFICIAL[a] RESULTS	ALKALINE SOLUTION		WATER SOLUTION		WATER SOLN ACIDIFIED	
	NO BUFFER	WITH BUFFER	NO BUFFER	WITH BUFFER	NO BUFFER	WITH BUFFER
6.05	5.84	6.38	4.94	5.96	5.72	6.12
8.59	8.76	8.91	7.88	9.62	9.00	9.36
12.28	12.32	15.36	11.38	13.52	12.08	12.80
6.40	6.34	7.80	5.19	6.12	6.00	6.36
8.42	8.38	8.83	7.04	8.60	8.00	8.58
6.40	6.34	7.72	5.04	6.00	5.72	6.12
5.03	4.68	5.54	4.24	4.90	4.60	4.86
4.91	4.86	5.66	3.92	4.60	4.01	4.26
4.85	4.76	5.66	4.38	5.00	4.56	4.92
5.16	5.00	5.98	4.50	5.36	5.00	5.32
6.91	6.62	8.42	6.38	7.06	6.54	6.78

[a] All results except those listed under "Official Results" were obtained on the flame photometer.

West, Folse, and Montgomery.* Only the solutions prepared by the official method, and without the buffer, gave consistent results that compared favorably with the A.O.A.C. method. A tabulation of these results is given in Table 1.

A series of 12 samples in the 0–50 p.p.m. range was checked on the flame photometer at two closely adjacent wavelengths, 769.9 and 767 mμ. Two sets of tests were made at each setting, using solutions prepared by the official method and the same solutions with the buffer added. The results of these tests are shown in Table 2.

Table 3 indicates the varied composition of the samples listed in Table 2. It will be noted that the sample with the greatest variation between the two analyses is the least complex of the set.

Table 4 shows a comparison of analyses obtained by the official method and by the flame photometer. The two results for each sample were found on a single solution prepared by the A.O.A.C. procedure.

PROCEDURE FOR ANALYZING MIXED FERTILIZERS FOR K_2O CONTENT EMPLOYING FLAME PHOTOMETER

Weigh 2.5 g of sample into a 250 ml volumetric flask; add 50 ml of satd NH_4 oxalate soln, and dil. with ca 100 ml of distd H_2O. After boiling starts, digest this soln on the hot plate for 30 min.; cool slightly, add an excess of NH_4OH and cool to room temperature. Dil. the soln to 250 ml, mix thoroly, and filter through a dry filter paper. Transfer a 10 ml aliquot from soln of material contg up to and including 8 per cent K_2O, and a 5 ml aliquot from soln of material contg over 8 per cent and up to and including 16 per cent K_2O, to a 200 ml volumetric flask and dil. to the mark with distd H_2O. Mix thoroly and transfer to 5 ml beakers for testing with the flame pho-

* *Anal. Chem.*, 22, 667 (1950).

TABLE 2.—*Comparison of results by official and flame photometric methods. Results as per cent K_2O*

SAMPLE NUMBER	OFFICIAL RESULTS	WITH BUFFER				WITHOUT BUFFER			
		769.9 mμ	DIFF.	767 mμ	DIFF.	769.9 mμ	DIFF.	767 mμ	DIFF.
1	7.92	7.76	−0.16	7.71	−0.21	8.15	+0.23	8.00	+0.08
2	6.85	6.54	−0.31	6.63	−0.22	7.15	+0.30	7.04	+0.19
3	8.42	8.10	−0.32	8.05	−0.37	8.70	+0.28	8.50	+0.08
4	3.69	3.48	−0.21	3.44	−0.25	3.72	+0.03	3.68	−0.01
5	8.26	8.34	+0.08	7.92	−0.34	8.30	+0.04	8.20	−0.06
6	4.69	4.97	+0.28	4.49	−0.20	4.99	+0.30	4.75	+0.06
7	5.39	5.74	+0.35	5.43	+0.04	5.28	+0.11	5.54	+0.15
8	4.52	5.04	+0.48	4.60	+0.08	4.91	+0.39	4.75	+0.23
9	7.84	7.55	−0.29	7.30	−0.54	7.70	−0.14	7.85	+0.01
10	5.03	5.15	+0.12	4.65	−0.38	5.24	+0.11	5.11	+0.08
11	5.28	5.38	+0.10	5.18	−0.10	5.42	+0.18	5.26	−0.02
12	6.40	6.07	−0.33	5.93	−0.47	6.38	−0.02	6.44	+0.04

TABLE 3.—*Composition of samples*

SAMPLE NUMBER	1	2	3	4	5	6	7	8	9	10	11	12
Guarantees						*(Per Cent)*						
Mg	2.0	2.0	2.0	—	—	—	—	3.0	2.0	—	—	2.0
MnO	0.5	1.0	0.75	—	—	—	—	—	—	—	—	—
Cu	0.5	0.5	0.5	—	—	—	—	—	—	—	—	—
Fe	3.0	—	0.75	—	—	—	—	—	—	—	—	—
Zn	0.2	—	—	—	—	—	—	—	—	—	—	—
B	0.2	0.18	0.18	—	—	—	—	—	—	—	—	—
Cl	6.0	5.0	2.0	3.0	8.0	5.0	7.0	2.0	8.0	7.0	5.0	7.0
					Plant Food derived from							
Muriate potash	x	x	—	x	x	x	x	—	x	x	x	x
Nitrate of soda	x	—	x	—	—	—	—	—	—	—	x	x
Am. sulfate	x	x	x	—	x	x	x	—	x	x	x	x
Uramon	—	—	—	—	—	—	—	—	—	—	—	x
Pot. and mag. sulfate	—	x	x	—	—	—	—	x	x	—	—	—
Pot. carbonate	x	—	—	—	—	—	—	x	—	—	—	—
Tankage	x	x	x	—	x	—	x	—	—	x	x	x
Super phos.	x	x	x	—	—	x	x	—	x	x	x	x
Am. super phos.	—	x	x	x	x	x	x	—	x	x	x	—
Castor pomace	x	x	x	x	—	—	x	—	x	x	x	x
Tobacco stems	x	x	x	—	—	—	—	—	—	x	—	—

TABLE 4.—*Comparison of results*

FLAME	A.O.A.C.	DIFFERENCE
6.16	6.03	+0.13
8.88	8.81	+0.07
5.04	5.01	+0.03
8.38	8.28	+0.10
7.78	7.68	+0.10
5.96	6.05	−0.09
10.32	10.36	−0.04
10.32	10.51	−0.19
5.29	5.24	+0.05
5.05	4.29	+0.06
5.17	5.16	+0.01
2.26	2.13	+0.13
4.26	4.19	+0.07
2.12	2.25	−0.13
6.80	6.79	+0.01
4.98	5.04	−0.06
2.94	3.10	−0.16
3.33	3.45	−0.12
5.08	5.04	+0.04
4.48	4.58	−0.10
5.43	5.43	—
4.69	4.66	+0.03
4.71	4.77	−0.06
10.42	10.46	−0.04
10.96	11.08	−0.12
6.15	6.05	+0.10
5.67	5.74	−0.07
5.10	5.22	−0.12
5.12	5.43	−0.16
6.91	6.89	+0.02
6.06	5.86	+0.20
10.44	10.17	+0.27
5.48	5.59	−0.11
5.48	5.51	−0.03
7.70	7.84	−0.14

tometer. Aspirate each soln into the flame and balance the instrument by adjusting the transmission dial. Obtain the percentage of K_2O from the standard curve.

These dilutions give a good spread on the scale and are reproducible. Each division on the transmission scale represents 0.1 per cent K_2O; percentages to the second decimal place can be estimated accurately. In routine work it has been found most efficient to group samples to be analyzed according to their guarantee. This procedure saves time and eliminates much manipulation.

The procedure for the preparation of sample, as given in detail above,

is the official method; thus the preparation of solutions for the flame and gravimetric procedures is the same. From this point on, the times necessary to complete the analyses are considerably different. By employing the flame photometer, a set of 40 samples can be finished and reported within an hour. Another eight hour period is necessary to complete the same number of samples with the gravimetric procedure.

SUMMARY

The flame photometer method is a rapid and accurate procedure for screening mixed fertilizers for potash content. This method cuts the cost of each analysis by reducing the man-hours required and eliminates the use of the most expensive material, *viz.*, platinum.

It has been found that dilute solutions (maximum, 50 p.p.m. as K_2O), prepared by the official procedure, give accurate and reproducible results. A wavelength of 767 mμ and as narrow a slit opening as possible should be used. The burner should be cleaned before each series of tests is run and the flow of oxygen and fuel should be kept as constant as possible. The wet batteries should be kept at full charge to prevent galvanometer drift.

While making a series of tests, it is important to check the instrument frequently with a standard solution. This should be done with the standard which corresponds to the guarantee of the particular sample being analyzed.

USE OF FLAME PHOTOMETRY TO DETERMINE POTASSIUM AT 404 MILLIMICRONS*

By A. T. BLACKWELL, C. L. YEAGER, and M. KRAUS (The Davison Chemical Corporation, Baltimore, Maryland)

The present official method for determining potassium in fertilizers and fertilizer materials is long, time-consuming, and costly. The use of flame photometry for this and other purposes is, understandably, increasing, a fact which is evident from the number of recent publications on the determination of the alkali metals in water (1), soils (2), and fertilizer (3). The purpose of the present paper is to report on the development of an accurate, simple, and rapid method for determining potassium in fertilizers.

The official method of preparing sample solutions presents serious drawbacks when used to determine potash in fertilizers by flame photometry. Schall, *et al.* (3), reported interferences from certain anions and cations which, however, were often of slight effect because of the large

* Presented at the annual meeting of the Association of Official Agricultural Chemists, September 29, 30, and Oct. 1, 1952, at Washington, D. C.

sample dilutions used. The problem is complicated because fertilizers vary considerably in chemical composition; one may contain 8 per cent P_2O_5, another 20 per cent; one may have KCl, another sulfate of potash, etc. The A.O.A.C. method now used for preparing sample solutions removes very little of the phosphate and sulfate ions which are the most objectionable interferences in this work. Accordingly they should be removed from the sample solutions.

The apparatus used was the Beckman Model DU spectrophotometer with the No. 9200 atomizer burner assembly and the photomultiplier attachment. The burner was operated with acetylene and oxygen gases at $3\frac{1}{2}$ and 17 p.s.i. respectively. In some experimental work done using the oxy-hydrogen flame, the flame background is much lower; however, emission is not proportional to the potash content as is the case with the oxy-acetylene flame.

PROCEDURE

Prepare standard solns for use in analyzing fertilizer samples as follows:

Accurately weigh 0.7915 g of KCl, C. P., and transfer to a one l Pyrex volumetric flask. Add 6 g 20% superphosphate, 100 ml 5% $Ba(OH)_2$ soln, several drops phenolphthalein indicator, and heat to *gentle* boiling. The soln should be shaken occasionally at the start to prevent caking on bottom. If the soln becomes acid at any time during the 10 min. boiling period, add successive 5 ml portions of 5% $Ba(OH)_2$ soln to make definitely alkaline. Do not add a large excess of $Ba(OH)_2$ because it must be removed from the soln. Next add 100 ml of 1% soln of $(NH_4)_2CO_3$ and boil *gently* for 20 min. to ppt. the excess Ba and to expel excess $(NH_4)_2CO_3$ and NH_4OH. Wash down sides of the flask with H_2O, cool to room temperature, dilute to vol. with H_2O, and mix. After filtering thru No. 40 or 42 Whatman filter paper or equivalent, the soln is ready for use and contains 500 p.p.m. K_2O. (Never use this soln unless it is free from precipitates.) Keep the soln stoppered and near the same temperature from day to day to prevent change in concn.

Superphosphate is added to compensate for the chief interfering elements in fertilizer samples, possible occlusion effects, vol. changes due to presence of precipitates, and solubility effects.

A standard soln of 1000 p.p.m. contains 1.583 g of KCl or 1.8501 g of K_2SO_4 per liter of soln. Prepare these solns by dissolving the potash salt in H_2O only.

Prepare standards for testing salts such as kainit and/or sulfate of potash-magnesia from a sample of the salt itself of known K_2O content.

Weigh samples and transfer to proper size flasks as shown in Table 1. Samples of potash salts are dissolved by boiling in H_2O only. Add to the mixed fertilizer samples 50 ml of 5% $Ba(OH)_2$ soln and a few drops of phenolphthalein indicator. Heat to gentle boiling with occasional shaking. If the soln is acid after boiling, add successive 5 ml portions of 5% $Ba(OH)_2$ soln, allowing time between addns for complete reaction, until the soln remains alk. after boiling for 5 min.* Then add 50 ml of 1% $(NH_4)_2CO_3$ soln and boil for 20 min. (5, 6). Wash down with H_2O, cool to room temperature, dil. to vol. with H_2O, and mix. Just prior to testing with the flame photometer, filter thru No. 40 Whatman filter paper. The photometer is standardized at 50% transmission with the standard soln (see Table 1).

* Foaming during preparation of the sample soln is prevented by addn of 2 ml of 2% alcoholic soln of Tetradecanol, a product of Carbon and Carbide Chemicals Corp.

TABLE 1.—*Recommended testing procedure data*

EXPECTED K_2O CONTENT	SAMPLE WEIGHT	SAMPLE VOLUME	STANDARD SOLUTION	FACTOR FOR CONVERTING PER CENT TRANSMISSION TO PER CENT K_2O
per cent	*grams*	*ml*	*p.p.m.*	*per cent*
3–8	2.5	250	500	0.1
9–16	2.5	500	500	0.2
17–32	1.25	500	500	0.4
Muriate	2.5	1000	1000	0.8
Sulfate of potash	2.5	1000	1000	0.8

DISCUSSION

With the proposed method for preparation of solution, jet clogging is no longer a problem. Lowering the concentration of interfering ions to levels that are without significant influence on potassium emission is very satisfactorily accomplished by addition of sufficient $Ba(OH)_2$ and boiling the solution to precipitate the sulfates. At the same time soluble monocalcium phosphate is converted to the insoluble dicalcium and dibarium phosphates. The reactions are:

$$2\ Ba(OH)_2 + (NH_4)_2SO_4 + Ca(H_2PO_4)_2 \rightarrow BaSO_4 + CaHPO_4 + BaHPO_4 + 2NH_4OH + 2H_2O$$

$$\text{Excess } Ba(OH)_2 + (NH_4)_2CO_3 \rightarrow BaCO_3 + 2NH_4OH$$

Excess $(NH_4)_2CO_3$ and NH_4OH are expelled during the boiling. Sodium is not removed and, when present in large amounts, may cause high results; however, results in Table 2 indicate that the difference in sodium content of 50 and 60 per cent K_2O muriate of potash was of little significance in the sample concentrations recommended in Table 1. Scott (4) reported that the radiation effect of sodium on potassium is not linear.

Calculation.—Since the emission of potassium in linear from 300 to above 4000 p.p.m. K_2O, a standard curve may be drawn by plotting transmission against p.p.m. on linear graph paper. If the schedule shown in Table 1 followed, no curve is required and the per cent transmission is multiplied by the factors given in the table. The formula for calculating when using the curve is:

$$\frac{\text{p.p.m. } K_2O \times \text{sample volume}}{\text{Sample weight} \times 10{,}000} = \text{Per cent } K_2O$$

Discussion of results in Table 2.—Samples marked[a] are the same except that the 7.02 K_2O by flame photometry result was found after adding 880 p.p.m. of MgO derived from $MgCl_2$ to the solution flask before treatment with $Ba(OH)_2$ and $(NH_4)_2CO_3$. Analysis of the filtrate after the prescribed treatment showed that the magnesia content had been reduced to 144 p.p.m. The K_2O result was 0.04 per cent lower by flame analysis. (Gravimetric analysis was not made on this solution.)

TABLE 2.—*Comparative K_2O results by flame photometry and A.O.A.C. method*

GRADE	FLAME	A.O.A.C.	GRADE	FLAME	A.O.A.C.
10–6–4	3.50	3.57	Ford '52	63.18	63.00
4–12–4	4.21	4.26	4–12– 8	8.00	8.04
3–12–6	6.23	6.20	5–10– 5	5.03	5.07
4–12–8	7.28	7.34	3–12– 6	5.83	5.83
5–10–10	10.28	10.25	7– 7– 7	7.20	7.12
5–10–10	10.18	10.02	5–20–10	10.50	10.35
Magruder #1	10.64	10.57	4–12–12	12.25	12.19
Magruder #2	23.21	23.49	12–12–12	12.38	12.42
0–14–14	14.40	14.21	0–14–14	14.19	14.02
8–16–16	15.79	15.48	8–16–16	15.33	15.30
Ford '52	17.40	17.26	0–20–20	21.48	21.46
Muriate	51.28	51.18	0–20–20	18.90	18.84 S[a]
Muriate	50.48	50.21	0–20–20	24.79	24.68 S[a]
Muriate	61.04	61.16	3–12– 6	7.06[a]	6.86[b]
Muriate	60.40	60.55	3–12– 6	7.02[a]	——

Average of 28 results by flame: 19.74; by A.O.A.C.: 19.69

[a] S, see text
[b] Omitted from average.

The two gravimetric results marked "S" were obtained on the solutions prepared for the flame analysis. Twelve filtrates from flame solutions were tested and showed that less than 0.05 per cent P_2O_5 remained in solution. Qualitative tests indicated that very small amounts of calcium remained in the solutions.

The average of 28 results by flame analysis is 0.05 per cent higher than by the A.O.A.C. method; however, in the opinion of the authors, this is of no significance, since most of the gravimetric results represent only one analysis on daily production samples. Two or more tests were run on samples of muriates, the Magruders, and Ford's 1952 collaborative potash samples (5). The results by flame photometry are from one solution preparation but represent an average of readings by two analysts.

SUMMARY

Results of this study indicate that the precision and accuracy of flame photometry equals that of the A.O.A.C. procedure at a saving of more than half the time.

It is hoped that these data may induce other analytical laboratories to do much more confirmatory work in order to help establish the validity of the flame photometric procedure as an official routine method of determining potash in fertilizers.

ACKNOWLEDGMENT

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REFERENCES

(1) WEST, P. W., FOLSE, P., and MONTGOMERY, D., *Anal. Chem.*, 22, 667 (1950).
(2) TOTH, S. J., and PRINCE, A. L., *Soil Sci.*, 67, 439 (1949).
(3) SCHALL, E. D., and HAGELBERG, R. R., *This Journal*, 35, 757 (1952).
(4) SCOTT, R. K., MARCY, V. M., and HRONAS, J. J., "Analysis of Water," presented at the A.S.T.M. meeting, Atlantic City, June 19, 1951.
(5) (See p. 649).

POTASSIUM ANALYSIS OF TWELVE YEARS' MAGRUDER CHECK SAMPLES BY FLAME PHOTOMETRY*†

By E. D. SCHALL (Department of Agricultural Chemistry, Purdue University Agricultural Experiment Station, Lafayette, Ind.)

Flame photometry has become widely used during the past six years to determine sodium and potassium in a variety of materials including among others, milk (1), soils (4), plant ash (5), and natural waters (6). In general, the potassium or sodium concentration in these materials is relatively low, usually in the order of 10 to 50 p.p.m. Where high concentrations are involved, it is necessary to dilute to this range, since the usual atomizer design is such that concentrated solutions are not handled very successfully. When used to determine potash in fertilizers, in which the potassium content is relatively high, it is necessary to dilute the sample several fold in order to come within this range (3). Although flame methods are much faster than gravimetric procedures, the steps involved in accurately diluting the sample are in themselves laborious and time-consuming, and contribute significantly to the over-all time required for the routine analysis of a large number of samples. From the standpoint of convenience, it is desirable to work in a higher concentration range and to eliminate much of the dilution required in the present procedures, providing this can be done without reducing accuracy. The first objective of the present work was to evaluate a procedure in which fertilizer samples containing 15 per cent or less of potash could be analyzed without dilution beyond that required in the usual preparation of the same as outlined under section **2.40(a)** in *Methods of Analysis* (2).

A second objective of the study was to investigate the adaptability of the Beckman Model B spectrophotometer as a flame photometer. The literature contains several reports of the successful use of the Model DU in flame analyses, and the reliability of this spectrophotometer is widely accepted. The Model B has been available a relatively short time and consequently its performance is less well known. The Model 9220 flame attachment for the DU is interchangeable with the Model B, although

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each spectrophotometer requires a special mounting platform to insure correct optical alignment.

The Model B incorporates several convenient features, and it appeared that a study of its performance characteristics would be of sufficient general interest to warrant presentation. Since the unit is line-operated, battery maintenance is eliminated. It employs a direct reading galvanometer rather than the familiar null point principle used in the Model DU and some of the other flame photometers. The Model B with flame attachment is available at a cost of approximately two-thirds that of the DU with this attachment.

The fertilizer samples selected for this study were the Magruder check samples distributed monthly by the F. S. Royster Guano Co. Samples collected during the past twelve years were available with the exception of those in which the containers had rusted unduly or, in some cases, where the original sample had been exhausted. The results as compiled in the Magruder reports were also available to compare with the flame photometer values and to give a basis for estimating the accuracy of the latter procedure.

EXPERIMENTAL

Apparatus.—The flame photometer consisted of a Beckman Model B spectrophotometer with a Beckman Model 9220 flame attachment. The 500 megohm resistor regularly furnished with the spectrophotometer was replaced by one of 10,000 megohms resistance to obtain increased sensitivity. Commercial tank oxygen and acetylene were used. A transformer type voltage stabilizer was also installed to reduce the effect of line voltage fluctuations.

Standard solutions.—A stock soln contg 5000 p.p.m. of potassium was prepared by dissolving 9.5345 g of KCl, reagent grade, (dried for 3 hrs at 110°) in distd H_2O and diluting to a vol. of one l. Standards contg 100, 200, 300, 400, 500, 600, and 750 p.p.m. potassium were prepared by appropriate diln. The standards also contained 2.0 g of NH_4 oxalate, 0.3 g of monobasic NH_4 phosphate, 1.9 mg of NaCl, and 0.1 g of Sterox SE (Monsanto Chemical Co.) per 250 ml. (Sterox SE is a noncationic wetting agent added to promote a uniform rate of atomization of the solns).

Preparation of sample solutions.—Solns for flame analysis were prepared by weighing 1.5060 g of the fertilizer sample into a 250 ml volumetric flask, adding 50 ml of satd NH_4 oxalate soln and 125 ml H_2O, and boiling for 30 min. If necessary, one drop of tributyl phosphate was added to control foaming. When it was cool, 0.1 g of Sterox SE was added; the soln was dild to vol. with distd H_2O and thoroly mixed.

Filtering was unnecessary if the solns were allowed to stand several hrs. Less than 5 ml was required for flame analysis; this amount was easily decanted from the clear supernatant liquid. If it is desirable to complete the analysis immediately, solns can be filtered.

Diln of these solns was required only if the original sample contained more than 15 per cent potash. When necessary, this was done conveniently by quantitatively transferring the boiled sample to a larger volumetric flask of appropriate size before diluting to vol. In this manner all pipetting and the possibility of errors arising therefrom were eliminated.

Analytical procedure.—Reference curves covering the range of 0 to 750 p.p.m were obtained by aspirating the standard solns and plotting relative intensity *vs*

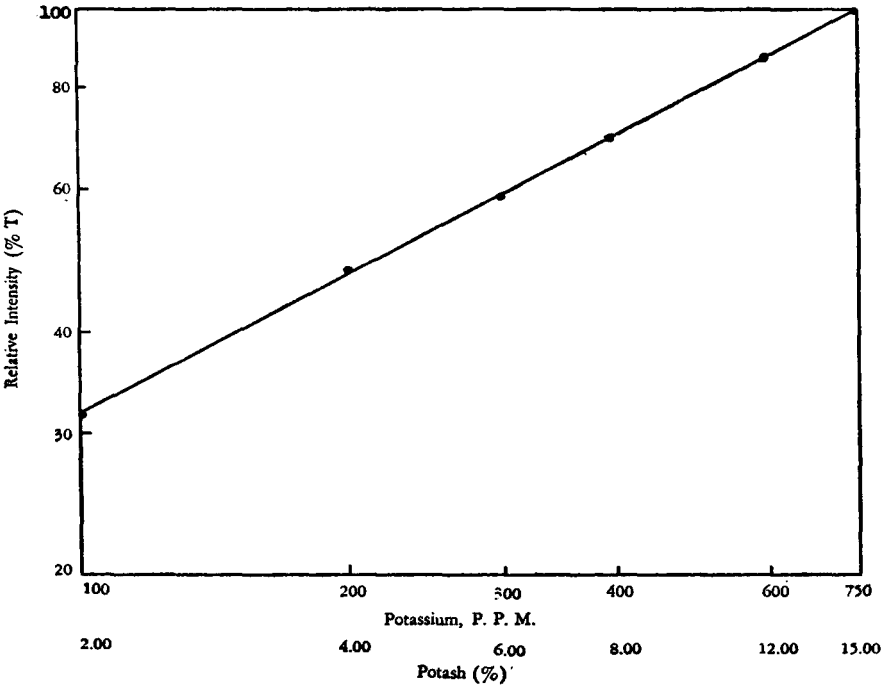

FIGURE 1.

concn at 767 mμ. For convenience, the graduations on the transmission scale (per cent *T*) were used, although the readings were not transmission values. If the plot is made on logarithmic paper a straight line relationship exists (Fig. 1). The curve is constant and easily reproducible on succeeding days by resetting one standard to the desired reading.

The spectrophotometer was operated with the sensitivity control in position 2. Oxygen was supplied at 15 and acetylene at 3 p.s.i. The flame height control was adjusted to give a flame of max. height while still maintaining a zero background reading with distd H_2O. A slit width of approximately 0.30 mm. was used under the above conditions.

The analysis of the sample was obtained by determining the response of the solution and converting this value to potash from the reference curve (Fig. 1). For greatest accuracy, a standard having approximately the same concn as the sample should be checked before and after each reading. The accuracy can also be improved by taking the average of several readings to eliminate random error, and it is imperative that this should be done with high potash samples where a dilution factor is used.

Effects of other ions.—Solns of fertilizer samples contain several ions which may affect the emission intensity of potassium. In high phosphate materials this anion will probably be present in relatively high concns. Ammonium salts, in addition to those present in the sample, are added in preparing the soln. Sodium may also be

present, although the amount is usually relatively low. Since the degree of interference varies with flame temperature and the relative concn of the various ions present in the soln, it was necessary to evaluate the effect of extraneous ions under conditions identical with those used in the analysis.

Ammonium oxalate in concns used in preparing sample solns resulted in an increase in the apparent potassium content. Since NH_4 oxalate increases the readings and is always added to the sample, 2.0 g per 250 ml was added to all solns, including standards, before proceeding with interference tests. Ammonium hydroxide, nitrate, and chloride had no detectable effect in the presence of oxalate. Phosphate, added as mono- or dibasic NH_4 phosphate, in concns equivalent to those present in a soln prepared from a fertilizer contg 12 per cent P_2O_5, resulted in readings slightly higher than the true value (Table 1). Fertilizers containing 6 per cent and 24 per cent P_2O_5 gave values identical with those at 12 per cent; thus it appears that the phosphate effect reaches a plateau at a low concn.

Sodium in concns of 100 p.p.m. causes an increase in the readings, but this effect appears to be canceled when the phosphate ion is also present. The same values were obtained with phosphate as with a mixture of phosphate and sodium (Table 1).

TABLE 1.—*Effect of interfering ions*

COMPOUND ADDED[a]	CONCENTRATION	POTASSIUM FOUND (P.P.M.)[c]			
None	—	100	300	600	750
$NH_4H_2PO_4$	1000 p.p.m. PO_4[b]	100	310	615	775
$(NH_4)_2HPO_4$	1000 p.p.m. PO_4	100	310	615	775
NaCl	100 p.p.m. Na	100	310	610	755
$NH_4H_2PO_4$ *plus* NaCl	1000 p.p.m. PO_4 100 p.p.m. Na	100	310	615	775

[a] All solns, including standards, contain 2.0 g of ammonium oxalate/250 ml.
[b] Equivalent to a 12% P_2O_5 fertilizer (1.506 g sample).
[c] P.p.m. K × 0.02 = % K_2O (1.5060 g sample).

RESULTS AND DISCUSSION

The Magruder samples analyzed were those received by this laboratory during the past twelve years. Since the moisture content had decreased significantly from original values, the potash values found could not be compared directly with the averages compiled in the Magruder reports. It was necessary to determine the present moisture level by heating a 2 gram sample for five hours at 98°C. in a water-jacketed oven, and to adjust the potash values to the original moisture content given in the reports.

Comparison of the flame photometer results with the Magruder averages for 80 samples showed good agreement, with the flame values generally slightly lower. The results, grouped according to potash content, are summarized in Table 2. (A negative average difference indicates flame photometer results lower than the Magruder values.)

The electrical circuit of the Model B is efficiently damped and galvanometer fluctuations due to slight flame irregularities are usually less than

TABLE 2.—*Comparison of flame photometer and Magruder results*

RANGE POTASH	NO. OF SAMPLES	AV. DIFF. POTASH	STANDARD DEVIATION
per cent		*per cent*	*per cent*
2.00– 4.99	11	−0.12	0.12
5.00– 5.99	20	−0.10	0.13
6.00– 8.99	27	−0.12	0.13
9.00–11.99	5	−0.28	0.12
12.00–19.99	12	−0.23	0.30
20.00–62.50	5	+0.04	0.30

one-half scale division. Successive readings can be reproduced to this value with proper operation, and one-half division represents 0.1 per cent potash in the procedure outlined. Operation at the high sensitivity required in flame photometry makes the instrument more responsive to voltage fluctuations, and this may cause difficulty if well-regulated power is not available.

As a matter of interest, the sodium concentration of solns prepared for flame analysis was determined. The maximum found was 145 p.p.m. in one sample. Twelve samples contained between 50 and 100 p.p.m. and the remaining 67 less than 50 p.p.m. An average of 33 p.p.m. was found and this was the basis for including 30 p.p.m. in all standard solns. This is equivalent to approximately 0.8 per cent NaCl in the fertilizer. If these samples, covering a period of 12 years, can be considered representative, it would appear that interference from sodium in the flame photometric determination of potash is negligible.

SUMMARY

1. Fertilizer samples ranging from 2.00 to 62.50 per cent potash were analyzed by the flame photometer, employing a Beckman Model B spectrophotometer with flame attachment. The standard deviation of the difference between the flame and Magruder values was 0.20 per cent.

2. Fertilizer samples containing less than 15 per cent potash were analyzed without dilution beyond that required in the preparation of sample solutions by the official method. Dilution was required where the potash content was greater than 15 per cent.

3. Phosphate and oxalate ions increased the apparent potassium content under the conditions employed in this investigation. Interference from these anions was compensated by the addition of each to the standard solutions.

ACKNOWLEDGMENT

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REFERENCES

(1) KEIRS, R. J., and SPECK, S. J., *J. Dairy Sci.*, **33**, 413 (1950).
(2) *Methods of Analysis*, 7th Ed., A.O.A.C., Washington, D. C., 1950, page 17.
(3) SCHALL, E. D., and HAGELBERG, R. R., *This Journal*, **35**, 757 (1952).
(4) TOTH, S. J., and PRINCE, A. W., *Soil Sci.*, **67**, 439 (1949).
(5) TOTH, S. J., PRINCE, A. W., WALLACE, A., and MIKKELSON, D. S., *ibid.*, **66**, 459 (1948).
(6) WEST, P. W., FOLSE, P., and MONTGOMERY, D., *Anal. Chem.*, **22**, 667 (1950).

CHEMICAL METHOD FOR AVAILABLE FERTILIZER NITROGEN IN UREA-FORMALDEHYDE COMPOSITIONS*

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The use of certain urea-formaldehyde (U-F) polymers as a source of nitrogen is an important development in recent fertilizer research. They are produced by the acid-catalyzed condensation of urea and formaldehyde under conditions that ensure the reaction of more than one mole of urea per mole of formaldehyde, as shown by Yee and Love (1). The products are believed to be a mixture of straight chain U-F compounds ranging from methylene diurea to those containing more than six urea molecules.

By controlling reaction conditions, the U-F materials can be prepared with different rates of nitrification to suit the nitrogen requirement of particular crop conditions. The U-F polymers, prepared by the authors, contain about 38 per cent total nitrogen, three-fourths of which is insoluble in cold water. In the soil, the insoluble portion provides nitrogen which becomes slowly available. The water-soluble portion contains urea and a low molecular weight polymer, both of which yield readily available nitrogen.

Nitrification studies are generally accepted procedures for measuring the rate at which nitrogen becomes available to plants. However, they are time-consuming, requiring six to twelve weeks for completion. There are two chemical methods given by the A.O.A.C. for determining the activity or quality of cold-water-insoluble nitrogen (2). The values obtained by either method serve only to distinguish between the better and the poorer sources of water-insoluble nitrogen. It has been shown that the neutral permanganate method, the more widely used of the two, is not reliable for all types of U-F products (3).

In 1948, Clark, *et al.* (4), demonstrated a correlation between water

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solubility and nitrification, and suggested a technique for measuring availability based upon two consecutive water extractions of a one gram sample at 30°C. This procedure has the disadvantage of requiring forty-eight hours for the extractions. In studies designed to provide a faster method of analysis, the authors investigated several of the variables affecting solubility of U-F polymers in water. From the results of this work, a solubility method was developed for the rapid estimation of the agronomic availability of these insoluble fertilizer nitrogen compounds.

The method requires two determinations for calculating the Availability Index (A. I.). The first is the per cent of cold-water-insoluble nitrogen by the A.O.A.C. method. The second is the per cent nitrogen, insoluble in a hot aqueous phosphate buffer solution, and is determined on another portion of sample. The nitrogen in the insoluble residues is determined by the usual A.O.A.C. macro-Kjeldahl procedure. The percentage of the cold water-insoluble nitrogen which dissolves in the hot buffered solution is an index of the availability of the cold-water-insoluble nitrogen.

METHOD FOR THE DETERMINATION OF THE AVAILABILITY INDEX (A I) OF U-F POLYMERS

APPARATUS

(a) *Water bath.*—The bath should have circular openings in the cover through which 400 ml beakers can be suspended. The heat capacity of the bath should be such that a bath temperature of 99–100°C. is maintained.

REAGENTS

(a) *Monopotassium phosphate.*—KH_2PO_4. Anhydrous, reagent grade.

(b) *Dipotassium phosphate.*—K_2HPO_4, anhydrous, reagent grade.

(c) *Phosphate buffer solution.*—0.063 *M*. Dissolve 14.3 g of KH_2PO_4 and 91.0 g of K_2HPO_4 in one l of H_2O. Dil. 100 ml of this soln to 1000 ml with H_2O. The *p*H of the dild soln should be 7.5.

(d) *Hydrochloric acid solution.*—0.5 *N*.

(e) *Sodium hydroxide solution.*—0.5 *N*, standardized.

(f) *Methyl red indicator.*—0.5%. Dissolve 1 g of methyl red in 200 ml of alcohol.

PROCEDURE

Preparation of Sample.—Crush the polymer sample to pass 20 mesh Tyler standard sieve, avoiding fine grinding.

Determination of Cold-Water-Insoluble Nitrogen, $IN_{25°}$ [(2) Method **2.34**].—Weigh rapidly and accurately a sample of 1.0 to 1.4 g into a 50 ml beaker. Add 20 ml of H_2O at 25° ±2 C. and allow to stand for 15 min. with occasional stirring. Transfer the supernatant liquid to 11 cm Whatman No. 2 filter paper and wash residue 4 or 5 times by decantation with H_2O at 25°C. Finally transfer all residue to filter and complete washing until filtrate measures 250 ml. Det. per cent nitrogen in wet paper and residue by the usual Kjeldahl procedure [(2) Method **2.23**].

Determination of Hot Water-Insoluble Nitrogen, HWIN (Buffer).—Weigh rapidly and accurately a sample contg 0.3 g of $IN_{25°}$. (The sample weight may vary ±5 mg but must be accurately weighed to ±0.0005 g). Place sample in a 400 ml beaker and add 250 ml of boiling dild buffer soln from a Pyrex graduate. Stir, cover beaker with a watch glass, and suspend in the boiling H_2O bath so that the liquid in the

beaker is below the H_2O line in the bath. Stir the soln gently for about 5 sec. after 10, 20, and 30 min., then remove at once from the bath and filter without delay thru a 15 cm Whatman No. 12 folded filter. Wash the insol. residue on to the filter with near-boiling H_2O, and wash the filter four times from the top down. (Total wash H_2O will be about 75–100 ml.) Det. per cent nitrogen in the wet paper and residue by the usual Kjeldahl digestion and distillation, using an 800 ml flask [(2), Method 2.23].

Calculation of AI of the $IN_{25°}$.—The Availability Index is:

$$AI = \frac{(\%\ IN_{25°} - \%\ HWIN) \times 100}{\%\ IN_{25°}},$$

where $IN_{25°}$ is cold-H_2O-insoluble nitrogen, and HWIN is hot-buffer-insoluble nitrogen.

EXPERIMENTAL

Figure 1 shows that the Availability Index can be used for predicting the agronomic availability of the A.O.A.C. water-insoluble nitrogen in U-F polymers.

The A.O.A.C. method for water-insoluble organic nitrogen was satisfactory for determining the water-insoluble nitrogen at 25°C. The use of alcohol was omitted because the material wets readily.

Some of the variables affecting the determination of hot water-insoluble nitrogen were studied by a preliminary method which employed 250 ml of hot water containing 0.1 g of $CaCO_3$ as a neutralizing agent. A sample of 1.0 g of U-F polymer was digested in a hot bath for varying times (15, 30, and 60 min.) at temperatures of 70, 90, and 100°C. Also, intermittent hand stirring was compared with continuous mechanical stirring. The solutions were filtered rapidly, the insoluble residue was washed with water

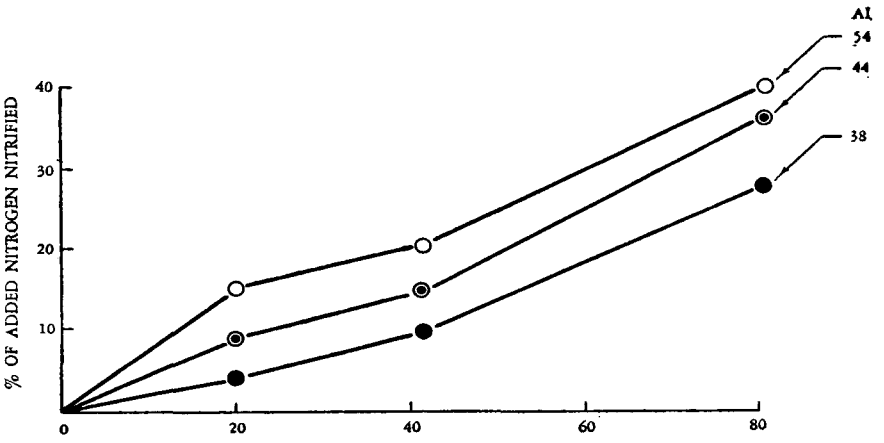


FIG. 1.—Nitrification rates compared with the availability indexes for the water insoluble fractions in U-F polymers.

at the temperature of the bath, and nitrogen was determined in the insoluble residue. Solubility (and hence A. I.) increased with time of digestion, higher temperature of the bath, and continuous agitation. From results of this preliminary study, the procedure selected (because of its satisfactory precision, simplicity, and close agreement with nitrification data) was digestion of a 1.0 g sample for 30 minutes at 100°C., with mild hand stirring at 10 minute intervals. The solution was filtered promptly, and the filter was washed with near-boiling water to remove soluble material.

Neutral salts had a negligible effect on the percentage of hot-water-insoluble nitrogen. Typical fertilizer salts, including $(NH_4)_2SO_4$, NH_4NO_3, $NaNO_3$, KCl, and K_2SO_4, were tested. However, when acid salts, such as monocalcium phosphate and monoammonium phosphate were present, the A.I. of some samples was appreciably increased. A preliminary water wash to remove acidity from the sample resulted in higher and variable results for the percentage of nitrogen insoluble in the hot water solution. Therefore, a dilute aqueous phosphate buffer solution at *p*H 7.5 was substituted for water and $CaCO_3$ in the digestion. The use of the buffer solution made prior removal of acidity unnecessary, since the buffer kept the solution slightly alkaline.

Six determinations of the percentage of hot-water-insoluble nitrogen of U-F polymer Samples A and B were made to determine the precision of the buffer procedure. As shown in Table 1, runs 1 and 2 contained no added salt. Monocalcium phosphate was included in runs 3 and 4, and a mixture of salts was included in runs 5 and 6 without significant effect on the A. I. The U-F polymer A had a mean value of 62.5 A. I. and a standard deviation of 0.8. U-F polymer B had a mean value of 38.0 and a standard deviation of 1.2. This is a low availability sample, and the results show more scatter from the mean than do those of sample A, a high-availability sample.

TABLE 1.—*Availability index by buffer method*

RUN NO.	PRODUCT A $IN_{38}°$=28.3%		PRODUCT B $IN_{38}°$=31.3%		ADDED SALTS
	HWIN (%)	AI	HWIN (%)	AI	
1	10.8	62.0	18.9	39.5	None
2	10.7	62.0	19.2	38.5	None
3	10.8	62.0	19.5	37.5	0.2 g $Ca(H_2PO_4)_2$
4	10.7	62.0	19.9	36.5	0.2 g $Ca(H_2PO_4)_2$
5	10.4	63.0	19.6	37.0	Mixture[a]
6	10.2	64.0	19.2	38.5	Mixture[a]
Mean		62.5		38.0	
Std. Dev.		0.8		1.2	

[a] Salt mixture: 0.2 g each: $Ca(H_2PO_4)_2$, $NH_4H_2PO_4$. 0.1 g each: NH_4NO_3, KCl, $(NH_4)_2SO_4$.

The effect of varying particle size is shown in Fig. 2. (The entire sample was crushed to pass the mesh indicated.) Decreasing the particle size results in an increase in A. I.; however, the A. I. for 20 mesh, Sample C, was only slightly less than for 35 mesh or even for 100 mesh.

A significant increase in A. I. occurs with decreasing weight of sample.

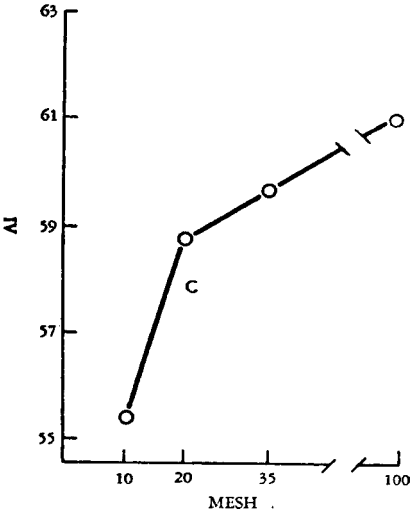


FIG. 2.—Effect of particle size on availability index by buffer method.

Sample C showed an A. I. of 58.5 when a 1.0 g sample was used, and 65.0 for a 0.5 g sample. Figure 3 shows that a definite sample weight is necessary in the hot buffer digestion in order to obtain comparable A. I. values. The final method specifies a sample containing 0.3 g of $IN_{25°}$.

Availability index of mixed fertilizers.—In a preliminary study of the A. I. of mixed fertilizers, the method was applied successfully to a 20–10–10 grade mixed fertilizer prepared from ammoniated triple superphosphate, KCl, U-F polymer, and inert siliceous filler. All of the insoluble nitrogen at 25°C. was derived from sample C, which comprised 50 per cent of the mixture. Portions of the sample were crushed to pass each of three screen sizes, *viz.*, 20, 35, and 100 mesh Tyler sieves. By using twice as much sample of the mixture as of Sample C for determining hot-water-insoluble nitrogen, equal weights of Sample C were compared. As shown in Fig. 4, the A. I. of the mixture agreed well with that of Sample C. The official method of sample preparation requires that mixtures which segregate, as this one did, must pass a 35 mesh sieve. At this mesh size, the A. I. of Sample C was 59.5, and that of the mixture was 60.5. Agreement was as good for 100 mesh.

The buffer method, using a sample equivalent to 0.3 g of $IN_{25°}$ for the

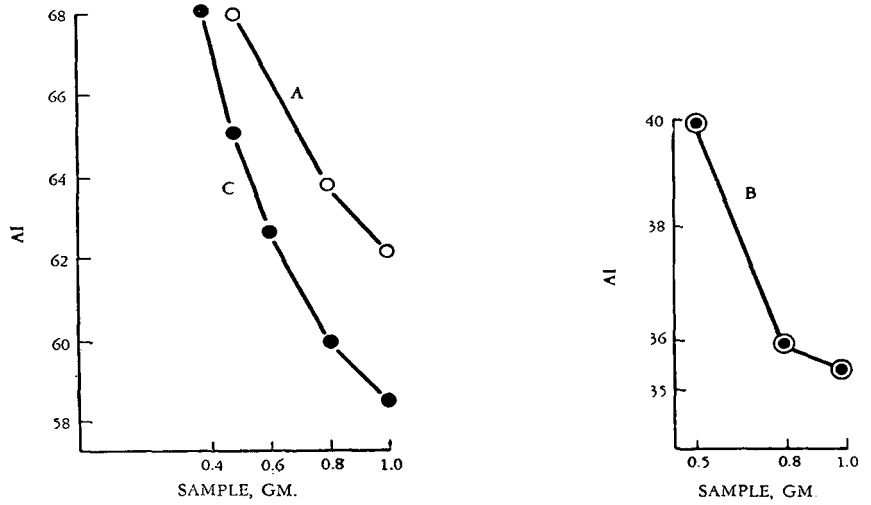


FIG. 3.—Effect of sample weight on A. I. by buffer method.

determination of HWIN, was applied to 20–12–8 and 10–6–4 grade fertilizers in which a representative U-F polymer was the sole source of $IN_{25°}$. The sample weight used for the determination of $IN_{25°}$ was increased so that not less than 0.5 g of U-F polymer was present. The A. I.

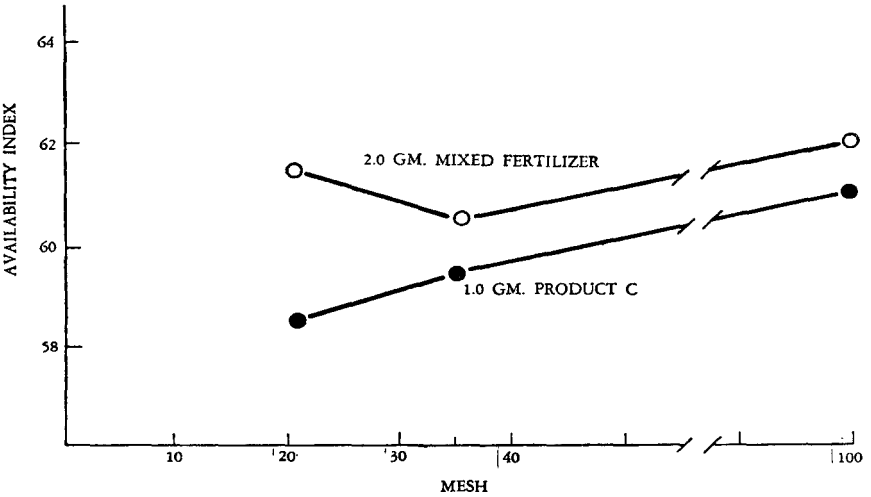


FIG. 4.—Comparison of availability index of product C and a 20–10–10 grade mixed fertilizer containing 50 per cent product C.

TABLE 2.—*Availability index of simple mixed fertilizers*

SAMPLE 35-MESH[a]	TOTAL N PER CENT	IN_{24}°		HWIN		AI
		G SAMPLE	PER CENT	G SAMPLE	PER CENT	
U-F Polymer[b]	37.5	1	27.6	1.087	11.5	58.5
20-12-8	19.4	2	12.6	2.380	5.40	57.0
10-6-4	9.8	4	6.16	4.870	2.54	59.0

[a] Ref. (2, 2.2.).
[b] U-F polymer used in the fertilizer mixtures.

found for the fertilizers was nearly the same as that of the polymer. Analytical data are shown in Table 2.

Determination of the amount of U-F polymer in a fertilizer mixture.—For a simple mixture that contains only U-F polymer as the source of water-insoluble nitrogen, an A.O.A.C. determination of water-insoluble nitrogen is made. The ratio of this percentage to the percentage of water-insoluble nitrogen in the original U-F polymer establishes the amount present. This is shown in Table 3. The same conclusion can be reached by the determination of the amount of formaldehyde recovered by distillation from dilute P_2O_5 solution (5). The ratio of 0.5 shows that the fertilizer contains 50 per cent of product C. Other materials that yield formaldehyde must be absent.

The presence of large amounts of hot water-insoluble urea-formaldehyde resins will be indicated by abnormally low A. I. values. For example, A.I. results of 2 and 10 were obtained on two samples of U-F resin scrap. Furthermore, decreasing the sample weight from 1.0 to 0.3 g in the hot buffer digestion did not increase the solubility of the nitrogenous materials. These products gave misleading values for availability by the A.O.A.C. neutral permanganate method. For example, the sample that had the A. I. of only 10 gave an activity of 76 per cent by the neutral permanganate method.

SUMMARY AND CONCLUSIONS

A simple, rapid, and reproducible method has been developed for predicting the agronomic value of U-F polymers. The new technique is superior to existing methods, and shows a close correlation with time-consuming

TABLE 3.—*Estimation of U-F polymer content in a simple fertilizer mixture*

	PRODUCT C	FERTILIZER MIXTURE D[a]	RATIO D/C (CALCD)
Per cent IN_{25}°	28.1	13.2	0.47
Per cent formaldehyde	27.0	13.6	0.50

[a] 50% of the mixture is Product C.

soil nitrification tests. The procedure requires two determinations: (*a*) nitrogen insoluble in cold water, in accordance with the Official Method of the A.O.A.C., and (*b*) nitrogen insoluble in a hot buffered phosphate solution. The percentage of the cold water-insoluble nitrogen which dissolves in the hot, buffered solution is an index of the availability of the cold water-insoluble nitrogen. It is recommended that the A.O.A.C. consider the proposed method for the determination of available fertilizer nitrogen in urea-formaldehyde polymers.

REFERENCES

(1) YEE, J. Y., and LOVE, K. S., *Proc. Am. Soil Sci. Soc.*, **11**, 389 (1946).
(2) *Methods of Analysis*, 7th Ed., A.O.A.C. Washington, D. C., (1950) **2.34–2.36.**
(3) RUBINS, E. J., and BEAR, F. E., *Soil Sci.*, **54**, 411 (1942).
(4) CLARK, K. G., YEE, J. Y., LOVE, K. S., and BOYD, T. A., *Ind. Eng. Chem.*, **43**, 871 (1951).
(5) *Unpublished method* (from the authors' laboratory).

THE IDENTIFICATION OF AZO DYES BY SPECTROPHOTOMETRIC IDENTIFICATION OF THEIR REDUCTION PRODUCTS*

II. COMPOUNDS WHICH GIVE NEUTRAL OR ACIDIC PRODUCTS ON REDUCTION

By L. S. HARROW and J. H. JONES (Department of Health, Education, and Welfare, Food and Drug Administration, Washington 25, D. C.)

In an earlier publication, the authors reported methods for the identification of azo dyes which give simple amines or diamines on reduction (1). This report describes similar methods for the identification of the amphoteric or acidic products obtained on reduction of certain azo colors.

In most cases the reduction products may be treated as a mixture of three classes of compounds:

(*a*) The basic components which can be either steam distilled or extracted from alkaline solution.

(*b*) The neutral components (usually amphoteric) which can be either steam distilled or extracted from neutral solutions.

(*c*) The acidic components, such as sulfonic acids, which cannot be steam distilled or extracted from aqueous solution.

The reduction products of an azo color may be systematically examined for each of the three classes of compounds by application of standard separation techniques followed by spectrophotometric examination of each fraction separated.

* Presented at the annual meeting of the Association of Official Agricultural Chemists, September 29 and 30, and October 1, 1952, at Washington, D. C.

Since the reduction products are identified spectrophotometrically, it is necessary to have available for comparison the spectra obtained from solutions of authentic samples of the aminosulfonic acids, amino-naphthols, amino-pyrazolones, etc., suspected to be present. Many of these compounds are available in most laboratories. Authentic samples of other compounds, such as the amino-naphthol sulfonic acids or amino-pyrazolone sulfonic acids, may be readily synthesized.

Most aromatic compounds can be identified by comparison of their absorbancy curves with the absorbancy curves of suitable reference compounds in the same solvent. Absorbancy ratios are often useful in distinguishing between compounds that have similar curves. In many cases, mixtures of compounds, or single compounds in solutions which show "background" absorption can be identified by application of the variable reference titration procedure. Application of this technique to the identification of reduction products from certain azo colors is discussed in detail elsewhere (2, 3).

The procedures for reduction of the dyes, and for separation of the basic components, were described in the previous report. After the basic components are isolated, the aqueous solution of the reduction products is neutralized with mineral acid, saturated with salt, and steam distilled. This treatment will separate steam-volatile amphoteric compounds. The residue from the steam distillation is extracted with ether to separate other neutral compounds. The aqueous residue from the ether extraction is made acidic and passed through a chromatographic column. Fractions of the eluate are collected and examined separately.

The identification of basic and neutral compounds obtained by the reduction of an azo color is usually fairly simple. Ordinarily, only one such compound is present, and it can usually be separated cleanly from the other reduction products.

Similarly, if only one acidic compound is obtained on reduction, identification is usually obtained easily. In many cases, two acidic compounds are obtained on reduction. The chromatographic step in the proposed procedure ordinarily does not separate them completely. Its primary purpose is to "clean up" the solution so that satisfactory ultraviolet curves may be obtained. When two acidic compounds are formed on reduction, the spectrophotometric curve will be that of a mixture whose components are often different enough to permit identification of each component. If the two curves overlap appreciably, the variable reference technique may be used to identify the components.

METHOD

APPARATUS

(a) *Spectrophotometer.*—A spectrophotometer capable of isolating a wave band of 5 mμ, or less, in the 220–400 mμ region.

(b) *Chromatographic column.*—A glass tube, constricted at one end, ca 15×100

mm, contg a 40 mm bed of Solka-Floc BW 40 supported by a pledget of glass wool or a medium porosity fritted glass disk.

REAGENTS

(a) *Sodium hydrosulfite.*—$Na_2S_2O_4$, C. P.
(b) *Titanium trichloride solution.*—Ca 0.1 *N*.
(c) *Standard reference solutions.*

REDUCTION

Water soluble colors.—Place 5–10 mg of color in a small beaker and dissolve in min. amount of H_2O. Heat soln on a steam bath, and add ca 1 mg portions of $Na_2S_2O_4$ until all the color has disappeared, or until there is no further change in color on heating for an addnl 15 min. Agitate the soln after each addn of $Na_2S_2O_4$.

Oil soluble colors.—Dissolve 5–10 mg of color in a small amount of hot alcohol. Add 2 ml of 10 per cent (w/v) NaOH, and reduce with $TiCl_3$ soln under a stream of CO_2. Add 2 mg of $Na_2S_2O_4$ to stabilize the reduction products.

SEPARATION OF COMPONENTS

Basic components.—When reduction is complete, transfer the soln to a separatory funnel and extract with two 40 ml portions of ether. (Where it is desired to det. the nature of the basic component only, isolate the basic component by steam distillation.) Proceed with the purification and spectrophotometric detn of the basic component as described in the previous report (1).

Neutral components.—Transfer the alk. aq. residue to a 300 ml round-bottom flask, dil. to ca 150–200 ml with H_2O, adjust to *p*H 6–8 (litmus) with dil. HCl soln, add sufficient salt to saturate the soln (ca 80 g), and steam distill. Collect and reserve 20 ml fractions of the distillate for spectrophotometric examination.

Cool residue in the distilling flask to room temperature, transfer to a separatory funnel, and extract with two 40 ml portions of ether. (Retain aq. layer for identification of the acidic components.) Transfer the combined ether extracts to a 150 ml beaker, add 20 ml of H_2O, and evap. the ether on a steam bath, using a gentle air stream to hasten the evapn. Save the aq. soln for spectrophotometric examination. The solns obtained from the steam distillation and ether extraction contain the volatile and non-volatile neutral components, respectively.

Acidic components.—Acidify aq. layer from the ether extraction with 1 ml of concd HCl; pass through the chromatographic column, and elute with distd H_2O. Collect 20 ml fractions of eluate and reserve for spectrophotometric examination. These fractions contain the acidic components and any neutral components not separated by the previous treatment of the sample.

SPECTROPHOTOMETRIC EXAMINATION

Acidify (litmus) the fractions collected in the separation procedures, heat for 15–20 min. on a steam bath to remove traces of ether, cool, and dil. (as little as possible) to a convenient vol. Det. the absorbancy curves of the solns between 220 and 400 mμ. Make the solns alk. (litmus) with 10% NaOH soln and dil. to convenient vol. (as little as possible). Det. the absorbancy curves of the alk. solns between 220 and 400 mμ. Compare the absorbancy curves of the various fractions with the absorbancy curves of solns of the possible reduction products.

EXPERIMENTAL

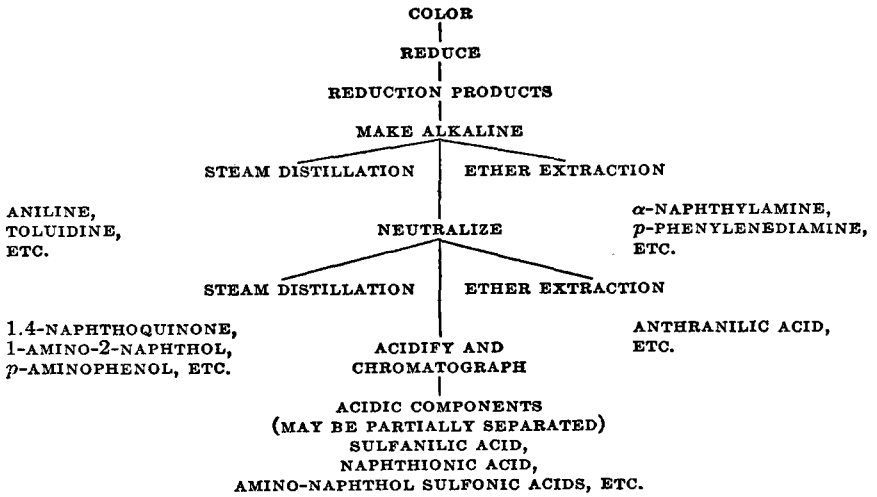
The preparation of a typical amino-naphthol sulfonic acid needed as a reference compound can be illustrated by the following procedure:

Preparation of 1-amino-2-naphthol-3,6-disulfonic acid.—Crude 2-naphthol-3,6-disulfonic acid was purified by the procedure of Forster and Keyworth (4). The purified product was coupled with diazotized *p*-toluidine to produce the azo dye, 1-(4-methyltolylazo)-2-naphthol-3,6-disulfonic acid. The color was isolated, and then reduced with $Na_2S_2O_4$ in basic soln. The *p*-toluidine was removed by extraction with ether, and the 1-amino-2-naphthol-3,6-disulfonic acid was precipitated by the addition of HCl.

Many of the amino compounds needed as reference materials can be prepared by similar procedures, starting with suitable intermediates.

DISCUSSION

The proposed separation procedure is shown in the following schematic diagram:



Since it would be impossible to describe all the applications of the proposed procedure, a few typical examples will be discussed.

The neutral compounds most often obtained by reduction of azo colors are the aminophenols and aminonaphthols. The ultraviolet absorbancy curves of 1-amino-2-naphthol, 1,4-naphthoquinone, and *p*-aminophenol are shown in Figs. 1, 2, and 3. (When 1-amino-4-naphthol is the primary reduction product, it appears to oxidize quantitatively to 1,4-naphthoquinone on steam distillation from neutral solution.)

Anthranilic acid is one of the most widely used of the non-volatile, neutral compounds. Its absorbancy curve is shown in Fig. 4.

The ultraviolet absorbancy curves of several acidic compounds obtained on reduction of typical azo colors are shown in Figs. 5–11. Many of

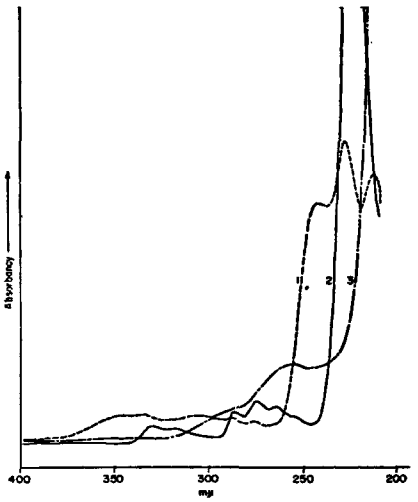


FIG. 1.—Absorption curves of 1-amino-2-naphthol (10 mg/l). *Curve 1:* in 95% alcohol; *Curve 2:* in 0.1 *N* HCl; *Curve 3:* in 0.1 *N* NaOH.

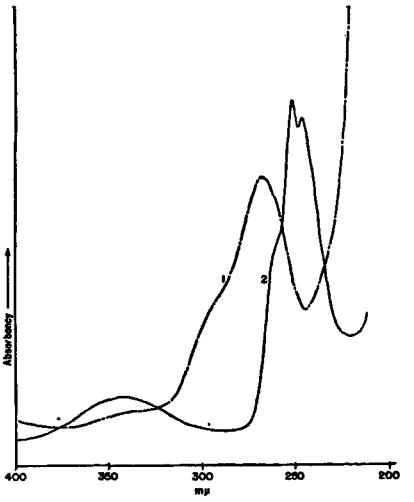


FIG. 2.—Absorption curves of 1,4-naphthoquinone (10 mg/l). *Curve 1:* in 0.1 *N* NaOH; *Curve 2:* in 0.1 *N* HCl.

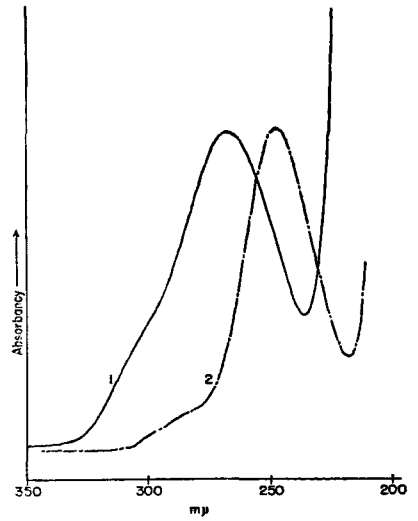


FIG. 3.—Absorption curves of *p*-aminophenol (10 mg/l). *Curve 1:* in 0.1 *N* NaOH. *Curve 2:* in H_2O.

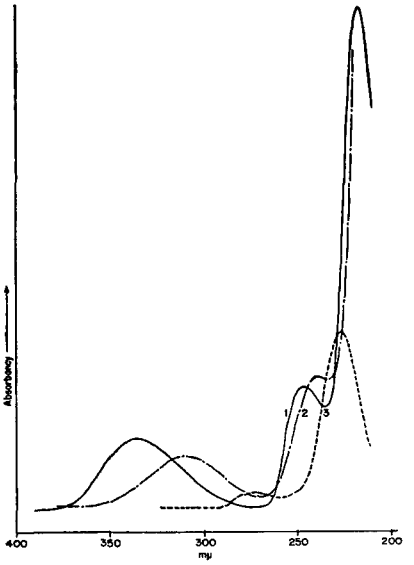


FIG. 4.—Absorption curves of anthranilic acid (10 mg/l). *Curve 1:* in 95% alcohol; *Curve 2:* in 0.1 *N* NaOH; *Curve 3:* in 0.1 *N* HCl.

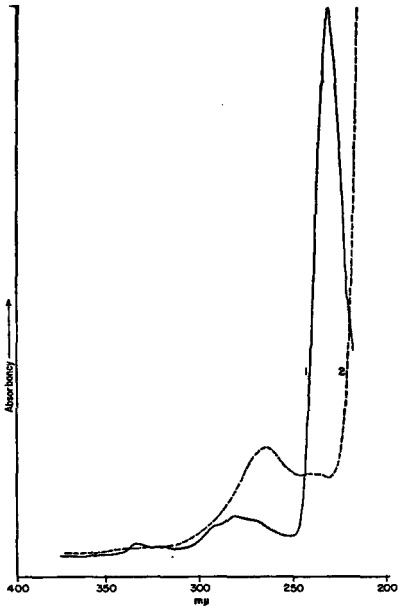


FIG. 5.—Absorption curves of 1-amino-2-naphthol-6-sulfonic acid (10 mg/l). *Curve 1:* in 0.1 *N* HCl; *Curve 2:* in 0.1 *N* NaOH.

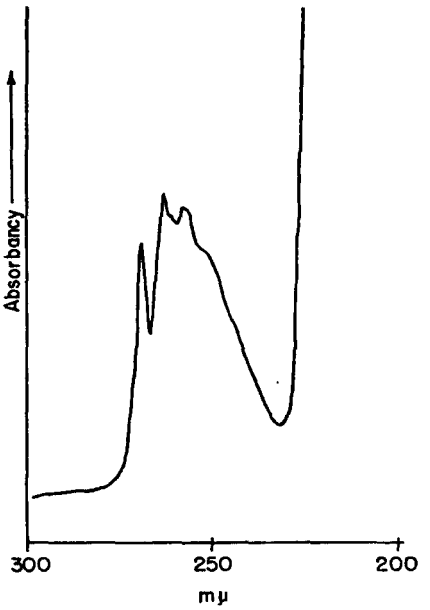


FIG. 6.—Absorption curve of sulfanilic acid (250 mg/l in 0.1 *N* HCl).

these compounds may be identified by simple inspection of the curves obtained from the acid solutions.

As can be seen from Figs. 5, 7, and 8, the absorbancy curves of the closely related compounds, 1-amino-2-naphthol-6-sulfonic acid, 1-amino-2-naphthol-3,6-disulfonic acid, and 1-amino-2-naphthol-6,8-disulfonic acid are quite similar. However, there are differences in the location of the peaks and in the absorbancy ratios between peaks.

The spectrophotometric curves of the amino-pyrazolones obtained by reduction of FD&C Yellow No. 5, and Ext. D&C Yellow No. 3, are shown in Figs. 10 and 11. (The spectra of the rubizonic acids (5) obtained by oxidation of the pyrazolones with Na_2O_2 are also useful in the identification of the pyrazolone colors.)

In Table 1 are listed a number of azo colors from which one or more neutral or acidic reduction products have been isolated and identified by the proposed procedure. In many cases, the samples were analyzed as unknowns by one of the authors (L.S.H.).

TABLE 1.—*Identification of reduction products*

COLOR	NEUTRAL OR ACIDIC COMPOUND SEPARATED	METHOD OF SEPARATION
FD&C Yellow No. 5	Sulfanilic acid	Chromatographic column
	1-(4-Sulfophenyl)-3-carboxy-4-amino-5-hydroxypyrazole	Chromatographic column
FD&C Yellow No. 6	Sulfanilic acid	Chromatographic column
	1-Amino-2-naphthol-6-sulfonic acid	Chromatographic column
FD&C Red No. 1	1-Amino-2-naphthol-3,6-disulfonic acid	Chromatographic column
FD&C Red No. 2	1-Amino-2-naphthol-3,6-disulfonic acid	Chromatographic column
	1-Amino-2-naphthol-4-sulfonic acid	Chromatographic column
FD&C Red No. 4	1,2-Naphthoquinone-4-sulfonic acid	Chromatographic column
D&C Red No. 5	1-Amino-2-naphthol-3,6-disulfonic acid	Chromatographic column
D&C Red No. 10	1-Amino-2-naphthol	Steam distilled from neutral salt solution
	2-Aminonaphthalene-1-sulfonic acid	Chromatographic column
D&C Red No. 35	1-Amino-2-naphthol	Steam distilled from neutral salt solution
D&C Red No. 36	1-Amino-2-naphthol	Steam distilled from neutral salt solution
Ext. D&C Yellow No. 3	1-(4-Sulfophenyl)-3-methyl-4-amino-5-hydroxypyrazole	Chromatographic column
C.I. 370 (Congo Red)	1,2-Diaminonaphthalene-4-sulfonic acid	Chromatographic column
Ext. D&C Red No. 17	1-Amino-2-naphthol-6,8-disulfonic acid	Chromatographic column
D&C Red No. 31	1-Amino-2-naphthol-3-carboxylic acid	Chromatographic column

TABLE 1.—(*continued*)

COLOR	NEUTRAL OR ACIDIC COMPOUND SEPARATED	METHOD OF SEPARATION
D&C Red No. 14	Anthranilic acid	Ether extraction from neutral salt solution
	1-Amino-2-naphthol	Steam distilled from neutral salt solution
C.I. 185	1-Amino-2-naphthol-6,8-disulfonic acid	Chromatographic column
D&C Red No. 17	1-Amino-2-naphthol	Steam distilled from neutral salt solution
D&C Red No. 18	1-Amino-2-naphthol	Steam distilled from neutral salt solution
FD&C Orange No. 1	1,4-Naphthoquinone	Steam distilled from neutral solution
	Sulfanilic acid	Chromatographic column
D&C Orange No. 3	1-Amino-2-naphthol-6,8-disulfonic acid	Chromatographic column

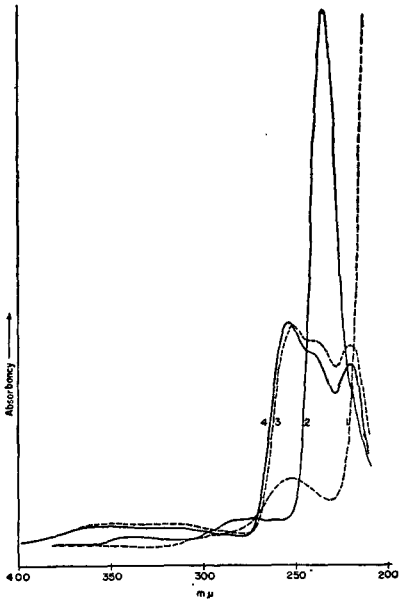


FIG. 7.—Absorption curves of 1-amino-2-naphthol-3,6-disulfonic acid (11.2 mg/l). *Curve 1:* in 0.1 *N* NaOH; *Curve 2:* in 0.1 *N* HCl; *Curve 3:* in H_2O; *Curve 4:* in 95% alcohol.

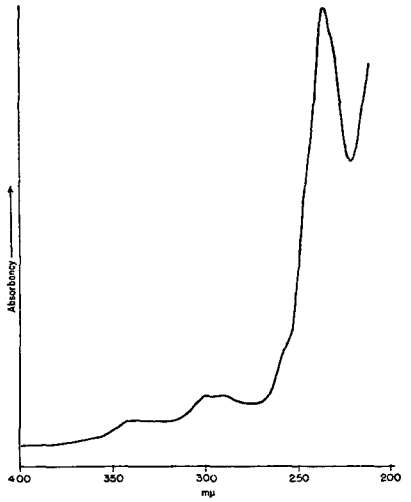


FIG. 8.—Absorption curve of 1-amino-2-naphthol-6,8-disulfonic acid (10 mg/l in 0.1 *N* HCl).

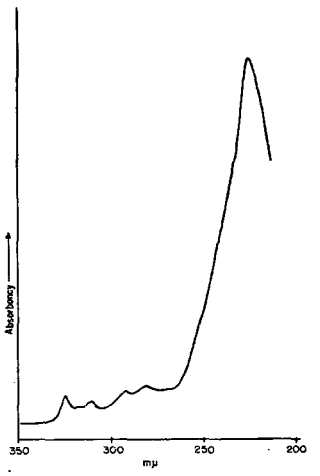


FIG. 9.—Absorption curve of 1,2-diaminonaphthalene-4-sulfonic acid (10 mg/l in 0.1 *N* HCl).

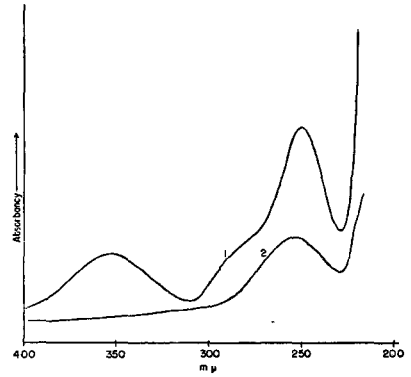


FIG. 10.—Absorption curves of 1-(4-sulfophenyl)-3-carboxy-4-amino-5-hydroxypyrazole (10 mg/l). *Curve 1:* in 0.1 *N* NaOH; *Curve 2:* in 0.1 *N* HCl.

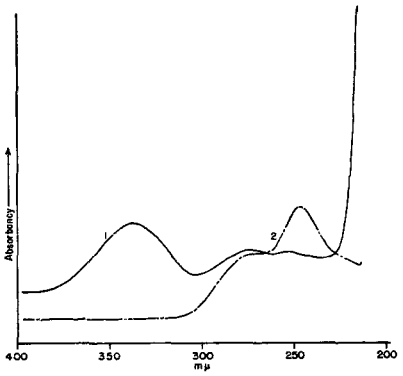


FIG. 11.—Absorption curves of 1-(4-sulfophenyl)-3-methyl-4-amino-5-hydroxypyrazole (10 mg/l). *Curve 1:* in 0.1 *N* NaOH; *Curve 2:* in 0.1 *N* HCl.

SUMMARY

Procedures have been described for the separation and spectrophotometric identification of the water-soluble neutral and acidic compounds obtained by reduction of azo compounds. These methods are applicable to samples containing only a few milligrams of azo color.

Examples of practical applications of the procedures are given.

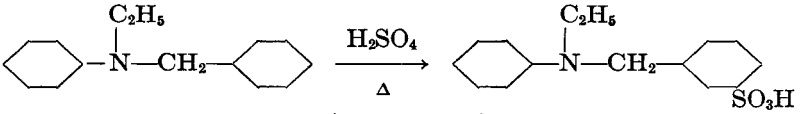
REFERENCES

(1) JONES, J. H., and HARROW, L. S., *This Journal*, **34**, 831 (1951).
(2) JONES, J. H., CLARK, G. R., and HARROW, L. S., *ibid.*, **34**, 135 (1951).
(3) JONES, J. H., and HARROW, L. S., Paper delivered at Conference on Analytical Chemistry and Applied Spectroscopy. Pittsburgh, Pa., March, 1952.
(4) FORSTER, B. B., and KEYWORTH, C. N., *J. Soc. Chem. Ind.*, **46**, 25T (1927).
(5) KNOOR, F., *Ann.*, **238**, 189, 192 (1887).

THE COMPOSITION OF COMMERCIAL ETHYLBENZYLANILINE SULFONIC ACID*

By KEITH S. HEINE, JR., and JOHN H. JONES (Division of Cosmetics, Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C.)

Ethylbenzylaniline sulfonic acid is produced commercially by the sulfonation of ethylbenzylaniline. For years it was assumed that the product of this reaction was chiefly the *p*-sulfonic acid. The specifications for the certifiable colors produced from this compound call for the compounds that would be produced from the *p*-isomer (1). However, in 1942 it was shown (3) that laboratory sulfonation of ethylbenzylaniline at 60°C. in 30 per cent fuming sulfuric acid for three hours produces mainly the *m*-isomer, some of the *p*-isomer (ca 15 per cent), and a small amount of the *o*-isomer.



(+some *para*)

Fierz-David (3) separated the isomeric ethylbenzylaniline sulfonic acids by an involved crystallization procedure. The structures of the *m*- and *p*-fractions obtained were determined by converting the benzyl portion of the molecule to the corresponding sulfonated benzoic acids which

* Presented at the annual meeting of the Association of Official Agricultural Chemists, September 29, 30, and October 1, 1952, at Washington, D. C.

were identified as the amides. The identity of the *o*-fraction was established by synthesis of the *o*-isomer starting with *o*-toluenesulfonyl chloride. While there appears to be little doubt as to the basic conclusions drawn by Fierz-David, the separations used can hardly be expected to give the quantitative composition of the product. Since the commercial sulfonation procedure is somewhat different from that used by Fierz-David, the composition of the material he examined may not be the same as that of the commercial product.

The work undertaken here had as its objective the development of a satisfactory quantitative procedure for the determination of composition of commercial samples of ethylbenzylaniline sulfonic acid. It was assumed that the isomeric composition of the commercial product could be determined by spectrophotometric methods if suitable standard samples of the various isomers were available.

EXPERIMENTAL

PREPARATION OF THE ISOMERIC ETHYLBENZYLANILINE SULFONIC ACIDS

Ortho-Isomer.—The *o*-isomer was prepared by a procedure very similar to that described by Fierz-David (3). In a 500 ml two-neck flask, fitted with a condenser and a separatory funnel, 38 g of freshly vacuum-distd *o*-toluenesulfonyl chloride (b.p. 130–132°C. at 18 mm Hg) and 70 g of freshly distd phosphorus oxychloride (b.p. 105–107°C.) were placed. The mixt. was heated to reflux, and 30 g of bromine was added drop-wise over a period of 6 hrs. The reaction flask was irradiated with a strong ultraviolet light (250 watts) thruout the addn of the Br. After this addn was completed, the reaction mixt. was refluxed for an addnl 1.5 hrs. The reaction mixt. was then distd until the distillation temperature reached 107°C. The remaining brown residue was mixed with several times its vol. of petr. ether. The light brown crystalline solid obtained by this treatment was recovered by filtration, and was recrystallized from petr. ether. The purified product was a white crystalline solid which melted at 60.5 to 61.5°C. (Reported, 60–61°C.). The yield was ca 16 g, or 30 per cent of theoretical.

Anal.—Br: Calcd, 28.3%, Found, 29.7%; Cl: Calcd, 13.1%, Found 12.3%.

Benzylbromide *o*-sulfonyl chloride (10 g) was refluxed with a mixt. of 17 g of absolute alcohol and 0.7 g of H_2O for 2 hrs. After the alcohol was removed by distillation, the remaining viscous brown material was poured directly into 12 g of N-ethylaniline, and the mixt. was warmed on the steam bath for five hrs. The reaction mixt. was cooled, dild with ca 3 vols of ether, and extracted with four 50 ml portions of 2 *M* Na_2CO_3. On acidification of the combined alk. extracts with dil. HCl, a fine white crystalline ppt was obtained. The solid material was recovered by filtration, washed with cold dil. HCl, and dried. The crude product was crystallized from H_2O until its ultraviolet spectrum did not change on further crystallization. The yield of the crude *o*-isomer was 7 g, or 65 per cent of the theoretical yield based on the amount of benzylbromide *o*-sulfonyl chloride used.

Anal.—Eq. Wt.: Calcd for $C_{15}H_{17}O_3NS$, 291; Found, 291.

Para-Isomer.—Benzylbromide *p*-sulfonyl chloride was prepared by the procedure described above, using *p*-toluenesulfonyl chloride (purified by recrystallization from petr. ether) in place of *o*-toluenesulfonyl chloride. The purified material was a crystalline solid which melted at 70.0 to 71.5°C.

Anal.—Br: Calcd, 28.3%; Found, 28.5%. Cl: Calcd, 13.1%; Found, 12.7%.

The benzylbromide *p*-sulfonyl chloride was condensed with N-ethylaniline as described under the preparation of the *o*-compound. The crude *p*-compound was dissolved in about 300 ml of 10% Na_2CO_3 soln, and the soln was extracted several times with an equal vol. of ether. The aq. soln was heated to boiling, decolorizing carbon was added, and the soln was filtered and evapd to a vol. of ca 100 ml. The concd soln was made slightly acid, and was cooled. The material pptd as an oil which eventually crystallized. The crude material was recrystallized from H_2O until its ultraviolet absorption spectrum remained unchanged upon further crystallization. The yield of the *p*-isomer was about 7–10 per cent of theoretical. The recrystallized *p*-isomer contained 2 moles of water of crystallization.

Anal.—Eq. Wt.: Calcd for $C_{15}H_{17}O_3NS \cdot 2H_2O$, 327; Found, 327. The *volatile matter* at 135°C. was 10.9 per cent; calcd value for 2 *moles of water*, 11.0%.

Meta-Isomer.—The *m*-isomer was prepared by repeated recrystallization of commercial ethylbenzylaniline sulfonic acid. Approximately 1500 ml H_2O was heated to boiling; 800 g of ethylbenzylaniline sulfonic acid and 100 g of decolorizing carbon were added. The mixt. was filtered, the filtrate was cooled to room temperature, and the material which crystallized was recovered by filtration. Crystallization was repeated 6 times with a recovery of about 85 per cent at each step. The spectrophotometric properties (ultraviolet) of the recovered material were constant after the third recrystallization.

Anal.—Eq. Wt.: Calcd for $C_{15}H_{17}O_3NS$, 291; Found, 293. Volatile matter at 135°C., 1.4%.

The amide of the *m*-isomer was prepared as directed by Fierz-David (3). Attempts to recrystallize the product from alcohol or benzene were unsuccessful. When the solvent was evapd, a light amber-colored, glass-like residue remained. After standing for several weeks, the mass crystallized into long needles. These crystals recrystallized readily from alcohol. The recrystallized material melted at 98–99°C. (Reported, 98–99°C.).

RECRYSTALLIZATION OF COMMERCIAL ETHYLBENZYLANILINE SULFONIC ACID

For experimental purposes, it was necessary to have considerable quantities of the pure *p*- and *m*-isomers. The *m*-isomer was obtained in good yield by the above procedure. Since the synthesis of the *p*-isomer described above is not a convenient method for the preparation of substantial quantities of the compound, an investigation was undertaken to find procedures for obtaining this isomer from the commercial material.

Fierz-David (3) obtained the *p*-isomer of ethylbenzylaniline sulfonic acid by crystallizing out most of the *m*-isomer and recovering the *p*-isomer from the filtrate as a sodium salt. It was found that this procedure did not give a consistently significant yield of the *p*-isomer.

Crystallization experiments indicated that the solubilities of the two isomers in H_2O were very nearly the same and that the filtrate obtained on recrystallization of mixtures of the two isomers usually contained them in approximately equal amounts. It was difficult, therefore, to obtain a fraction containing more than 50% of the *p*-isomer by fractional crystallization of the commercial material from water. Occasionally (but not consistently) the *p*-isomer could be obtained in a fairly pure form by seeding a saturated solution containing both isomers. Either of the isomers could readily be purified by recrystallization from H_2O, once a fraction relatively rich in the desired isomer was available. Conversion of the isomers to the sodium salts did not appear to give any better results in the separation of the isomers by recrystallization.

The method finally developed for the separation of the two isomers is based on the observation that when a 50-50 mixture of the two acids is crystallized from alco-

hol, the *m*-isomer usually crystallized out first. The filtrate from the alcohol recrystallization is, therefore, richer in the *p*-isomer. The *p*-isomer can be recovered from the alcoholic solution by adding water and evaporating the alcohol.

The isolation procedure gave fairly constant results. As a matter of routine, however, the approximate composition of the filtrate and precipitate obtained in each step of the separation was determined from the ultraviolet spectra of the material in acid solution (Fig. 1). The approximate isomeric composition of the mixture is easily checked by comparing the curves obtained with those of the pure isomers. Monitoring of the recrystallization in this manner saved considerable time; it could be readily determined whether any fraction obtained should be saved, discarded, or reworked.

On several occasions, very small amounts of the *o*-isomer were isolated by reworking the filtrates obtained on recrystallizing the fractions rich in the *p*-isomer.

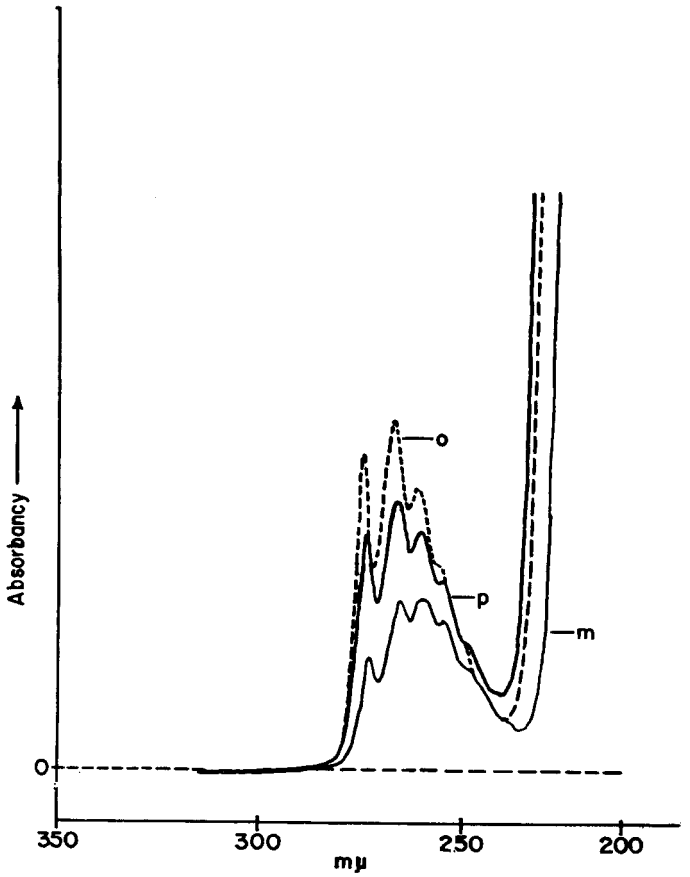


FIG. 1.—Ultraviolet absorbancy curves of the ethylbenzylaniline sulfonic acid. Concn: 200 mg/liter; solvent: 0.1 *N* HCl; cells: 1 cm.

The largest amount of *o*-isomer obtained was only 0.5 g from a 300 g sample of the commercial material.

Separation Procedure.—The commercial material, 300 g, was added to one l of cold H_2O; the mixture was stirred for ca 30 min. and filtered. The filtrate, which was usually dark in color, was discarded. The residue was digested at about 75°C. with 700 ml H_2O for one hr and the mixture was filtered while hot. The undissolved material was chiefly the *m*-isomer; it was either discarded or saved for the preparation of the purified *m*-isomer. The filtrate was cooled slowly to room temperature and filtered again. The crystals which separated usually consisted of an approximately 50-50 mixture of the isomers. An addnl crop of the 50-50 mixture could be obtained by evaporating the filtrate to a small vol. The mixture of the isomers was thoroly air-dried, and sufficient boiling 95% ethyl alcohol was added to dissolve most of the material. The hot soln was filtered, cooled to room temperature, and filtered again. Ordinarily, both ppts consisted chiefly of the *m*-isomer and were discarded. The filtrate was dild with an equal vol. of H_2O and was heated on the steam bath until the odor of alcohol disappeared. The remaining aq. soln was cooled and the material which separated was collected by filtration. This crystalline material usually contained a high percentage of the *p*-isomer. Evaporation of the filtrate to a small vol. gave a second crop of crystals which in most cases were suitable for combination with the first crop. The usual yield was about 20 g of the relatively pure *p*-isomer from a 300 g batch of the commercial material.

The relatively pure *p*-isomer fractions from several runs were combined, and the material was recrystallized from H_2O until the ultraviolet spectra of the product was the same as that of the *p*-isomer synthesized from *p*-toluene sulfonic acid.

ANALYSIS OF COMMERCIAL SAMPLES

The infrared spectra of the three isomers were determined, using suspensions of the material in CS_2 containing aluminum stearate (Fig. 2).

When the infrared spectra of commercial samples of ethylbenzylaniline sulfonic acid were compared with the infrared spectra of the three isomeric compounds, it was apparent that the commercial material consisted chiefly of the *m*-isomer mixed with substantial amounts of the *p*-isomer (Fig. 2). It could be concluded from the infrared data that the amount of the *o*-isomer present in the commercial samples of ethylbenzylaniline sulfonic acid was of the order of 1 per cent.

It had originally been assumed that it would be necessary to use infrared spectrophotometry to analyze commercial samples of ethylbenzylaniline sulfonic acid. Inspection of the ultraviolet curves of the isomeric compounds (Fig. 1) indicates that it is possible to determine the composition of the mixture of any two of these isomers from the ultraviolet spectra of the mixture in acid solution. The results obtained in analyses of known mixtures of *m*- and *p*-isomers, using the variable reference technique (2), were within ± 2 per cent of the calculated values.

The ultraviolet spectrophotometric determination was applied to 19 samples of commercial ethylbenzylaniline sulfonic acid. The results are shown in Table 1. The ultraviolet curves obtained in the course of these analyses indicated that only the *m*- and *p*-isomers were present in substantial amounts. The *o*-isomer, if present at all, was in amounts of less

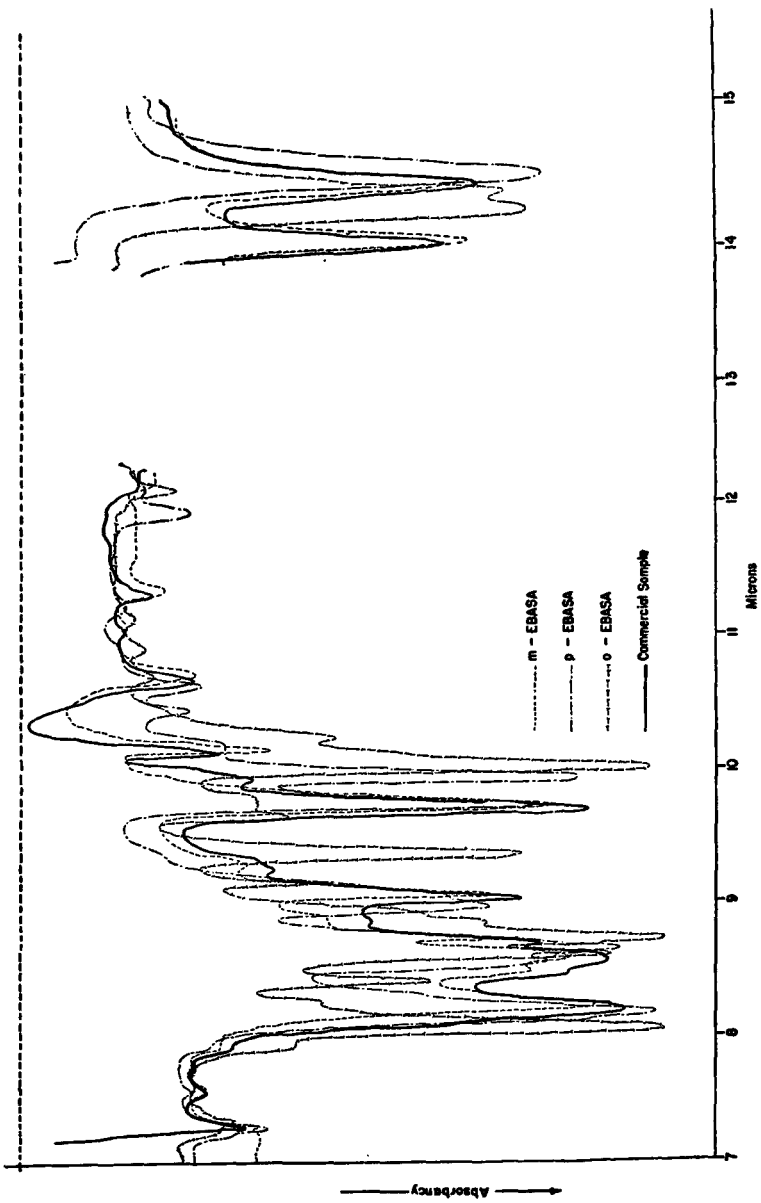


FIG. 2.—Infrared curves—ethylbenzylaniline sulfonic acids. Concn: 10 mg/ml; solvent: CS_2-aluminum stearate; cells: ca. 0.4 mm.

than 2 per cent. These analyses also indicated that the commercial samples contained little (less than 1 per cent) of aromatic material other than ethylbenzylaniline sulfonic acid.

TABLE 1.—*Analysis of commercial ethylbenzylaniline sulfonic acid*

SAMPLE	VOLATILE MATTER	TOTAL EBASA	ISOMERIC COMPOSITION[a]	
			PARA	META
	per cent	*per cent*	*per cent*	*per cent*
1	17.1	79.7	13.3	86.7
2	17.3	85.9	11.5	88.5
3	20.4	78.1	8.7	91.3
4	28.7	65.8	9.0	91.0
5	30.0	67.2	9.2	90.8
6	22.7	71.0	12.5	87.5
7	16.9	80.4	14.05	85.9
8	14.2	88.0	15.5	84.5
9	4.58	91.1	14.6	85.4
10	4.60	96.4	15.3	84.6
11	4.51	91.4	17.8	82.2
12	4.21	93.5	16.8	83.2
13	4.30	91.5	10.7	89.3
14	1.50	87.1	16.0	84.0
15	5.30	95.5	15.0	85.0
16	4.61	93.0	15.2	84.8
17	4.49	84.1	16.3	83.7
18	4.49	93.6	16.9	83.1
19	4.47	93.0	14.7	85.3

[a] Determined from ultraviolet spectra by variable reference technique.

As shown in Table 1, the total sulfonic acid content of commercial samples of ethylbenzylaniline sulfonic acid varied from 65 to 95 per cent. The major compound in all samples was the *m*-isomer. The percentage of the total of sulfonic acid present as the *p*-isomer varied from 9 to 18 per cent. These samples were obtained from four different manufacturers. There was no evidence from this limited number of samples that the isomeric composition of the material produced by any one manufacturer differed consistently from that of any other producer.

SUMMARY

Standard samples of the three isomeric ethylbenzylaniline sulfonic acids have been prepared, or have been obtained by purification. Using these samples as standards, it has been shown that the *m*-isomer is the chief component of commercial samples of ethylbenzylaniline sulfonic acid. The commercial samples examined contained 9–18 per cent of the *p*-isomer and 2 per cent or less of the *o*-isomer.

REFERENCES

(1) S.R.A., F.D.C. 3—Coal-Tar Color Regulations.
(2) JONES, J. H., CLARK, G. R., and HARROW, L. S., *This Journal*, **34**, 135 (1951).
(3) BLANGEY, L., FIERZ-DAVID, H. E., and STAMM, G., *Helv. Chim. Acta*, **25**, 1162 (1942).

STUDIES ON COAL-TAR COLORS. XIII. D&C RED NO. 33*

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Chromatographic analyses of commercial samples of Acid Fuchsin D (Colour Index No. 30), certifiable as D&C Red No. 33, show that such samples may contain three colors. In addition to D&C Red No. 33, there may be present Chromotrope 2R (Colour Index No. 29) and very small amounts of an unidentified blue color. This paper describes the preparation, analysis, and optical properties of pure D&C Red No. 33 and Chromotrope 2R, and gives a procedure for the determination of Chromotrope 2R in commercial samples of D&C Red No. 33.

EXPERIMENTAL

Purification of intermediates.—Aniline was purified by distillation over zinc dust.

H acid (1-naphthol-8-amino-3,6-disulfonic acid) was recrystallized from H_2O three times, and was dried first at 80°C. and then in an Abderhalden dryer at the temperature of boiling alcohol.

Anal.—N: Calcd, 3.85%; Found, 3.80%. S: Calcd, 17.60%; Found 17.69%.

A portion of this material was converted to the *p*-toluidide salt and was recrystallized several times, then was hydrolyzed to H acid with NaOH soln. The *p*-toluidine was removed by extracting the alk. soln with ether. The remaining soln was evapd under vacuum on a steam bath until H acid began to crystallize. The material was cooled, and the H acid was collected in a Büchner funnel and dried. The ultraviolet absorption curves of the two purified samples of H acid were identical.

Preparation of D&C Red No. 33.—Aniline (0.1 mole) was diazotized in the manner described by Fierz-David (1) except that, after diazotization was complete, the excess nitrous acid was destroyed with sulfamic acid. The diazonium compound was then coupled with 0.110 mole of the purified H acid in the manner described in the Colour Index (2). The dye was salted out, filtered, and dried, first in air and then at 135°. A chromatogram of this material, using the method described below, showed that only one dye was present.

Purification to remove colorless impurities was accomplished by converting the dye to the *n*-octadecylamine salt and recrystallizing from 50% alcohol soln. Octadecylamine was chosen as the base for this purpose because it forms a solid salt, insoluble in H_2O, but readily recrystallized from alcohol or aq. alcohol.

* Presented at the annual meeting of the Association of Official Agricultural Chemists, September 29, 30, and October 1, 1952, at Washington, D. C.

The *n*-octadecylamine salt of the dye was then decomposed by the addn of the theoretical quantity of 0.45 *N* NaOH soln. The free *n*-octadecylamine pptd quantitatively and was removed by filtering the material thru a very retentive filter paper, over stainless steel gauze. The vol. of the dye soln was reduced by evapg under vacuum until the dye began to ppt from soln. Acetone (ca a five-fold vol.) was added until no more dye pptd. The dye was then filtered, and was dried in air at room temperature and finally at 135°.

Anal.—N: Calcd, 8.80%; Found, 8.99%; S: Calcd, 13.81%, Found 13.71%; Na (as Na_2SO_4): Calcd, 9.84%, Found, 9.79%. Titration of the dye with standard $TiCl_3$ soln showed a purity of 99.8%.

SPECTROPHOTOMETRIC DATA—PURE DYE

Spectrophotometric measurements were made with a General Electric recording spectrophotometer, using a wavelength band of 8 mμ, and with a Cary recording spectrophotometer. The *p*H values of the solns were measured with a Beckman *p*H meter.

Approximately 0.15 g of purified color was weighed into a small weighing bottle, dried overnight at 135° and then at 100° and 2–3 mm pressure in an Abderhalden dryer for three hrs, cooled in a desiccator and reweighed. The sample was then dissolved in one l of distd H_2O, and 10 ml aliquots of this soln were put into 100 ml volumetric flasks. Appropriate quantities of HCl, NaOH, and buffers were added, and the solns were made to vol. with H_2O. The spectrophotometric data obtained with the Cary instrument from these solns are shown in Fig. 1.

At *p*H levels of 4, 6, 8, and 10, the spectrophotometric curves are identical, with peak absorbancy at 530 ±2 mμ. When 0.1 *N* NaOH soln or 0.1 *N* HCl soln is used as the solvent, the curves differ in shape, in the wavelength of maximum absorption, and in absorptivity.

Solutions of the dye in 0.04 *N* NH_4 acetate, in concns from 4 to 14 mg/l, follow Beer's law. The extreme variation for 15 measurements was about 1 per cent.

When solns of the color in H_2O are stored in the dark, they are stable for at least two weeks.

ANALYSIS OF COMMERCIAL SAMPLES

Commercial D&C Red No. 33 was paper-chromatographed on strips of Whatman No. 1 filter paper, using solvent systems 1 through 5 (Table 1). Solvent No. 1 was found to be the most effective and rapid, and was therefore chosen for use. With this solvent, three colored components were separated. The major fraction, identified as D&C Red No. 33, was a broad red band about half-way up the paper; a much smaller red band was located above the main fraction, and the third fraction remained at the base of the strip. The third fraction was a blue color which was estimated as less than 1% of the total dye present, and was therefore not investigated further.

Theoretical considerations indicated that the red subsidiary dye might be Chromotrope 2R, Colour Index No. 29, a non-certifiable dye. H acid is prepared by alkali fusion of 1-naphthylamine-3,6,8-trisulfonic acid (Koch Acid) (3); if the temperature of fusion exceeds 190°, or if the fusion is too prolonged, some chromotropic acid (1,8-dihydroxy-naphthalene-3,6-disulfonic acid) is formed. Since Chromotrope 2R is made by coupling

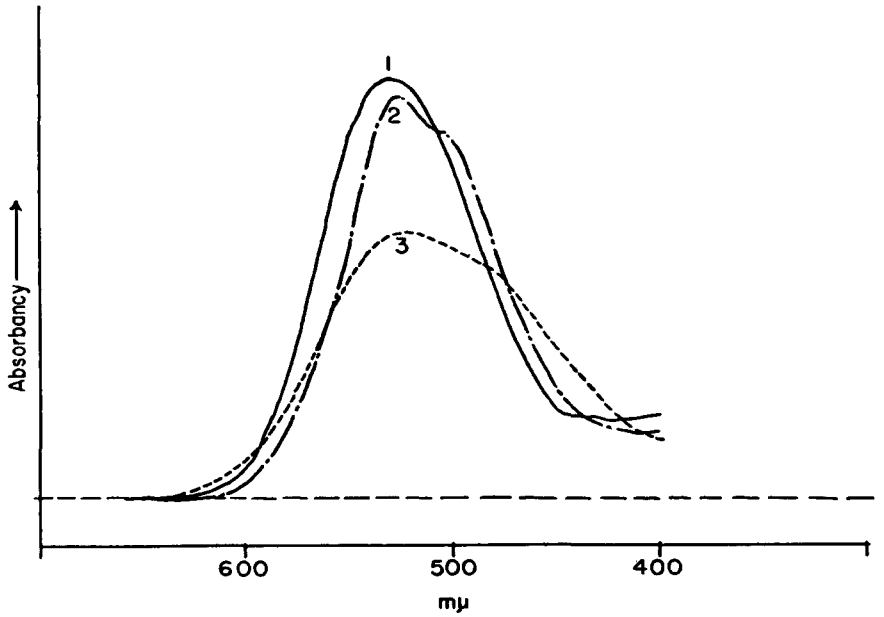

FIG. 1.—Absorption curves of D&C Red No. 33 in water at various *p*H levels (concn of 13.3 mg per liter). Curve 1.—*p*H levels of 4, 6, 8, and 10. Curve 2.—*p*H 1 (estimated). Curve 3.—*p*H 13 (estimated).

TABLE 1.—*Solvent systems*

Solvent No. 1	Saturate water with isoamyl alcohol and add ammonium hydroxide to give a 1 + 99 by vol. solution
Solvent No. 2	Saturate water with *n*-butanol and add ammonium hydroxide to give a 1 + 99 by vol. solution
Solvent No. 3	*n*-Butanol, two parts by vol.; water, one part; alcohol, 0.5 part
Solvent No. 4	80% phenol by wt; 20% water
Solvent No. 5	Distilled water
Solvent No. 6	Benzyl alcohol, 2 parts by vol.; ethyl alcohol, 2 parts; water, 1 part
Solvent No. 7	Benzyl alcohol, 2 parts by vol.; methyl cellosolve, 2 parts; water, 1 part
Solvent No. 8	*n*-Butanol, 2 parts by vol.; water, 1 part; alcohol, 0.5 part
Solvent No. 9	*n*-Butanol, 2 parts by vol.; methyl cellosolve, 2 parts; water, 1 part
Solvent No. 10	Ethyl alcohol, 4 parts by vol.; water, 1 part

diazotized aniline with chromotropic acid in alkaline solution (2), it would be formed with D&C Red No. 33 if the H acid used contained chromotropic acid.

Comparison of the R_F value of the red subsidiary dye with the R_F value of a commercial sample of Chromotrope 2R, chromatographed simultaneously, tentatively confirmed this identification. A larger sample of commercial D&C Red No. 33 was chromatographed, and the subsidiary dye was extracted from the paper with water. Spectrophotometric curves obtained from this solution and from a solution of a known sample of Chromotrope 2R were identical.

In order to verify the identity of the subsidiary dye completely, a sample of Chromotrope 2R was synthesized from purified intermediates. Samples of the synthesized material were chromatographed simultaneously with samples of commercial Chromotrope 2R using solvent systems 6 through 10 shown in Table 1. The best solvent found was No. 6. With this solvent the commercial sample separated into two colored components: a main fraction and a small amount of red subsidiary dye. When chromatographed with solvent No. 6, the laboratory preparation showed no colored impurities. Qualitative absorbancy measurements of both samples of Chromotrope 2R gave identical curves. Spectrophotometric curves of a solution of the laboratory preparation of Chromotrope 2R, and of the subsidiary dye separated from a commercial sample of D&C Red No. 33 are shown in Fig. 2.

DETERMINATION OF CHROMATROPE 2R IN D&C RED NO 33

The pure dye content of the laboratory sample of Chromotrope 2R was obtained by titration with standard $TiCl_3$ solution. The absorbancy for 1 mg/liter of pure Chromotrope 2R at the peak, 508 mμ, was then determined and used to evaluate the amount of this impurity separated from samples of commercial D&C Red No. 33.

The paper chromatography of commercial samples of D&C Red No. 33, streaked near the base of sheets of 9×9 inch Whatman No. 1 filter paper, was very satisfactory for the separation of the dye into its components but was not wholly satisfactory for quantitative analysis because of the small quantity of dye which could be applied to the paper.

Chromatography on a column of Solka-Floc (powdered cellulose) was found to be much more satisfactory for quantitative work. A column was prepared by pouring a dil. slurry of Solka-Floc into a column 18–24 mm×100 cm so that it settled to a height of about 50 cm. It was first washed with a 20% salt soln and about 5 ml of a 0.1% dye soln, dild with about 20 ml of 20% salt soln, was then added. When this soln had just passed into the Solka-Floc, the sides of the column were washed with a little more of the 20% salt soln, and the chromatogram was then developed with 10% salt soln contg 1% NH_4OH. The subsidiary dye came down first, and the main fraction moved more slowly, while the blue dye remained at the top

of the column. The subsidiary dye, when it had advanced to a point about 10 cm ahead of the main fraction, was eluted with 5% salt soln contg 1% NH_4OH. Since the blue dye was present in such small concn, no attempt was made to elute the D&C Red No. 33 and the blue dye separately. The remaining dyes were therefore washed out with H_2O.

The soln of the subsidiary dye was neutralized with acetic acid and examined spectrophotometrically.

Three batches of commercial samples were analyzed in duplicate. Stock solns of each dye were prepared and an aliquot of each was chromatographed on a column of Solka-Floc. The fractions of D&C Red No. 33 and Chromotrope 2R were ana-

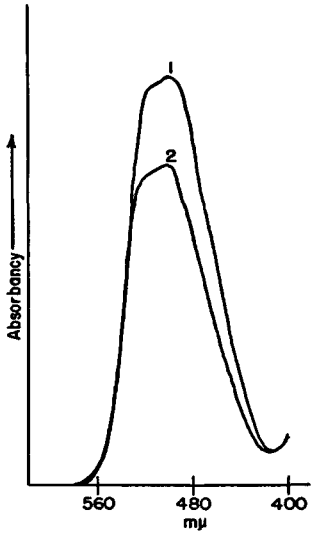

FIG. 2.—Absorption curves of chromotrope 2R in water buffered with ammonium acetate. Curve 1.—Laboratory preparation (concn ca 7 mg/liter). Curve 2.—Subsidiary dye separated from commercial sample of D&C Red No. 33.

lyzed spectrophotometrically by determining separately the absorbancy of each fraction at 530 mμ. Another aliquot of each stock soln, contg a mixt. of both dyes, was analyzed directly. Recoveries are based on a comparison of the sum of the absorbancies of the chromatographed fractions at 530 mμ with the absorbancy of the original soln at 530 mμ. The recoveries were very nearly quantitative as shown in Table 2.

The weight in mg of each dye present in the chromatographed fractions was calcd from the spectrophotometric data, using the previously detd unit absorbancies at 530 mμ for D&C Red No. 33 and at 508 mμ for Chromotrope 2R. From these values, the percentage of each dye present in the original sample was calcd. The results are given in Table 3.

DISCUSSION

Absorption curves, in the visible and ultraviolet regions, of D&C Red No. 33 and Chromotrope 2R in acid, base, and neutral solutions are suf-

TABLE 2.—*Recovery data*

SAMPLE	ABSORBANCY AT 530 mμ OF D&C RED NO. 33	ABSORBANCY AT 503 mμ OF SUBSIDIARY COLOR	TOTAL ABSORBANCY	ABSORBANCY AT 530 mμ OF UNTREATED	PER CENT RECOVERY
1	0.468	0.055	0.523	0.525	99.6
	0.454	0.057	0.511	0.529	96.6
2	0.581	0.005	0.586	0.590	99.3
	0.581	0.005	0.586	—	99.3
3	0.520	0.006	0.526	0.528	99.6
	0.520	0.006	0.526	—	99.6
Lab. prep.	0.634	—	0.634	0.635	99.8
D&C Red No. 33	0.635	—	0.635	—	100.0

ficiently different to permit identification when each dye is present alone. D&C Red No. 33 has one peak at 530 mμ. Chromotrope 2R has a double peak, the major one at 508 mμ. D&C Red No. 33 changes color at a *p*H below 4 and above 10; the color of Chromotrope 2R is unaffected by acid but is changed by base (Fig. 3). However, the differences in the absorption curves of D&C Red No. 33 and Chromotrope 2R are not sufficient to permit detection of a small percentage of the subsidiary dye in commercial samples of D&C Red No. 33.

The dyes could not be separated by extraction. They both fall into Group 6 of the Koch extraction scheme (4).

Chromatographic analysis of commercial samples of D&C Red No. 33 is a simple yet sensitive method for the isolation of small amounts of

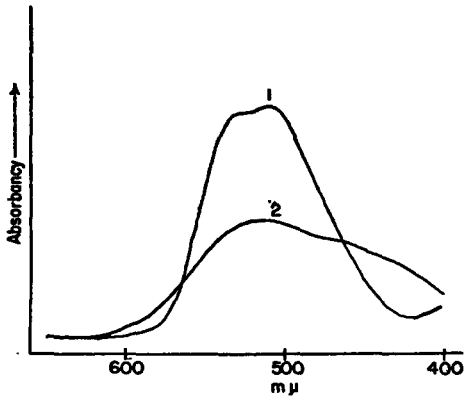

FIG. 3.—Absorption curves of chromotrope 2R in water (concn ca 10 mg/liter). Curve 1.—*p*H 1 and 7. Curve 2.—*p*H 13.

TABLE 3.—*Analysis of commercial D&C Red No. 33 for subsidiary dye*

SAMPLE	WEIGHT OF SAMPLE	D&C RED NO. 33 FOUND	CHROMOTROPE 2R FOUND
	mg	*per cent*	*per cent*
1	2.000	71.8	8.58
	2.000	69.7	8.75
2	10.000	89.1	0.80
	10.000	89.1	0.75
3	10.000	79.8	0.96
	10.000	79.8	0.96

colored impurities, and practically quantitative results can be obtained.

REFERENCES

(1) FIERZ-DAVID, H. E., and BLANGEY, L., *Fundamental Processes of Dye Chemistry*. Trans. from 5th Ed., Interscience Publishers, Inc., New York, 1949, p. 243.
(2) ROWE, F. M., *Colour Index of the Soc. of Dyers and Colourists*, Society of Dyers and Colourists, Yorkshire, England, 1924, p. 7.
(3) FIERZ-DAVID, H. E., and BLANGEY, L., *Fundamental Processes of Dye Chemistry*. Trans. from 5th Ed., Interscience Publishers, Inc., New York, 1949, pp. 213, 214.
(4) KOCH, L., *This Journal*, 26, 245 (1943).

THE SEPARATION AND DETERMINATION OF SULFONATED NAPHTHALENE INTERMEDIATES IN CERTIFIABLE COAL-TAR COLORS

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Many azo colors listed as certifiable for use in foods, drugs, and cosmetics under regulations promulgated by the Secretary of the Department of Health, Education, and Welfare (1) are manufactured from sulfonated naphthalene intermediates. Excessive amounts of these intermediates in samples of colors submitted for certification might be considered as evidence of poor manufacturing practice.

Until the present time, suitable methods for the determination of sulfonated naphthalene intermediates in coal-tar colors have not been available. To be of value, such methods must permit determination of the intermediates when present in the color to the extent of 0.2 to 1.0 per cent.

The proposed general method is relatively simple and rapid. It involves separation of the intermediates from the colors by column chromatogra-

phy followed by spectrophotometric determination of the compounds.

Chromatography has been shown by several workers to be a delicate tool for the quantitative separation of small amounts of material (2, 3). The characteristic fluorescence of naphthalene compounds excited by ultraviolet radiation offers a convenient means of following the progress of chromatographs. Spectrophotometric methods can be used to determine these intermediates even when only small amounts of material are available for analysis.

METHOD

REAGENTS

(a) *Adsorbent.*—Solka-Floc BW 40* or equivalent.

(b) *Eluting solutions.*—10% and 20% aq. solns of Na_2SO_4.

(c) *Standard intermediate solutions.*—Weigh 100 mg of the intermediate into a 100 ml volumetric flask and dil. to vol. with H_2O. Make dilns to give final solns which contain 10 mg/l and 1 mg/l of the intermediates, respectively, in 0.1 *N* HCl.

APPARATUS

(a) *Short chromatographic tube.*—A glass tube 2.5 cm in diam., 40 cm long, reduced and sealed at one end to a short length of 8 mm diam. glass tubing.

(b) *Long chromatographic tube.*—A glass tube 2.5 cm in diam., 115–120 cm long, reduced and sealed at one end to a short length of 8 mm diam. glass tubing.

(c) *Ultraviolet light source.*—R. V. Black Light 110–120 V, 8 watt, 60 cycle, made by Vogel Luminescence Corporation, San Francisco, California, or equivalent.

(d) *Spectrophotometer.*—A spectrophotometer, capable of isolating a 2 mμ band width between 220 and 350 mμ, accommodating cells 10 cm in length.

(e) *Cells.*—Matched sets of 1 cm and 10 cm quartz cells.

DETERMINATION

Preparation of chromatographic column.—Slurry ca 1 part of Solka-Floc in 10 parts H_2O and allow to stand at least 10 min. Place small pledget of glass wool in large end of tube and force to constricted end with H_2O. Clamp the column vertically in a ring stand and rapidly pour in the well-stirred Solka-Floc slurry until tube is ca two-thirds full. When ca half of the excess H_2O has drained from the column, the actual height of adsorbent in the column should be apparent. (The actual adsorbent height required for each case will vary with color under examination. A preliminary trial will indicate whether the short or long column is needed. If the short column is used, the best adsorbent height is ca 12 cm. When the long column is needed, a height of ca 50 cm should be used.) Add small portions of the adsorbent slurry until correct height is attained. At no time should the liquid level be allowed to drain below the top of the adsorbent material. A short length of rubber tubing fitted with a pinch clamp may be slipped over the narrow end of the column and used as a valve to regulate flow of liquid. When the H_2O level is approximately the height of the adsorbent, add ca 50 ml of eluting soln. (The 10% Na_2SO_4 soln is used for the simultaneous separation of naphthionic acid and 2-naphthol-3,6-disulfonic acid from FD&C Red No. 2. In all other experiments described, the 20% soln was used.) When the liquid level has again reached the top of the adsorbent column, it is ready to receive the sample.

Separation of intermediate from color.—Weigh 100 mg of sample into a 50 ml beaker, add 20 ml of eluting soln and 1 g of the Solka-Floc adsorbent, and stir the

* Obtained from Brown Co., 500 Fifth Avenue, New York 18, New York.

mixt. thoroly. Carefully pour the slurry into the column, so as not to disturb the top surface of the adsorbent. When the liquid level has reached the adsorbent level, carefully rinse the beaker with small portions of eluting soln and add them to the column. When the liquid level has again reached the adsorbent level, slowly add the eluting soln until the chromatograph tube is nearly full.

Direct the ultraviolet light source at the column from a distance of ca 20 cm. The presence of a naphthalene intermediate is indicated by the appearance of a fluorescent band (the color of the band varies with the intermediate under study). As the chromatogram is developed, the intermediate band will precede the coal-tar color band thru the chromatographic column. Collect the eluted fluorescent material in a 100 ml volumetric flask. When all of the fluorescent material has been collected, add 1 ml of concd HCl to the flask and dil. to vol. with H_2O. (Use above soln for spectrophotometric examination in all cases except that of the 2-naphthol-3,6-disulfonic acid, for which dil. a 10 ml portion of the first soln to 100 ml with H_2O for the final detn. In these experiments, the addnl diln was used to overcome the interference caused by extraneous materials eluted.)

Place the soln to be examined in a 10 cm quartz cell and det. its absorbancy curve from 210 to 400 mμ. Det. the absorbancy curve of the standard intermediate soln under the same conditions.

CALCULATIONS

Using rectangular coordinate graph paper, plot the determined absorbancy values against wavelength. Using a straight edge, draw a "base-line" between the two "background wavelengths."

Det. the "base-line" absorbancy value for the unknown soln at the peak wavelength. From a similar plot det. the "base-line" absorbancy of the standard soln, and calc. the per cent intermediate in the 100 mg sample by the formula:

$$\text{Per cent } C = \frac{A \text{ base-line unknown}}{A \text{ base-line standard}} \times \frac{\text{Conc of standard solution (mg/l)}}{10}$$

A practical example of the "base-line" calculation is illustrated below:

The absorbancy curves of a standard solution of 2-naphthol-6-sulfonic acid (10 mg per liter in 0.1 *N* HCl, 10 cm cell) and of a solution of 2-naphthol-6-sulfonic acid separated by the proposed method from a 100 mg sample of FD&C Yellow No. 6 are shown in Figs. 1 and 2, respectively. Points *C* and *B* in the figures are the "background wavelengths" referred to in the description of the calculations. The "base-line" is the line *CB*. The "base-line" absorbancy is the difference between the peak absorbancy, *D*, and absorbancy on the line *CB* at the same wavelength, i.e., *A*.

Per cent 2-Naphthol-6-sulfonic acid

$$= \frac{DA \text{ unknown}}{DA \text{ standard}} \times \frac{\text{Concn of standard soln (mg/l)}}{10}$$

$$\text{Per cent} = \frac{5.9}{11.3} \times \frac{10}{10} = 0.52$$

EXPERIMENTAL

The intermediates used in this work were recrystallized from water until a maximum absorptivity was obtained. Weighed amounts of the purified intermediates were added to known amounts of the dyes studied and the recovery was tested by the proposed methods. The results of these experiments are summarized in Tables 1–7.

TABLE 1.—*Recovery of naphthionic acid from FD&C Red No. 2*

WEIGHT DYE	NAPHTHIONIC ACID		RECOVERY
	ADDED	FOUND	
mg	*mg*	*mg*	*per cent*
100[a]	—	—	—
100	1.0	0.89	89
100	1.0	0.97	97
100	1.0	0.94	94
100	0.5	0.49	98
100	0.5	0.47	94
100	0.5	0.45	90
100	0.2	0.18	90
100	0.2	0.22	110
100	0.2	0.19	95

[a] Blank.

TABLE 2.—*Simultaneous determination of naphthionic acid and 2-naphthol-3,6-disulfonic acid in FD&C Red No. 2*

WEIGHT DYE	NAPHTHIONIC ACID		2-NAPHTHOL-3,6-DISULFONIC ACID (R-ACID)		RECOVERY	
	ADDED	FOUND	ADDED	FOUND	NAPTHIONIC ACID	R-ACID
mg	*mg*	*mg*	*mg*	*mg*	*per cent*	*per cent*
100[a]	—	—	—	—	—	—
100	1.0	0.97	0.2	0.19	97	95
100	1.0	0.95	0.2	0.18	95	90
100	1.0	0.94	0.2	0.19	94	95
100	0.5	0.47	0.5	0.45	94	90
100	0.5	0.46	0.5	0.51	92	102
100	0.5	0.49	0.5	0.46	98	92
100	0.2	0.08	1.0	0.88	90	88
100	0.2	0.20	1.0	0.91	100	91
100	0.2	0.18	1.0	0.90	90	90

[a] Blank.

TABLE 3.—*Recovery of 2-naphthol-6,8-disulfonic acid from D&C Orange No. 3*

WEIGHT DYE	2-NAPHTHOL-6,8-DISULFONIC ACID		RECOVERY
	ADDED	FOUND	
mg	*mg*	*mg*	*per cent*
100[a]	—	—	—
100	1.0	0.99	99
100	1.0	0.91	91
100	0.5	0.46	92
100	0.5	0.5	100
100	0.2	0.2	100
100	0.2	0.19	95

[a] Blank.

TABLE 4.—*Recovery of 2-naphthol-3,6-disulfonic acid from FD&C Red No. 1*

WEIGHT DYE	2-NAPHTHOL-3,6-DISULFONIC ACID		RECOVERY
	ADDED	FOUND	
mg	*mg*	*mg*	*per cent*
100[a]	—	0.31	—
100[a]	—	0.27	—
100	1.00	1.31	102
100	1.0	1.29	100
100	0.5	0.78	98
100	0.5	0.78	98
100	0.2	0.51	110
100	0.2	0.51	110

[a] Blank.

TABLE 5.—*Recovery of 2-naphthol-3,6-disulfonic acid from D&C Red No. 5*

WEIGHT DYE	2-NAPHTHOL-3,6-DISULFONIC ACID		RECOVERY
	ADDED	FOUND	
mg	*mg*	*mg*	*per cent*
100[a]	—	0.06	—
100[a]	—	0.05	—
100	1.0	1.04	98
100	1.0	1.03	98
100	0.5	0.53	96
100	0.5	0.56	100
100	0.2	0.25	100
100	0.2	0.21	80

[a] Blank.

TABLE 6.—*Recovery of 2-naphthol-6-sulfonic acid from FD&C Yellow No. 6*

WEIGHT DYE	2-NAPHTHOL-6-SULFONIC ACID		RECOVERY
	ADDED	FOUND	
mg	*mg*	*mg*	*per cent*
100[a]	—	—	—
100	1.0	0.99	99
100	1.0	1.0	100
100	0.5	0.49	98
100	0.5	0.50	100
100	0.2	0.18	90
100	0.2	0.21	105

[a] Blank.

DISCUSSION

The conditions prescribed for the determination of several intermediates in certain certifiable colors are listed in Table 8.

TABLE 7.—*Recovery of 2-aminonaphthalene-1-sulfonic acid from D&C Red No. 10*

WEIGHT DYE	2-AMINONAPHTHALENE-1-SULFONIC ACID		RECOVERY
	ADDED	FOUND	
mg	*mg*	*mg*	*per cent*
100[a]	—	—	—
100	1.0	0.96	96
100	1.0	0.97	97
100	0.5	0.49	98
100	0.5	0.49	98
100	0.2	0.18	96
100	0.2	0.19	98

[a] Blank.

All naphthalenic intermediates used in the manufacture of certifiable colors have characteristic spectra in the ultraviolet region (4), and most of them fluoresce under ultraviolet radiation. Thus it should be possible to extend the proposed method to the determination of many of these compounds in samples of the colors submitted for certification.

A preliminary investigation has been made of the application of the method to intermediates other than those listed in this paper. The only difficulty encountered was in the case of the determination of 1-amino-8-naphthol-3,6-disulfonic acid in D&C Black No. 1 and D&C Red No. 33.

TABLE 8.—*Determination of individual sulfonic acids present in a mixture*

INTERMEDIATE	COLOR	SUGGESTED BASE-LINE WAVE LENGTHS, mμ	APPROXIMATE LOCATION OF PEAK, mμ	CHROMATOGRAPHIC CONDITIONS	
				COLUMN	SODIUM SULFATE ELUANT
					per cent
Naphthionic acid	FD&C Red No. 2	270, 310	285	long	10
2-Naphthol-3,6-disulfonic acid	FD&C Red No. 2	220, 250	237	long	10 (1 to 10 dilution of eluant)
2-Naphthol-3,6-disulfonic acid	FD&C Red No. 1	220, 250	237	short	20 (1 to 10 dilution of eluant)
2-Naphthol-3,6-disulfonic acid	D&C Red No. 5	220, 250	237	short	20 (1 to 10 dilution of eluant)
2-Naphthol-6,8-disulfonic acid	D&C Orange No. 3	270, 310	289	long	20
2-Naphthol-6-8-disulfonic acid	FD&C Yellow No. 6	254, 310	282	long	20
2-Amino-naphthalene-1-sulfonic acid	D&C Red No. 10	270, 300	288	short	10

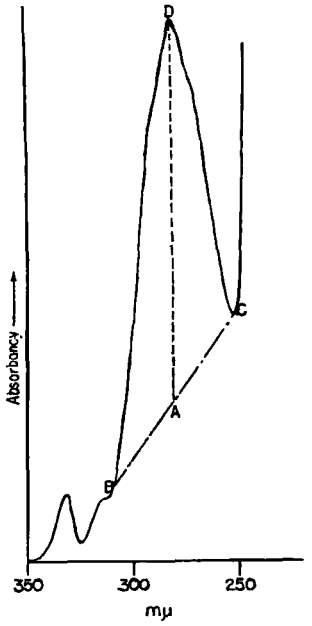

FIGURE 1

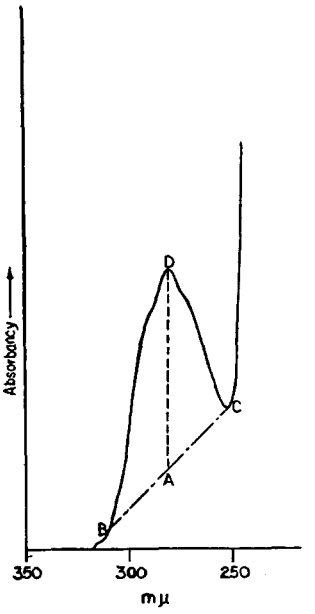

FIGURE 2

This particular intermediate seems to undergo some type of decomposition under the influence of ultraviolet radiation. Partial recoveries, ranging from 20 to 60 per cent, are obtained when the proposed method is applied.

It appears that the proposed procedure may be used in certain cases to determine the individual sulfonic acids present in a given mixture of intermediates. For example, naphthionic acid and 2-naphthol-3,6-disulfonic acid can be readily separated from each other and from FD&C Red No. 2 by chromatography in 10 per cent sodium sulfate solution. The 2-naphthol-3,6-disulfonic acid band moves through the column at a faster rate than naphthionic acid and can be detected as a separate greenish-blue band in front of the blue band of naphthionic acid (see Table 8).

An attempted separation of the closely related isomers, 2-naphthol-3,6-disulfonic acid and 2-naphthol-6,8-disulfonic acid, was not successful. These compounds were obtained, however, in a chromatographic fraction sufficiently free from interfering materials for spectrophotometric differentiation and determination by the variable reference technique (5).

The chromatographic separation and determination of the sulfonated benzene compounds has not been investigated. The compounds of this class usually encountered as color intermediates do not fluoresce; thus it is not easy to check the progress of the chromatographic separation.

Should these compounds appear in the fraction of eluate containing the naphthalene components, their presence can be readily detected by inspection of the spectrophotometric curve of that fraction.

SUMMARY

A general method is proposed for the determination of sulfonated naphthalene intermediates in coal-tar colors. Accurate and reproducible results were obtained when the method was applied to the determination of five such intermediates in several certifiable coal-tar colors.

Preliminary results indicate that it should be possible to extend the proposed method to the determination of similar compounds in other dyes and to the determination of individual sulfonated naphthalene compounds in certain mixtures.

REFERENCES

(1) S.R.A., F.D.C. 3, Coal-Tar Color Regulations.
(2) GRAICHEN, C., *This Journal*, **34**, 795 (1951).
(3) TILDEN, D., *ibid.*, **35**, 423 (1952).
(4) HARROW, L. S., and JONES, J. H., *ibid.*, **36**, 914 (1953).
(5) JONES, J. H., CLARK, G. R., and HARROW, L. S., *ibid.*, **34**, 135 (1951).

THE SEPARATION OF CERTAIN ANTHRAQUINONE DYES BY PAPER CHROMATOGRAPHY

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

Paper chromatography was found of value in the course of an investigation of analytical methods (1) employed for the estimation of D&C Green No. 5 and External D&C Violet No. 2 (2). Since these two dyes are made from the same intermediates, the contamination of one by the other is possible. In theory, at least, either dye may also contain D&C Green No. 6, D&C Violet No. 2, and "monosulfonated D&C Green No. 5." The interrelationship of these five anthraquinone dyes is shown by the structural formulas given in Fig. 1. This paper presents a method for the separation of mixtures of these dyes.

METHOD

APPARATUS

(a) *Glass jar.*—12×25×25 cm adapted to 8″×8″ paper (3). Employ auxiliary glass troughs to hold the mobile solvent, supported at proper height by a glass grid.

(b) *Filter paper.*—Whatman No. 1 in 8″×8″ sheets.

(c) *Glass sprayer.**—For application of stationary solvent to paper.

* Available from University Apparatus Company, Berkeley, California.

REAGENTS

(a) *Stationary solvent.*—Refined soybean oil in ethyl ether, A.C.S. grade (1 + 99, v/v).

(b) *Mobile solvent.*—Methyl cellosolve and H_2O (4+1 v/v).

(c) *Mixture of dyes.*—Prepare four mixt. of 5 dyes, (A) D&C Green No. 5, (B) "monosulfonated D&C Green No. 5," (C) D&C Green No. 6, (D) External D&C Violet No. 2, and (E) D&C Violet No. 2, by dissolving them in dimethylformamide so that each ml contains 1 mg of each dye, as follows:

Mixture No. 1 to contain A, D, C, and E
Mixture No. 2 to contain A, B, C, D, and E
Mixture No. 3 to contain A and C
Mixture No. 4 to contain A, B, and C

DETERMINATION

Rule the paper with a hard pencil 1 in. from bottom of sheet, spacing dots at 1½ in. intervals along the line, starting 1 in. from either side. By means of capillary pipets, spot various mixts. at the marked intervals along the line. The spots should

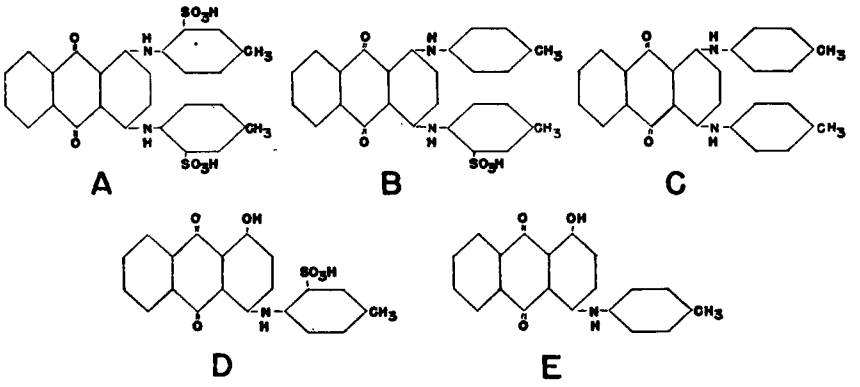

FIG. 1.—Formulas of the five anthraquinone dyes. A.—D&C Green No. 5; B.—"Monosulfonated D&C Green No. 5"; C.—D&C Green No. 6; D.—External D&C Violet No. 2; E.—D&C Violet No. 2.

not be more than 7 mm in diam. Clip top edge of the paper to rod which is to be used to suspend paper in jar, invert, and clip bottom edge of paper to an auxiliary glass rod supported in a well-ventilated hood. Impregnate filter paper with stationary solvent (Reagent a) by spraying rapidly and uniformly in horizontal strips, beginning at marked base line and continuing to opposite edge or "top" of paper. The entire sheet from the base line to the top must be uniformly covered. Unclip the auxiliary glass rod, transfer paper to jar so that lower edge dips into the mobile solvent (Reagent b), and seal cover with cellophane tape. Allow to stand until the mobile solvent front approaches (but does not reach) top of paper (ca 2½ hrs). Remove paper from jar and hang from rod in hood until dry.

DISCUSSION

Solvent systems in which the lower alcohols and water were used on plain paper (no soybean oil), whether neutral, acid with acetic, or alkaline

TABLE 1.—*Chromatographic data and R_F values*

SOLVENT NUMBER[a]	R_F VALUES													
	MIXTURE 1				MIXTURE 2					MIXTURE 3		MIXTURE 4		
	DYE A G[b]	DYE D V[b]	DYE C G	DYE E V	DYE A G	DYE B G	DYE D V	DYE C G	DYE E V	DYE A G	DYE C G	DYE A G	DYE B G	DYE C G
1	.98	.90	.00	.10	.98	.90	.94	.00	.10	.98	.00	.98	.89	.00
2	.94	.87	.00	.03	.93	.84	.87	.00	.03	.91	.00	.92	.84	.00
3	.98	.92	.14	.24	.98	.86	.90	.14	.24	.98	.12	.98	.86	.12
4	.95	.98	.00	.34	.93	.98	.97	.00	.33	.93	.00	.93	.98	.00
5	.99	.92	.04	.06	.99	.90	.94	.03	.09	.99	.03	.99	.90	.04

[a] Mobile Solvent Phase: 1—Ethanol+water, 4+1(v/v); 2—Methanol+water, 4+1 (v/v); 3—Methyl Cellosolve+water, 4+1 (v/v); 4—Acetonitrile+water, 13+7 (v/v); 5—Acetone+water, 4+1 (v/v). Immobile Solvent Phase: Soybean Oil+Ethyl Ether, 1+99 (v/v).

[b] Color of Dye: G=Green, V=Violet.

with ammonia, caused the dyes to move on the paper but did not separate them completely. Other solvents, e.g., collidine or pyridine with water, produced streaks on the paper. As the amount of water was increased, the sulfonated dyes streaked and the unsulfonated dyes ceased to move. However, when a vegetable oil was used as a stationary solvent, the dyes separated into two groups. The three sulfonated dyes moved materially farther on the paper than the two unsulfonated dyes. The oil also caused a distinct separation of three sulfonated dyes. Without the stationary solvent, water plus ethanol or acetone did not separate the two sulfonated green dyes. From the data in Table 1 it will be noted that acetonitrile with water causes the sulfonated dyes to separate in an order different from that observed with the other solvents listed.

Mineral oil as the stationary solvent was found unsatisfactory. Vegetable oils, other than soybean, were not used in this work. Previous work (4), however, indicates that any of the refined oils, liquid at the temperature used, would probably be satisfactory.

In Table 1 are given data obtained with five solvent systems. Methyl cellosolve is preferred.

SUMMARY

Mixtures of three water-soluble and two water-insoluble anthraquinone dyes can be separated in one step by paper chromatography. The paper is pre-impregnated by spraying it with an ethyl ether solution of the stationary phase (soybean oil); methyl cellosolve with water (4 + 1) is the mobile phase.

REFERENCES

(1) *Official Methods of Analysis*, Association of Official Agricultural Chemists, Washington, D. C., 7th Ed., 1950, **34.21 (b), 34.21 (e),** and **34.32.**
(2) Coal Tar Color Regulations, Department of Health, Education and Welfare, S.R.A., F.D.C. 3.
(3) MITCHELL, L. C., *This Journal*, **35,** 920 (1952).
(4) MITCHELL, L. C., and PATTERSON, W. I., *ibid.*, **36,** 553 (1953).

THE EFFECT OF STANDING, HANDLING, AND SHIPPING ON FREE LIQUID, SOLIDS, AND SALT CONTENT OF OYSTERS*

By G. T. DAUGHTERS and E. M. HOSHALL,† (Food and Drug Administration, Department of Health, Education, and Welfare, Baltimore, Maryland)

I. CHESAPEAKE BAY AREA OYSTERS

There has long been a decided difference of opinion as to the "free liquid" (drained liquid) content of Chesapeake Bay area oysters as affected by standing, handling, and shipping operations. Therefore a series of practical tests was planned to determine whether under normal conditions of standing, handling, and shipping, the oyster would "bleed" or "leak" and thus increase its free liquid content or whether it would "soak up" and decrease in free liquid content. The proposed plan follows:

EXPERIMENTAL

(1) The oysters (standards in all cases) will be authenticated as to source, and each shipment will involve only one lot from one boat or truck.

(2) Food and Drug Inspectors will observe all operations involved in receipt, shucking, washing, and packing of oysters. Plant equipment will be used, but operations will be timed, and volumes of water controlled so that the conditions set forth in the standards‡ will be fulfilled.

(3) Shucking pots will be filled to the maximum allowed by the standards.

(4) Total time of exposure to fresh water in washing and blowing operations will comply with standard requirements.

(5) The oysters will be drained on plant skimmers to a point where the free liquid content does not exceed the 5 per cent requirement of the standards.

(6) Immediately after draining, the oysters will be packed in 1 pt cans, closed, iced, and shipped, or held iced for shipment in accordance with the schedule shown in Table 1.

(7) A total of 13 gallons will be prepared for each authentic sample, and each gallon (8 pts) will constitute a subdivision. **(a)** One gallon (8 pts) will be tested for free liquid at the plant within 15 min. of packing. **(b)** Twelve gallons (96 pts) will be iced and sent to collaborating laboratories at periodic intervals, as shown in Table 1, for examination for drained liquid, total solids, and salt content.

ANALYTICAL

Collaborators were requested to make the following determinations on the oysters as soon as practicable after receiving them:

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* Presented at the annual meeting of the Association of Official Agricultural Chemists, September 29, 30, and Oct. 1, 1952, at Washington, D. C.

† The work was directed by the authors of record. The following inspectors and chemists contributed materially, and special thanks are due those who worked overtime in order to conform to time schedules: J. L. Carroll, H. H. Fradin, W. B. Logan, H. R. Surgen, H. E. Gakenheimer, J. P. Traynor, and S.M. Walden, all of Baltimore, and M. A. McEniry, F. J. McNall, D. J. Miller and W. C. Woodfin, of St.Louis, Cincinnati, Buffalo, and Pittsburgh Districts. In addition, P. J. Jones and J. J. McAuliffe of Baltimore District participated in the studies reported in Part II of this paper.

‡ Food & Drug Administration, S.R.A., F.D.C. 2, Rev. 1, Jan. 1949.

Determination	*Method*
Condition of oysters: frozen, uniced, cans leaking, oysters stale, decomposing, etc.	Organoleptic inspection
Preparation of Sample	*Methods of Analysis, A.O.A.C.*, 7th Ed., **18.1**
Drained Liquid (at 45°F. ±2°F.)	*Methods of Analysis, A.O.A.C.*, 7th Ed., **18.3**
Total Solids	*Methods of Analysis, A.O.A.C.*, 7th Ed., **18.4**
Salt	*Methods of Analysis, A.O.A.C.*, 7th Ed., **18.7**

The inspectors were instructed to make a determination of drained liquid on subdivision 1 (8 pints) of each sample at the plant, within 5 minutes after packing, then composite the gallon, ice it, and forward to Baltimore District for analysis.

In accordance with the preceding plan, a series of 7 authentic samples of oysters (standards), of 104 pints each, was prepared during the period Nov. 27–30, 1951, at 7 different commercial packing establishments. Disposition of the authentic samples for analysis is shown in Table 1.

TABLE 1.—*Disposition of authentic samples*

SAMPLE #48, PACKED: NOV. 27, 1951 (P DAY) SOURCE: TANGIER SOUND			REMAINING SIX (6) SAMPLES[a]		
SUB NO.	SHIPPED TO	DISTANCE SHIPPED MILES (APPROX)	SAMPLE NO.	DATE PACKED (P DAY)	SOURCE OF OYSTERS
1	Baltimore, Md.	180	12	Nov. 27	Tangier Sound
2	Pittsburgh, Pa.	511	13	Nov. 29	Nanticoke River
3	Cincinnati, Ohio	827	10	Nov. 28	Tangier Sound
4	St. Louis, Mo.	1122	11	Nov. 29	Tangier Sound
5	Pittsburgh, Pa.	511	99	Nov. 28	Harris Creek
6	Cincinnati, Ohio	827	03	Nov. 30	Manokin River
7	St. Louis, Mo.	1122			
8	Pittsburgh, Pa.	511			
9	Cincinnati, Ohio	827			
10	St. Louis, Mo.	1122			
11	Pittsburgh, Pa.	511			
12	Cincinnati, Ohio	827			
13	St. Louis, Mo.	1122			

[a] Disposition of the remaining six (6) samples was the same as that shown for #48.

RESULTS

In Table 2 are listed the collaborator's results on the seven authentic samples for drained liquid, and in Table 3 those for total solids and salt. Each of the results shown is the average of duplicate determinations.

In two cases, the free liquid as determined at the plant ran slightly higher than the specified 5 per cent (Table 2).

All of the collaborators reported that they used the Waring blendor,

TABLE 2.—*Results of analysis of authentic oyster packs. Per cent drained liquid*

SUB. NO.	DISTRICT	SAMPLE NUMBER													
		48		12		13		10		11		99		03	
		DATE EXAM.	DR. LIQUID	DATE EXAM.	DR. LIQUID	DATE EXAM.	DR. LIQUID	DATE EXAM.	DR. LIQUID	DATE EXAM.	DR. LIQUID	DATE EXAM.	DR. LIQUID	DATE EXAM.	DR. LIQUID
		P	5.3[a]	P	3.0[a]	P	2.9[a]	P	2.3[a]	P	4.0[a]	P	5.5[a]	P	4.5[a]
1	Balt.	P+2	1.1	P+2	1.2	P+1	1.1	P+1	1.1	P+1	1.1	P+1	2.8	P+3	1.4
2	Pitts.	P+2	1.2	P+2	1.4	P+1	0.9	P+1	1.8	P+1	0.6	P+1	3.5	P+3	1.2
3	Cinn.	P+3	3.4	P+3	2.3	P+2	1.2	P+2	1.4	P+2	1.3	P+2	4.6	P+3	0.7
4	St. L.	P+3	0.8	P+2	0.8	P+3	0.9	P+2	2.3	P+3	0.7	P+3	1.5	P+3	2.3
5	Pitts.	P+8	1.0	P+8	1.0	P+7	0.7	P+7	0.7	P+7	0.6	P+7	2.0	P+7	0.9
6	Cinn.	P+8	2.3	P+8	0.9	P+7	0.8	P+7	2.2	P+7	1.0	P+7	3.6	P+8	1.7
7	St. L.	P+9	1.0	P+9	0.2	P+8	0.5	P+8	0.4	P+8	0.5	P+8	1.2	P+8	0.7
8	Pitts.	P+13	1.2	P+13	1.2	P+13	0.7	P+13	1.2	P+13	0.7	P+1	1.6	P+12	0.8
9	Cinn.	P+13	2.8	P+13	1.2	P+13	1.5	P+12	2.2	P+13	0.8	P+12	1.7	P+12	1.0
10	St. L.	P+13	0.9	P+13	1.1	P+14	0.6	P+13	0.8	P+14	0.6	P+13	1.5	P+13	0.9
11	Pitts.	P+18	1.9	P+18	1.1	P+17	3.3[c]	P+17	1.5	P+17	1.9[c]	P+17	1.2	P+16	1.1[c]
12	Cinn.	P+17	2.4[d]	P+17	1.8[d]	P+18	5.1[c,d]	P+18	3.8[c,d]	P+18	4.9[c,d]	P+19	5.1[c,d]	P+17	1.6[c,d]
13	St. L.	P+19	—[b]	P+20	—[b]	P+19	3.2[c]	P+19	1.7	P+19	1.5[c]	P+20	1.8[c]	P+18	1.1[c]
Average (1–13)			1.7		1.2		1.6		1.6		1.2		2.5		1.2
Average[e]			1.6		1.1		0.9		1.4		0.8		2.3		1.2
Decrease in Dr. Liq.			3.7		1.9		2.0		0.9		3.2		3.2		3.3

[a] At plant, within 15 min. after packing; [b] Frozen solid on receipt, can leaking; [c] Frozen solid on receipt; [d] Oysters decomposing; [e] Defective samples excluded.

TABLE 3.—*Results of analyses of oysters. Per cent total solids and salt*

SUB NO.	SAMPLE NUMBER																				
	48			12			13			10			11			99			03		
	DATE EXAM.	TOTAL SOLIDS	SALT	DATE EXAM.	TOTAL SOLIDS	SALT	DATE EXAM.	TOTAL SOLIDS	SALT	DATE EXAM.	TOTAL SOLIDS	SALT	DATE EXAM.	TOTAL SOLIDS	SALT	DATE EXAM.	TOTAL SOLIDS	SALT	DATE EXAM.	TOTAL SOLIDS	SALT
		per cent	*per cent*		*per cent*	*per cent*		*per cent*	*per cent*		*per cent*	*per cent*		*per cent*	*per cent*		*per cent*	*per cent*		*per cent*	*per cent*
1	P+2	15.1	0.25	P+2	15.5	0.28	P+1	15.8	—[a]	P+1	14.9	0.24	P+1	14.7	—[a]	P+1	14.0	—[a]	P+3	15.5	0.30
2	P+2	14.8	0.24	P+2	15.2	0.28	P+1	15.5	0.19	P+1	14.4	0.26	P+1	15.0	0.21	P+1	13.9	0.22	P+3	15.3	0.29
3	P+3	15.0	0.23	P+3	15.1	0.27	P+2	16.0	0.20	P+2	14.8	0.26	P+3	15.2	0.21	P+2	13.3	0.22	P+3	15.7	0.29
4	P+3	14.9	0.23	P+3	15.5	0.26	P+3	15.4	0.20	P+2	14.4	0.26	P+3	15.2	0.22	P+3	13.6	0.22	P+3	15.5	0.29
5	P+8	14.8	0.23	P+8	15.1	0.26	P+7	15.0	0.20	P+7	14.6	0.26	P+7	14.9	0.22	P+7	13.6	0.23	P+7	14.9	0.30
6	P+8	15.1	0.23	P+8	14.7	0.28	P+7	15.6	0.19	P+7	14.6	0.26	P+7	14.7	0.21	P+7	13.7	0.23	P+8	15.4	0.30
7	P+9	15.5	0.24	P+9	15.1	0.26	P+8	15.5	0.19	P+8	14.4	0.27	P+8	14.9	0.21	P+8	13.5	0.24	P+8	14.5	0.31
8	P+13	15.0	0.23	P+13	15.0	0.26	P+13	15.0	0.19	P+13	14.2	0.27	P+13	14.9	0.21	P+13	14.0	0.23	P+12	15.1	0.31
9	P+13	14.8	0.23	P+13	15.0	0.27	P+13	15.0	0.19	P+12	14.4	0.26	P+13	15.0	0.22	P+12	14.1	0.24	P+12	15.3	0.30
10	P+13	14.7	0.23	P+13	14.5	0.27	P+14	15.0	0.18	P+13	14.6	0.27	P+14	14.8	0.22	P+13	13.9	0.24	P+13	15.2	0.30
11	P+18	14.8	0.23	P+18	14.8	0.27	P+17	15.2[c]	0.18	P+17	14.3	0.27	P+17	14.8[c]	0.22	P+17	13.9	0.27	P+16	15.2[c]	0.30
12	P+17	14.6[d]	0.25	P+17	15.0[d]	0.27	P+18	15.2[c]	0.18	P+19	14.6[c,d]	0.26	P+18	14.8[c,d]	0.22	P+19	14.0[c,d]	0.26	P+17	15.0[c,d]	0.30
13	P+19	14.8[b]	0.23	P+20	14.8[b]	0.27	P+19	15.3[c]	0.18	P+20	14.6[c]	0.27	P+19	14.8[c]	0.22	P+20	13.8[c]	0.26	P+18	14.9[c]	0.30
Av.		14.9	0.23		15.0	0.27		15.3	0.19		14.5	0.26		14.9	0.22		13.8	0.24		15.2	0.30

[a] Salt not determined.

[b] Frozen solid on receipt, can leaking.

[c] Frozen solid on receipt.

[d] Oysters decomposing.

Methods of Analysis, 7th Ed., **18.1**, in the preparation of their samples for analysis. Their comments relative to the condition of the samples on arrival are shown by the superscripts in Tables 2 and 3.

DISCUSSION

Free or drained liquid.—The average drained liquid for each of the seven (7) authentic samples was less than the drained liquid found within fifteen minutes after packing. In five out of 89 determinations, the drained liquid found was greater than that determined in the plant by the inspectors; however, in all five cases the oysters were more than fifteen days old and were in a frozen and/or decomposing condition on arrival at the collaborating laboratory. Although the oysters were iced immediately following packing and were kept iced until shipment, a sudden cold spell starting December 13 was responsible for freezing of the oysters in transit.

In no single case did an authentic sample of oysters in good condition "bleed" or "leak" water as a result of handling, storing, or shipping. As shown by the last horizontal line in Table 2, the oysters, instead, absorbed or took up from 0.9 per cent to 3.7 per cent of the drained liquid found present within 15 minutes of packing.

Total solids and salt.—These determinations were included to determine if handling, storing, shipping, or age of oysters had a material effect on their total solids and salt content.

Maximum, minimum, and average figures for total solids and salt are summarized in Table 4 in order to show the variation in the authentic samples.

TABLE 4.—*Total solids and salt variation in authentic samples*

SAMPLE	PER CENT TOTAL SOLIDS			PER CENT SALT			SOURCE OF SAMPLE
	MAX.	MIN.	AV.	MAX.	MIN.	AV.	
48	15.5	14.6	14.9	.25	.23	0.23	Tangier Sound
12	15.5	14.5	15.0	.28	.26	0.27	Tangier Sound
13	16.0	14.3	15.3	.20	.18	0.19	Nanticoke River
10	14.9	14.2	14.5	.27	.24	0.26	Tangier Sound
11	15.2	14.7	14.9	.22	.21	0.22	Tangier Sound
99	14.1	13.3	13.8	.27	.22	0.24	Harris Creek
03	15.7	14.5	15.2	.31	.29	0.30	Manokin River[a]
(All 7)	16.0	13.3	14.8	.31	.18	0.24	

[a] Actually a tidal inlet with very little land area draining fresh water into it.

None of the collaborators' results are excluded from Table 4, although the total solids of old and frozen oysters may be slightly lower because of decomposition. The range, and the maximum and minimum figures for the different collaborating laboratories indicate a need for continuing the

study of methods for the determination of total solids, particularly with respect to preparation of the sample for analysis.

In general, oysters from the Bay area have higher total solids than those from the rivers, although the river beds from which the oysters were taken are near the bay.

SUMMARY

Seven authentic packs of standard oysters, of 13 subdivisions each, were shipped to points from 180 to 1122 miles from the point of packing, and were analyzed over a period of from one to nineteen days from the date of packing. Analysts at four different collaborating points found that Chesapeake Bay area oysters, packed according to Federal Standards, do not increase in drained liquid (free liquid) content. Instead, the oysters were shown to have decreased in drained liquid content in all cases.

These findings conclusively disprove any claim that Chesapeake Bay area oysters "bleed" or "leak" during standing, handling, and shipping, with consequent increase in drained liquid content.

The total solids and salt content of the authentic packs, within the variations of the analytical methods, remained uniform during the test. Useful regulatory data relative to maximum, minimum, and average total solids and salt content for standard oysters from the authenticated areas, for the time of year indicated, were obtained from this experiment.

ACKNOWLEDGMENT

Grateful acknowledgment is made to the seven oyster packers in the Chesapeake Bay area for providing facilities, and participating in the experiment.

II. RIVER OYSTERS (PRELIMINARY STUDIES)

During the course of regulatory operations in late 1952, and in spite of the convincing data presented in Part I showing that Chesapeake Bay area oysters do not bleed when packed according to the standards, some packers still insisted that "river" oysters would bleed. In particular, they specified that oysters from the Rappahannock, York, and Potomac Rivers would bleed after packing.

This initial investigation, limited by facilities and time, followed closely the plan followed in Part I for authentication, receipt, shucking, washing,

TABLE 1.—*Authentic packs of river oysters*

SAMPLE NO.	DATE PACKED	SOURCE OF RAW STOCK	NO. PINTS PACKED
72	2/4	Rappahannock River	48
84	2/11	Potomac River	48
85	2/12	York River	48

and packing of oysters. A total of 48 pints of standard oysters was prepared for each of three authentic packs (See Table 1).

All analyses were made at the Baltimore District laboratory except the determination of drained liquid "within fifteen minutes of packing," which was done by the inspection team preparing the authentic packs.

RESULTS

Table 2 lists the results on the authentic samples for drained liquid, and Table 3 for total solids and salt. Each of the results shown is the average of duplicate determinations.

TABLE 2.—*Results of analysis of authentic oysters*
Per Cent Drained Liquid

SUB NO.	SAMPLE #72		SAMPLE #84		SAMPLE #85	
	DATE EXAM.	DR. LIQUID	DATE EXAM.	DR. LIQUID	DATE EXAM.	DR. LIQUID
		per cent		*per cent*		*per cent*
1	2/4[a]	3.2[b]	2/11[a]	4.2[b]	2/12[a]	5.7[b]
2	2/5	0.9	2/13	2.0	2/13	4.0
3	2/9	1.6	2/16	1.6	2/16	1.8
4	2/12	3.1	2/19	1.4	2/19	1.4
5	2/16	1.7	2/24	2.3	2/24	1.7
6	2/19	3.3	2/27	3.3	2/27	2.2
Av. Drained Liquid		2.3		2.5		2.8
Decrease in Drained Liquid		0.9		1.7		2.9

[a] Date packed. [b] Drained liquid within 15 min. of packing.

TABLE 3.—*Results of analysis of authentic oysters*
(Total Solids and Salt)

SUB NO.	SAMPLE #72			SAMPLE #84			SAMPLE #85		
	DATE EXAM.	TOTAL SOLIDS	PER CENT SALT	DATE EXAM.	TOTAL SOLIDS	PER CENT SALT	DATE EXAM.	TOTAL SOLIDS	PER CENT SALT
1	2/5	11.18	0.08	2/13	13.51	0.08	2/13	12.49	0.09
2	2/5	11.33	0.07	2/13	13.78	0.09	2/13	12.28	0.07
3	2/9	11.16	0.09	2/16	13.99	0.08	2/16	12.70	0.08
4	2/12	11.14	0.09	2/19	14.30	0.08	2/19	12.57	0.08
5	2/16	11.13	0.09	2/24	14.16	0.09	2/24	12.38	0.11
6	2/19[a]	11.32	0.09	2/27[a]	14.19	0.09	2/27[a]	11.89	0.13

[a] Analyst reported oysters normal in appearance, and edible, but somewhat stale in odor.

DISCUSSION

Free or drained liquid.—The average drained liquid for each of the three authentic samples was less than the drained liquid found within fifteen

minutes after packing. In only a single instance was the amount of drained liquid greater than that determined by the inspector in the plant. This was Sample #72, where the inspector packed with 3.2 per cent drained liquid and fifteen days later the analyst found 3.3 per cent drained liquid. This difference is considered insignificant.

Except for the lone case cited, the authentic samples of oysters did not "bleed" or "leak" water as a result of handling, shipping, and storage over a period of sixteen days. Instead, as shown by the last horizontal line of Table 2, the oysters took up or absorbed 0.9, 1.7, and 2.9 per cent respectively, of the drained liquid found present within fifteen minutes of packing.

Total solids and salt.—The results shown on Table 3 indicate that neither handling, storage, shipping, nor age of oysters had any material effect on their total solids and salt content.

SUMMARY

Based on the analysis of three authentic samples of standard oysters of 48 pints each, analyzed over a period of from one to sixteen days from time of packing, it was found that the "river" oysters, packed according to Federal standards, did not increase in drained liquid (free liquid) content. Instead, the oysters were shown to have decreased in drained liquid content.

DETERMINATION OF YEAST-FERMENTABLE SUGAR IN BEER*

By ALEX P. MATHERS and JOHN E. BECK (Alcohol and Tobacco Tax Division Laboratory, Bureau of Internal Revenue, Washington, D. C.)

The yeast-fermentable sugar content of beer has received considerable attention recently. For beers containing 0.2 gram or less of fermentable material per 100 ml, methods based on the determination of "real extract" (1, 2), or the reducing sugar content before and after "end fermentation" (3) are not very accurate. The "real extract" varies between 3 and 5 grams per 100 ml (often higher), whereas the yeast fermentable sugar of the so called "dry beers" may be of the order of 0.1 gram per 100 ml. The component measured may therefore be 30 to 50 times greater than the yeast-fermentable sugar, and any error in the determination is totally reflected in the weight of the latter. The above also applies to the determination of copper reducing materials present in beer, which, calculated as maltose, may comprise 1 to 2 per cent by weight of the beer.

* Presented at the annual meeting of the Association of Official Agricultural Chemists, September 29, 30, and October 1, 1952, at Washington, D. C.

The procedures in the following method, which involve sampling, dealcoholization, yeasting, and fermentation, are similar to those of the methods mentioned above. However, the final results are based on a determination of the alcohol produced in an "end fermentation" of the dealcoholized beer. The weight of ethyl alcohol multiplied by two will approximate the weight of sugar fermented. (The theoretical weight of anhydrous maltose required to produce one gram of ethyl alcohol is 1.86 grams, but in practice approximately 2 grams of maltose are required for each gram of alcohol produced.) In this procedure, the percentage of error for the alcohol is identical with the percentage error of yeast-fermentable material reported as maltose.

The dealcoholization of the beer is accomplished in the manner prescribed in the determination of the "real extract" (4), except that the sample is measured rather than weighed.

METHOD

REAGENTS

(a) *Starch solution.*—**39.20.**

(b) *Potassium dichromate solution.*—0.1 *N* **39.2, 39.22.**

(c) *Sodium thiosulfate solution.*—0.1 *N* **39.35, 39.36.**

(d) *Ceric solution.*—Ca 0.1 *N*. Triturate 11 g ceric ammonium nitrate with 25 ml of 60% perchloric acid for 5 min. [This step is essential (5).] Add 75 ml of 60% perchloric acid and 100 ml of H_2O to dissolve the ceric salt. Reflux the soln for 30 min., cool, and filter. Standardize the ceric soln against the thiosulfate soln as follows: pipet 20 ml of ceric soln into flask, add 200 ml of H_2O and 0.3 to 0.5 g of KI. After 5 min., titrate the liberated iodine with thiosulfate, using starch indicator. Ceric salts in perchloric acid tend to liberate oxygen on standing; thus standardization is required periodically (5).

DETERMINATION

Dealcoholization.—Evap. 300 ml of sample on H_2O bath or asbestos plate, at a temperature not exceeding 80°C., to about ¾ of the original vol. Cool and make to original vol. with H_2O. Pipet 100 ml of dealcoholized sample into a distg flask and distill according to A.O.A.C. procedure **11.4.** (Complete dealcoholization should be checked by one of the chemical methods below.)

End Fermentation.—Ferment the remaining 200 ml of dealcoholized beer with 1 g active compressed brewer's yeast at 15–24°C. for forty-eight to seventy-two hrs, or until fermentation is complete. Equip fermentation flask with H_2O or Hg seal. Use 100 ml of fermented sample for distilling alcohol as above. This distillate is used for the chemical determinaton of ethyl alcohol.

Sample Size.—The amount of sample and reagents are based on an alcoholic content of 0.1 g per 100 ml. The oxidation by either method must be carried out with an excess of oxidizing agent equivalent to ca 5 ml of 0.1 *N* sodium thiosulfate in order to force the reaction to completion in the specified time. If the final titration indicates that this excess is lacking, the sample size must be decreased or the amount of oxidizing agent increased.

Dichromate Oxidation.—$2K_2Cr_2O_7 + 3C_2H_5OH + 8H_2SO_4 \rightarrow 2Cr_2(SO_4)_3 + 3CH_3COOH + 11H_2O + 2K_2SO_4$. Pipet 10 ml of 0.1 *N* dichromate soln into a flask, add 5 ml of concd H_2SO_4, and cool. Into this soln pipet 5 ml of sample, connect

to H_2O cooled condenser, and reflux for 15 min. Cool, add 200 ml of H_2O, and 0.3 to 0.5 g of KI. Titrate the liberated iodine with 0.1 *N* sodium thiosulfate, using starch indicator. One ml of 0.1 *N* dichromate soln equals 0.001150 g of ethyl alcohol.

Ceric Oxidation.—$12Ce^{++++} + C_2H_5OH + 3H_2O \rightarrow 12Ce^{+++} + 2CO_2 + 12H^+$. Pipet 20 ml of ceric reagent and 5 ml of sample into a flask and reflux for 30 min. Cool, add 200 ml of H_2O, 0.3 to 0.5 g of KI, and titrate the liberated iodine with thiosulfate, using starch indicator. One ml of 0.1 *N* ceric soln equals 0.000383 g of ethyl alcohol.

For both ceric and dichromate oxidations, 1 mg of ethyl alcohol equals 2 mg of yeast-fermentable sugar as maltose.

DISCUSSION

The determination of ethyl alcohol by dichromate oxidation is essentially that of Hoepner (6). A number of modifications have been proposed, such as the use of diphenylamine or *o*-phenanthroline indicators, and use of ferrous sulfate, potassium ferrocyanide, or other reducing agents for titrating the excess oxidizing agent as well as spectrophotometric determination of the excess dichromate. The chromic ions which are formed on reduction of the chromate have a light blue color which could be mistaken for the iodine starch color, but repeated checks indicate that the end point can be readily determined within one drop of 0.1 *N* thiosulfate. The accuracy of the method for solutions of low alcoholic content is generally reported as 97 per cent.

Other organic materials are oxidized by dichromate solution but when the test is carried out as outlined above, alcohol is the only volatile organic material present in detectable amounts. When ordinary laboratory reagents and 5 ml of water were refluxed for 15 minutes with the sulfuric acid-dichromate solution, the oxidation value of the dichromate solution dropped from 0.100 *N* to 0.098 *N*. This may have been due to impurities in the sulfuric acid, water, or potassium dichromate which were oxidized on heating. For this reason the oxidizing solution should be checked for stability to heat, so that appropriate corrections may be applied in the alcohol determination.

Lindenberg and Guillemet (7) state that in 30 per cent or less sulfuric acid solution, ethyl alcohol is quantitatively oxidized to acetic acid and water by dichromate, but that in 75 per cent sulfuric acid, it is oxidized to carbon dioxide and water. The sulfuric acid concentration here is well below 30 per cent. Refluxing the sample in oxidizing solution for 10 minutes, 30 minutes, or letting it stand at room temperature for 3 hours without heating, gave the same titration values. The oxidizing solution should be diluted to between 2 and 4 per cent sulfuric acid before addition of the potassium iodide.

In recent years, ceric salts have been used as oxidizing agents with increasing frequency. Kolthoff and Sandell (8) mention some of the advantages of ceric solutions in oxidimetry. Smith and Fly (5) discuss ammonium hexanitratocerate as a primary standard, and Smith (9) has

provided a complete summary of cerate oxidimetry to 1942.

Despite the difficulty in preparation, and lack of stability of the ceric solution, this method possesses two advantageous features: (*a*), the ceric compound is of high molecular weight and undergoes a positive valence change of only one (twelve ceric ions are consumed per molecule of alcohol); (*b*), upon completion of the titration with thiosulfate, the solution is water-white, and this makes the end point very sharp. Alcohol determinations on a number of samples, varying from 0.0000 to 0.2000 g of alcohol per 100 ml, gave an accuracy of better than 97 per cent

Samples of beer were dealcoholized (*a*) by heating in an evaporating dish on a steam bath at 100°C. until the volume had decreased to $\frac{1}{3}$ of the initial volume; and (*b*) by distilling the alcohol from a flask over wire gauze until the same volume decrease occurred. After an end fermentation, these samples gave identical alcohol values with those dealcoholized at 80°C. Some material precipitated at the higher temperature, but there was no apparent adverse effect on the determination. These methods of dealcoholization are not recommended, however, without further study.

The amount of yeast-fermentable sugar, calculated as maltose, from the alcoholic content of an "end-fermented" beer determined chemically, was always between the high and low values based on differences in "real extract" or "copper reducing materials."

SUMMARY

Alcohol in low concentration can be determined accurately by ceric ion oxidation in perchloric acid.

Both ceric and dichromate oxidations for the determination of alcohol in "end-fermented" beer are shown to be feasible.

A method is described for determining the yeast-fermentable sugar in beer based on the alcohol produced in an "end-fermentation."

REFERENCES

(1) *Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Washington, D. C., 1950, Sec. **10.35.**
(2) *Book of Methods*, Am. Soc. Brewing Chemists, 5th Ed. (1949).
(3) Allen, A. H., *Commercial Organic Analysis*, Vol. 1, 5th Ed., P. Blakiston's Sons & Co., Philadelphia, p. 210, 1923.
(4) *Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Washington, D. C., 1950, Sec. **10.8.**
(5) SMITH, G. F., and FLY, W. H., *Anal. Chem.*, **21**, 1233 (1949).
(6) HOEPNER, K., *Chem. Abstr.*, **14**, 713 (1920); *Chem. Abstr.*, **16**, 1549 (1922).
(7) LINDENBERG, A. B., and GUILLEMET, R., *Compt. rend.*, **230**, 2104 (1950).
(8) KOLTHOFF, I. M., and SANDELL, E. B., "Textbook of Quantitative Inorganic Analysis," The Macmillan Company, New York. Reprinted 1941, p. 553.
(9) SMITH, G. F., "Cerate Oxidimetry," G. Frederick Smith Chemical Co., Columbus, Ohio, 1942.

SPECTROPHOTOMETRIC DETECTION OF CERTAIN TYPES OF ADULTERANTS IN VANILLA

By RICHARD M. ROBERTS (Standard Vanilla Company, Los Angeles, California)

A wide variety of flavoring materials is said to occur as adulterants in pure vanilla and vanillin-fortified vanillas. Among the synthetics, essential oils, botanical extracts, etc., so mentioned, are many constituent oxygenated and olefinic compounds of the side-chain aromatic (benezenoid) type, which undergo reactive solution in concentrated sulfuric acid (1). (For brevity, this reaction will be broadly termed as sulfonation, although reaction mechanics frequently indicate either sulfation, molecular dehydration, polymerization, or oxonium-complex formation.)

In many cases the reaction products are chromophores or "ultrachromophores," which are relatively stable unless exposed to hydrolysis. Selected U.S.P. chemicals, in smaller than milligram quantities, were dissolved in 2 ml of 95 per cent ethanol and treated with concentrated sulfuric acid to a volume of 25 ml. The absorbancies of the solutions were then recorded on the spectrophotometer through the available range of 325 to 600 mμ. The readings throughout the spectral region employed were found to be reproducible and, with two exceptions, relatively stable after a lapse of thirty minutes. Figures 1–7 illustrate the absorbance versus wavelength curves of some of the better known sulfonated synthetic sophisticants of vanilla, many of which also occur as constituents of important flavoring materials.

Curves are given for cinnamic acid (Fig. 4-c) and eugenol (Fig. 5-d), even though acidic compounds cannot be normally isolated by the method to be outlined. This is done because the flavor-wise important neutral esters of these compounds are normally obtained in the final isolate. On treatment with sulfuric acid, these esters hydrolyze, and if the substituent group is a short-chained (less than ten carbon atoms) aliphatic radical, the absorbance characteristics of the solution will be essentially those of the aromatic group, since most aliphatic acids and alcohols form only weakly absorbing reaction products with sulfuric acid. This same behavior was noted in esters of anisyl alcohol, anisic acid, and cinnamic alcohol.

Equimolar solutions of isoeugenol and eugenol produce identical curves on sulfonation, which may be attributed to an allylic shift occurring in the eugenol reaction system resulting in the quantitative formation of isoeugenol. Thus, short-chained aliphatic esters of both phenols produce identical curves when in equimolar concentration.

Isolates of vanillas, obtained by a modified Hess-Prescott coumarin method (2), were sulfonated and found to contain trace constituents which evolved a straw-yellow solution (red in the case of *Vanilla Tahitensis*). The absorbance versus wavelength curves were recorded and com-

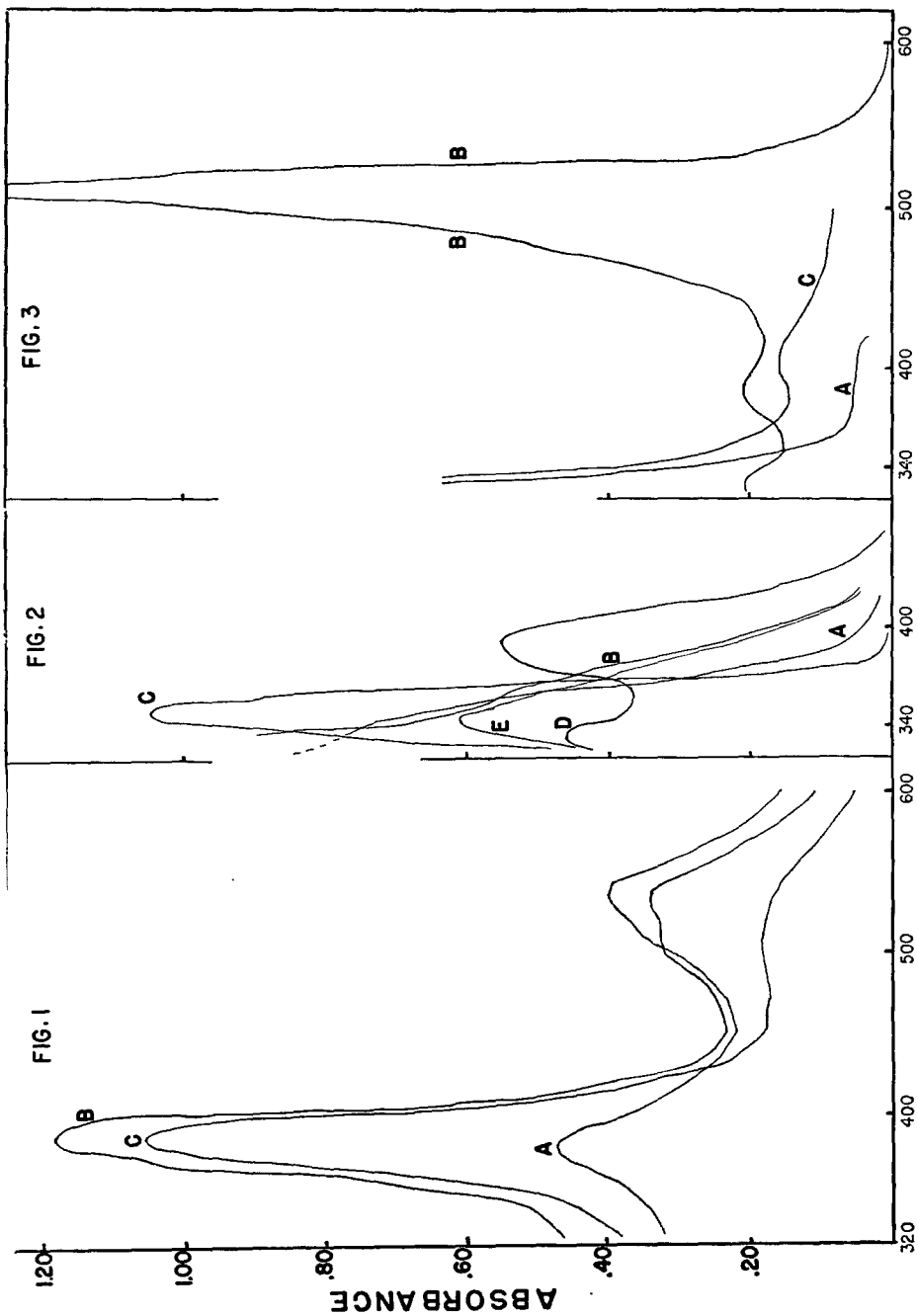

FIG. 1.—*alpha*-Substituted cinnamaldehydes. *A*. *alpha*-Butylcinnamaldehyde (.41 mg/25 ml); *B*. *alpha*-Amylcinnamaldehyde dimethylactal (.72 mg/25 ml); *C*. *alpha*-Hexylcinnamaldehyde (.59 mg/25 ml).

FIG. 2.—Benzaldehyde and related compounds. *A*. Benzaldehyde (.88 mg/25 ml); *B*. *p*-Tolualdehyde (.79 mg/25 ml); *C*. Anisaldehyde (.10 mg/25 ml). *D*. Piperonal (.15 mg/25 ml); *E*. Veratraldehyde (.12 mg/25 ml).

FIG. 3.—Phenyl aliphatic aldehydes. *A*. Phenylacetaldehyde (.85 mg/25 ml); *B*. Phenylpropionaldehyde (.66 mg/25 ml); *C*. 1-Methyl-1-

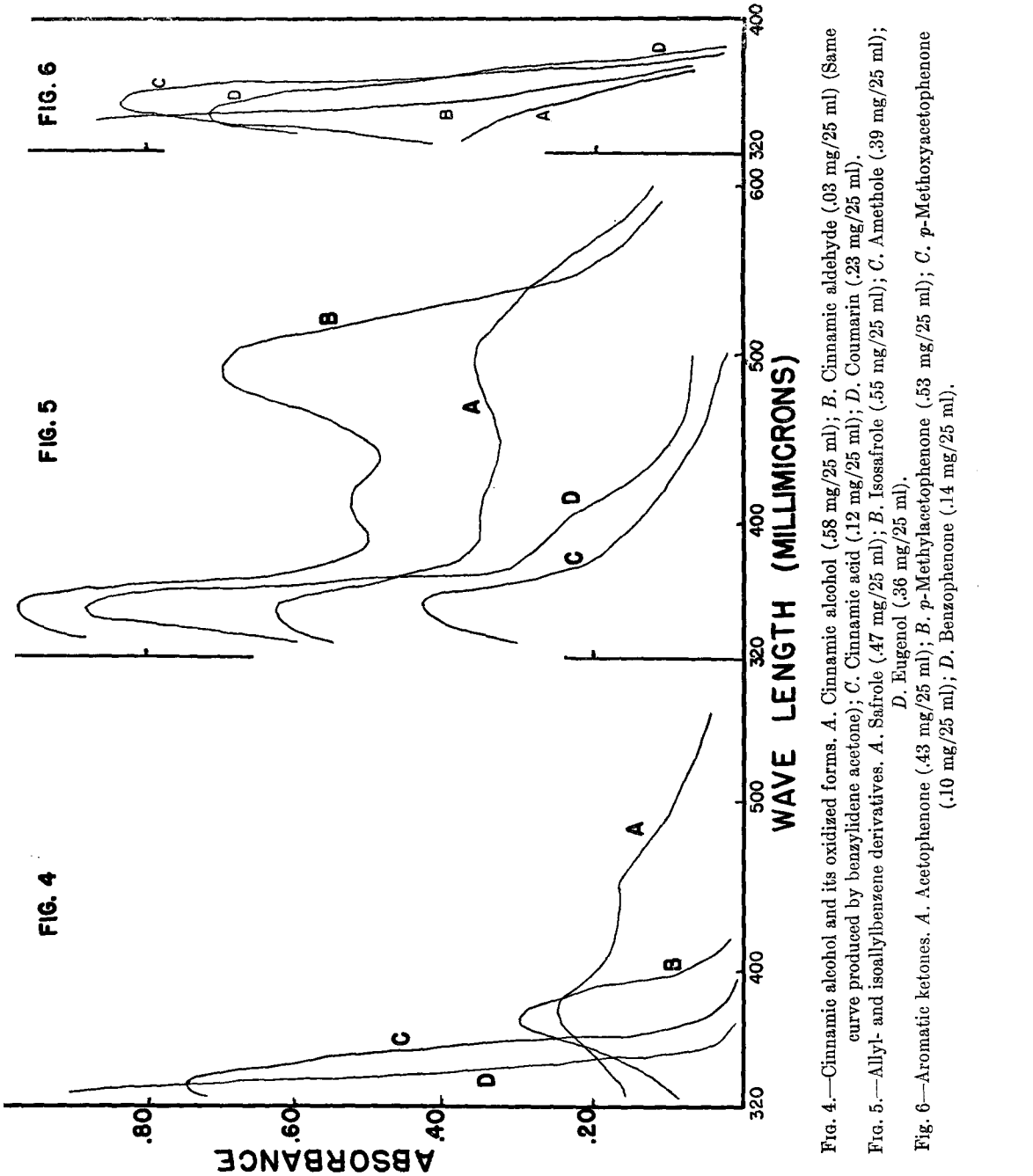

Fig. 4.—Cinnamic alcohol and its oxidized forms. *A*. Cinnamic alcohol (.58 mg/25 ml); *B*. Cinnamic aldehyde (.03 mg/25 ml) (Same curve produced by benzylidene acetone); *C*. Cinnamic acid (.12 mg/25 ml); *D*. Coumarin (.23 mg/25 ml).

Fig. 5.—Allyl- and isoallylbenzene derivatives. *A*. Safrole (.47 mg/25 ml); *B*. Isosafrole (.55 mg/25 ml); *C*. Amethole (.39 mg/25 ml); *D*. Eugenol (.36 mg/25 ml).

Fig. 6—Aromatic ketones. *A*. Acetophenone (.43 mg/25 ml); *B*. *p*-Methylacetophenone (.53 mg/25 ml); *C*. *p*-Methoxyacetophenone (.10 mg/25 ml); *D*. Benzophenone (.14 mg/25 ml).

pared with those of the sophisticant compounds. This comparison suggested that the presence of sulfuric acid-chromogenic adulterants in vanilla could be demonstrated graphically from the curve distortion they produced.

The sulfonated vanilla isolates were found to produce rather featureless curves, exhibiting single maxima below 340 millimicrons. It was found that the absorbance at the maximum is roughly proportional to the amount of vanilla solids used in preparing the flavor, if the degree of exhaustion is held constant. Under identical extraction conditions, four vanilla extracts were obtained using the same quantity of vanilla solids (32.5 grams per 500 ml). From these, the "coumarin" residues were isolated and sulfonated. The absorbancies at the maxima were found to vary in random order by only .06, although the vanilla beans extracted (*Vanilla Fragrans*, Ex Mexico) represented extremes in quality (see Table 1).

TABLE 1.—*Absorbancies of vanilla extracts*

GRADE	MOISTURE (W/W)	WICHMANN LEAD NUMBER	WET WEIGHT EXTRACTED PER 500 ML	MAXIMUM ABSORBANCE
	per cent		*grams*	
Superior	40.0	.661	54.2	.290
Buena	30.8	.697	45.2	.338
Mediana	25.7	.743	43.7	.280
Cuts	18.8	.826	40.0	.321

To determine whether curve characteristics varied significantly with the type of extractives encountered, cured vanilla specimens from most of the commercial cultivation areas were extracted and tested. With the exception of *Vanilla Tahitensis* (see below), all produced similarly patterned curves (see Figures 8 and 9) which exhibited good reproducibility and stability over an observed period of thirty minutes. These extracts were prepared on a wet weight basis, so that the intensities of absorbance at the maxima vary considerably, depending on the moisture content of the samples tested.

Commercial extracts, concentrates, and sugars, representing the production of seventeen different manufacturers, were also tested. The curves obtained fitted well the pattern displayed by the laboratory extracts.

The ages of the laboratory and commercial vanillas varied from a few hours to several years. Curve anomalies which could be directly associated with this factor were not apparent.

Anisyl alcohol, a sulfuric acid labile constituent absent in other geographic types, is found in Tahiti vanilla beans (3) in concentrations as high as 1 per cent by weight. This results in an obscured sulfonation

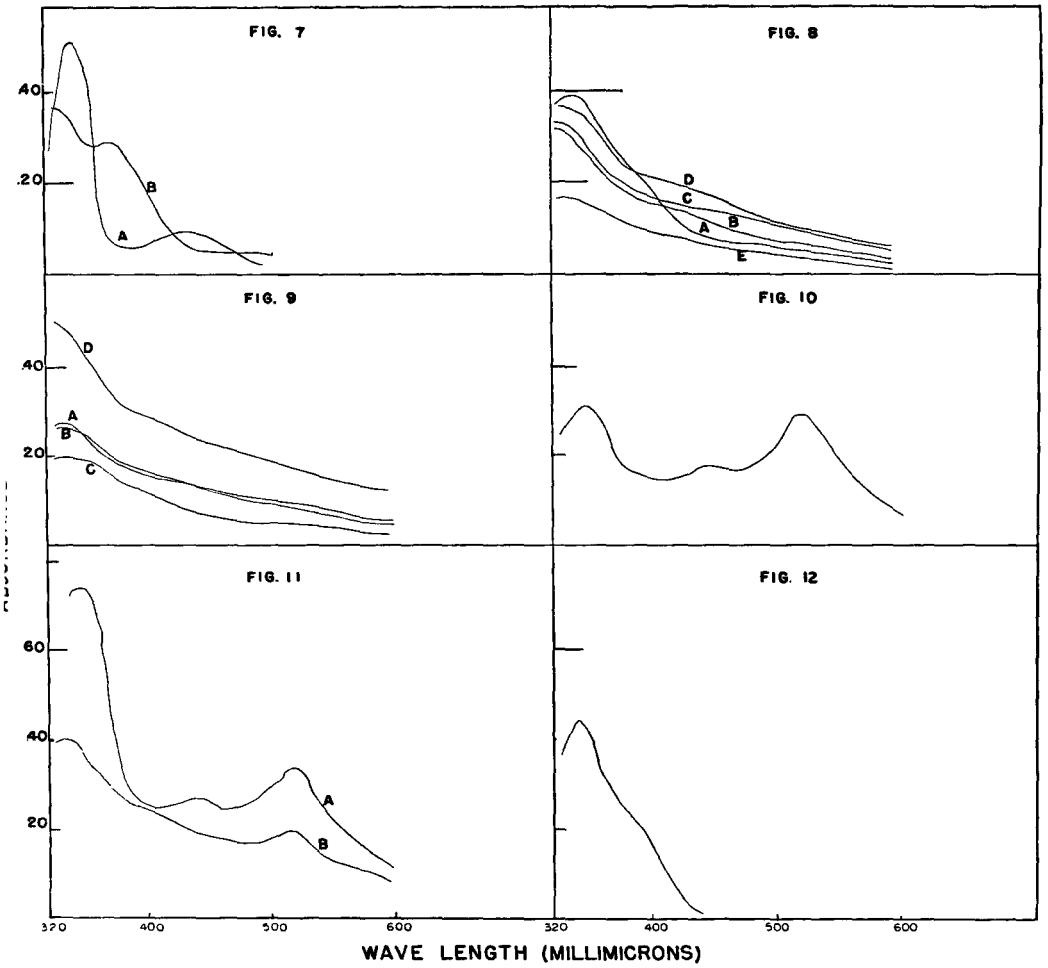

FIG. 7.—Naphthalene compounds. *A*. *alpha*-Methyl naphthyl ketone (.08 mg/25 ml). *B*. *beta*-Napthol methyl ester (.38 mg/25 ml).

FIG. 8.—Sulfonated isolates of vanilla (single strength) from various geographic areas. *A*. Dominica; *B*. Java; *C*. Mexico; *D*. Madagascar; *E*. Seychelles.

FIG. 9.—Sulfonated isolates of vanillas (single strength) from various geographic areas. *A*. Reunion; *B*. Guadeloupe; *C*. Comoro; *D*. Puerto Rico.

FIG. 10.—Sulfonated anisyl alcohol. Concentration 1.08 mg/25 ml.

FIG. 11.—Sulfonated Tahiti isolates (bisulfite procedure). *A*. 100% Tahiti (single strength vanilla). *B*. 25% Tahiti: 75% Mexican (double strength vanilla).

FIG. 12.—Sulfonated vanillin. Concentration: .08 mg/25 ml.

system, unless the Tahiti extractives are blended down sufficiently (ca 15 per cent) with other types. In the latter case, no special treatment is necessary, since the characteristic *p*-methoxybenzylsulfate absorbance (see Figure 10) is readily recognized and interpretable, even though additively modified by other sulfonated natural constituents. Where Tahiti extractives constitute more than 15 per cent of the vanilla, reduction of the anisyl alcohol concentration in the "coumarin" isolate is required. This can be accomplished, although not quantitatively and with limiting effects, by further extracting the ethereal isolate with sodium bisulfite, neutralizing, and re-extracting with ether. Figure 11 illustrates the typical results obtained after such manipulation.

It is of interest to note that the absorbance curves of Tahiti vanillas afford no indication of the presence of piperonal. Differentiation between naturally occurring and added piperonal is thus possible, which is apparently not the case when the Labat color test (4) is used.

METHOD

EQUIPMENT AND REAGENTS

(a) *Spectrophotometer.*—Beckman Model B, equipped with matched Pyrex glass cells (1 cm).

(b) *Sulfuric Acid.*—A. C. S. grade, sp. gr. 1.84.

(c) *Ethanol.*—U. S. P. grade, 95%.

(d) *Sodium Hydroxide.*—Reagent grade, 2% and 20% (w/v) solns.

(e) *Sodium Bisulfite.*—Reagent grade, 15% (w/v) soln. Prepare fresh before use.

(f) *Sodium Sulfate.*—Anhydrous, reagent grade.

(g) *Lead Acetate* (neutral).—Reagent grade, 8% (w/v) soln.

(h) *Sodium Carbonate.*—Reagent grade, satd (20°C.) soln.

PROCEDURE FOR LIQUID EXTRACTS

Select or interpolate sample size from Table 2. (Where extractive concn is not known, lead number data may be used in estimating the extractive content.) Place aliquot in 250 ml conical flask and dil. with H_2O to ca 100 ml. Fit flask with 10 mm suction tube rising at least 10 in. above the flask and breather tube whose capillary tip is ca 2 in. above the liquid. Add a few boiling chips and evacuate flask; clamp off breather tube so that air flow into the flask produces only a faint dimple on the liquid surface. When the liquid comes to a spontaneous boil, turn on 250 watt infrared heater mounted 3 in. under the flask. Reduce vol. to ca 25 ml and add 2 ml of 20% NaOH; wash flask contents into 100 ml volumetric flask contg 25 ml of 8% $Pb(OAc)_2$ with boiling H_2O. Fill almost to mark and mix by swirling gently. Cool under tap, make to mark with H_2O, and mix thoroly. Filter with suction thru Whatman No. 5 filter paper, coated with several g Celite analytical filter aid. Pipet 50 ml of filtrate into one of four 250 ml separatory funnels and extract with four 15 ml portions of ether. Dephenolate combined ether extracts with at least four 15 ml portions of 2% NaOH, discarding the aq. layers. It may be necessary to use extra portions of base with samples heavily fortified with vanillin. In this case, dephenolate until the next to last aq. layer is entirely colorless. In the absence of anisyl alcohol (or if present in low concn) wash ether with 15 ml of H_2O to remove residual base.

If anisyl alcohol is present in interfering concn, extract ether with three 15 ml portions of 15% $NaHSO_3$. Without overlong delay, neutralize the combined aq. layers with satd Na_2CO_3 and extract with four 15 ml portions of ether. Wash combined ether extracts with 15 ml of H_2O.

Run ether soln, obtained by either procedure, into dry 250 ml Erlenmeyer flask thru funnel fitted with small plug of glass wool covered with a thin layer of anhydrous Na_2SO_4. Wash plug with about 5 ml of ether and evap. to dryness *in vacuo*. Dissolve fragrant residue in 2 ml of 95 per cent ethanol and add ca 10 ml concd H_2SO_4. Mix by swirling and pour into acid-rinsed 25 ml volumetric flask. Rinse Erlenmeyer flask with several addnl smaller portions of acid and add to volumetric flask until mark is almost reached. Cool under tap and make to mark with acid. Immediately record the absorbance of the reaction mixt., referred to acid, on spectrophotometer thru available range.

If sample is adulterated so that off-scale readings are obtained, the sulfonation mixt. may be dild with acid until an on-scale curve is obtained. This curve should be of value in identifying the adulterant, if desired.

PROCEDURE FOR VANILLA SUGARS

Using Table 2, weigh powdered sample to nearest 0.1 g and place in paper thimble. Extract in the Soxhlet for 4 hrs with sufficient 95% ethanol so that the boiler flask contains about 75 ml of liquid prior to receiving the siphonage. On completion, remove thimble and max. amount of refluxate; then reduce boiler liquid to ca 25 ml by distg into empty extraction tube. Remove and cool boiler flask and allow to stand for 15 min. in ice bath. Filter (Whatman No. 40) by gravity into a 250 ml conical flask. Rinse the boiler with two 5 ml portions of ethanol and pour thru filter. Dil. the filtrate with H_2O to ca 100 ml and de-alcoholize to 50 ml in the evaporator. Again dil. to 100 ml and continue as in regular procedure with further dealcoholization, etc.

TABLE 2.—*Proportions for aliquoting*

CONCENTRATION BEANS PER GAL. OR 8 LBS	ALIQUOT SIZE
ounces	*ml or g*
13.35 (U.S.P.)	60.0
16.00 (Calif.)	50.0
26.70 (Dbl. Str.)	30.0
133.52 (10 Fold)	6.0

DISCUSSION

Sample defecation is employed to prevent the formation of troublesome emulsions which often form when undefecated samples are shaken with ether. The filtration of the lead acetate-vanilla mixture was found to be time consuming, especially with Tahiti extracts. This was remedied by adjusting the *p*H of the alcohol-free sample with sodium hydroxide before the addition of lead acetate.

The vanillin must be removed, since this phenolic aldehyde forms an intensely absorbing sulfonated chromophore (see Fig. 12), which completely obscures the system. Unfortunately, dephenolation limits the scope

of detection, particularly since several substituted phenols and aromatic carboxylic acids are said to find effective use as vanilla adulterants. Dephenolation with strong base is preferred, rather than with ammonium hydroxide, which forms ether-insoluble hydrobenzamides with some aldehydes. The participation of traces of vanillin in the sulfonation reaction is possible, since absorbance curves of vanillin and of vanilla isolates have roughly coincident maxima.

If anisyl alcohol is present in the extract tested and its concentration is reduced by the bisulfite extraction procedure, overlong standing of the latter should be avoided. The anisyl alcohol saturating the aqueous phase is slowly oxidized to anisaldehyde, evidenced by an increased absorbance of the ultraviolet maximum (345 mμ for both compounds) with a corresponding reduction of the maximum of 515 mμ (anisyl alcohol), even though the aqueous phase is out of contact with the ethereal (source) phase. This effect is not apparent after two hours of standing, but on overnight exposure to aqueous oxidation, the ultraviolet under-curve area is considerably increased.

Solution of the isolated residue in ethanol was found to be necessary since treatment with acid alone produced turbidity. The same difficulty is experienced when the water is not held to a subcritical level. Varying the amount of ethanol added from one to two milliliters resulted in only minor fluctuations in the absorbance curves; the larger portion was selected because it facilitated manipulation.

The absorbance of U.S.P. ethanol was compared with that of ethanol purified with *m*-phenylenediamine dihydrochloride. The absorbance of blank solutions of both were identical at all wavelengths. The absorbance of a U.S.P. ethanol blank was then compared with that of undiluted acid. The highest absorbance of the blank, referred to acid, was only 0.005 at 330 mμ. For this reason, the use of a blank may be omitted.

In contrast with the other synthetics tested, cinnamaldehyde and *beta*-naphthol methyl ether produced unstable sulfonation products. The cinnamaldehyde chromophore faded on standing, while the napththol ether exhibited transient absorbance, reflecting intermediate compound formation. Thus it is recommended that absorbance readings be taken immediately after treatment with acid.

Vanillas containing polyhydric solvents, such as glycerol or 1,2,-propanediol, were found to behave in the same manner as alcoholic extracts. Several of the commercial vanillas tested were of the former type.

APPLICATION

To illustrate the application of the method, curves are shown for adulterated vanillas which were actually taken from commerce.

Figure 13 resulted from the sulfonation of a double strength Tahiti vanilla (bisulfite extracted). The curve (Fig. 13-A) went off scale, so that

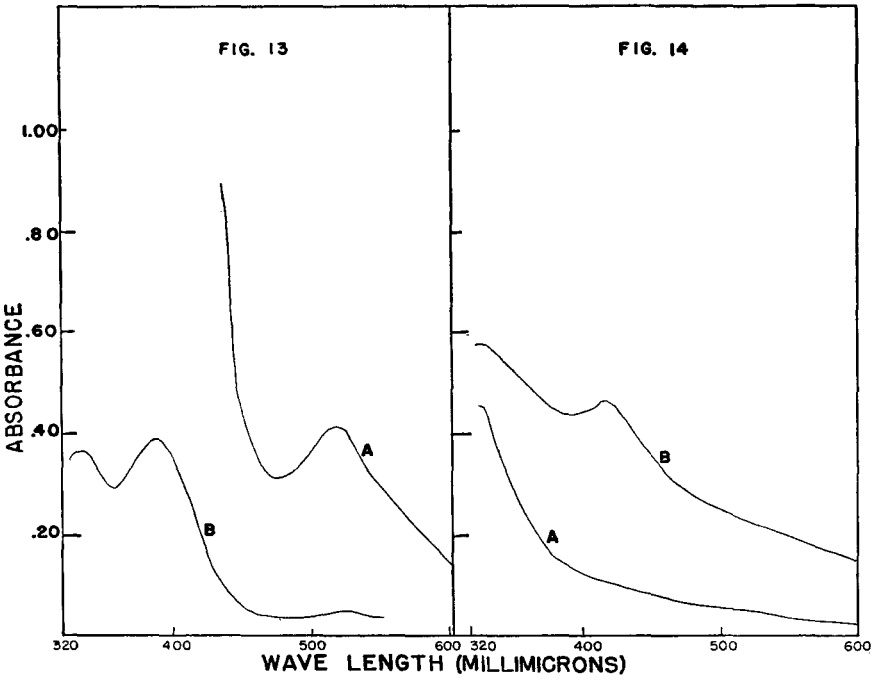

FIG. 13.—Sulfonation curve of adulterated double strength Tahiti vanilla. *A*. Absorbance of undiluted sulfonated isolate; *B*. Same diluted to 10% solution.

FIG. 14.—Sulfonation curves of two vanilla-vanillin flavors. *A*. Vanilla originally supplied consumer according to specifications. *B*. Same vanilla later supplied with unknown additives.

dilution with acid to a 10 per cent solution was necessary in order to obtain the curve in Figure 13-B. The resemblance of the latter to that of piperonal is obvious.

Figure 14 reflects a case history of adulteration for economic purposes, in which the manufacturer probably substituted unknown flavoring materials for vanilla extractives. This substitution was effected (after the second shipment, Fig. 14-A) in the third shipment to the consumer (Fig. 14-B).

The further possibilities of this technique, as applied to other flavoring extracts, are now being studied. Encouraging results have already been obtained with true fruit extracts of strawberry and raspberry.

ACKNOWLEDGMENTS

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Brothers, Inc., Zink and Triest Company, and Howard M. Bollinger and Arthur W. Steers of the Los Angeles District of the U. S. Food and Drug Administration.

REFERENCES

(1) HAMMET, L. P., *Physical Organic Chemistry*, McGraw-Hill Book Co., New York, 1940, p. 45.
(2) HESS, W. H., and PRESCOTT, A. B., *J. Am. Chem. Soc.*, **21**, 256 (1899).
(3) WALBAUM, H., *Semi-Annual Report*, 142, Schimmel and Co., Oct. 1909.
(4) LABAT, A., *Bull. Soc. Chim.*, **5**, 742 (1919); *Bull. Soc. Pharm. (Bordeaux)*, **57**, 259 (1919).

THE DETECTION AND ESTIMATION OF PARSNIP ADULTERATION IN PREPARED HORSERADISH BY INFRARED SPECTROPHOTOMETRY*

By JONAS CAROL and L. L. RAMSEY (Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C.)

Infrared absorption spectrophotometry has become one of the most valuable analytical tools available to the chemist, and it is being used in the Food and Drug Administration laboratories to solve many diffcult analytical problems. The estrogenic, androgenic, and adrenal cortex hormones; penicillin and other antibiotics; coal-tar dye intermediates; and organic insecticides are some of the types of products that have been successfully examined by this procedure.

An infrared spectrum provides both quantitative measurement and qualitative identification of a substance in one operation; in fact, the infrared spectrum is the most positive single proof of identity of a substance. No two compounds, with the exception of mirror-image optical isomers, have identical infrared spectra. Frequently a slight difference in structure will result in a marked difference in absorption spectra. As an example , all of the isomers of benzene hexachloride have different spectra, and each can be determined quantitatively in mixtures.

The present problem required proof of adulteration of prepared horseradish with ground parsnip root. The samples were found by microscopic examination to contain less than 50 per cent of horseradish root. Prepared horseradish was formerly frequently adulterated with ground parsnip root. As parsnips have a bland flavor, these adulterated products usually contain added synthetic oil of mustard to produce the desired pungency. It was hoped that the presence of synthetic oil of mustard could be proved by showing a difference in the infrared spectra of volatile oil of horseradish and allyl isothiocyanate (synthetic oil of mustard). A sample of volatile

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* Presented at the annual meeting of the Association of Official Agricultural Chemists, September 29, 30, and October 1, 1952, at Washington, D. C.

oil of horseradish was prepared as follows:

Six hundred g of authentic ground horseradish root was steam-distilled in an all-glass apparatus until the distillate was no longer cloudy. The distillate was made alk. with $NaHCO_3$ and extracted twice with ether. The combined ether extract was washed with H_2O, dried over anhyd. Na_2SO_4; the ether was removed by careful evapn to minimize loss of volatile oil. The infrared absorption spectrum of a film of the oil was recorded from 2–15 μ, and was found to be almost identical with a spectrum of a similar but somewhat thicker film of allyl isothiocyanate, shown in Fig. 1.

This indicates that the volatile oil of horseradish is essentially allyl isothiocyanate and that it probably would not be possible to detect addition of synthetic oil of mustard to prepared horseradish by this method.

Volatile oil of parsnips was prepared from authentic ground parsnips by the same procedure. Its infrared spectrum (2–15 μ) obtained as above, and shown in Fig. 2, is distinctly different from that of allyl isothiocyanate. The very strong absorption band at 4.75 μ, due to the isothiocyanate group, is absent in the parsnip oil spectrum, whereas the strong 6.65 μ band of the parsnip oil spectrum is absent in that of allyl isothiocyanate. Because of these differences in spectra, direct detection and determination of parsnip adulteration appeared to be possible.

The samples of commercially prepared adulterated horseradish containing 50 and 80 per cent ground parsnip (by microscopic estimation) were steam distilled as above, and the spectra of the oils were recorded from 2–15 μ (Fig. 3). These spectra, with the exception of a band at 4.75 μ, are almost identical with the spectrum of the oil of parsnip, and indicate that the prepared "horseradishes" consist largely of parsnips with varying amounts of horseradish and/or synthetic oil of mustard. Using baseline measurements of absorption at 4.75 and 6.65 μ, the relative amounts of parsnip and horseradish (or oil of mustard) were calculated, and agreed closely with the microscopic estimation.

These studies were confined to only three authentic samples of horseradish, two roots and one commercially prepared (grated) product, and one authentic sample of parsnips (roots). In view of the wide difference in the infrared spectrum of the volatile oil of the two products, however, it is believed that the method can be accepted as a basis of differentiating these materials.

The present work was exploratory and time did not permit the develop-

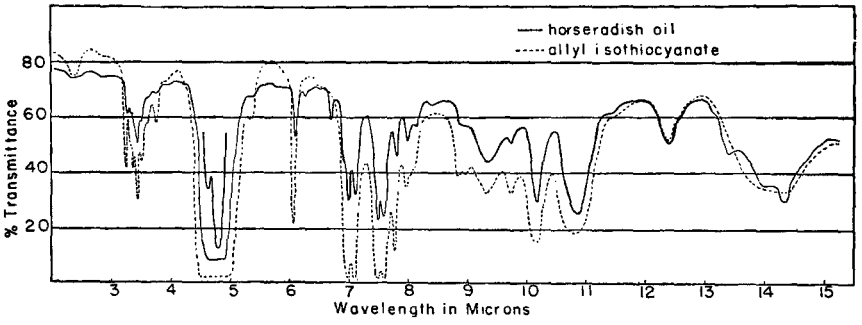

FIG. 1.—Comparison of infrared spectra of volatile horseradish oil and allyl isothiocyanate.

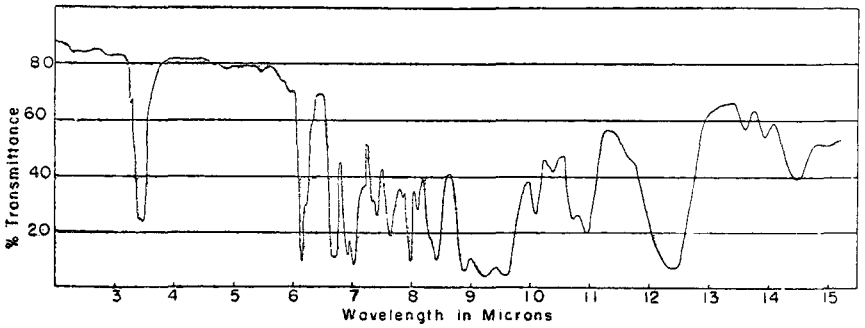

FIG. 2.—Infrared spectrum of volatile parsnip oil.

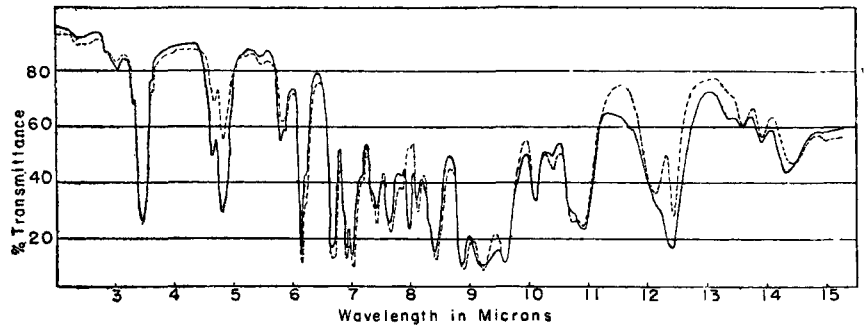

FIG. 3.—Infrared spectra of the oils of adulterated horseradish.
——— Approximately 50% parsnip. --- Approximately 80% parsnip.

ment of a more finished analytical procedure. If extension of the investigation is contemplated at a future time, the spectra should be obtained from carbon disulfide solutions in cells, instead of from films of the oil. These solutions should be prepared with a definite ratio of volume to weight of sample, so that accurate quantitative measurements can be made.

QUALITATIVE PAPER CHROMATOGRAPHY OF SUGARS IN PLANTS

TECHNIQUE AND REAGENTS

By KENNETH T. WILLIAMS and ARTHUR BEVENUE (Western Regional Research Laboratory, Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture, Albany, California)

Paper chromatography is now recognized as an important tool in many branches of analytical chemistry. In particular, it has been of tremendous value to the biochemist where only minute amounts of material may be

available or necessary for study. It has opened up new vistas in the study of the sugars present in plant life; this is especially true of the sugars that are present in the plant in only very small amounts. Prior to the advent of paper chromatography, it was not possible to identify such sugars, except in a few instances where identity could be made by the tedious extraction and purification of the sugar from a large quantity of raw material. Separations of homologous series of sugars are possible by chromatography. The technique is useful not only for identifying compounds, but also as a means for isolating and purifying each sugar found in the plant. Although the development of paper chromatography has not reached the stage where such identification is wholly valid, it has been and will probably continue to be of great value in providing new information on the characterization and distribution of the sugars present in plant materials. If it is necessary to establish more definite proof of the identity of the compound, the classical methods for the determination of its chemical and physical constants must be used.

For several years the authors have used paper chromatographic techniques to investigate the sugars in various plant materials (28–30). A group of procedures for making such investigations has evolved from their own experience and from the most applicable published work. It is believed that these procedures will be of value to investigators just entering this field and may also be of considerable help to those experienced in chromatographic work. Details of the various operations are presented in this report.

PROCEDURE

Preparation of the plant extracts.—Fresh plant material is finely chopped and quickly added to sufficient 95% hot ethyl alcohol (freshly redistilled) to give, with the H_2O naturally present in the plant, an approximate alcohol concn of 80%. One to 2 g $CaCO_3$ is added and the mixt. is heated for 1 hr, with frequent stirring, on a steam bath. The supernatant liquid is filtered through a folded filter paper. The residue is covered with 80% ethyl alcohol, reheated for 30 min. on the steam bath, and filtered. The residue is blended for 5 min. in a Waring blendor with sufficient 80% ethyl alcohol to make a slurry, then filtered, and washed sparingly with hot 80% ethyl alcohol. All filtrates and washings are combined.

Dried plant material is ground to pass a 40 mesh screen. The extraction process is similar to that used for fresh material, with the exception that the blending step is omitted and 80% alcohol is used throughout the procedure.

Any precipitates formed after the extracts have cooled to room temperature should be removed by filtration.

The quantity of the plant extract to be selected for subsequent chromatographic study will vary considerably with the material. For example, total sugars in fresh white potato may be only 1%, whereas in dehydrated carrot they may be as great as 60%. The aliquot should be of such amount that the final reconstituted concentrate, to be used for chromatographic applications, is 1–3% with respect to each component sugar.

The selected aliquot of the plant extract is evapd on a steam bath to remove the alcohol. If necessary, distd H_2O may be added to prevent the concentrate from go-

ing to dryness. Since the extract will contain such materials as organic acids, amino acids, and electrolytes in addn to sugars, it is not advisable to permit the sample to go to complete dryness because of possible reactions between the sugars and one or more of these substances. The concentrate is filtered, with suction, through a mat of Celite analytical filter aid,* and the filtrate is made to 100 ml with distd H_2O.

There may be substances in this soln that interfere with the identification of the sugars by paper chromatography. To remove many of these impurities the soln is treated with ion-exchange resins in the following manner: resin columns are prepared in two Tswett tubes, each 25 mm i.d. and 300 mm in length. One tube is charged with 7–15 g of cation exchange resin Amberlite IR-120 (H) AG and the other with 10–20 g of anion exchange resin Amberlite IR-4B AG (31). Experience has shown that a selected amount of cation exchange resin will require ca 1.5 times as much anion exchange resin. Before other resin combinations are used, it should be shown that they do not retain or destroy sugars (23).

The solution is added to the cation exchange resin column, maintaining the liquid level above the resin level until the last portion of the liquid has passed through the column. The eluate from the cation exchange column is allowed to drip directly into the anion exchange column, to minimize the amount of time the solution is acidic. When the liquid level in the anion resin column rises above the resin level, the outlet of this column is opened and adjusted to maintain the liquid level above the resin until the last portion of the liquid is received from the cation column. The rate of flow from each of the columns should be adjusted, by means of a screw clamp on rubber tubing attached to the Tswett column outlet, to approximately 3 ml/min. Occasional checks with *p*H indicator paper should be made of both the cation and the anion resin eluates. The *p*H of the eluate from the anion exchange resin column should be between 6 and 7. If the *p*H of the anion resin eluate drops below 6, either the solution is passing through the column too rapidly or insufficient resin has been used to remove the electrolytes. If the *p*H is above 7, the cation resin is not removing all of the cations.

The deionized soln is divided into two portions. One portion is evapd to obtain the proper sugar concn and chromatographed. The other portion is subjected to complete fermentation by bakers' yeast prior to the chromatographic process. The detailed procedures are described in the following paragraphs:

One portion of the deionized soln is evapd to near dryness on a steam bath. Towards the end of the evapn period the soln is transferred to a 10 ml beaker or crucible and the vol. is reduced to 2–3 ml. Evapn is then completed at room temperature, under vacuum, in a desiccator contg concd H_2SO_4. The dried residue is now ready for proper diln and application to the paper chromatogram. Preferably, it should be kept at freezing temperature until desired for chromatographic studies.

The fermentable sugars are removed from the second portion (about 50 ml) of the deionized soln in the following manner: 10 g of fresh bakers' yeast is suspended in 80 ml of distd H_2O. The suspension is centrifuged and the supernatant liquid is discarded. The yeast is washed four times by this procedure and then suspended in 100 ml of distd H_2O. Washing of the yeast is necessary for the removal of small amounts of reducing substances present in the yeast cake. A buffer soln is prepared contg 15 ml of glacial acetic acid and 34 g of sodium acetate trihydrate in an amount of distd H_2O to make 500 ml of soln. The soln to be fermented is placed in a flask, together with 2 ml of the buffer, 2 ml of 0.2 *M* KH_2PO_4, 6 ml of the yeast suspension, and 5 drops of Difco invertase soln. After mixing, the flask is lightly plugged with cotton and the mixt. held at 30°C. for 24 hrs. (Trace amounts of the fermentable

* Mention of manufacturers and commercial products does not imply that they are endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.

sugars may still be present if shorter periods of fermentation are used. Such amounts would not be measured by the conventional quantitative methods of sugar analysis but they would be revealed on the paper chromatogram.) The mixt. is agitated several times during the fermentation period. The fermented soln is filtered with suction through a mat of Celite. The filtrate is deionized and concd as described above.

Certain sugars that are difficult to separate by paper chromatography may be quantitatively separated by fermentation with bakers' yeast. For example, in a galactose-glucose mixture, the glucose can be removed by this type of fermentation. Also, the ketoheptoses follow fructose closely on the chromatogram, and the fructose interferes in spray tests for the 7-carbon sugars. The fructose interference can be removed by the same fermentation technique.

Generally, there are relatively greater quantities of the fermentable than the non-fermentable sugars in plants. Therefore, it is of considerable advantage to use the fermentation procedure so that larger amounts of the nonfermentable sugars can be used without overloading the paper.

Chromatographic terminology.—A review of the literature on paper chromatography will show that the word "develop" has been used to denote two different processes. In some papers it refers to the use of the solvent mixture for separating the sugars on the paper chromatogram; in other papers it refers to the reagents used for staining the sugar spots on the chromatogram. The authors have therefore refrained from using this term and have used the words "irrigation" and "spray," defined as follows:

"Irrigation" refers to the flow of the solvent mixture on the paper chromatogram to partition the different sugars.

"Spray" refers to the reagent used to react with the sugars on the chromatogram to form a colored spot, and also to the process of adding this reagent to the paper chromatogram.

"Descending paper chromatography" (5) refers to the procedure whereby one end of the paper is immersed in a solvent mixture contained in a trough and the other portion of the paper hangs freely from this trough, allowing the solvent to flow downward, resolving the unknown sugar mixture as the solvent front progresses and eventually drips from the end of the paper. In some sugar studies, the chromatogram may be stopped before the solvent front drips off the end of the paper.

"Ascending paper chromatography" (32) refers to the procedure whereby the irrigating solvent is placed in the bottom of the chromatographic cabinet, the paper is placed upright in the solvent in such a manner that the sugar spots are above the surface, and the solvent flowing upward resolves the sugar mixture.

"R_F value" (5) is a ratio obtained as follows: measure the distance the sample spot (use center of spot as terminal point of distance) has moved from its origin on the paper. Also, measure the distance the solvent

front has moved from the same origin. The quotient of "distance of spot movement"/"distance of solvent front movement" is the R_F value.

"R_Z value" is a ratio of the distance moved by two sugars. That is, if the control sugar is glucose, then R_Z value becomes "R_G value," and this value will be the quotient of "distance moved by the unknown spot"/"distance moved by the glucose spot." For example, tetramethyl *d*-glucose has been used for a reference compound (2).

"Chromatographic cabinets" are the sealed containers in which the paper chromatograms are suspended and irrigated with a solvent mixture, to effect the separation of the various sugars.

Description of chromatographic cabinets.—We have used stainless-steel cabinets contg two stainless steel troughs in which four full-size filter paper sheets ($18\frac{1}{2}'' \times 22''$) can be irrigated. These cabinets are designed for descending chromatography by which method long periods of irrigation can be used without disturbing the chromatograms. They are used when a large number of samples are to be chromatographed simultaneously, or for collecting a sufficient quantity of a sugar for addnl study. The troughs are 65 cm long. The sides of the troughs are tapered so that the top width is ca 7 cm and the bottom width is ca 6 cm; the depth is ca 4 cm. It is desirable to have a small flange on the edge of the troughs in addn to the tapered sides because it is necessary for the paper to hang free from the side of the trough at some point above the origin spots of the sugars. The end of the paper which is to be immersed in the solvent trough is bolted (preferably with stainless steel bolts) between two 1-in. strips of stainless steel which add sufficient weight to hold the paper in the trough.

A well-fitted lid is necessary to maintain vapor equilibrium in the cabinet. There should be a hole in the lid directly over each of the troughs through which the irrigating solvent can be added. The holes are tightly sealed during the period of irrigation.

Borosilicate precipitation jars with indented handles make very satisfactory cabinets for general use. A strip of window glass can be placed on the handles and sealed in place to support the solvent trough. For this type of cabinet, a borosilicate baking dish can be used as the solvent trough. Also, a white enameled instrument tray ($9 \times 5 \times 2$ in. deep) can be used for solvent mixtures that do not contain acids. However, any fracture in the enamel on this type of tray, exposing the iron core, will produce rust which will cause streaking on the paper chromatogram. A piece of window glass, or preferably a desiccator lid with a hole for a stopper, is used for a closure. Split rubber tubing is used for a gasket.

Preparation of cabinet and chromatogram for irrigation.—The solvent mixt. is added to the cabinet 4–24 hrs in advance of actual use. When a two phase solvent mixt. is used, such as H_2O-satd butanol, the H_2O-rich phase is placed in the bottom of the cabinet, and the butanol phase is placed in the trough. When a one phase mixt. is used, a portion of it should also be placed in the bottom of the cabinet. Removing the lid to insert the paper disturbs the vapor equilibrium in the cabinet, but this is not a serious factor where chromatograms are irrigated for more than 16 hrs. This problem can be avoided, however, by loading the bottom of the cabinet with part of the irrigant, placing the paper in the dry trough, and sealing the cabinet. Later (4–24 hrs), the solvent mixt. is introduced into the trough through a hole in the lid of the cabinet (9, 11).

Whatman No. 1 filter paper is satisfactory for the chromatographic separation of sugars (19). For qualitative paper chromatography, it has not been found neces-

sary to pretreat the paper for any impurities that might be present. However, contamination from handling of the paper should be avoided.

As a guide for spotting the samples and known sugars, a penciled line is drawn parallel to and about 7–8 cm from one end of the paper. This distance may vary with the depth of the trough to be used. It is imperative that the position of the line be placed so that when the paper is suspended in the trough, the sugar spots are outside and below the edge of the top of the trough. Division marks about 2 cm apart are made on this line. The other end of the paper may be triangulated with the apex at the center of the sheet, or it may be trimmed with pinking shears to give a saw-toothed effect. This will promote more even drainage and, therefore a more uniform movement of the solvent down the paper. It is reported that this also permits a faster movement of the solvent front (11). The paper is now ready for the application of the sugar spots. They are placed on the division marks (2 cm apart) so that there will be sufficient space to allow movement down the paper without contamination from one another.

The known sugar solns are prepared in concns of about 3%. In general, if a crystal of thymol is added to each of the sugar solns they will remain stable, under refrigeration, for several months. Some sugars, such as galactose, maltose, sucrose, and especially some of the ketoheptoses, eventually change when kept under such conditions and they will then reveal more than one spot on the paper chromatogram.

Applications of known sugar solns and plant extracts are made on the paper by means of a platinum wire loop. A set of loops that will spot volumes from 1 to 5 microliters are desirable. Such loops are commercially available or they may be formed from Pt wire. After the application of each soln, the loop must be thoroly rinsed in distd H_2O, and as a further precaution it may be heated to redness in a gas flame. A practical amount may be from 2 to 3 microliter spots contg 20–50 mmg of each component sugar. This is not considered to cover the workable range, because both greater or smaller amounts may be necessary with an unknown soln, and only trial and error will determine the proper quantity. To increase the concn on the unknown sample spot, multiple applications may be made on the same spot. However, to avoid increased diffusion of the spot area, each applied spot should be thoroly dried (at room temperature) prior to the superimposition of the next application. Also, the spots should be allowed to dry thoroly before the paper is placed in the chromotographic cabinet.

Solvent mixtures for irrigation.—Unfortunately, no solvent is known that will distinctly separate all sugars on the paper chromatogram. This phase of the technique has received considerable attention and various organic solvent mixtures have been recommended for the separation of certain sugars or classes of sugars (10, 11). However, each investigator still must determine the most suitable irrigant for his particular problem. In fact, most of the irrigants and sprays mentioned are modifications of the mixtures originally reported.

If the solvents used in the irrigants are not of high quality, purification may be necessary (20). Freshly redistilled ethyl alcohol and distd H_2O should be used. Impurities in the reagents may produce streaked chromatograms, or they may add spots of unknown identity to the finished chromatogram.

The following discussion of solvents is based on their use at about 25°C. Recent work (9) has shown that faster and better separations may be obtained with some irrigants at raised temperatures, up to 37°C. From a study of various solvent mixtures, the following have been chosen as applicable to the type of work discussed in this paper; each is more suitable for a given purpose:

A solvent mixt. found most useful for the initial investigation of plant extracts is one of 10 parts 1-butanol, 1 part ethanol, and 2 parts H_2O (17). The difficulty of

a second phase is generally avoided if this mixt. is prepared by mixing the butanol and ethanol and then adding the H_2O. It should be shaken thoroly before using. Under normal conditions chromatograms are irrigated with this mixt. for periods of 48–72 hrs. To obtain a definite separation of such sugars as galactose and glucose, at least 72 hrs is necessary. This solvent mixt. is also excellent for long periods of irrigation, 4–45 days or more, when investigating oligosaccharides (25, 26, 28). Well-defined spots are obtained, since the diffusion of the sugars with this irrigant is not great. Mannose, fructose, and sorbose will not separate distinctly with this mixt., regardless of the time of irrigation. A more definite separation of mannose and fructose can be made by replacing the H_2O fraction of the mixt. with a satd soln of boric acid. Apparently the boric acid does not affect the mannose, but it does form a complex with fructose, giving the latter sugar completely different chromatographic properties. The use of boric acid in the irrigant mixt. may be of value in identifying other sugar compounds on the chromatogram (4).

Another useful irrigant is a mixt. of 8 parts ethyl acetate, 2 parts pyridine, and 1 part H_2O (11). It is about three times faster in resolving power for sugars than the butanol-ethanol-H_2O mixt. It will provide considerable information on 16-hr chromatograms. Galactose and glucose can be separated within this period of time. Freshly prepared mixtures should be used, because the ethyl acetate will slowly hydrolyze.

A third mixt. consists of 3 parts benzyl alcohol, 1 part glacial acetic acid, and 3 parts H_2O; two phases are formed (11). After it has been thoroly shaken and allowed to stand for several hrs, the H_2O-rich phase is added to the bottom of the chromatographic cabinet; the alcohol-rich phase is added to the trough. Mannose and fructose can be distinctly separated on the chromatogram with this solvent. It is considerably slower in resolving power than the aforementioned mixt. However, much information can be obtained after a 24 hr period of irrigation. It should be freshly prepared because the alcohol in the mixt. will eventually esterify.

Water-satd 1-butanol may be used as an irrigant (19). The alcohol should be agitated thoroly with excess H_2O and allowed to stand for 16–24 hrs, during which time the excess H_2O should separate from the H_2O-satd solvent. The excess H_2O may be removed by means of a separatory funnel. This irrigant should be prepared and used in a room maintained at a nearly uniform temperature. A tangible decrease in temperature will cause pptn of H_2O from the mixt., resulting in a streaked chromatogram, and similar results will be obtained if excess H_2O is not removed from the mixt. Such chromatograms cannot be correctly interpreted (24). These precautionary measures apply to any organic solvent that is to be satd with H_2O.

Procedure for locating the sugar spots on the chromatogram.—On completion of the irrigation period, the chromatogram is removed from the cabinet, suspended from hooks in a hood by means of large (1¼″) binder clips, and dried at room temperature, instead of at elevated temperatures, to avoid the hazard of overheating some sugars. Chromatograms contg such solvents as butanol and ethanol are completely dry within 1 hr or less. Papers irrigated with such solvents as collidine or phenol should be dried for a period of 24–48 hrs. Papers irrigated with mixtures contg pyridine should be thoroly washed with anhydrous ethyl ether; otherwise, during the subsequent heating period, the residual pyridine in the paper may react with some of the sugars present on the paper.

The spray reagent is applied as a fine mist, evenly distributed, over the entire chromatogram and on both sides of the sheet, until the paper is just wet. The addn of too much spray soln will cause drainage and the sugars will tend to migrate. In this laboratory the spray is applied to the paper by means of a glass atomizer, using air pressure.

Although the primary purpose of the spray reagent is to locate the position of the sugar spots on the chromatogram, it may also serve to identify a sugar or a group of sugars. There are many sprays described in the literature; some are good, some are of little value. Only those found most useful by the authors are described.

Silver nitrate spray.—This reagent consists of a mixt. of 100 ml 0.1 *N* $AgNO_3$, 5 ml concd NH_4OH, and 50 ml of 10% NaOH (7). It is sensitive to many reducing substances, and is effective for all reducing sugars, but reacts only slightly with nonreducing di- and higher saccharides. Because of its sensitivity to reducing substances that are not sugars, it is used primarily for exploratory work. For example, this spray will reveal the polyols (8); some of these alcohols travel at the same rate as some sugars.

Some spots will appear on the silver-sprayed chromatogram almost instantaneously at room temperature. Nevertheless, the sprayed sheet should be immediately heated 2–5 min. at about 105°C. to obtain the max. effect of this reagent. Immediately after heating, the chromatogram is washed with H_2O, followed by a rinse with 0.1 *N* Na thiosulfate to remove the excess Ag and prevent the entire chromatogram from becoming black. This rinsing procedure can be most conveniently accomplished by suspending the sheet with several binder clips above a sink, and adding the H_2O to the top of the sheet, (followed by the thiosulfate rinse) by means of an ordinary wash bottle. The delivery tube of the wash bottle should be capable of delivering a rather heavy stream of liquid. A final wash with ethanol will hasten the drying period of the chromatogram. With proper washing and subsequent drying, the spots are black on a gray or white background.

During the rinsing treatments the paper becomes very tender and must be handled gently to prevent tearing.

Resorcinol spray.—This reagent consists of 0.1% resorcinol in 0.5 *N* HCl (20) and is used for the detection of ketohexoses and any oligosaccharides and polysaccharides which contain a ketose sugar. The sprayed chromatogram is heated 2–5 min. at 85°C. The spots of the simpler sugars will appear first, colored either pink or deep red brown; the sugars of higher molecular wt will appear more slowly.

3,5-Dinitrosalicylic acid spray.—This reagent consists of 0.5% 3,5-dinitrosalicylic acid in 5% NaOH soln (10, 18), and is used for the detection of aldohexoses, ketohexoses, aldopentoses, methyl aldopentoses, and such disaccharides as maltose, lactose, cellobiose, gentiobiose, and melibiose. The sprayed chromatogram, heated 5 min. at 105°C., will show brown sugar spots on a yellow background. It is not sensitive to quantities of sugars less than 5 mmg. It does not detect such fructose-containing sugars as sucrose, raffinose, stachyose, and melezitose.

Aniline spray.—This reagent consists of two solns, A and B, which are mixed in equal vols at the time of use. Solution A contains 5 ml aniline (preferably redistilled over zinc), 200 ml glacial acetic acid, and 100 ml distd H_2O. Solution B contains 50 g distd trichloroacetic acid and 200 ml distd H_2O (22). The sprayed chromatogram is heated for about 5 min. at 105°C. The color formed with the pentoses will be purple. The color reaction with aldohexoses, ketohexoses, and other sugars will vary from a pale buff to a dark brown. The principal values of this spray are its sensitivity (1–2 mmg) and the apparently specific color produced with the pentoses.

Orcinol spray.—This reagent consists of 0.5 g orcinol and 15.0 g of trichloroacetic acid in 100 ml of H_2O-satd 1-butanol. The color reaction of this spray has been shown to be useful for the detection of ketoheptoses on paper chromatograms (1, 12). Sprayed chromatograms should be heated for 20 min. at 105°C. to obtain a maximum color reaction. The chromatogram will show rich blue spots for the ketoheptoses, and yellow spots for all of the other mono-, oligo-, and polysaccharides which contain a ketose sugar.

It should be mentioned that some of these spray reagents will give spots with

such substances as the uronic acids, phosphate esters, sugar acids, etc. However, since all of the solns studied are treated with ion-exchange resins, such interferences are removed.

INTERPRETATION OF THE CHROMATOGRAM

Some workers have identified the sugar spots on chromatograms by the use of R_F values. To establish the R_F value of a known sugar, of course requires replicate chromatograms to determine the tolerance within which this value will appear consistently. Unless the replicate chromatograms are prepared under the same fixed conditions, a given R_F value can not be reproduced. The limit to which these conditions can be controlled will be governed by such factors as constancy of paper quality, impurities in the reagents, substances other than sugars in the unknown material, constancy of composition of the irrigating solvents, temperature control, the distance between the starting point of the unknown spot and the source of the irrigant, and perhaps other factors (3, 6, 13–16, 24, 33). Because of the difficulty of duplicating conditions, some workers have selected an R_Z instead of an R_F value.

It is evident from the most recent literature that investigators are relying less upon R_F values for the identification of unknown substances. Instead of trying to duplicate a given chromatogram, known pure sugars are included in each chromatogram. Known and unknown spots in juxtaposition are examined further to verify their similarity. Any attempt to identify sugars by paper chromatography should include at least three different solvent mixtures for irrigants and as many spray reagents as are applicable (21). Two different sugars may appear to be identical on a chromatogram prepared with one irrigant, whereas a second, or a third, irrigant will separate them distinctly. Such data must be obtained before any definite interpretation is made. Also, as evident from the above discussion on spray reagents, a considerable amount of confirmatory evidence may be obtained from the use of supplemental sprays.

Another factor which must be considered is the rate of movement of sugars on the paper chromatogram. Generally, this rate is governed by the molecular size of the sugar; the larger the molecule, the slower the sugar will travel. For this reason, when a sprayed chromatogram reveals a circular or an elongated spot at or near the origin, a new series of chromatograms of the substance under investigation should be irrigated for longer periods of time (26, 28). Chromatograms of this series should be removed periodically from the cabinet and sprayed to determine if the spot at the origin is a single entity or a group of different substances. Unless this precaution is taken, slow-moving oligosaccharides, such as stachyose and raffinose, cannot be identified. Some of the chromatograms of such a series should be removed from the chamber before the solvent front reaches the end of the paper, so that fast-moving sugars will not be washed off. Such sugars as glyceraldehyde, dihydroxyacetone, and some of the

pentoses and the hexoses can be lost if the solvent is allowed to drain from the paper for any appreciable length of time.

Additional techniques are useful, and sometimes necessary, for the confirmation of the identity of some sugars. The use of fermentation to remove certain sugar groups and the use of boric acid to form certain sugar complexes have been mentioned. In addition, epimerization studies may prove valuable for confirmatory work (9). A method for the identification of such ketose sugars as stachyose, raffinose, and sucrose is the treatment of the unknown sample spot on the paper (prior to the irrigation period) with a solution of invertase (27). If the unknown sample contains any of the ketose sugars mentioned above, the simpler sugars formed by hydrolysis will appear on the finished chromatogram. If the unknown is a polysaccharide, containing fructofuranoside linkages, this comparatively rapid procedure may not produce complete hydrolysis. Such material should be eluted from the chromatogram, hydrolyzed by the more conventional procedure, and rechromatographed. The application of the enzyme solution to the paper chromatogram is not necessarily limited to that of invertase.

The analyst need not limit his investigations to the procedures described herein. Exploratory work may produce a better irrigant or an improved spray for the particular problem under study. Paper chromatography is a flexible analytical tool which may be subjected to numerous variations in technique and reagents. Its final value depends upon the skill of the analyst in interpreting the results obtained by these various techniques.

REFERENCES

(1) BEVENUE, A., and WILLIAMS, K. T., *Arch. Biochem. and Biophys.*, **34**, 225 (1951).
(2) BROWN, F., HIRST, E. L., HOUGH, L., JONES, J. K. N., and WADMAN, W. H., *Nature*, **161**, 720 (1948).
(3) BURMA, D. P., *J. Indian Chem. Soc.*, **28**, 631 (1951).
(4) COHEN, S. S., and SCOTT, D. B. M., *Science*, **111**, 543 (1950).
(5) CONSDEN, R., GORDON, A. H., and MARTIN, A. J. P., *Biochem. J.*, **38**, 224 (1944).
(6) HANES, C. S., and ISHERWOOD, F. A., *Nature*, **164**, 1107 (1949).
(7) HEFTMANN, E., and LEVANT, A. J., *J. Biol. Chem.*, **194**, 703 (1952).
(8) HOUGH, L., *Nature*, **165**, 400 (1950).
(9) HOUGH, L., JONES, J. K. N., and WADMAN, W. H., *J. Chem. Soc.*, p. 1702, 1950.
(10) JEANES, A., WISE, C. S., and DIMLER, R. J., *Anal. Chem.*, **23**, 415 (1951).
(11) JERMYN, M. A., and ISHERWOOD, F. A., *Biochem. J.*, **44**, 402 (1949).
(12) KLEVSTRAND, R., and NORDAL, A., *Acta Chem. Scand.*, **4**, 1320 (1950).
(13) KOWKABANY, G. N., and CASSIDY, H. G., *Anal. Chem.*, **22**, 817 (1950).
(14) ———, *ibid.*, **24**, 643 (1952).
(15) MARTIN, A. J. P., *Partition Chromatography in Annual Review of Biochemistry*, **19**, 517–542, Annual Reviews, Inc., Stanford, California, 1950.
(16) MARTIN, A. J. P., and MITTELMANN, R., *Biochem. J.*, **43**, 353 (1948).
(17) MCCREADY, R. M., private communication.

(18) McCready, R. M., Walter, E. D., and Maclay, W. D., *Food Technol.*, **4**, 19 (1950).
(19) Partridge, S. M., *Nature*, **158**, 270 (1946).
(20) ———, *Biochem. J.*, **42**, 238 (1948).
(21) ———, *Biochem. Soc. Symposia (Cambridge, Eng.) No. 3*, Cambridge University Press, 1950, pp. 52–61.
(22) Reid, W. W., *J. Sci. Food Agr.*, **1**, 234 (1950).
(23) Roseman, S., Abeles, R. H., and Dorfman, A., *Arch. Biochem. and Biophys.*, **36**, 232 (1952).
(24) Smith, E. L., *Biochem. Soc. Proc.*, 1952, p. xxxvi. Bound with *Biochem. J.*, 50(5), (March, 1952).
(25) White, L. M., and Secor, G. E., *Arch. Biochem. and Biophys.*, **36**, 490 (1952).
(26) ———, *ibid.*, **43**, 60 (1953).
(27) Williams, K. T., and Bevenue, A., *Science*, **113**, 582 (1951).
(28) ———, *Cereal Chem.*, **28**, 416 (1951).
(29) ———, *This Journal*, **34**, 817 (1951).
(30) Williams, K. T., Potter, E. F., and Bevenue, A., *ibid.*, **35**, 483 (1952).
(31) Williams, K. T., Potter, E. F., Bevenue, A., and Scurzi, W. R., *ibid.*, **32**, 698 (1949).
(33) Wynn, V., *Nature*, **164**, 445 (1949).

ASH DETERMINATIONS IN FOODS WITH AN ALKALINE BALANCE

VI. REACTION OF SODIUM CARBONATE WITH CALCIUM PHOSPHATES IN THE ASHING OF MILK

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Experiments dealing with the remarkable reactions between mixtures of pure calcium phosphates and alkali carbonates at ashing temperatures were published in 1943 (1). In brief, they showed that under certain conditions, sodium and potassium carbonates reacted with calcium phosphates to form complex alkali-containing, water-insoluble compounds; this contradicts the usual conceptions of the water solubility of the alkali salts. The next step would naturally be the extension of the experiments to the ashing of typical agricultural products which contain calcium, sodium, potassium, and phosphorus, to observe if such reactions have their counterpart when natural products are ashed.

Milk is a product suitable for such experiments. Its ash contains both sodium and potassium; it has a generous proportion of calcium and phosphorus and little magnesium to confuse the calcium reactions. It also contains sulfates and chlorides. These anions are more volatile than phos-

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phate; it remains to be seen if they can affect the reactions of the phosphous pentoxide and the bases.

Milk and milk products sour readily and the lactic acid produced is frequently neutralized with alkaline earth hydroxides or sodium carbonates. Sodium lactate burns to sodium carbonate at ashing temperatures. The effect should be, in the main, as if calcium phosphates were heated with sodium carbonates as in the experiments cited. Unneutralized milk has an alkaline balance and contains both sodium and potassium compounds; it would be expected, in the light of previous experiments, that part of these bases would be found in the water-insoluble ash. Hillig (2) has noted that part of the sodium added to milk in the form of sodium lactate is rendered insoluble, and is not recovered in the water-soluble ash. Neutralization would increase the alkali/phosphate ratio in milk ashes in proportion to the degree of neutralization. It was shown previously that the reactions described were sensitive to both temperature and the alkali/phosphate ratio. Therefore, the factors governing the previously described experiments would be expected to prevail in these.

The present experiments are not intended primarily to provide methods for the detection of sodium neutralization, but to study the fundamental reactions that the ash constituents of milk, as representative of a general food, undergo in the ashing process, and the effect a sodium neutralizer can have. The temperature ranges used in the previous experiments are impossible here because 500°C. is about the minimum temperature that will produce clean milk ashes. However, it was predicted that curves illustrating the reaction in milk ashes would duplicate, more or less, the temperature charts of the previous publication between 500 and 700°C.

EXPERIMENTAL

Two dried skimmed milks* made from the same original slightly soured milk (approximately 0.1% lactic acid by titration) were on hand. One had been dried without neutralization, and the other had been slightly over-neutralized with soda before drying. The ash content and composition of the ash (550°C.) of these two milks are given in Table 1.

Two-gram portions of these milks were ashed at 500°C. When necessary, the carbonaceous ash was disintegrated with H_2O, dried down, and re-ashed at 500°C. until a clean carbon-free ash resulted.

The ashes were then heated at selected temperatures from 500 to 700°C. for 15 min. (Neutralized milk ashes have a tendency to sinter or fuse at ca 600°C.) After each ash had been heated for 15 min. at the selected temperature, the Pt dish was covered with an aluminum cover, cooled in a desiccator, and weighed. The ash was then treated with about 50 ml of H_2O and the mixt. heated to incipient boiling for about 5 min., or heated on an active steam bath for ca 10 min. During this time the insol. residue was pulverized with a rubber-tipped glass rod. The mixt. was then transferred to a 100 ml volumetric flask with hot H_2O, shaken well, and allowed to

* Prepared under the personal observation of Fred Hillig, Division of Food, Food and Drug Administration.

TABLE 1.—*Composition of ash of two samples of dried skimmed milk*

	A	B
	DRIED SKIMMED MILK FROM SLIGHTLY SOURED MILK, DRIED WITHOUT NEUTRALIZATION	DRIED SKIMMED MILK FROM MILK A, SLIGHTLY OVER-NEUTRALIZED BEFORE DRYING
	per cent	*per cent*
Ash at 550°C.	7.95	10.52
CaO	24.78	18.33
K_2O	22.80	17.72
Na_2O	9.75	22.88
MgO	3.01	2.00
P_2O_5	27.97	22.01

stand on the steam bath with intermittent shaking for ca 10 min. The contents of the flask were then cooled, made to mark, and filtered on a dry filter; the filtrate was returned to the filter until a clear or almost clear filtrate resulted.

Water-soluble phosphates were detd in the filtrate by the volumetric phosphomolybdate method. The H_2O-insoluble residue was transferred from the flask to the filter with 100 ml of hot H_2O and was washed on the filter with another 100 ml of hot H_2O. The H_2O-insoluble residue and filter paper were ignited at ca 550°C. and then heated for 15 min. at 700°C. After weighing, the H_2O-insoluble residue (hereafter referred to as insoluble ash) was dissolved in dil. HCl and made to 100 ml in a volumetric flask. Sodium and potassium oxides were detd in aliquots of this soln by the magnesium-uranyl acetate and sodium colbaltinitrite methods respectively. The data are shown in Fig. 1.

The weight of the total ash (curve A, unneutralized milk) decreased slowly and regularly with increased temperature, which checks the data first reported in the third paper of the series (3). The low water soluble P_2O_5 at nearly all temperatures seems noteworthy and contrary to previous experience. The water-insoluble sodium amounts to about 70–80 per cent of that available in the milk; it is more than twice the water-insoluble potassium although the total potassium in the milk is more than twice the total sodium. This seems to mean that, under these circumstances, sodium is more reactive than the potassium. Increase in temperature caused only a very moderate increase in the water-insoluble sodium and potassium. The increase in the insoluble ash with temperature reflects the changes in the water-insoluble alkali curves. These data show that the insoluble ash of unneutralized milk contains some of the complex calcium-alkali-phosphates previously described.

The B curves show the results obtained with the neutralized dried skim milk. As expected, the total ash is higher than that of the unneutralized milk. Increased temperatures caused weight changes in the total ash similar to that of the unneutralized milk. The soluble P_2O_5 was practically the same in both milks—both very low. The insoluble potassium is slightly greater in the neutralized milk, and increased temperature increased it slightly; the two curves are practically parallel and show low values for

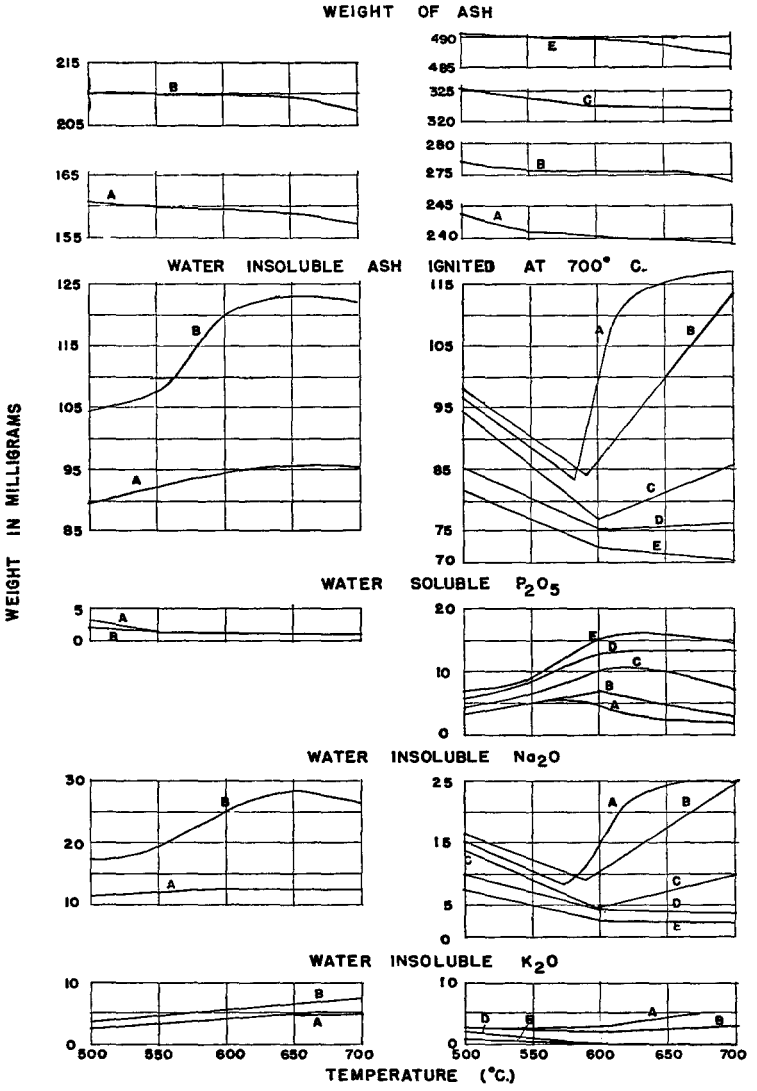

FIG. 1 FIG. 3

FIG. 1.—(Left-hand portion). Composition of the ash of 2 g portions of dried skim milk and neutralized dried skim milk. *Curves A:* Unneutralized skim milk; *Curves B:* From same milk as A, but neutralized with soda before drying.

FIG. 3.—(Right-hand portion). Composition of the ash from 2 g portions of neutralized dried skim milk with added sodium lactate. *Curves A:* Neutralized dried skim milk with added sodium lactate corresponding to 0.25% lactic acid in the original fluid milk. *Curves B, C, D, and E:* Same with added sodium lactate corresponding to 0.52, 0.85, 1.41, and 2.43% lactic acid, respectively.

potassium. Fig. 1 shows that the greatest difference between the ashes of the two milks lies in the sodium fraction of the insoluble ashes, and in the decided increase with temperature, particularly above 550°C. The practical disappearance of the water-soluble P_2O_5 and the decided increase in insoluble sodium above 550°C. distinguish these milk experiments from previous ones where pure calcium phosphates and sodium carbonate were used. Different and as yet unexplained reactions must have occurred.

There was considerable "scatter" in the sodium oxide and water-insoluble ash determinations. This difficulty in duplication is a reminder of similar difficulties in previous work with the pure compounds. "Crusting" was prevented by the leaching with water and crushing of the carbonaceous ashes before re-ashing to a clean ash. Particle size of the ash at 500°C. and before the final heating at the selected temperature, seemed to have an influence on the final results. Small clumps of ash and carbon were disintegrated as much as possible with a rubber-tipped glass rod, but such disintegration could not be uniform in any one experiment or from one experiment to another. No doubt the effectiveness of the contact between the insoluble calcium phosphates and the sodium carbonate from the added sodium lactate affected the degree of penetration of the sodium into the calcium phosphate molecule, and therefore some differences in the composition of the insoluble ash is not unexpected. Another factor that might not be uniform was the hot water extraction. Hydrolysis to different degrees might occur during the extractions, and the efficiency of extraction may vary. The "scatter" of the points on some of the curves is therefore not too surprising. The lack of high reproducibility in determining water-soluble and water-insoluble ash, their respective alkalinities, and their phosphorus content has often been noted in food analysis during the past twenty-five years.

The finding that both sodium and potassium can be introduced into the insoluble ash of milk agrees with the experience of Hillig (2) who ashed only at 550°C. and therefore did not demonstrate that temperature also was a factor governing the amount of water-insoluble alkali. It was found previously that the character of the calcium-alkali-phosphorus complex could be varied by different alkali/phosphate ratios and temperatures when working with pure compounds. The results shown so far demonstrate that this is also true in the case of milk ash.

Figure 2 shows the percentage of carbon dioxide in some of the water-insoluble ashes of these milks and how heat affects its retention. The curves show that the introduction of a sodium neutralizer into the milk forces carbon dioxide, as well as sodium and potassium, into the water-insoluble ash. When the neutralized milk ash was heated to 600°C., even more carbon dioxide was found in the water-insoluble ash. This checks with the increased insoluble ash and sodium oxide shown in Fig. 1. The carbon dioxide in this case also seems to be more heat-resistant between 600 and 800°C. This appears to be significant and suggests the presence in the

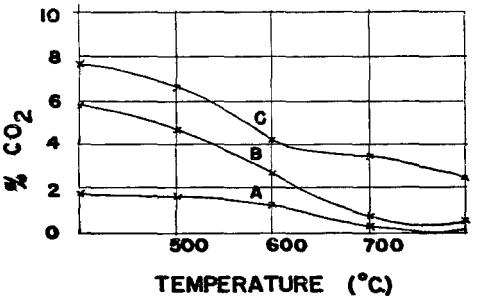

FIG. 2.—Per cent CO_2 in water-insoluble ash of unneutralized and neutralized dried skim milk. Each point represents 15 min. heating. *Curve A:* milk A, Table 1, ashed at 500°C. *Curve B:* milk B, Table 1, neutralized, ashed at 500°C. *Curve C:* milk B, Table 1, ashed at 600°C.

water-insoluble ash of some of the same kind of heat resisting carbonated calcium-sodium phosphates or "rhenanites" (as for example $4CaNaPO_4 \cdot Na_2CO_3$) described in the previous publication (1).

Sodium carbonate is used sparingly in the manufacture of dried milks for human use. Considerably larger quantities of alkalies or alkaline earths are used in the neutralization of sour cream before it is churned into butter. To learn what would happen if milk products were neutralized with greater amounts of sodium carbonate before ashing, various quantities of sodium lactate solutions were added to reconstituted milk made from two grams of slightly over-neutralized milk (sample B, Table 1). The amounts of added sodium lactate corresponded to the lactate that would have been produced by the neutralization of 0.25, 0.52, 0.85, 1.41, and 2.43 per cent of lactic acid produced in the original slightly over-neutralized fluid milk. The addition of sodium lactate to milk is not exactly comparable to the neutralization of sour milk. The souring of milk causes changes in its mineral composition that neutralization may not exactly reverse. It is impossible to follow the souring of milk by neutralization and then make the necessary analyses within reasonable time limits and therefore the addition of sodium lactate to a reconstituted milk from a supply of dried milk was the best substitute available. (The author hopes that these experiments will be sufficiently realistic to show the general effect of the addition of alkali and temperature on the composition of milk ash, particularly the water-insoluble portion.) The simulated acidities in some of these experiments are greatly in excess of those expected in commercial neutralized dried milks but could well be actually experienced in the analysis of dried butter milks if the creams had been neutralized with soda.

Figure 3 illustrates the data obtained. An inspection shows that the total ash increased in proportion to the added lactate, and that increased tem-

perature produced small decreases in weight in the same fashion shown in Fig. 1. But the composition of the water-insoluble ash is most emphatically influenced by the added alkali and by temperature, especially in the neighborhood of 600°C. The ashes sintered or fused near this temperature. Ashing temperatures are generally selected below the fusing temperature because it is difficult to burn carbon in a fused ash. In this case the carbon had been previously burned at 500°C. and did not cause trouble. The platinum dishes were more or less stained in the ashing, especially at 650–700°C., but this was tolerated because temperatures above 600°C. were the most interesting in this investigation.

The first point to be observed in Fig. 3 is that it was again difficult to duplicate experiments exactly. A smooth curve through all the points could not be drawn and estimated average curves are shown.

Potassium seems to be a minor constituent of the water-insoluble ash of milk and neutralized milk. With only small amounts of added sodium lactate (Fig. 1 and Curves A and B of Fig. 3), the potassium in the water-insoluble ash is appreciable, but rather low. Increased simulated neutralization causes it to disappear, especially above 600°C. This seems to be consistent with observations made in the previous paper (1). Increased alkali and higher temperatures apparently cause the insoluble calcium-potassium phosphate compound to be unstable.

The temperature of 575–600°C., where the milk ash begins to sinter, seems to be a very important one and indicates a change in the reactions of the inorganic compounds present in the water-insoluble ash. The insoluble ash, and sodium oxide "B" curves of Fig. 1 do not drop below the origin at 500°C., but in Fig. 3 the curves do drop, sometimes quite precipitately, between 500 and about 600°C. Above 600°C. some of the curves rise again more or less sharply to 700°C., the highest temperature employed (potassium carbonate volatilizes above 700°C.). This is particularly true on curves A and B but not on D and E. Curve C is intermediate. The soluble phosphorus increased, and then decreased on the first three curves; again the change of direction occurred at about 600°C. The rise was more rapid on curves D and E, but above 600° there was no decrease. Such changes were not experienced in previous investigations. The temperature of 575–600°C. seems to be critical. In the author's opinion, the curves indicate that neutralized milk ash at 600°C. is in a state of great flux. Before the reactions and the compounds produced in the water-insoluble ash of milk and neutralized milk could be further interpreted, it seemed desirable to learn more about the carbon dioxide in the insoluble ash.

Insoluble ashes were prepared in the same manner as those illustrated in Fig. 3. Various quantities of Na lactate solns were added to 2 g of unneutralized dried skim milk in a weighed Pt dish. The solns of lactate and the reconstituted milk were

evapd and ashed at 500°C. Several leachings with H_2O, evapns, and ashings were necessary to secure a carbon-free ash. The ashes were heated to 120°C. in an oven to prevent decrepitation on further ashing. Before the final heating at 500°C., the ash was disintegrated into a fine powder with a rubber-tipped policeman. It was next heated for 15 min. at the selected temperatures, dispersed with small vols. of H_2O, and then boiled gently with about 50 ml of H_2O for 5 min. The insoluble ash was filtered on a rapid filter and washed on the filter with hot H_2O till the filtrate amounted to 200 ml. The insoluble ash was washed from the filter back into the Pt dish with H_2O and the H_2O was evapd. The dish was then heated at 120°C. in an oven, and then in the muffle at 250°C. to remove the H_2O without decrepitation. After heating in the muffle, an aluminum cover was placed over the Pt dish, it was cooled in a desiccator, and the wt of the insoluble ash was obtained. The powd. insoluble ash was then mixed well in the dish with a glass rod, and its percentage of CO_2 was detd by the Van Slyke method. Milligrams of CO_2 in the H_2O-insoluble ash could then be calcd. The data obtained are shown by the curves of Fig. 4.

A notable characteristic of Fig. 4 is the difference in the carbon dioxide content of the insoluble ash of unneutralized milk, commercially neutralized milk, and milk with greater degrees of simulated neutralization. The insoluble ash of unneutralized milk contained very little carbon dioxide at any temperature. Commercial neutralization with sodium carbonates, as well as very moderate simulated neutralization, immediately introduced quite appreciable amounts. (Possibly this difference could be the basis for another method for the detection of sodium neutralization in milk or other dairy products, but the main interest of the moment is its help in studying the nature of these ash reactions.) Increase of temperature from 500° to 550° or 600°C. increased the insoluble carbon dioxide, but

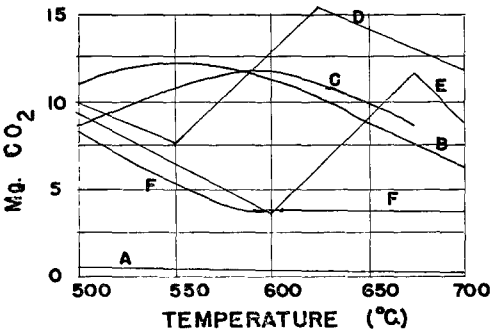

FIG. 4.—CO_2 in water-insoluble ash of dried skim milks. Sodium lactate added to unneutralized milk to simulate neutralization. *Curve A:* Unneutralized dried skim milk; *Curve B:* Milk neutralized with Na_2CO_3 (commercial production); *Curve C:* Same milk as A with added sodium lactate equivalent to 0.48% lactic acid in original fluid milk; *Curve D:* Same milk as A with added sodium lactate equivalent to 0.79% lactic acid in original fluid milk; *Curve E:* Same milk as A with added sodium lactate equivalent to 1.21% lactic acid; *Curve F:* Same with added sodium lactate equivalent to 2.37% lactic acid.

temperatures beyond 600°C. again depressed it (but not to zero). The fact that these temperatures mark a decided increase in the insoluble sodium favors the assumption that in these experiments the carbon dioxide is not all combined directly with sodium; part of it might be in combination with calcium as calcium carbonate which decomposes rapidly above 600°C. With a somewhat greater degree of simulated neutralization, the carbon dioxide content varies greatly with changes in temperature, but in accord with the fluctuations of the insoluble sodium curves of Fig. 3. This similarity is striking and can mean only that the carbon dioxide is directly connected as sodium carbonate with at least part of the insoluble sodium in the insoluble ash, notwithstanding the solubility of Na_2CO_3 in water. A still higher degree of neutralization causes a decided simultaneous decrease in both sodium and carbon dioxide in the insoluble ash of milk from 500 to 600°, with a leveling off above 600°C. These fluctuations in insoluble sodium and carbon dioxide are connected with the similar increase and decrease of the soluble P_2O_5 at the same temperatures, for the same degree of neutralization. These factors must be interrelated, and show that there is a change in the reactions of the constituents of the water-insoluble milk ash as the temperature of the ashing and the degree of neutralization, expressed by the alkali/phosphate ratio, are increased.

Unneutralized milk has a small alkaline balance. Its water-insoluble ash contains both sodium and a small amount of potassium. The evidence so far leads to the hypothesis that the insoluble ash is essentially $CaNaPO_4$ and $CaKPO_4$ with little or no soluble phosphates or carbonates. Perhaps their production can be visualized as follows:

(1) $$2CaHPO_4 + Na_2CO_3 = 2CaNaPO_4 + H_2O + CO_2$$

(2) $$2CaHPO_4 + K_2CO_3 = 2CaKPO_4 + H_2O + CO_2$$

There appears to be little difference whether the temperature is 500 or 700°C.

A very moderate neutralization, as in the commercial production of dried skim milk from slightly soured fluid milk, makes practically no change in the amount of soluble phosphorus or insoluble potassium produced, but increases the insoluble sodium and carbon dioxide materially at 500°C. Increased temperature increases the insoluble potassium only slightly and for practical purposes we can neglect it, especially for higher temperatures and greater degrees of neutralization. The greatest interest is in the composition of the insoluble sodium compounds.

Curves A and B of Fig. 2 show that the carbonate found in the insoluble ash of unneutralized and slightly neutralized milks is not very stable at temperatures above 600°C. But the insoluble ash of a slightly neutralized milk heated to 600°C. was much more stable (curve C, Fig. 2). It is the author's belief that the insoluble ash of a neutralized milk ashed at 500°C. is a mixture of $CaNaPO_4$, $CaKPO_4$, and to some extent, $CaCO_3$, the latter

probably derived from the reaction of sodium or potassium carbonates with the calcium chloride also found in milk.

(3) $$CaCl_2 + Na_2CO_3 \rightleftharpoons CaCO_3 + 2NaCl$$

Another reaction or reactions at 550°C., and particularly above it, must begin to force more sodium into the insoluble ash and to produce more heat resistant carbonates as follows:

(4) $$4CaHPO_4 + 3Na_2CO_3 = 4CaNaPO_4 \cdot Na_2CO_3 + 2H_2O + 2CO_2$$

(5) $$4CaHPO_4 + 3K_2CO_3 = 4CaKPO_4 \cdot K_2CO_3 + 2H_2O + 2CO_2$$

These are the basic double phosphates or "rhenanites" that have been discussed before. Neither reaction 4 nor 5 should produce any soluble phosphates, but only greater amounts of insoluble alkalies and heat-stable carbon dioxide which should in a measure account for the difference in curves B and C in Fig. 2. If the insoluble ashes represented by the higher temperature portion of curve B, Fig. 1, and curves B and C, Fig. 4 should be a mixture of one or both of these basic phosphates with calcium carbonate, the dropping of the carbon dioxide content noted at higher temperatures, without a corresponding drop in the insoluble sodium, might be accounted for.

The next points of interest are curves A and B on the insoluble sodium plot of Fig. 3 and curves D and E in Fig. 4. These represent intermediate neutralizations. A drop in these curves from 500° to 550° or 600°C. is followed by a sharp rise. The drop can be easily accounted for by postulating the formation of some hydroxyapatite according to:

(6) $$10CaHPO_4 + 6Na_2CO_3 = 3Ca_3(PO_4)_2 \cdot Ca(OH)_2 + 4Na_3PO_4 + 4H_2O + 6CO_2$$

This reaction was encountered in previous work. Data for soluble phosphates are shown in Fig. 3. The A, B, and C curves first rose, and then dropped from approximately 600° to 700°C. Residual soluble P_2O_5 was greatest with curve C. Such data could be accounted for by a limited production of trisodium phosphate at temperatures below 600°C., followed by a partial reaction that produced the basic mixed phosphates first encountered in Fig. 1. The insoluble ashes produced at 500–600°C. may contain quite a mixture of compounds, e. g., the mixed calcium, sodium, or potassium phosphates, basic mixed phosphates, hydroxyapatite, and calcium carbonate. It may be impossible to distinguish among all of them. At about 550–600°C., there is a most decided change. Whatever the nature of the insoluble ash up to 600°C., beyond this temperature the significant fact is the decided acceleration of the formation of insoluble sodium, with accompanying and corresponding carbonation. The carbon dioxide that is introduced is remarkably heat resistant. This can only mean a spectacular increase in the production of the basic mixed phosphates or rhenanites. The temperature of about 600°C., it will be remembered, is also the sintering point of milk ashes. This partial fusion of the

milk ash may greatly promote the formation of the rhenanites. Possibly part of this increase in the rhenanites may be due to reversion of the hydroxyapatite to basic mixed phosphates. By whatever reaction, there is no doubt about the decided formation of relatively stable basic sodium-calcium phosphate compounds between 600 and 700°C., if sufficient neutralizer has been added.

As the degree of simulated neutralization is increased still further, more soluble phosphates appear up to 600°C., but higher temperatures decrease rather than increase the soluble phosphates, insoluble sodium, and carbon dioxide. The formation of the rhenanites is suppressed and the insoluble ash is now essentially hydroxyapatite containing very moderate amounts of sodium and carbon dioxide. Such hydroxyapatites are well known.

We come now to a consideration of what part the chlorides and sulfur compounds of milk might play in the reactions of milk ash considered heretofore. Unneutralized, commercially neutralized, and simulated neutralized milks were ashed at temperatures from 500 to 700°C. and the water-insoluble ashes were tested for chlorides and sulfates. Most of the insoluble ashes contained no chlorides, and the few that did, contained only traces. It may be concluded that chlorine is not a material constituent of the insoluble ash of milk and probably takes but little part in forcing alkalis into the calcium phosphates of milk ashes.

Milk ash contains only between three and four per cent of sulfur calculated as the trioxide. The water-insoluble ash of unneutralized milk ashed at various temperatures between 500 and 700°C. contained no sulfur precipitable as barium sulfate. Similar ashes from neutralized and simulated neutralized milk did contain traces of sulfur as shown by small precipitates of barium sulfate weighing from 0.5 to 5.0 mg. It may be that sulfur can enter the calcium-sodium phosphate complex comprising the insoluble ash of agricultural products under some circumstances, but in the case of milk ashes its influence seems negligible.

Dairy products are neutralized with potassium carbonate only in rare instances, if ever, because of the high cost and the question of how it reacts in the water-insoluble ash of unneutralized milk is therefore of little practical importance. Milk ash does contain calcium-potassium phosphate, but the changes in reaction with degree of neutralization have not been investigated. No doubt they will be much the same as those experienced in the previous article (1).

Calcium and magnesium hydroxides are used singly or in admixture as neutralizers in dairy products. Hillig (2) demonstrated that calcium displaced the potassium and sodium in the water-insoluble ash of milk when calcium lactate was added before ashing at 550°C. The effect of temperature and ratio of added alkaline earth to phosphate can be determined only by experiments similar to those described in this paper.

The author also wishes to observe that neutralization is practiced in processing other foods besides milk products, for example the "dutching" of chocolate. The addition of magnesium acetate to flour, a food with an acid balance, before ashing and the subtraction of a MgO blank is a recognized analytical procedure, but the nature of the reactions that occur in the process are still unknown. The results are the same as those produced by a regular ashing, although logically they might not be. Some foods and food products may contain components that are alkaline in reaction and others that are acid. Ashing of the whole is really a partial or complete neutralization according to the relative proportions of the acid and alkaline portions, and reactions similar to those encountered here may occur. Perhaps the basis for a chemical determination of the presence of excess hulls in ground seeds, grains, or spices might be found by applying some of the experiences of this investigation.

The occurrence of sodium-calcium phosphate, potassium-calcium phosphate, basic sodium- or potassium-calcium phosphates, and hydroxyapatite in the water-insoluble ash of milk, under different conditions of ashing temperatures and alkali/phosphate ratios, can be shown by X-ray diffraction patterns. Such patterns can be checked against known preparations if they are available. Accordingly four insoluble ashes from milk were prepared and submitted to E. L. Gooden* for X-ray analysis. The insoluble ashes were prepared according to the following schedule:

A. Water-insoluble ash from unneutralized milk heated to 600°C. (curve A, Fig. 1).

B. Water-insoluble ash from unneutralized milk treated with sodium lactate solution equivalent to 2.37 per cent lactic acid in the original fluid milk. Heated to 600°C. (curves E and F, Figs. 3 and 4, respectively).

C. Water-insoluble ash from a commercially neutralized milk. Heated to 625°C. (curve B, Fig. 1).

D. Water-insoluble ash from unneutralized milk treated with sodium lactate solution equivalent to 0.79 per cent lactic acid in the original fluid milk. Heated to 625°C. (curves and A and D, Figs. 3 and 4).

In addition, diffraction patterns were made from a sample of "high temperature" sodium-calcium phosphate and one of hydroxyapatite obtained from W. L. Hill† (Fig. 5). Preparation A, a mixture of $NaCaPO_4$ and $KCaPO_4$, did not have a pattern at all similar to the high temperature (1400°C.) sodium-calcium phosphate of Hill (not shown). This was confusing until Hill recalled an English publication (4) containing patterns of two sodium-calcium phosphates, a "high temperature" and a "low temperature" preparation that differed materially in their diffraction pattern but were identical in chemical composition. Preparation A, a low temperature (600°C.) water-insoluble ash, had a pattern that was

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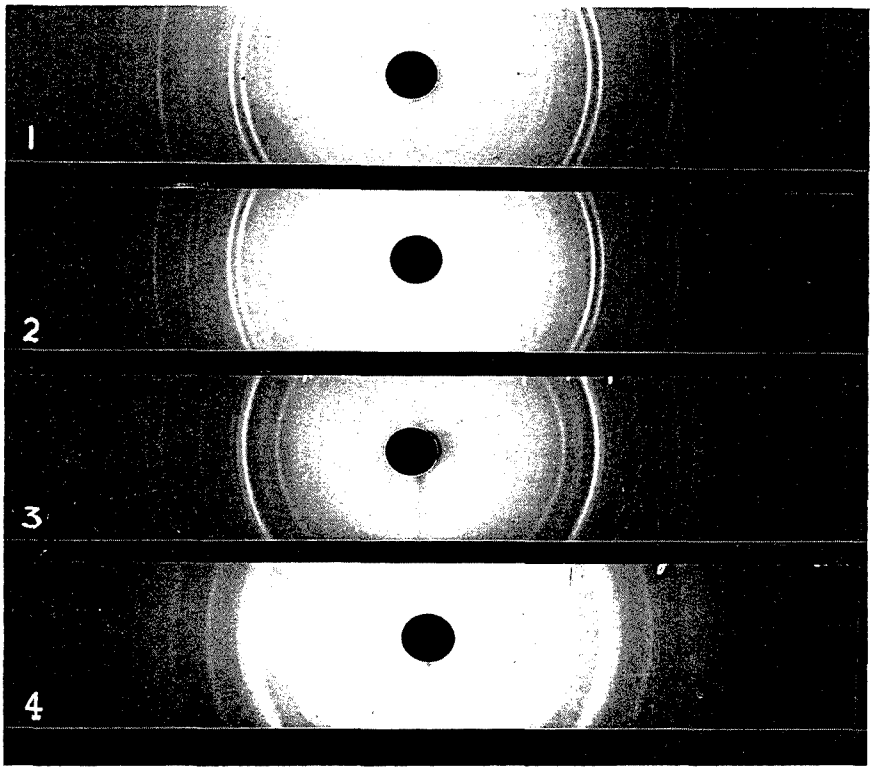

FIG. 5.—X-ray diffraction patterns. *Strip 1*, preparation A (water-insoluble ash from unneutralized milk, heated to 600°C.). *Strip 2*, preparation D (water-insoluble ash from unneutralized milk treated with sodium lactate equivalent to 0.79% lactic acid). *Strip 3*, hydroxyapatite (Hill preparation). *Strip 4*, preparation B (water-insoluble ash from unneutralized milk treated with sodium lactate equivalent to 2.37% lactic acid).

remarkably similar to the "low temperature" $NaCaPO_4$ of the English publication. Unfortunately, no sample of the low temperature variety was available to permit direct comparison of its diffraction pattern. Slight differences noted were taken as probably due to a small admixture of $KCaPO_4$.

Preparations C and D were almost identical and only D is reproduced (Fig. 5). It can be seen that pattern D bears a strong resemblance to pattern A but with small displacements. Apparently the sodium carbonate has entered into the alkali-calcium phosphate molecule, but the change in composition has resulted in only a small difference in crystal habit, as reflected by the small difference in the diffraction pattern. The compositional structure 4 $CaNaPO_4 \cdot Na_2CO_3$, suggests that the predominating

constituent is still $CaNaPO_4$ and no great change in crystal habit should be expected.

The diffraction patterns of preparation B and that of hydroxyapatite are seen to be very similar. The hydroxyapatite from the milk ash contains small intrusions of carbon dioxide but that of the Hill preparation does not. Any detectable differences in the two patterns may perhaps be ascribed to small differences in composition. It seems, then, that the assumption based on the chemical data here presented are corroborated in large part by the X-ray evidence.

SUMMARY

The water-insoluble ash of unneutralized milk contains mixed sodium-calcium and potassium-calcium phosphates with very little carbon dioxide. Sodium neutralizers introduce carbon dioxide into the insoluble ash. This carbon dioxide exists in the form of basic alkali-calcium phosphates or carbonated hydroxyapatite according to the degree of neutralization. Moderate neutralization may result in mixtures of hydroxyapatite, the above basic mixed phosphates and possibly small amounts of other compounds such as calcium carbonate as determined by temperature and the alkali phosphate ratio. The addition of still more sodium neutralizer produces an insoluble ash consisting essentially of hydroxyapatite containing small intrusions of sodium and carbon dioxide.

ACKNOWLEDGMENT

Grateful acknowledgment is due to E. L. Gooden, W. L. Hill, and S. B. Hendrick of the United States Department of Agriculture for assistance in the X-ray diffraction studies reported in this paper, and to F. J. Sabatino of this laboratory for drafting the figures.

REFERENCES

(1) WICHMANN, H. J., *This Journal*, **26**, 522 (1943).
(2) HILLIG, F., *ibid.*, **24**, 744 (1941).
(3) WICHMANN, H. J., *ibid.*, **25**, 481 (1942).
(4) *The Production, and Agricultural Value, of Silicophosphate Fertilizers.* Monograph No. 11.108, Ministry of Supply. London, (1951), page 77.

X-RAY EXAMINATION FOR THE DETECTION OF INTERNAL INSECT INFESTATION IN CORN

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Several recent reports (1–7) have been published on the problem of internal insect infestation of wheat. A similar problem exists for corn. Since the application of X-radiation appeared to be one of the most promising means of measuring internal insect infestation in wheat, similar techniques were applied to corn. It was also felt that a successful adaptation of X-ray procedures to corn would tend to show that these techniques could be applied generally to other food seeds.

Using the techniques described below, comparison was made between the recovery of insects by the cracking-flotation procedure which was considered to be the most reliable measure of insect infestation (1), and the amount of insect damage visible on the radiograph. As expected, one of the important phases of this study was the evaluation of the radiographic image as it related to internal insect damage and to normal seed structures.

PROCEDURES

CRACKING FLOTATION PROCEDURE

Mix grain to be examined by passing thru a Jones sampler, recombining the separations before each pass. After mixing, separate slightly more than 100 g and weigh to 100 g. Brush the samples (a small amount at a time) on a 5/8 in. No. 8 sieve, using a stiff-bristled brush to remove surface insects from sample.

Grind the screened corn in a Labconco (or equivalent) mill set at .062″. Transfer the cracked corn, including all the residue in the mill, to 2 l trap flask. Trap with gasoline, using 60% isopropyl alcohol (previously satd with gasoline) as the flotation medium, and filter on 10XX bolting cloth. If, on trapping off, a starchy residue remains, add an equal vol. of H_2O, add sufficient HCl to make 1% HCl, hydrolyze by heating, and transfer to bolting cloth with hot H_2O. Examine at 30×, counting only whole insects, insect heads, cast skins, and head capsules.

X-RAY PROCEDURE

Using a Westinghouse Model 475, grain inspection X-ray unit, the grain was placed 26 inches from the exit port, separated from the film (DuPont 504 or Eastman Type A) by a thin layer of opaque plastic. Radiographs were made by using an exposure of 5 milliamperes, 17 kilovolts, for 42 seconds. When corn kernels were unusually thin or thick, the exposure time was varied accordingly. The X-ray film was developed at 68°F. in Kodak X-ray liquid developer for 5 minutes with Eastman film, and 8 minutes with DuPont film. Compensation was made in developing time as the activity of the developer decreased.

INTERPRETATION OF THE RADIOGRAPH

The appearance of internal insect infestation in radiographs of corn closely resembles comparable damage in the radiographs of wheat (2).

However, because of the characteristic external and internal corn structures, there are numerous radiographic shadows, described below, which resemble insect damage. Shadows caused by plant structures were difficult (and at times impossible) to differentiate from those caused by insect damage. Suspect kernels were dissected to determine the cause of these shadows. The different areas of the corn kernel are shown in Figs. 1–9 and the nomenclature given in the figures is used in the following descriptions:

SHADOWS PRODUCED ON THE RADIOGRAPH BY NORMAL KERNEL STRUCTURES

(1) *Shadows at dent end of the kernel.*—(Fig. 1, and Fig. 10, Row 1). This portion of the kernel in "dent-type" corn invaginates, causing a shadow on the radiograph which may resemble insect tunneling.

(2) *Shadows resulting from the separation of the scutellum from the endosperm.*—(Fig. 2, and Fig. 10, Row 2). Such a separation rarely appears around the entire margin, as shown in Fig. 2, but only at one or more points. This separation causes a light gray radiographic shadow, often resembling fine insect tunneling. If insect damage is present in this area, it will probably be confirmed by the damage radiating from this margin and insect tunneling in the adjoining endosperm.

(3) *Shadows at the pedicel.*—(Fig. 3, and Fig. 10, Row 3). Radiographic shadows at this point may be due to the roughened grooves caused by the removal of the kernel from the cob. As shown in Fig. 3, this damage may extend back to and on either side of the scutellum. The extent of these tunnel-like shadows may be restricted to the very tip end or may extend some distance on either side of the scutellum.

(4) *Shadows appearing as transverse bands.*—(Fig. 4, and Fig. 10, Row 4). The exact cause of these shadows is not known but appears to be due to fractures in the endosperm. These tunnel-like bands are most commonly seen extending from one or both sides of the hypocotyl into the endosperm. The same type of shadow may be present at the top end of the plumule or even beyond the germ area. When the damage described crosses into the germ area, the intensity of the shadow decreases or may even disappear.

(5) *Shadows caused by shrinkage of the plumule and radicle from the scutellum.*—(Fig. 5, and Fig. 10. Row 5). The shadows here closely resemble those shown in Fig. 2 and rarely ever completely enclose the plumule/radicle margin, as shown in Fig. 5. The shrinkage may be confined either to the plumule or radicle.

(6) *Shadows caused by fracture lines.*—(Fig. 6, and Fig. 10, Row 6). This type radiographic shadow is caused by extensive fracturing of the endosperm without separation. For the most part there will be no progressive enlargement of these shadows so characteristic of insect tunneling.

(7) *Shadows caused by missing or rotten germ.*—(Fig. 7, and Fig. 10, Row 7). When a portion or all of the germ has been broken away or

chewed out by rodents, the area will approach the density of the background, depending upon the depth of the damage. This same type of shadow is also exhibited by kernels with rotten germ, especially those where the rot extends into the endosperm.

(8) *Shadows caused by mechanical breakage.*—(Fig. 8, and Fig. 10, Row 8). Fig. 8 exhibits only one type of mechanical breakage. This damage may occur on any part of the kernel. Mechanical breakage can be characterized and distinguished from insect damage by the presence of sharply defined straight edges.

SHADOWS PRODUCED ON THE RADIOGRAPH BY INSECT DAMAGE

A preliminary comparative study was made of mounted corn specimens and the radiograph of these same specimens. The radiograph was used as the guide for determining suspect kernels which were removed from the mount and examined microscopically to determine the origin of the shadows noted on the radiograph.

Minute damage (entrance holes, weevil egg plugs, and initial tunneling) for the most part did not register as shadows on the radiograph of corn. This is contrasted with prior experience with wheat (2) where such initial damage was visible on the radiographs.

The following describes the various types of insect damage in corn as indicated by the radiographic shadows:

(1) *Early infestation.*—(Fig. 11, Row 1). In all of these kernels, early damage is shown with three kernels also showing extensive tunneling. The damage shown here is early larval tunneling and is, for practical examination, the earliest stage of infestation in corn that can be determined radiographically.

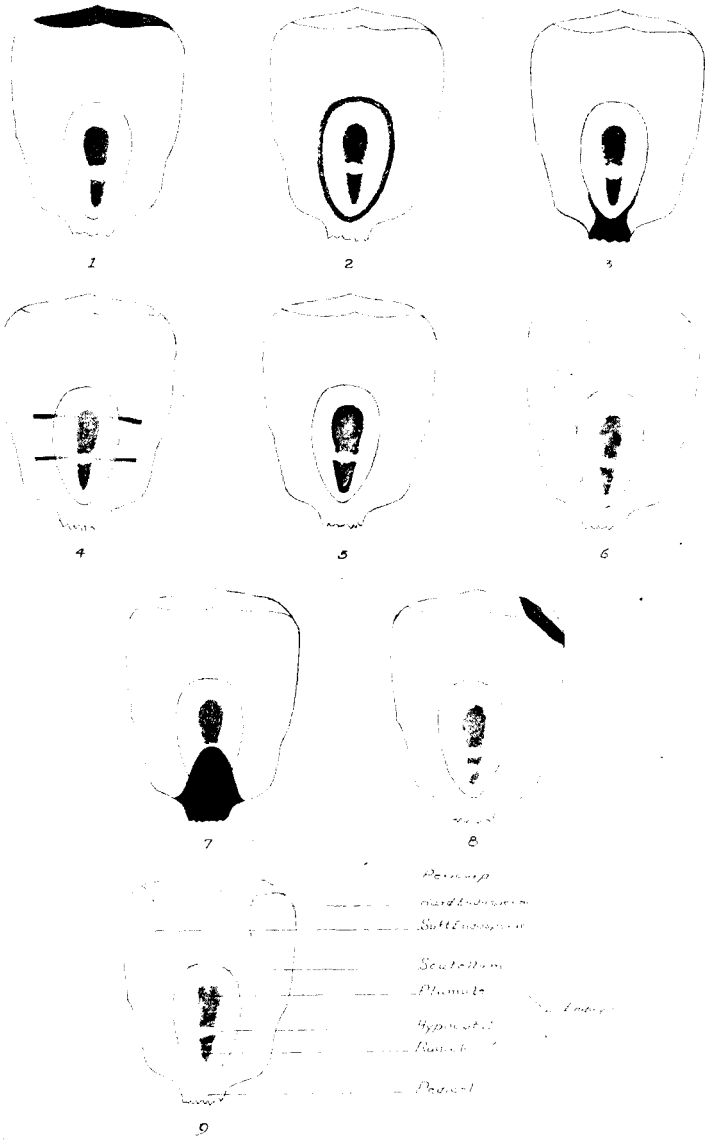
(2) *Advanced tunneling with insect stages.*—(Fig. 11, Row 2). A larva is shown in kernel 1, while kernels 4 and 5 show well-defined pockets with pupae or the early adult stage.

(3) *Exit holes.*—(Fig. 11, Row 3). This row of damage shows the very extensive tunnels which remain after the adult has emerged. The exit hole made by emergence of the adult will appear at some margin of the corn kernel. The presence of granular material within the tunnel indicates moth damage.

(4) *Multiple damage.*—(Fig. 11, Row 4). These kernels show a combination of the above and indicate that more than one insect has fed within a kernel. This multiple infestation appears to be more common in corn than in wheat.

COMPARISON OF METHODS

The results tabulated in Table 1 were obtained by utilizing the method previously described. Two 100 gram subdivisions were separated from each sample and X-rayed. These same subdivisions were then tested by



FIGURES 1–9

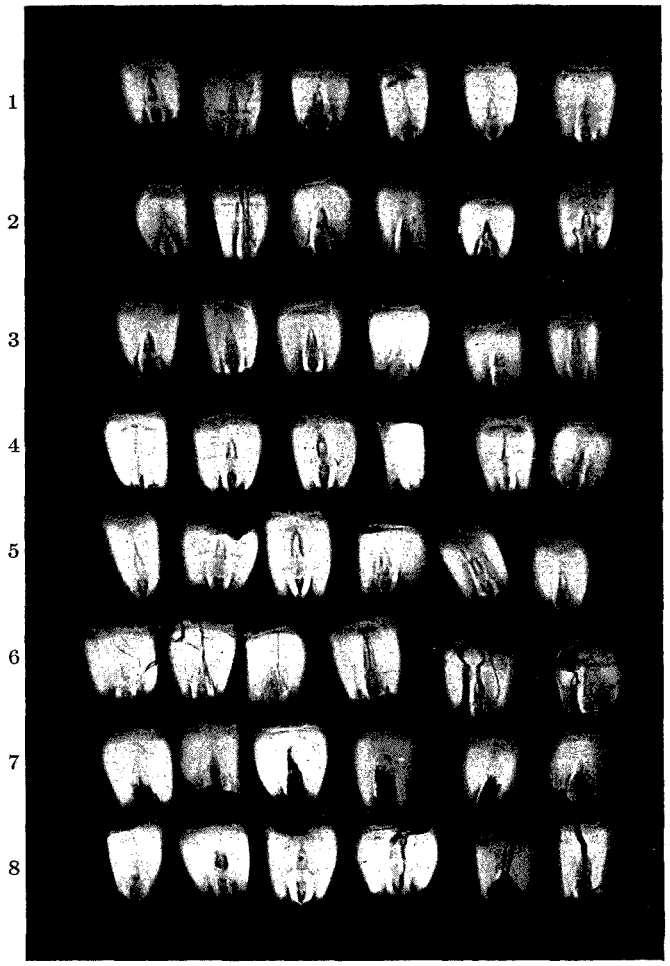


FIGURE 10

the cracking-flotation procedure and the results on the identical subdivisions were compared. Sample variation was therefore eliminated.

DISCUSSION OF RESULTS

It is obvious that there is close agreement between the cracking and X-ray results of the same subdivisions. As anticipated, there are some samples which show variation between the results by the two methods. Those cases where the cracking test shows greater contamination may

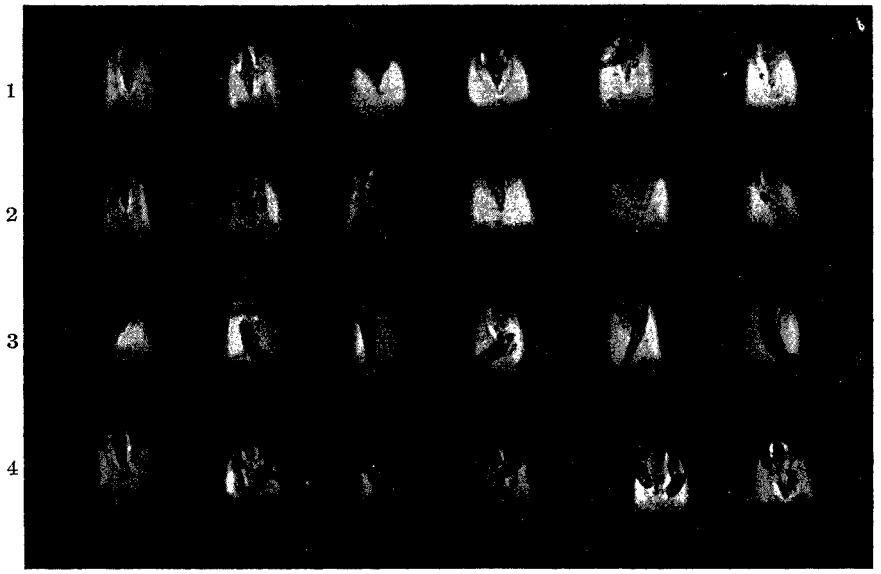


FIGURE 11

result from the fact that insect damage, where one or more insects were involved, were counted as a unit on the radiograph. Thus, if more multiple damage is present in a sample than is ordinarily found, the sample will show greater contamination by the cracking-flotation than by the X-ray method. Such multiple infestation was difficult to interpret or evaluate in the radiographs of corn; this was also true in the case of wheat. The same effect may also result from the presence of a large amount of incipient damage not visible on the radiographs of corn.

In the total of each column, it may be noted that the ratio was almost 1:1 (cracking *vs* X-ray). However, in wheat the ratio was 1:1.5 in favor of the X-ray method.

CONCLUSIONS

(1) Internal insect damage as shown by characteristic radiographic shadows can be differentiated for the most part from the characteristic shadows of normal corn structures and the abnormalities of the corn kernel.

(2) The gross insect damage visible on the radiograph correlates well with the cracking-flotation results.

(3) As compared with the previous methods of determining internal insect damage, the X-ray method is the most rapid for an accurate evaluation of internal insect damage.

TABLE 1.—*Detection of insects in corn by X-ray*

SAMPLE NO.	CRACK. TEST		TOTAL X-RAY DAMAGE	
	1	2	1	2
35	4	5	4	5
37	2	1	3	2
38	3	1	2	2
41	14	10	9	8
42	15	6	9	10
43	10	10	8	4
46	7	5	8	6
47	9	11	10	11
48	7	8	8	9
49	27	23	24	20
53	18	12	18	13
55	6	11	7	10
56	17	21	10	23
57	13	19	10	22
59	16	22	21	26
60	10	10	10	7
63	2	1	4	4
64	3	4	2	6
65	4	2	4	2
66	2	1	2	1
67	5	3	4	3
857	1	2	0	2
858	24	21	22	20
860	1	2	0	1
861	2	0	1	0
863	1	1	1	2
869	23	22	14	18
870	4	4	4	6
80	0	0	1	1
81	0	0	1	2
82	125	91	104	106
83	71	66	81	73
86	14	15	18	15
87	2	3	2	2
88	9	7	8	5
89	4	3	4	3
90	4	4	3	4
91	7	12	4	6
92	3	2	4	2
99	0	0	0	2
100	0	0	0	1
102	0	0	1	1
103	0	0	1	0
106	1	2	1	1
107	2	1	2	0
108	3	0	2	3
110	7	7	7	7
111	18	22	16	13
117	0	1	0	2
120	45	50	45	43
123	12	7	10	6
127	1	2	4	4
128	0	1	0	2
130	0	1	1	2
131	0	2	1	2
133	7	10	8	9

TABLE 1.—(*continued*)

SAMPLE NO.	CRACK. TEST		TOTAL X-RAY DAMAGE	
	1	2	1	2
134	6	5	5	8
135	5	9	5	7
136	0	0	1	2
138	1	0	2	3
140	1	1	1	1
142	1	2	2	2
150	3	0	3	2
151	2	3	2	3
153	0	3	4	3
163	7	9	4	7
164	5	2	4	2
165	3	0	2	1
170	2	2	3	2
171	2	1	2	1
172	2	2	2	3
174	1	2	1	1
180	3	2	3	2
187	6	4	6	3
197	0	0	0	1
198	0	0	2	3
199	0	0	1	1
200	0	0	1	1
1105	0	1	0	0
1117	4	6	4	6
1118	8	4	7	3
1119	5	4	5	4
1122	4	2	3	3
1123	0	1	1	1
1137	3	4	2	4
1138	5	4	4	3
1144	2	2	3	2
1145	25	30	13	12
1148	5	4	4	3
1149	4	2	3	2
1150	3	2	1	2
1151	4	2	5	2
1154	0	1	1	2
1155	0	0	1	3
1156	0	0	0	0
1158	0	0	1	1
1159	0	1	2	3
1160	0	2	1	3
1161	0	0	0	1
1162	0	0	1	0
1166	1	0	1	1
1173	0	0	1	0
1174	0	0	0	0
1176	5	5	3	4
1177	5	3	3	3
1178	0	0	0	0
1179	0	0	0	0
1180	0	0	1	0
1181	0	2	2	2
1182	6	4	4	3
	731	680	681	692

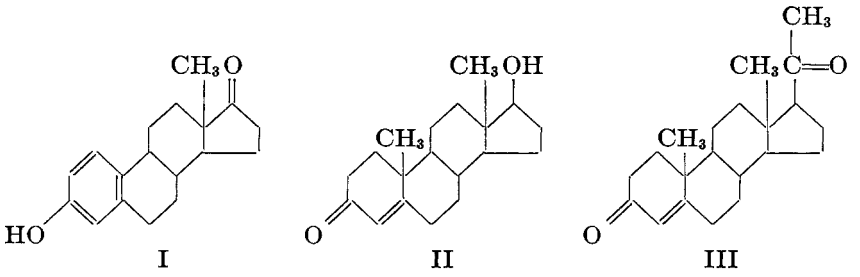
REFERENCES

(1) HARRIS, K. L., *et al.*, *This Journal*, **35**, 115 (1952).
(2) NICHOLSON, J. F., *et al.*, *ibid.*, **36**, 138 (1953).
(3) MILNER, M., LEE, M. R., and KATZ, R., *J. Econ. Entomol.*, **43**, 933 (1950).
(4) KATZ, R., LEE, M. R., and MILNER, M., *Non-Destructive Testing*, **8**, Fall, 1950.
(5) MILNER, M., *Milling Production*, **245**, 1a (Jan. 9, 1951).
(6) MILNER, M., LEE, M. R., and KATZ, R., *Food Technology*, **6**, 44 (1952).
(7) DUBOIS, D., *Milling Production*, **248**, 1a (Sept. 9, 1952).

THE CHROMATOGRAPHIC SEPARATION OF PROGESTERONE AND TESTOSTERONE*

By JONAS CAROL (Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C.)

The simultaneous administration of two or more different types of steroid hormones is a recent trend in endocrine therapy. As a consequence, pharmaceutical preparations are now being manufactured which contain estrogens and androgens, and estrogens, androgens, and progesterone. Usually these preparations are in tablet form or in aqueous suspension, and isolation of the steroids, free of excipient matter, presents no difficulty. The estrogens [estrone (I), α-estradiol, or mixed estrogenic substances], are weakly acid phenols and can be separated from the others by extraction from organic solvents with dilute alkali. Triple hormone preparations encountered to date, contained, in addition to the estrogen, equal quantities of testosterone (II) and progesterone (III).



I II III

Testosterone and progesterone differ only in the side chain at C_{17}. Both react readily with carbonyl reagents, and their ultraviolet absorption spectra are characterized by a single sharp maximum at 241 mμ (in alcohol) due to conjugation of the carbonyl at C_3 with the double bond at C_4. Although the infrared absorption spectra of the two compounds

* Presented at the annual meeting of the Association of Official Agricultural Chemists, September 29, 30, and October 1, 1952, at Washington, D. C.

(Fig. 1) are distinctly different, they are not entirely suitable for the determination of mixtures containing all proportions of each.

Because of the OH group at C_{17}, testosterone is decidedly more soluble in dilute alcohol than progesterone, and when the two are partitioned between 80 per cent alcohol and isooctane, testosterone appears chiefly in the alcoholic fraction, and progesterone in the isooctane. A chromatographic partition, using 80 per cent alcohol as the immobile solvent adsorbed on Celite, and isooctane as the mobile solvent, separated these two hormones sharply.

METHOD

APPARATUS

(a) *Adsorption tube.*—Select a 25 × 200 mm test tube of 3.85 to 4.00 sq. cm cross-sectional area by measuring the height of a 50 ml column of H_2O in it. Fuse a 6 cm length of 5–6 mm tubing to the bottom of the tube and slightly constrict this stem about 2 cm below the tube.

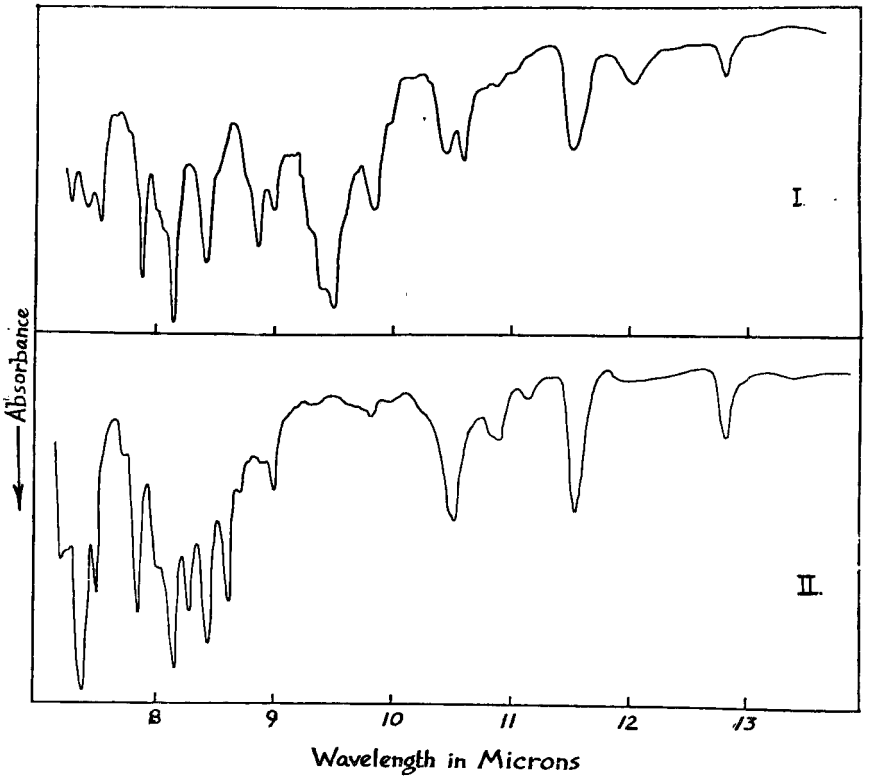


FIG. 1.—The infrared absorption spectra, 7–14 μ, of testosterone (I) and progesterone (II) in carbon disulfide. Concentration 100 mg/ml. Cell thickness 1.0 mm.

(b) *Isooctane reservoir.*—A separatory funnel, 500 ml, with 3 mm or larger bore stopcock lubricated only with H_2O. The stem should be ca 10 cm long.

(c) *Packing rod.*—Flatten the end of a glass rod to a circular head with a clearance of about 1 mm in the adsorption tube.

(d) *Leveling rod.*—A sharp-edged rod about 1.5 cm in diam.

REAGENTS

(a) 80% *alcohol.*—Dil. 80 ml of U.S.P. alcohol to 95 ml.

(b) *Isooctane.*—99% 2,2,4-trimethylpentane.

(c) *Celite No. 545.*—Johns-Manville diatomaceous earth.

DETERMINATION

Preparation of adsorption column.—Pack fine glass wool into the constricted stem of the adsorption tube so that when the tube is filled with isooctane, the rate of flow is between 2.5 and 3.0 ml/min. Before packing the column, fasten a piece of rubber tubing with attached screw clamp to the outlet to limit the flow during packing. Cover 8 g of Celite in a mortar with about 40 ml of isooctane, and distribute over the Celite from a pipet exactly 5 ml of 80% alcohol. Mix carefully with a pestle for several min. until the Celite appears uniformly wet. With the adsorption tube about ¼ filled with isooctane, transfer about ⅛ of the Celite mixt. to the tube. Form a flocculent suspension by slowly working the packing rod up and down, as a piston, through the Celite mixt. Gently compress the Celite with the packing rod. Open the screw clamp enough to permit slow drainage during packing of the tube. With a spatula, transfer the remainder of the Celite to the adsorption tube in about 5 portions; suspend each portion and gently pack as above. Finish off the top of the column by scraping down any Celite on the upper wall of the tube so as to form a sharply defined level surface on a column of ca 30 ml vol. over the initial pack. The Celite must be covered with isooctane at all times. Nearly fill the separatory funnel with isooctane satd with 80% alcohol and seal stopper with film of H_2O to prevent air leaks. Insert the stem of the funnel into the isooctane over the Celite, fully open the stopcock, and adjust the level of the isooctane to produce a flow rate of 2.0 to 2.5 ml/min. with the screw clamp fully open. Mark the level of the isooctane on the adsorption tube, close the stopcock, and remove the isooctane reservoir.

Separation.—Dissolve mixt. of testosterone and progesterone (dry and contg not more than 50 mg of total steroids) in 5 ml of isooctane. Add a drop of alcohol, if necessary, to obtain complete soln. Remove the rubber tubing from the adsorption tube, and when the isooctane *just stops* dripping from the tube, transfer the soln at once to the tube, allowing it to flow down the wall near the top of the Celite (a 5 ml pipet is convenient for this purpose). When the isooctane just stops dripping from the tube, complete the transfer in like manner with 3 more 5 ml portions of isooctane. Immediately place a 100 ml graduated cylinder under the tube, add isooctane to the level marked on the tube, and replace the isooctane reservoir, supporting it at a height to maintain that level when the stopcock is fully opened. When 100 ml of effluent has collected in the cylinder, replace with a second cylinder and collect 50 ml. Evap. the 100 ml of effluent to dryness on a steam bath and reserve the residue for the detn of progesterone. Discard the second 50 ml of effluent and collect an addnl 250 ml. Evap. this final fraction to dryness and reserve the residue for the detn of testosterone. Final detn of both steroids is made by either ultraviolet or infrared spectrophotometry.

Using the proposed method, three synthetic mixtures of testosterone and progesterone and two commercial preparations of aqueous suspensions

of these compounds were analyzed. Final determinations were made by infrared spectrophotometry, using CS_2 solutions of each steroid at 9.50 μ for testosterone and 7.40 μ for progesterone. The results (Table 1) show very good recovery for the synthetic mixtures and close adherence to labeled declaration for the commercial preparations.

TABLE 1.—*Recoveries of progesterone and testosterone*

SAMPLE	MG PROGESTERONE		MG TESTOSTERONE	
	ADDED	FOUND	ADDED	FOUND
Synthetic Mixture	10.0	10.1	10.0	10.1
	25.0	25.8	5.0	4.7
	5.0	5.1	25.0	24.4
Commercial Mixture	25.0[a]	25.9	25.0[a]	24.5
	25.0[a]	24.2	25.0[a]	24.0

[a] Labeled declaration on commercial sample.

COLLABORATIVE STUDY OF METHODS FOR ANALYSIS OF TOBACCO

NICOTINE AND MOISTURE

By C. O. WILLITS, MILDRED GASPAR, and J. NAGHSKI (Eastern Regional Research Laboratory, Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture, Philadelphia, Pennsylvania)

A report is hereby presented of data obtained in the initial collaborative study of methods for determining nicotine and moisture in tobacco, conducted by an informal committee of tobacco research chemists.* The studies were designed to determine which of several methods described in the literature and in common use would prove suitable either in their present form or in some modified form as standard procedures for the analysis of tobacco for moisture and nicotine. The collaborators submitted not only their analytical results but also detailed descriptions of their methods. They were requested to send all the values obtained so that a statistical analysis of the data could be made, both to compare the different procedures (parts of the methods) and to show the effects of these procedures on the precision and accuracy of the method.

Two samples were sent to each tobacco analyst who had signified a willingness to participate in the studies. One was an acidified aqueous

* A committee on standardization of analytical methods for tobacco products, established informally by a group of tobacco research chemists representing government, university, and industrial organization, as a result of a tobacco research conference at the Eastern Regional Research Laboratory in October, 1947.

so'ution of nicotine and the other was Pennsylvania cigar leaf ground finer than 100 mesh. By using the liquid sample, the effect of variables inherent in moisture analysis and extraction of nicotine from tobacco plant tissue were largely eliminated; this allowed a better appraisal of the methods for nicotine.

NICOTINE ANALYSIS OF LIQUID SAMPLES

Results of analysis for nicotine in the liquid samples were reported by 17 collaborators. Ten analysts reported 41 values by the spectrophotometric method (4) and 17 analysts reported 86 values by the gravimetric method (2). Tables 1 and 2 list statistical summaries of the results. In these tables, n is the number of nicotine values reported by each collaborator, $\bar{X}$ is the mean of his data, and s is the standard deviation. The median value is the middle value obtained when all the data are arranged in ascending or descending order. The median value was used in these studies in lieu of a true value, because the true nicotine value is unknown and a median value is less affected by high or low results than a mean value would be.

TABLE 1.—*Statistical summary of nicotine values obtained by gravimetric analysis of liquid samples*

COLLAB. NO.	n	$\bar{X}$	s	$\bar{X}$-MEDIAN
		per cent		
0	6	0.99	0.000	+0.01
2	8	0.88	0.003	−0.10
3	4	1.00	0.002	+0.02
5	8	0.93	0.022	−0.05
6	6	1.01	0.015	+0.03
8	8	0.98	0.006	0.00
9	2	0.98	0.000	0.00
10	5	0.94	0.010	−0.04
11	4	0.99	0.000	+0.01
12	4	1.00	0.010	+0.02
13	4	0.99	0.000	+0.01
14	4	0.99	0.000	+0.01
16	3	0.97	0.000	−0.01
18	6	0.99	0.000	+0.01
19	4	0.98	0.002	0.00
20	8	0.98	0.010	0.00
21	2	1.00	0.010	+0.02
17	86			
Mean		0.98	0.005	0.01

Median = 0.99%
$s_{\bar{x}}$ = 0.032%

The mean of the $\bar{X}$'s for values by the gravimetric and spectrophotometric methods is 0.98 and 0.97, respectively. These values are almost identical with the median of a composite of all the values submitted. This was expected, since the average difference between the median and the average, $\bar{X}$, of each analyst's values is 0.01 for both methods. The average of the $\bar{X}$'s for both methods falls on the median for all

TABLE 2.—*Statistical summary of nicotine values obtained by spectrophotometric analysis of liquid samples*

COLLAB. NO.	n	$\bar{X}$	s	$\bar{X}$-MEDIAN
		per cent		
0	6	0.97	0.000	−0.01
2	6	0.77	0.054	−0.21
5	8	0.97	0.002	−0.01
10	3	0.97	0.002	−0.01
14	2	0.98	0.000	0.00
16	1	1.03	0.000	+0.05
18	2	0.97	0.000	−0.01
20	8	0.97	0.003	−0.01
21	2	0.97	0.028	−0.01
22	3	1.06	0.122	+0.08
10	41			
Mean		0.97	0.021	0.00

Median = 0.97%

$s_{\bar{x}}$ = 0.076%

the nicotine values. Student's t test was applied to determine if there is a significant difference between the means of the values of the two methods so that the one in closest agreement with the best measure of the true value (median value) can be established.

Since the calculated t, 0.518, is less than 2.060 t at the 95% level, there is no significant difference between the two methods. From these experiments it can be assumed that the two procedures studied for the analysis of "pure" nicotine in solution under the conditions specified are satisfactory as referee methods. Therefore, in the analysis of a plant material for nicotine, any apparent differences in the results submitted by the collaborators will be due either to differences in the methods of moisture analysis, in the procedures for recovering the nicotine from the plant, or in variations of the procedure for nicotine analysis.

MOISTURE ANALYSIS

The data on moisture, comprising 72 analyses furnished by the 18 collaborators, are summarized statistically in Table 3. These data have been treated in the same manner as the data on nicotine with n, $\overline{X}$, s, and $\overline{X}$ median. Here the median, 15.75%, and the mean of the $\bar{x}$'s are identical, and the mean of the s is only 0.071. The values for moisture represent a range of more than 2% and, as expected, the $s_{\bar{x}}$ is 0.79; showing that although the average precision for the analysts (s = 0.071) is acceptable, the precision for all the analysts is poor.

Based on these results, it was judged best to correlate the values for nicotine on an "as is" basis to eliminate any inherent errors in the moisture analyses. In doing so, it is assumed that there was less difference in the moisture of the ground leaf samples "as received" by the collaborators than in the moisture values reported. It can at least be assumed that there is no greater difference in the values reported on the "as is" basis than there is in the moisture values found.

NICOTINE ANALYSIS OF GROUND TOBACCO LEAVES

Nineteen analysts collaborated in the analysis of nicotine in ground tobacco leaves by the gravimetric silicotungstic acid method, and reported 97 values. The

stf tistical summary of these analyses is given in Table 4. The median value of 3.90 is derived from all the 97 analyses and not from the analysts' means ($\bar{X}$). However, the average of the analysts' means, 3.86%, compares favorably with the median of all values, 3.90%. The small average standard deviation of 0.064, as compared with 0.177 for $s_{\bar{x}}$'s indicates high precision for individual analysts but shows less precision when an inter-laboratory comparison is made. The low $\bar{X}$ median (−0.04) shows an average high accuracy. Nevertheless, there does exist a range of values of +0.26 to

TABLE 3.—*Statistical summary of moisture values of samples of ground tobacco leaves*

COLLAB. NO.	n	$\bar{X}$	s	$\bar{X}$-MEDIAN
		per cent		
0	8	16.00	0.041	+0.25
2	4	16.85	0.221	+1.10
3	3	15.20	0.000	−0.55
5	4	16.13	0.080	+0.38
6	1	15.91	0.000	+0.16
8	8	15.44	0.268	−0.31
9	2	15.77	0.014	+0.02
10	2	15.80	0.042	+0.05
11	2	15.45	0.000	−0.30
12	4	15.08	0.066	−0.67
13	2	15.88	0.000	+0.13
14	4	16.47	0.037	+0.72
16	6	15.69	0.116	−0.06
18	6	15.56	0.102	−0.19
19	3	16.62	0.050	+0.87
20	8	13.30	0.144	−2.45
21	2	16.73	0.036	+0.98
22	3	15.68	0.030	−0.07
18	72			
Mean		15.75	0.071	0.00

Median = 15.75%
$s_{\bar{x}}$ = 0.793%

−0.36, or a variation of 0.62%, in the nicotine found in this sample, which contained approximately 4% nicotine, indicating a possible error of 15% due to causes other than the determination of the nicotine in the distillate.

To compare the relative merits of the gravimetric and the spectrophotometric methods, the statistical analysis of the 45 values for nicotine by the spectrophotometric method submitted by 9 collaborators is presented in Table 5. The principal difference in the values obtained by the two methods is the $s_{\bar{x}}$, which is 0.498 for the spectrophotometric method and only 0.177 for the gravimetric. This difference indicates a wider spread between laboratories in the spectrophotometric results. Inspection shows that this spread is due to two laboratories, since one is 1.33% lower than the median, and the other 0.50% higher than the others. There is no apparent justification for deleting these two values, and statistically they cannot be dropped, yet if they were eliminated, the $s_{\bar{x}}$ value would be 0.129, which compares favorably with the $s_{\bar{x}}$ value of 0.177 for the gravimetric analyses.

TABLE 4.—*Statistical summary of nicotine values obtained by gravimetric analysis of ground tobacco leaves*

COLLAB. NO.	n	$\bar{X}$	s	$\bar{X}$-MEDIAN
		per cent		
0	8	3.91	0.022	+0.01
2	8	3.57	0.147	−0.33
3	2	4.16	0.000	+0.26
3	3	3.75	0.069	−0.15
5	6	3.88	0.021	−0.02
6	6	3.98	0.015	+0.08
8	8	4.00	0.046	+0.10
9	2	4.04	0.050	+0.14
10	5	3.88	0.050	−0.02
11	6	3.76	0.386	−0.14
12	4	3.79	0.030	−0.11
13	4	3.84	0.017	−0.06
14	4	3.60	0.050	−0.30
16	6	4.14	0.024	+0.24
18	6	3.91	0.037	+0.01
19	4	3.54	0.065	−0.36
20	8	3.75	0.017	−0.15
21	3	3.83	0.016	−0.07
22	4	3.98	0.159	+0.08
19	97			
Mean		3.86	0.064	−0.04

Median = 3.90%
$s_{\bar{x}}$ = 0.177%

TABLE 5.—*Statistical summary of nicotine values obtained by spectrophotometric analysis of ground tobacco leaves*

COLLAB. NO.	n	$\bar{X}$	s	$\bar{X}$-MEDIAN
		per cent		
0	8	3.92	0.024	+0.02
2	8	2.57	0.159	−1.33
5	6	3.93	0.068	+0.03
10	3	4.00	0.040	+0.10
14	4	3.64	0.050	−0.26
16	2	4.40	0.192	+0.50
18	3	3.93	0.129	+0.03
20	8	3.73	0.052	−0.17
21	3	3.81	0.040	−0.09
9	45			
Mean		3.77	0.084	−0.13

Median = 3.90%
$s_{\bar{x}}$ = 0.498%

EVALUATION OF VARIABLES IN METHODS FOR DETERMINATION OF MOISTURE AND NICOTINE

Since each collaborator described his modification of the method used for analyses of moisture and nicotine, it was possible to summarize all the variables in the procedures and correlate the data with one or the other of the many alternate variables of the procedures. Tables 6, 7, and 8 show the more important pairs of alternate variables for the methods of moisture, nicotine extractions, and nicotine analysis procedures, as well as the variable used by each collaborator.

By plotting the values obtained when either of a pair of variables was used, it is possible to show graphically which of the two variables contributes to greater accuracy. This is done by plotting the deviations of the $\overline{X}$ values from the median values, and indicating the average for each set of deviations. If the averages of the deviations for each of a pair of variables are essentially the same, it can be assumed that neither one of that pair has a significant effect on the accuracy of the results. If, on the other hand, the averages of the deviations are considerably different for any pair of variables, it is an indication that the differences in procedure have a significant effect on the accuracy. That procedure yielding an average deviation closest to the median value is to be preferred, since it tends to produce more accurate results. Plotted values for deviations from the median provide an easy method for appraising the effect of a variable, and this appraisal can be checked by application of the Student's t test (3) for unequal populations.

$$t = \overline{X}' \sqrt{\frac{n_a n_b (n_a + n_b - 2)}{(n_a + n_b)[(\overline{X}_a - \overline{X}_b)^2 + (\overline{X}_b - \overline{X}_b)^2]}}$$

where $\overline{X}' = \overline{X}_a - \overline{X}_b$; n_a and n_b are the number of values for a and b; X_a and X_b are the means of the individual values for the two groups, a and b, respectively; and $\overline{X}_a$ and $\overline{X}_b$ are the means of values of the two sets of values for a and b, respectively.

MOISTURE

In the analysis for moisture, the only variable found to be critical was the time of drying. The plot of deviations for values for heating times less than and greater than 3 hours is given in Fig. 1. The t test shows that the drying time is critical at the 90% level.

NICOTINE

A similar analysis was made of the effect of variations in procedure on the nicotine values of ground tobacco leaf. Plots of the deviation of the $\overline{X}$'s from the median nicotine value were made for the 18 different variations of the procedure. Plots are presented of only the 7 variations whose means were shown to be significantly different. In Fig. 2 is shown a comparison of the deviation from the median nicotine percentage of values obtained when the A.O.A.C. nicotine still was used and values obtained when other stills were used. The means of the analyst's $\overline{X}$'s indicate that the values obtained when the A.O.A.C. still was used are more accurate. This is confirmed by the t test, since the t at the 95% level, 2.064, is less than the calculated t, 3.542. The values obtained by the Griffith-Jeffreys still or its Willits-Connelly modification (1) give greater precision.

A comparison of the deviations of analysts' $\overline{X}$ values, obtained by using either NaOH or $Mg(OH)_2$ in the nicotine still pot, from the median nicotine percentage (Fig. 3), shows that the values obtained with NaOH are the most accurate. This is confirmed by the t test, since the t value found, 3.949, is larger than 2.086, t, at the 95% level. In this tobacco sample, although almost all the alkaloid was nicotine,

TABLE 6.—*Variables in methods used by the collaborators for the gravimetric determination of nicotine*[a]

	COMPARISON	COLLABORATOR NO.																		
		0	2	3	5	6	8	9	10	11	12	13	14	16	18	19	20	21	22	*n*
HCl added in precipitating nicotine	More than 1 ml	x					x	x		x		x		x				x		7
	Less than 1 ml		x	x					x		x		x			x	x			7
Volume silicotungstic acid per mg nicotine	More than 0.2 ml			x	x	x		x				x		x						6
	Less than 0.2 ml	x	x				x		x	x			x			x	x	x		9
Mg nicotine precipitated	More than 25 mg	x	x				x	x					x					x		6
	Less than 25 mg			x	x	x			x			x			x				x	7
Precipitate digested	Yes		x		x									x	x		x		x	6
	No	x		x		x	x	x	x	x	x	x	x			x		x		12
Crystallization	At room temp.	x		x		x	x	x	x	x	x	x	x	x		x		x		13
	Refrigerated		x		x										x		x		x	5
Filtering medium	Filter paper	x		x		x	x	x		x	x	x	x	x		x		x	x	13
	Other		x		x				x						x		x			5
Precipitate washed with H_2O	Yes						x										x			2
	No	x	x	x	x	x		x	x	x	x	x	x	x	x	x		x	x	16
Volume of acid wash solution	More than 100 ml			x		x	x	x		x				x	x	x		x		9
	Less than 100 ml	x	x		x				x		x	x	x				x		x	9
Ignition	Muffle furnace		x	x	x	x		x			x	x	x	x	x	x	x		x	13
	Meker burner	x					x			x								x		4
Ignition temperature	More than 800°C.	x		x		x		x				x	x					x		7
	Less than 800°C.		x		x					x	x			x	x	x	x			8
Time of ignition	More than 30 min	x	x	x	x	x		x				x				x	x		x	10
	Less than 30 min						x			x	x		x					x		5

[a] x=variable used by collaborator.

TABLE 7.—*Variables in methods used by the collaborators in the spectrophotometric determination of nicotine and determination of moisture*[a]

	COMPARISON	COLLABORATOR NO.																		
		0	2	3	5	6	8	9	10	11	12	13	14	16	18	19	20	21	22	*n*
Spectrophotometric determination of nicotine																				
No. of observations on spectrophotometer	More than 3	x			x								x				x	x	x	6
	Less than 3		x						x					x	x					4
No. of distillates	More than 3	x	x										x		x		x	x		6
	Less than 3				x									x					x	3
Moisture																				
Moisture dish	With cover	x		x	x	x			x	x			x			x	x	x	x	11
	Without cover		x					x			x	x		x	x					6
Drying temperature	More than 105°C.		x						x	x	x		x		x	x		x		8
	Less than 105°C.	x		x	x	x		x				x		x			x		x	9
Drying time	More than 3 hr.	x	x	x	x			x					x	x		x		x	x	10
	Less than 3 hr.								x		x	x			x		x			5
Sample size	More than 2.5 g	x		x		x		x				x			x			x		7
	Less than 2.5 g				x				x		x			x			x		x	6
Oven	Mechanical	x				x		x						x	x	x	x			8
	Gravity convection		x	x	x				x	x	x	x	x	x						9

[a] x = variable used by collaborator.

TABLE 8.—*Variables in methods used by the collaborators for nicotine extraction*[a]

	COMPARISON	COLLABORATOR NO.																		
		0	2	3	5	6	8	9	10	11	12	13	14	16	18	19	20	21	22	*n*
Sample size	More than 2 g	x		x		x	x	x			x	x		x	x			x		10
	Less than 2 g				x				x	x			x			x	x		x	7
Sampling	Weighing directly	x		x		x	x	x	x	x	x	x		x		x	x	x	x	14
	By difference		x		x								x		x					4
Stills	A.O.A.C.			x			x	x				x		x						5
	Others	x	x		x	x			x	x	x		x		x	x	x	x	x	13
Steam supply	House	x	x	x	x	x	x	x	x		x	x			x					11
	Lab. generated									x			x	x		x	x	x	x	7
Steam purified by scrubbing	Yes	x																		1
	No		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	17
Steam source	Tap H_2O	x	x				x		x	x	x	x			x	x		x		10
	Distilled H_2O												x	x			x		x	4
Alkali	NaOH			x	x	x	x	x	x	x				x		x			x	10
	MgO		x	x							x	x	x				x	x		7
	Others	x				x									x					3
Alkali per 100 ml of residue	More than 0.1 mole			x	x			x			x	x		x	x					7
	Less than 0.1 mole		x				x						x			x	x	x		6
Sodium chloride	Added			x	x		x		x					x						5
	Not added	x	x	x		x		x		x	x	x	x		x	x	x	x	x	14
Hydrochloric acid in receiver	More than 2 ml			x		x	x			x	x	x		x	x	x		x	x	11
	Less than 2 ml	x	x		x				x				x				x			6
Time of distillation	More than 45 min	x		x		x	x	x				x		x		x	x	x		10
	Less than 45 min		x		x				x	x	x		x		x				x	8

[a] x=variable used by collaborator.

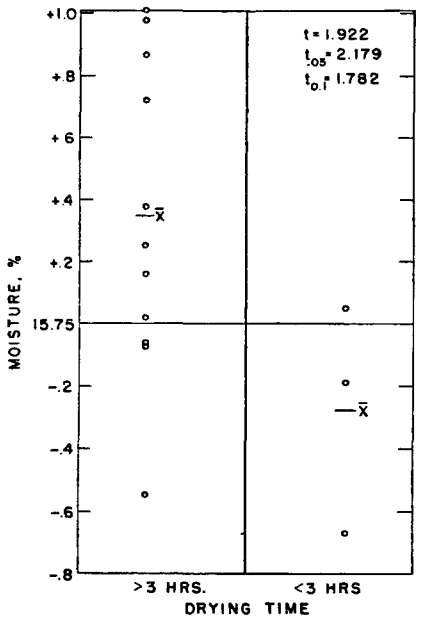


FIG. 1.—Deviations of the analysts' $\overline{X}$'s from the median value for moisture in ground tobacco leaf as influenced by drying time.

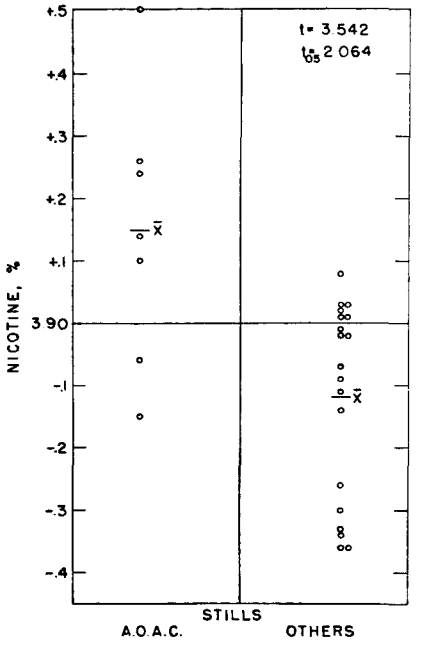


FIG. 2.—Deviations of the analysts' $\overline{X}$'s from the median value for nicotine in ground tobacco leaf as affected by the type of still.

the values obtained with MgO are, in all cases, lower than the median percentage of nicotine.

The concentration of alkali used in the nicotine still pot, measured in moles of alkali per 100 ml of residue left in the pot at the end of the distillation, has a significant effect on the accuracy of the results. Figure 4 shows that for concentrations of alkali greater than 0.1 mole, the accuracy is significantly better than that obtained for concentrations of less than 0.1 mole. This is confirmed by the t test, inasmuch as the calculated t, 4.678, is much larger than the t, 2.110, at the 95% level. This result indicates that unless care is taken to have an adequate concentration of alkali in the still, nicotine will be incompletely distilled.

The importance of the addition of sodium chloride to the still pot is demonstrated in Fig. 5. Here it is shown that the analyst's $\overline{X}$ values obtained when NaCl was used were much more accurate than those obtained when the NaCl was omitted. The t test shows that there is a significant difference, since the calculated t, 3.183, is larger than t, 2.074, at the 95% level. The fact that most of the low values were obtained when NaCl was not used indicates that the lower boiling temperatures of these still pot mixtures were not high enough to distill all the nicotine.

In these studies, the size of the sample taken for nicotine had a significant effect on the accuracy of the results obtained. Figure 6 shows that samples larger than 2 g gave more accurate values than were obtained when smaller samples were used.

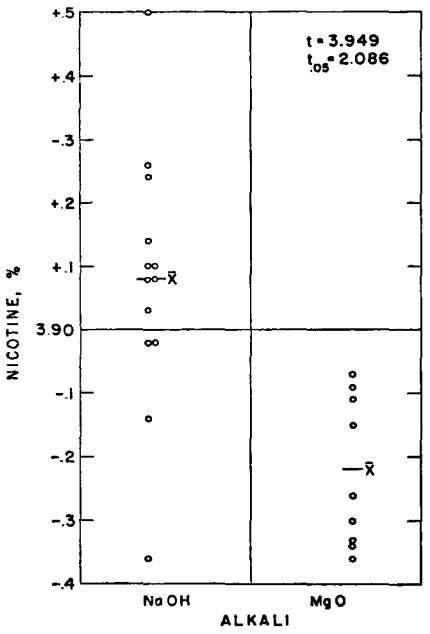


FIG. 3.—Deviations of the analysts' $\bar{X}$'s from the median value for nicotine in ground tobacco leaf as affected by the type of alkali.

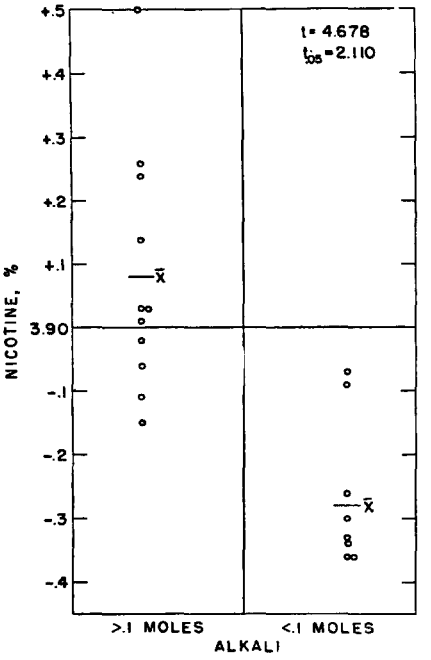


FIG. 4.—Deviations of the analysts' $\bar{X}$'s from the median value for nicotine in a ground tobacco leaf as affected by the concentration of alkali in residue.

This is confirmed by the t test, since the calculated t, 2.402, is much larger than t at the 95% level.

As expected, more accurate values were obtained when the liquid in the receiver was acid enough to convert *all* the nicotine to the less volatile salt. It can be seen from Fig. 7 that the values obtained when the receiving liquid contained more than 2 ml of acid were considerably higher and more accurate than when less acid was used. This difference is confirmed by the t test, since the calculated t, 2.281, is larger than t at the 95% level.

Another factor that appeared to have an important effect on the nicotine values was the amount of silicotungstic used to precipitate the nicotine. In Fig. 8 it is shown that the use of less than 0.2 ml of a 120 g/l solution of silicotungstic acid per mg of nicotine gave low and less accurate values. This is perhaps one of the most important of the factors that affect the accuracy of the results, since the calculated t is much larger than that for the 95% level.

DISCUSSION

In this collaborative study of methods for the determination of moisture and nicotine in tobacco, the statistical analysis of the data and the conclusions drawn can be interpreted only as indications of cause and effect on the values found. To draw more positive conclusions, it would have

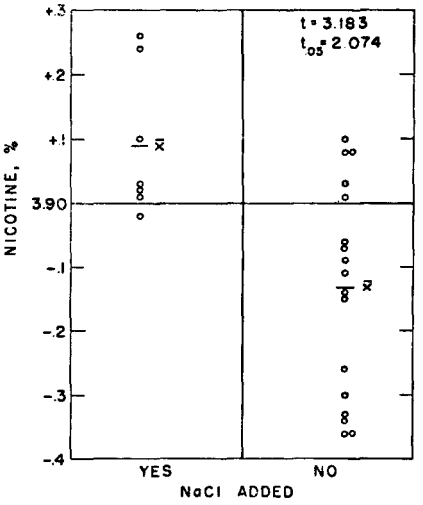


FIG. 5.—Deviations of the analysts' $\bar{X}$'s from the median value for nicotine in ground leaf tobacco as affected by the addition of salt to still pot.

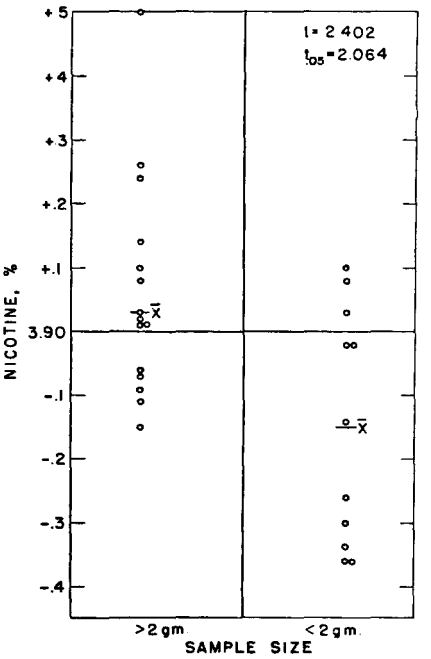


FIG. 6.—Deviations of the analysts' $\bar{X}$'s from the median value for nicotine in ground tobacco leaf as affected by the size of sample.

been necessary to adjust the data for each variable studied in terms of the data for the other variables. Because of the large number of variables considered and the relatively small number of analytical values for one variable, adjustment of the data to compensate for the effect of other variables would reduce the values of a particular series to such a small number that a statistical analysis would be valueless. The number of points in the figures do not necessarily agree with the number of collaborators who participated, as shown in Tables 6, 7, and 8 since often one collaborator submitted more than one set of data and in a few instances the values submitted were not included because they were received after the statistical analysis had been completed.

This study indicated that in the moisture analysis of ground tobacco leaf, conditions which do not influence the accuracy of the analyses are:

(1) Covered moisture dishes.
(2) Drying temperatures above or below 105°C.
(3) Sample size larger than or less than 2.5 g.

The time of drying had a slight effect on the moisture values. Drying

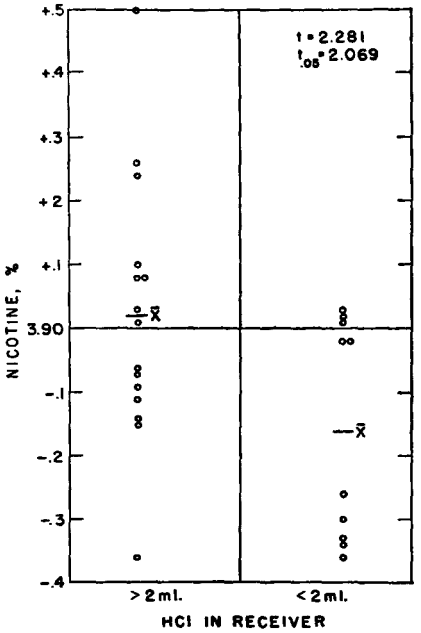


FIG. 7.—Deviations of the analysts' $\bar{X}$'s from the median value for nicotine in ground tobacco leaf as affected by the amount of hydrochloric acid in the receiver.

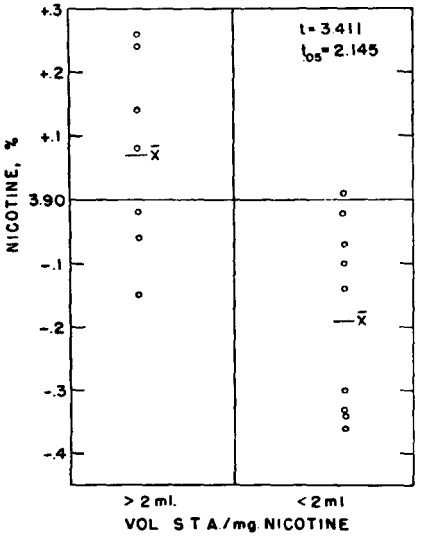


FIG. 8.—Deviations of the analysts' $\bar{X}$'s from the median value for nicotine in ground tobacco leaf as affected by the amount of silicotungstic acid used to precipitate the nicotine.

for more than 3 hours tended to give higher values.

In determinations of nicotine the A.O.A.C. silicotungstic acid gravimetric method and the spectrophotometric procedures gave equally precise and accurate values.

In the gravimetric procedure, the conditions that apparently do not affect the results within the ranges normally used are:

(1) Time of distillation.
(2) Test solutions that contain more or less than 25 mg of nicotine.
(3) Filtering medium.
(4) The use of more than or less than 100 ml of acid wash.
(5) Digestion of the precipitate on the steam bath.
(6) Ignition temperatures above or below 800°C.

Conditions that appear to have an effect on the results are:

(1) The sample should weigh more than 2 g.
(2) The use of sodium hydroxide in the still pot yields more nearly accurate results.
(3) More than 0.1 mole of alkali should be used in the still pot.

(4) Sodium chloride should be added to the distillation mixture.
(5) Until further data are obtained, the use of the A.O.A.C. still is preferred to all other stills. (Because of insufficient data, comparison between the A.O.A.C. and Griffith-Jeffrey stills could not be made.)
(6) The receiver should contain not less than 2 ml of concentrated hydrochloric acid.
(7) A minimum of 0.2 ml of silicotungstic acid should be used to precipitate each milligram of nicotine in the test solution.

SUMMARY

The data obtained suggest procedures that should be included in a standard method for nicotine analysis, but they should be confirmed by further study. The following conditions, however, have been fairly well defined:

(1) Care must be exercised in the time of drying (moisture analysis). This may be correlated to moisture of sample and temperature used.
(2) A sample larger than 2 grams should be used for nicotine analysis.
(3) Magnesium oxide will give lower values for total alkaloids as nicotine than sodium hydroxide.
(4) The still should contain more than 0.1 mole of alkali per 100 ml.
(5) The still pot mixture should contain sodium chloride.
(6) The receiver should contain more than 2 ml of concentrated hydrochloric acid.
(7) More than 0.2 ml of silicotungstic acid solution should be added per mg of nicotine.

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REFERENCES

(1) BEINHART, E. G., WOODWARD, C. F., WILLITS, C. O., RICCIUTI, C., BADGETT, C. O., and WILLAMAN, J. J., *Nicotiana rustica*, U. S. Dept. of Agriculture, Bur. Agr. Ind. Chem., AIC 335 (Eastern Regional Research Laboratory), 1952 (Processed).
(2) *Official Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Washington, D. C., 1950, p. 69.
(3) SNEDECOR, G. W., *Statistical Methods Applied to Experiments in Agriculture and Biology*, 4th Ed., Iowa State College Press, Ames, Iowa, 1946.
(4) WILLITS, C. O., SWAIN, M. L., CONNELLY, J. A., and BRICE, B. A., *Anal. Chem.*, 22, 430 (1950).

THE USE OF ION EXCHANGE RESIN IN THE QUANTITATIVE CHEMICAL DIFFERENTIATION BETWEEN NICOTINIC ACID AND NICOTINAMIDE*

By JAMES P. SWEENEY and WALLACE L. HALL (Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C.)

In the examination of commercial vitamin products, it is sometimes important to differentiate between nicotinic acid and nicotinamide. For example, because of an unpleasant physiological reaction to nicotinic acid in some patients, there is a clinical preference for the amide (1). In research, accurate information as to identity and purity, where either or both of these compounds are being studied, is also necessary.

A number of methods for this differentiation are available. However, they either lack sensitivity or are too complex for routine use (2–6). It is more feasible to make an accurate analysis of a mixture of the two compounds, when one is present in small amounts compared with the other, if a preliminary separation of the two is made.

Kato and Shimizu (7) used the synthetic anion exchange resin, IRA-400, in the determination of nicotinamide and found that it is not adsorbed by the resin. The present authors have observed, further, that at *p*H 4.5 to 5, nicotinic acid is quantitatively adsorbed by IRA-400. This

* Presented at the annual meeting of the Association of Official Agricultural Chemists, September 29, 30, and Oct. 1, 1952, at Washington, D. C.

fact appeared to offer a simple means for the separation of nicotinic acid and nicotinamide.

EXPERIMENTAL

Solutions containing the two forms of the vitamin, buffered at *p*H 5, were added to ion exchange tubes containing IRA-400 in the basic form. The nicotinamide was removed by washing with H_2O, and the adsorbed nicotinic acid was subsequently eluted with *N* HCl. The nicotinamide and nicotinic acid content of the resulting solutions was determined by the Koenig reaction (8), using CNBr and sulfanilic acid as described previously (9).

TABLE 1.—*Separation of nicotinic acid and nicotinamide*

NICOTINIC ACID ADDED	NICOTINAMIDE ADDED	NICOTINIC ACID RECOVERED	NICOTINAMIDE RECOVERED
(*Micrograms*)	(*Micrograms*)	(*per cent*)	(*per cent*)
5000	50	98.0	97.5
950	50	100.0	102.0
900	100	97.1	98.0
700	300	98.6	102.0
500	500	102.0	98.0
300	700	96.6	101.5
100	900	101.0	98.7
50	5000	150.0	97.5

Table 1 shows results obtained when solutions containing known amounts, in varying proportions, of nicotinic acid and nicotinamide are tested. The results demonstrate that it is possible to make an accurate determination of one part of nicotinamide in the presence of 100 parts of nicotinic acid. As further evidence of this separation, Tobias acid can be substituted for sulfanilic acid. When this is done the nicotinic acid color, after 3–4 minutes, is orange, while that of the nicotinamide is pink (6). On the other hand, some difficulty was experienced in the analysis of mixtures containing one part nicotinic acid in the presence of 100 parts nicotinamide, and recoveries of nicotinic acid amounted to 150 per cent of that added.

In an attempt to discover the reasons for this discrepancy, the following experiments were carried out. Solutions of nicotinamide from two sources were prepared; the first was the U.S.P. Reference Standard nicotinamide, and the second a sample of nicotinamide tablets shown by another method of differentiation to contain only the amide (6). In each case the solution was passed through an ion exchange column, and the column was washed with H_2O until free of nicotinamide. The resulting solution and washings were passed through a second column which was also washed free of the amide. This solution and washings were then passed through a third col-

umn, which in turn was washed free of nicotinamide. Each of the columns was then eluted with *N* HCl and the nicotinic acid content of the eluate was determined. Results are given in Table 2.

TABLE 2.—*Tests with nicotinamide*

SAMPLE	WEIGHT OF SAMPLE	NICOTINIC ACID FOUND (PER CENT)		
	MG	TUBE 1	TUBE 2	TUBE 3
U.S.P.Ref. Std. nicotinamide	5	0.47	0.41	0.45
Nicotinamide tablets	5	1.16	0.52	0.55

Apparently a small amount of nicotinic acid is found in the U.S.P. standard under these conditions. If we may assume complete adsorption in the first tube, the amounts occurring in the second and third tubes can be explained only on the basis of hydrolysis of the amide form. Values for the nicotinamide tablet sample indicate that a similar degree of hydrolysis occurred in the second and third tubes, and the higher value for the first tube suggests that the sample actually contained a small amount of the acid form.

To investigate this point further, solutions containing varying amounts of nicotinamide were passed over columns containing 2 g of the resin. In addition, a column containing 5 g was used for comparison. The amount of nicotinic acid eluted from each of these washed columns is given in Table 3.

TABLE 3.—*Tests with nicotinamide*

IRA-400	NICOTINAMIDE	NICOTINIC ACID FOUND	
gram	*mg*	*mg*	*per cent*
2	0.25	0.002	0.80
2	0.5	0.00425	0.85
2	2.0	0.014	0.70
2	5.0	0.038	0.76
5	5.0	0.100	2.0

Within the range of concentrations used, the amount of nicotinic acid increased as the amount of nicotinamide added to the column was increased, and the ratio remained nearly constant for a given amount of resin. When the amount of IRA-400 was increased to 5 g, the nicotinic acid formed was also increased in the same proportion.

In an attempt to correct for the hydrolysis of nicotinamide by the resin, samples of nicotinamide were tested before and after the addition of 1 per cent of nicotinic acid. Results are listed in Table 4. Corrected recoveries

TABLE 4.—*Recoveries of nicotinic acid*

WT. SAMPLE	SAMPLE	NICOTINIC ACID IN ORIG. SAMPLE	NICOTINIC ACID ADDED	NICOTINIC ACID FOUND AFTER ADDITION	NICOTINIC ACID RECOVERED (COLUMNS 5-3)	RECOVERED
mg		*mg*	*mg*	*mg*	*mg*	*per cent*
5	Nicotinamide tablets	0.046	0.05	0.095	0.049	98.0
5	Nicotinamide tablets	0.050	0.05	0.095	0.045	90.0
5	Nicotinamide tablets	0.043	0.05	0.093	0.050	100.0
5	Nicotinamide tablets	0.063	0.05	0.110	0.047	94.0
5	Nicotinamide, U.S.P.	0.030	0.05	0.078	0.048	96.0
5	Nicotinamide, U.S.P.	0.030	0.05	0.082	0.052	104.0

of the nicotinic acid ranged from 90 to 104 per cent of the amount added and indicate that it is possible to make an analysis of a vitamin preparation containing only one part of nicotinic acid in the presence of 100 parts of nicotinamide, provided that a correction is made for tube hydrolysis. However, tube hydrolysis may be ignored in the analysis of routine samples containing usual amounts of nicotinic acid and nicotinamide, since the amount of hydrolysis is well within the limits of experimental error.

Table 5 shows results obtained by application of the method to pharmaceutical products. The amounts of nicotinic acid and nicotinamide found are compared with the total amount found by the A.O.A.C. method (9).

TABLE 5.—*Comparison of results*

SAMPLE	NICOTINIC ACID FOUND (MG)	NICOTINAMIDE FOUND (MG)	TOTAL (COL. 2+3)	TOTAL NICOTINIC ACID BY A.O.A.C. METHOD
1*b*	5.0	5.3	10.3	10.3
2*b*	5.3	5.25	10.55	11.0
3	10.5	40.7	51.2	51.7

METHOD

REAGENTS

(a) *Sodium hydroxide soln.*—4 per cent.

(b) *Hydrochloric acid.*—*N* soln.

(c) *Anion exchange resin.*—IRA-400* (basic form).

(d) *Buffer, pH 5.*—Mix 9.7 ml of 0.1 *M* citric acid soln with 10.3 ml 0.2 *M* disodium phosphate soln.

Other reagents are the same as those described previously (9).

APPARATUS

(a) *Ion exchange tube.*—Similar to those used in the thiochrome detn of thiamine.

* Rohm and Hass Co., Philadelphia 5, Pa.

Preparation of Ion Exchange Tubes.—Place a pledget of glass wool over upper end of capillary with aid of glass rod. Add ca 25 ml H_2O; then add 2 g IRA-400. Wash with 10 ml 4% NaOH, allowing last portion to remain in tube for 10 min. Next wash tube with H_2O until the eluate is green to bromthymol blue (*p*H 7).

Preparation of Sample.—Prepare sample as given previously (9).

ION EXCHANGE PROCEDURE

Pipet an aliquot of the sample soln into a small beaker and adjust to *p*H 5 by the addn of a few drops of the buffer soln. Decant the contents of the beaker into the ion exchange tube. Elute the nicotinamide with H_2O, and collect washings in a volumetric flask. For usual amounts of nicotinamide, up to 500 mmg, 100 ml of H_2O is sufficient for washing. Quantities as large as 5 mg of nicotinamide require ca 300 ml of H_2O. Most efficient washing is obtained if the rate of flow of the H_2O is 3–5 ml per min. When sufficient H_2O has been used, test the washings for nicotinamide by collecting 1 ml in a test tube. Make alk. with dil. NH_4OH and add 2 ml 10% CNBr. No color should develop after the tubes stand one min. With samples contg a very high ratio of nicotinamide to nicotinic acid, pass an addnl 50 ml of H_2O thru the tube, after the nicotinamide test is negative, as an added precaution. After elution of nicotinamide, elute the nicotinic acid with 40 ml of hot *N* HCl. Determine nicotinic acid and nicotinamide in the usual manner (9).

SUMMARY

A method that offers a means for the ready separation and independent determination of nicotinic acid and nicotinamide is described by which the analysis of mixtures containing only one part of one form of the vitamin in the presence of one hundred parts of the other is made possible. This represents a ten-fold increase in sensitivity over methods in which the two forms of the vitamin are not separated.

REFERENCES

(1) *New and Nonofficial Remedies*, R. T. STORMONT, Ed., J. B. Lippincott Co., Philadelphia, Pa., 1950, p. 484.
(2) CIUSA, W., *Ann. chim. appl.*, **39**, 93 (1949).
(3) LAMB, F. W., *Ind. Eng. Chem., Anal. Ed.*, **15**, 352 (1943).
(4) MELNICK, D., and OSER, B. L., *ibid.*, **15**, 355 (1943).
(5) WOLLISH, E. G., SCHMALL, M., and SHAFER, E. G. E., *Anal. Chem.*, **23**, 768 (1951).
(6) SWEENEY, J. P., and HALL, W. L., *ibid.*, **23**, 983 (1951).
(7) KATO, M., and SHIMIZU, H., *Science*, **114**, 12 (1951).
(8) KOENIG, W., *J. Prakt. Chem.*, **69**, 105 (1904).
(9) SWEENEY, J. P., *This Journal*, **34**, 99, 380 (1951).

NOTES

A CONSTANT TEMPERATURE WATER BATH FOR USE IN DETERMINATION OF AVAILABLE PHOSPHORIC ACID IN FERTILIZERS*

By J. A. SHRADER and H. R. ALLEN (Kentucky Agricultural Experiment Station, Lexington, Kentucky)†

An electrically heated, constant temperature water bath with a continuous agitation device, needed for determination of available phosphoric acid in fertilizers, was designed by the authors‡ and was built in the University of Kentucky shops. It has been in use for twenty months without mechanical difficulty.

The bath has double sides and bottom, with $\frac{1}{2}''$ of cork insulation between them. Inner sections are of stainless steel and outer sections are of galvanized iron. Inside dimensions are $50'' \times 19\frac{1}{2}'' \times 8''$ deep. Two special features are the clamps which support the flasks, and the type of motion imparted to solutions in the flasks. The clamps consist of two pieces of stainless steel curved to fit the necks of the flasks. One piece is attached to a horizontal stationary tube and the other piece is attached to a movable rod inside this tube. The flask is released by pushing on a handle attached to

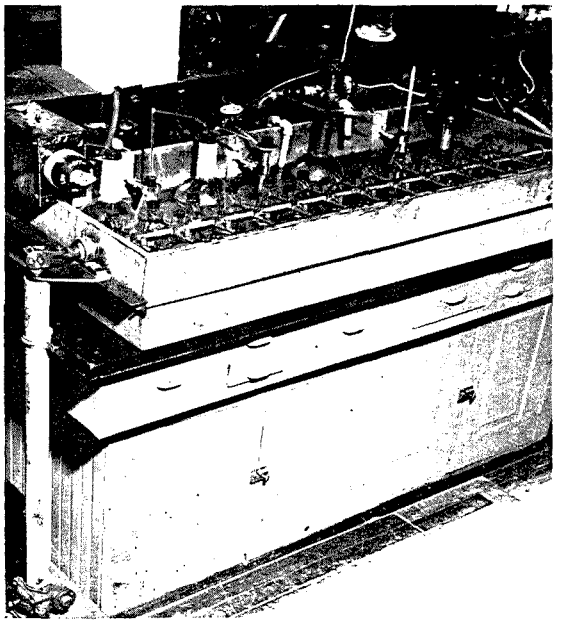

FIG. 1.—Apparatus.

* Presented at the annual meeting of the Association of Official Agricultural Chemists, September 29, 30, and Oct. 1, 1952, at Washington, D. C.

† The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

‡ The authors will be glad to supply sources of materials required, and details of construction, upon request.

the movable rod, which compresses a spring in the rear part of the stationary tube. Clamps for 24 flasks are attached to the central shaft.

A swirling motion is imparted to the solns in the flasks by a combination reciprocating and rocking motion of the flask-supporting shaft. The reciprocating motion is obtained by attaching the supporting shaft to a crankpin by means of a connecting rod. The crankpin is placed about $\frac{1}{2}''$ from the center of the crankshaft and this gives a 1″ reciprocation of the supporting shaft. The rocking motion is accomplished by setting the crankpin at an angle of 5° from the perpendicular. By means of a combination crankshaft and universal joint the angular-set crankpin imparts the desired motion to the flask-supporting shaft.

The crankshaft is connected to a $\frac{1}{3}$ H.P. motor through a speed reducer (on floor, Fig. 1) which gives an operating speed of 120 r.p.m. The central shaft extends through the end walls of the bath and is supported on each end by a bearing which is in turn supported by an iron shelf bolted to the outer walls of the bath.

The central shaft is placed closer to one side of the bath, thus increasing space on the other side to accommodate thermometers, the thermoregulator, circulating pump, and three 1 liter wash bottles in which washing water is heated. Erlenmeyer flasks holding the thermometers and the thermoregulator are held in place by screw clamps which are soldered to strips of stainless steel.

Water in the bath stands at a height of $3\frac{1}{2}''$ and is obtained from the laboratory hot-water supply; thus the time required to attain a temperature of 65° is materially shortened. This requires that water be emptied from the bath after it is used by a siphon from the pipe extending over the side center of bath; there are no openings in the bottom. Two flexible-type heating coils, each 11 ft. long, of 1500 watt capacity, are the additional source of water heating. These coils are on the bottom of the bath and are covered by a false bottom of stainless steel in which holes 1″ in diameter are spaced 3″ apart. A mercury plunger relay is mounted in the control box between the power line and the Fenwal thermoregulator.

Some water splash developed because half of the flasks are close to the side of the bath. This was corrected by placing a stainless steel mesh screen, 6″ high, between the flasks and the side of the bath. The flask holders should be designed for either 200 or 250 ml Erlenmeyer flasks. To prevent electrolytic action, it is desirable to have all metal parts in contact with water made of the same metal.

A LARGE CAPACITY CONSTANT AGITATION BATH FOR USE IN CITRATE-INSOLUBLE PHOSPHORIC ACID DETERMINATIONS*

By W. P. MATTHEWS† (North Carolina Department of Agriculture, Raleigh, N. C.)

In order to shorten the time between collection and analysis of some 10,000 fertilizer samples, it became necessary to handle larger groups of samples in the citrate-insoluble P_2O_5 determination. It was decided to substitute constant agitation of the samples in the digestion bath for the manual shaking at five-minute intervals, and to heat the bath by electricity rather than by gas. The described bath is the result

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* Presented at the annual meeting of the Association of Official Agricultural Chemists, September 29, 30, and Oct. 1, 1952, at Washington, D. C.

† Acknowledgment is hereby gratefully made of aid and ideas from other members of the laboratory, especially Mr. R. T. Teague and Mr. J. J. Filicky.

The author will be glad to supply sources of materials required, and details of construction, upon request.

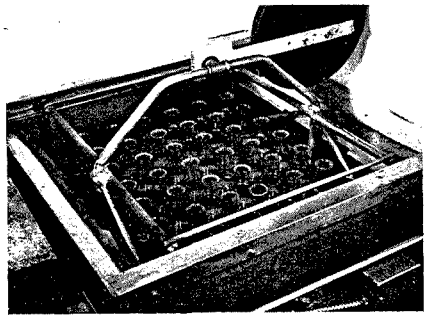

FIGURE 1

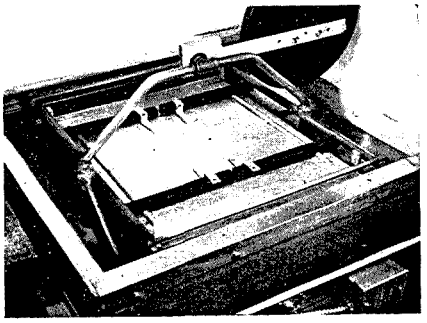

FIGURE 2

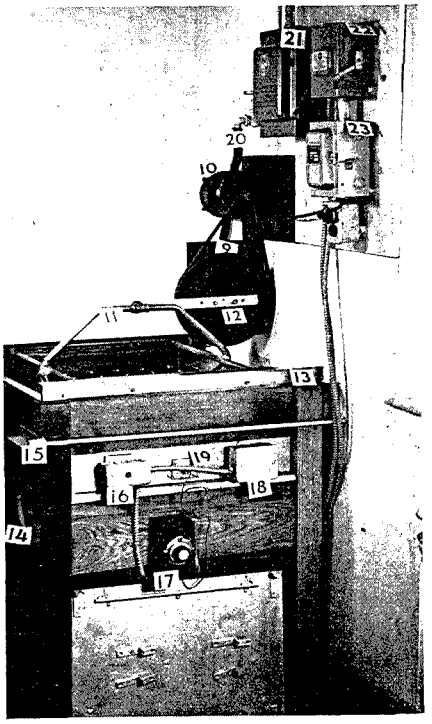

FIGURE 3

of these needs, and has been used for the past year with a great deal of satisfaction.

A heavy sheet copper bath, measuring 24″×24″×9″ deep, was constructed. Its wooden frame was made from a sturdy table from which the top was removed, and a plywood shelf was inserted 9″ from the upper ends of the legs.

Agitation of the flasks containing the ammonium citrate and the sample is obtained by rolling a platform back and forth on ball bearings, a horizontal distance of one inch. This platform is made from a ¼″ piece of sheet brass and has soldered to it 42 copper cups for holding the 125 ml Erlenmeyer flasks. The platform is suspended, by brass rods bolted into each corner, from the cross arms on which the bearings are attached. The bearings roll between 1½″ brass angles, which are adjusted to hold the platform assembly firmly in line. The entire assembly is moved back and forth at the rate of 175 round trips per min. This movement is accomplished with a ⅓ H.P., 1725 rpm motor, coupled with a V-belt to a 10″ pulley (Fig. 1). The large wheel has attached to it a ball-bearing cam which gives the desired horizontal movement. The cam and platform assembly are joined by a flat drive rod which is attached at the top of the arched support. Operation is practically noiseless.

The large pulley and cam, with its support, was taken from a discarded Ross-Kershaw shaking apparatus; however, an equally good, if not better, arrangement may be made by using a 10:1 speed reducer and a machined cam to fit the reducer shaft.

The flasks are all closed simultaneously by placing over them a piece of sponge

rubber cemented to $\frac{3}{4}$" plywood. The sponge rubber is prevented from absorbing the liquid in the flasks by a rubber dam which is stretched over it and cemented to the edges of the plywood (Fig. 2).

Heating is accomplished by use of a 6000 watt element in the bottom of the bath, and a 2000 watt element screwed into the side. A thermostat with a ±5°C. differential is employed to control the larger element, while a fine adjustment Fenwal thermostat controls the 2000 watt heater more closely. Both heaters are used to bring the water in the bath to the required 65°C. initially, but only the smaller one is needed to maintain the temperature.

The volume of water needed to cover the flasks sufficiently is approximately 13 gallons. Thirty minutes is ample time for heating the bath from room temperature to 65°C. before placing the samples and filter papers in the flasks. No shaking by hand to break up the paper is necessary, nor are the flasks removed from the bath in order to add the sample.

The motor, speed reduction unit, and switches are fastened to the wall in this installation, while the thermostats are mounted directly on the bath or frame (Fig. 3). A lever-operated valve empties the water through an outlet in the bottom of the bath directly into the drain line when cleaning is required.

This bath requires little attention during digestion, and its increased capacity has enabled the laboratory to maintain an accelerated schedule while actually decreasing the physical effort expended on this analysis.

Analysis of samples run in both the constant agitation and "hand shaking" baths show very good agreement.

A NEW FLOTATION METHOD FOR THE DETERMINATION OF INSECT INFESTED WHEAT

By DOROTHY B. SCOTT (Division of Microbiology, Food and Drug Administration Department of Health, Education, and Welfare, Washington, D. C.)

Following the publication of a flotation procedure for detecting internal infestation in wheat (1), this laboratory has been investigating several aspects of the method. The effect on recovery by changes in the salt used, specific gravity of the salt solution, and manipulation of the sample are under investigation. The preliminary data obtained from these investigations indicate that the results are influenced by the type of wheat involved, type of insect causing the damage, and the type of damage in the wheat being tested. The results also seem to indicate that the kind of salt used has little bearing on recovery, but the use of salt solutions of higher specific gravity than that suggested by Apt (2% $Fe(NO_3)_3 \cdot {}_9H_2O$ increases the recovery of insect-damaged kernels. Under practical conditions of use, the increased recovery of infested wheat kernels must be balanced against a similar increase in the number of normal kernels which also float. Thus there is the practical problem of weighing any increase in the per cent of insect damaged kernels recovered against the floating of undamaged kernels which must then be picked over before an accurate count can be obtained on the number of insect damaged kernels.

One approach to this problem is to coat the kernels with a volatile silicone* so that the kernels are less readily wet when immersed in the salt solution. The silicone coating is colorless and of molecular thickness.

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* The use of a volatile silicone was suggested by Dr. Henry Fischbach, Division of Pharmaceutica Chemistry, Food and Drug Administration. The "silicone" used was a mixture of dimethyldichlorosilane and methyltrichlorosilane.

METHOD

Fold double a piece of metal screening about 5″ square and then into a pocket to hold a small wad of absorbent cotton between a double layer of wire gauze so that none of the cotton projects out of the wire. Pour approximately 2 ml of the volatile silicone (General Electric SC-77) on the cotton in the screen and drop immediately into a large-mouth, screw-top, 1 qt. Mason jar contg 300 g of wheat. (HCl fumes from the silicone make the use of a hood or strong current of air advisable.) Screw the top of the jar in place and expose the grain to the fumes for one min., agitating continuously. Remove the wire screen and wad of cotton from the jar, and immediately fill it to the neck with 20% NaCl soln. Gently agitate the jar and contents by turning it upside down and back again several times. Remove the cap and skim off the floating grains of wheat. Screw the cap on again and repeat, turning and skimming several times.

Spread the floating grains out on absorbent paper as they are removed. Examine the floated grains on sandpaper, rolling them so that all sides can be examined.

RESULTS

Using this procedure, it is possible to float out for examination most of the kernels which have been bored by insects and which are obviously insect eaten. Grains which are shriveled, those with glumes attached, and some with the pericarp partly detached and other grains with air pockets also float. The number of floated kernels without obvious external insect damage varied from 7 to 35 g from original samples of 300 g each. Some of the floating kernels with no obvious external evidence of insects contain insects within the kernel. The silicone procedure resulted in higher percentage recoveries of visibly infested kernels with lower numbers of floating kernels not visibly infested than flotation procedures on samples not treated with the silicone.

CONCLUSIONS

A method utilizing a volatile silicone for the flotation of insect damaged wheat is given.

Because of the active interest in infested wheat and the possibility that the principle of coating the kernels with a silicone may be of interest to other investigators, this progress report is presented and work will be continued.

REFERENCES

(1) APT, A. C., *Milling Production*, May, 1952.

A POLAROGRAPHIC ELECTROLYSIS VESSEL

By ARTHUR D. ETIENNE (Alcohol and Tobacco Tax Division Laboratory, U. S. Internal Revenue Service, Washington, D. C.)

This note describes a simplified electrolysis vessel for polarographic analysis which can be readily fashioned by anyone reasonably adept at glass blowing. The vessel has numerous advantages, particularly when many samples are to be analyzed at constant temperature. The vessel consists of two parts, the cap with external electrode, and the sample cell (Fig. 1).

The external electrode consists of a 50 ml round bottom flask with a bent 10 mm diam. side arm which forms the salt bridge. The side arm has two sintered glass disks, sealed in at the bottom and near the top to support the KCl-agar mixture. The upper disc prevents the KCl-agar mixture from blowing back if the nitrogen is

inadvertently turned on while the capillary is in position. The side arm and nitrogen inlet tubes are ring-sealed thru the top of an outer section of a 34/28 standard taper Pyrex joint to form a cap for the sample cell. The sleeve for the dropping Hg electrode is also sealed into the cap and positioned as shown in Fig. 1, top view.

The sample cell is made from the inner section of a 34/28 standard taper Pyrex joint sealed off so that its capacity is 30 ml to the bottom of the grind.

The cell bath, Fig. 2, is fashioned from a 500 ml Erlenmeyer flask into which is sealed an inner tube slightly larger than the sample cell. The water outlet is placed at the top of the bath so that water will completely fill it. The cell is held in position in the bath by means of a rubber gasket. The space between the cell and inner tube is filled with water, and water from a constant temperature bath is circulated through the cell bath by a motor driven pump.

In operation, the sample is measured into the cell and placed in the constant temperature bath where it is bubbled with nitrogen introduced through the sin-

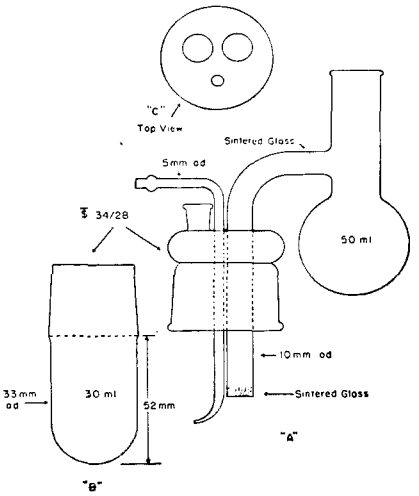

FIG. 1.—Electrolysis vessel.

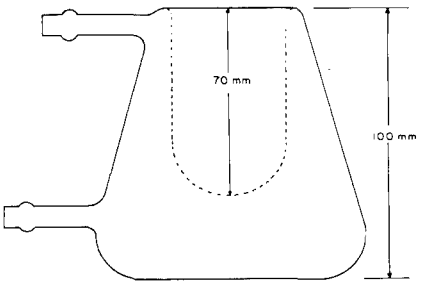

FIG. 2.—Cell bath.

tered glass tube. (The sintered glass greatly increases the rate of de-oxygenating the sample while it is brought to temperature.) The sample and cell is then placed in the cell bath and held in position under the cap by means of a platform which may be swung under the cell bath. Nitrogen is again bubbled through the sample for several minutes to remove all oxygen from the vessel; the capillary is then inserted in the sleeve and prevents any further contamination with oxygen. When not in use, a cell containing saturated KCl is positioned under the cap.

After an analysis, the electrode assembly is rinsed with a stream of distilled water and excess is removed by blotting with tissue. To catch the rinse water, a 10 cm^2 funnel, mounted in a block of wood, is positioned under the assembly. To prevent the loss of Hg down the drain, the stem of the funnel is sealed off to form a trap, and a side arm carries off the water.

This electrolysis vessel has the following advantages:

(a) By using the outer section of the standard taper joint for the cap, the possibility of the sample coming in contact with the joint is greatly reduced.

(b) The complete analysis is conducted in an atmosphere free from oxygen.
(c) The entire electrode assembly can be clamped in position with the bath and cell assembly as the only movable parts.
(d) The assembly is easily cleaned and rinsed.
(e) Additional samples are readily positioned and held at constant temperature.

THE SPECTROPHOTOMETRIC IDENTIFICATION OF DEHYDROACETIC ACID IN CHEESE

By CHARLES F. BRUENING (Food and Drug Administration, Department of Health, Education, and Welfare, Chicago, Illinois)

Recently a qualitative test and a quantitative procedure for dehydroacetic acid (DHA) in cheese were studied collaboratively and found satisfactory (1). The qualitative test was adapted from the quantitative salicylaldehyde colorimetric method of Woods, *et al.* (2), and the quantitative procedure was essentially the spectrophotometric method of Hogan and De Long (3).

In those instances where further confirmatory evidence of the presence of DHA in cheese is desired, two additional tests are now proposed. The first is based on an observation that DHA in 0.1 *N* NaOH solution gives a maximum absorption peak at 292 mμ, whereas in the quantitative procedure (3), referred to above, the measurement is made in 0.04 *N* HCl at 307 mμ. Thus, as the solvent is changed from 0.04 *N* HCl to 0.1 *N* NaOH, the absorption peak shifts from 307 to 292 mμ. Eight commercially packaged cheese samples of different varieties, containing from 20 to 250 p.p.m. of added DHA, all gave an absorption peak at approximately 292 mμ. Nine DHA-free cheeses gave no discernible inflections or peaks at 292 mμ.

The second test is concerned with the relative heights of the absorption peaks at 292 and 307 mμ. The ratio of absorbancy at 292 mμ to that of 307 mμ varies over a very narrow range for cheese containing DHA, ranging from 0.70 for an aq. solution of the pure acid to a maximum of 1.04 for one cheese containing 20 p.p.m. of DHA. In eleven other samples, the maximum was 0.91. In cheeses containing no DHA, absorption at these two wavelengths is very low; the corresponding ratios are very variable and usually several-fold greater.

It was also observed that DHA in 0.1 *N* NaOH gave an additional absorption peak at 231 mμ in contrast to that at 225 mμ in 0.04 *N* HCl. These peaks were not included in the proposed test because in this part of the ultraviolet spectrum, many cheeses give high background absorption which obscures the DHA peaks.

REFERENCES

(1) RAMSEY, L. L., *This Journal*, **36**, 744 (1953).
(2) WOODS, L. A., SHIDEMAN, F. E., SEEVERS, M. H., WEEKS, J. R., and KRUSE, W. T., *J. Pharmacol. Exptl. Therap.*, **99**, 84 (1950).
(3) HOGAN, D., and DE LONG, R. F., (Marathon Corporation). *Private communication*, Sept. 25, 1950.

BOOK REVIEWS

The Intelligent Use of the Microscope. By C. W. OLLIVER. viii+192 pp. Illus., Index. Chemical Publishing Co., Inc., New York, N. Y., 1953. Price $4.00.

The author defines the title of his book in the introduction in which he states that the intelligent use of the microscope involves knowing "the rules which can, and those which cannot, be ignored according to circumstances." Herein lies the value of this compact treatise on the microscope. For the beginning student or for the specialist who uses the microscope as an adjunct to the technique of his particular field, the book is a practical guide to getting the most out of the compound microscope.

The book is limited wisely to instrumental technique and does not cover microscopic technique involving material or slide preparation, which is adequately covered in many texts dealing with the application of the instrument to the particular science. As such, the book will be a valuable addition to the microscopist's reference shelf since it expands upon the material often covered only briefly in other texts.

The chapter on illumination, which is one of the most important phases of the intelligent use of the microscope, is especially noteworthy. This is covered in a practical manner and the reader can evaluate and select the best type of illumination for the particular job. The material is summarized in table form for ready use.

A thorough discussion of filters as an aid to ordinary visual observation, as well as in photomicrography, adds to the value of this text. Although of much importance to photomicrography, which the author deals with in a separate chapter, the expeditious use of color filters can be equally important in obtaining detail and contrast in ordinary observation.

The author concludes with a chapter on the newer subject of phase contrast microscopy. The latter is treated briefly for the beginner but includes an excellent bibliography for those who wish to pursue the subject further.

WM. V. EISENBERG

Microscopy for Chemists. By HAROLD F. SCHAEFFER, D. Van Nostrand Co., Inc., New York, N. Y., 1953. viii+264 pages. Illustrated. Price $4.50.

The ever-increasing variety of problems capable of being solved by microscopic procedures emphasizes the need for systematic training in the use of the microscope. The purpose of this volume is to give an understanding of the construction and basic principles of the microscope and the accessories or modifications adapting it to use in specialized fields. It has been prepared for use either as a classroom text or as a source book for the chemist who must depend upon his own resources in learning the techniques of microscopy.

The first half of the book consists of a discussion of principles and procedures, whose scope is indicated by the titles of the chapters: I. Principles governing lenses; II. Getting acquainted with the microscope; III. Properties of objectives and oculars; IV. Illumination: Equipment and principles; V. Permanent records: Preparation of sketches and photomicrographs; VI. Quantitative microscopy; VII. Polarized light: Principles, sources, and applications; VIII. Chemical procedures on a microscopic scale: General; IX. Chemical procedures on a microscopic scale: Inorganic reactions; X. Chemical procedures on a microscopic scale: Organic reactions.

Study problems are given at the end of each chapter, and thirty-two laboratory experiments related to each of these subjects are given in the latter half of the book. A condensed bibliography lists 115 references to more detailed texts and papers dealing with specialized fields.

While the author does not present an exhaustive study of chemical microscopy,

the information given should enable the chemist to adapt various published micro or macro methods to his own field, or to develop new procedures.

ALBERT H. TILLSON

Manual of the North American Smut Fungi. By GEORGE WILLIAM FISCHER. Ronald Press Co., New York, N. Y., 1953. 343 pages. Price $8.75.

This text is written primarily for those interested in plant pathology, mycology, or agronomy for the purpose of identifying the various forms of smut fungi found in North America.

The data assembled in the book bring together the most recent facts known about the smuts, as well as those scattered through reports of earlier records. These data cover the description of 22 genera of smuts, including 276 species which are found on some 242 species of host genera.

The author begins the manual with an index to the host genera with a key to the species of smut fungi. This index is followed by a key to the genera of the smut fungi. Following this key, the next portion of the text comprises a description of the 22 genera. With each description of these genera, there is an accompanying key to the known species. Each species listed is then described. These descriptions include the various synonyms, the hosts on which it occurs, the characteristic appearance on these hosts, and its geographical distribution.

An outstanding feature of the manual is the numerous illustrations of the spore forms of the smuts described. There are 136 illustrations showing the characteristic spores and some showing the appearance on the host genus. These illustrations are of considerable aid in identifying the smut fungi.

This manual has been written primarily for the identification of the smut fungi, and in order to facilitate this purpose, the genera, with their species, have been arranged in alphabetical order. In this way, easy reference can be made to some particular genus. No reference to family affiliation has been given because the author considers that such detail requires considerable research and time-consuming efforts.

The book is well organized and the contents are presented in such a manner that it should prove a useful aid to all botanists and especially to those who specialize in the study of the smut fungi.

F. ALLEN HODGES

Quantitative Chemical Analysis. By CHARLES W. FOULK, HARVEY V. MOYER, and WILLIAM M. MACNEVIN. McGraw-Hill Book Co., Inc., New York 36, N. Y., 1952. 484 pages. Price $5.00.

The stated aim of the authors is "to develop in the student an understanding of the science of chemical measurement, not simply an ability to analyze a few materials." This is as important to the experienced analyst as to the beginner.

The text has the first twenty chapters (280 pages) devoted to a broad discussion of the principles of chemical analysis, followed by thirty-three laboratory exercises covering gravimetric, volumetric, colorimetric, and electrometric measurements.

It is the first section of the book in which members of this Association will be particularly interested. Here will be found a clear discussion of the principles of analytical chemistry. Chapters of particular interest include the chemical balance and its use; the common operations of quantitative analysis; the operations of gravimetric analysis, analytical precipitates; volumetric solutions; solutions of acids, bases, and salts; oxidation-reduction reactions; precipitation and complex formation; errors in chemical analysis; oxidation-reduction potentials; and colorimetric analysis.

R. A. OSBORN

Aids to Analysis of Foods and Drugs, 7th Edition. By J. R. NICHOLLS. Bailliere, Tindall and Cox, 7 & 8 Henrietta Street, London, W.C. 2, England, 1952. 516 pp. Price 12*s*, 6*d*. ($1.80).

The scope and content of this book is the same as that of the previous edition which was reviewed in *This Journal*, **26**, 290 (1943). Almost 100 pages have been added, chiefly in the form of a 54-page initial chapter covering the main constituents of foods and the general methods of food analysis.

It is rather difficult to assess the value of this book to the food and drug chemist in this country. It certainly cannot be placed in the hands of a beginner in this field because of a wide difference in stress and point of view of the English chemist. First, there is a difference in emphasis on the various fields. Much attention is given throughout the book, as well as in the miscellaneous chapter, to preservatives which are now rarely encountered in our domestic foods. Materials that are now of real concern to the American food chemist, such as organic pesticidal residues, are not mentioned at all. Second, there are also important differences in methods, as for example in the determination of extraneous material. Seven pages are devoted to the topic "Dirt in Milk," and the very complicated procedure which is presented is in marked contrast to the simple sediment test used in this country. The Babcock test is now incorporated in this edition, but a false impression of the importance of this test in the United States would be obtained from the fact that of the eleven pages devoted to the determination of fat in milk, only one page is devoted to the Babcock method. Third, there is the omission of tests which are very useful in this country, such as the determination of squalene as an index of the quantity of olive oil in mixtures, and methods for the determination of egg content of various foods. Finally, references to "United States Standards" frequently quote the old advisory standards without so designating them. The drug chapter is concerned primarily with products which appear in the British Pharmacopeia, and the most modern drugs which are discussed are the barbiturates. Not even the sulfonamides are mentioned. The references, as expected, are predominately to *The Analyst* and coverage of that Journal is fairly complete. Coverage of other journals in the food and drug analysis field, however, is rather sketchy.

The main value of this book will be as a "refresher course" to the experienced analyst who will be aware of these differences. This volume is very readable and contains a remarkable amount of information on "classical" food chemistry which is well worth the price quoted.

WILLIAM HORWITZ

Carl Alsberg, Scientist at Large. Edited by JOSEPH S. DAVIS. Stanford University Press, Stanford, California, 1948. xi+182 pp. Price $2.00.

This book is the outcome of a desire of groups of friends of Carl Alsberg at Berkeley and Stanford Universities to pay tribute to him in an enduring fashion. In selecting contributors, the Editor stated that the book should be designed "not only for Carl's many friends, but also for scientists, scholars, administrators, and their potential successors, to many of whom his life and career seemed to us to have a distinct message." Death among the contributors, together with interruptions caused by the war, delayed completion of the project. The lapse of time between inception and completion was regretted by the Editor "because each year has taken its toll of those who would have read the book with keenest appreciation." However, it can be agreed that the elapsed "eight years is a short time in the lives of men, and the subject is almost timeless." To those who now remain, fortunate enough to have known and worked with Carl Alsberg, the book completes an understanding of the man, and to those as well as to others less fortunate, it depicts the career of the research scientist turned administrator.

BUREAU OF CHEMISTRY CHIEFS OF BRANCH LABORATORIES, MARCH 1913

(See Review of Carl Alsberg, "Scientist at Large")

(1) A. L. Sullivan. (2) W. D. Bigelow. (3) Carl L. Alsberg. (4) D. B. Bisbee. (5) R. W. Hilts. (6) R. W. Balcom. (7) W. C. Burnet. (8) A. S. Mitchell. (9) A. L. Knisely. (10) W. J. McGee. (11) Wm B. Alwood. (12) W. L. Dubois. (13) R. E. Doolittle. (14) A. L. Winton. (15) H. L. Schulz. (16) B. R. Hart. (17) C. S. Brinton. (18) F. W. Liepsner. (19) F. G. Smith. (20) A. E. Taylor. (21) A. W. Hansen. (22) S. H. Ross. (23) R. S. Hiltner. (24) H. M. Loomis. (25) M. C. Albrech.

The first chapter on "The Making of the Man" fittingly goes to beginnings. It is by Alfred L. Kroeber, Emeritus Professor of Anthropology, University of California, a life-long friend. It recites ancestral influences, boyhood experiences, scholastic environment, and their bearing on this scientist of pure investigative bent.

Alsberg's *Work in the Natural Sciences* is a subject of the chapter by Donald D. Van Slyke, Research Chemist, Rockefeller Institute of Medical Research. In it are reviewed the scientific activities before the scientist became the administrator, and from it is learned that Alsberg actively sought the appointment as Chief of the Bureau of Chemistry in the United States Department of Agriculture. He saw that whoever got the position "would have it in his power to have done, and to stimulate a great deal of good work"; and that the man "if he is a thorough scientist with good ideas, he will then be able to do an immense lot of good."

How well he fulfilled these ambitions is set forth in the chapter "Chief of the Bureau of Chemistry" by Fred B. Linton, his administrative assistant in the Bureau. Here one sees the conflict of opposing demands and the struggle between the urge to remain the scientist and at the same time to become the administrator. The metamorphosis was never entirely complete. His many achievements illustrating his growth as a director of scientific endeavors during the tenure of this office are placed in review. He resigned the position because he did not want to remain predominantly an administrator. He was influenced in making this decision because he could see little prospect of obtaining a sufficient appropriation by which the Bureau could carry out what he regarded as an adequate research program.

Next to pure research, Alsberg's chief interest was in teaching others to know. It is clearly evident in the chapter "University Professor and Administrator" by Robert D. Calkins, Vice-President and Director, General Education Board, an associate of Alsberg's at the Food Research Institute and a colleague at Berkeley, how Alsberg's unusual talents were combined in the dual role of directing research as expanded to new fields in economics at the Food Research Institute and of teaching as a Professor at Stanford. One learns from the chapter "Social Scientist beyond the University" by John D. Condliffe, Professor of Economics, University of California of those characteristics of Alsberg's which made his transition from cold physical science to the more humane social sciences so successful.

Three papers from the pen of Alsberg are included in the book. One is "Progress in Chemistry and the Theory of Population." It is explained that it was included for three reasons: "The subject attracted Alsberg's interest early and late; the treatment illustrates his way of bringing scientific knowledge to bear on a large social problem; and despite gains in understanding in the twenty-five years since the paper was written it is still germane to today's discussions."

The life work of Alsberg is reflected in the second paper, an address given at a Symposium on the Relation of the Social Sciences to the Natural Sciences at a meeting of the American Association for the Advancement of Science, in June, 1931, entitled, "What the Social Scientist Can Learn from the Natural Scientist." An insight into Alsberg's self-evaluation is gained from the third paper, a "Commencement Address at Reed College," June 16, 1938, which is in the nature of an autobiography. The book closes with a bibliography of Alsberg's published works of 212 references.

To those interested in the work of the Association of Official Agricultural Chemists it is surprising to find only one mention of the Association in the book and that in a quotation from a report by Alsberg as Chief of the Bureau of Chemistry. It is unfortunate that in a book dedicated to Alsberg as a "Scientist At Large," his work in the Association has been overlooked. He served that scientific organization as Secretary during most of the period of his eligibility as a member. One of his most important accomplishments in the interest of science was the establishment

of the Association's publications; he was the first editor of the Journal of the A.O.A.C., now in its 36th year and one of the leading publications in its field.

As Chief of the Bureau of Chemistry, Alsberg was associated with many of the members of the Association. One group for which a record is still available is that of the Chiefs of the Field Stations of the Bureau. The accompanying picture (March, 1913; front steps of the Bureau in Washington) is of that group. All are scientists, and among them were leaders in the development of methods of analysis and contributors to the sum total of fundamental knowledge so vital to the enforcement of Federal and State Food and Drug laws. The long record of their achievements in those early days of enforcement is written across the pages of the Proceedings of the Association.

H. A. LEPPER

Organic Reactions, Vol. VII. ROGER ADAMS, Editor-in-Chief. John Wiley & Sons, Inc., 440 Fourth Avenue, New York 16, N. Y., 1953. viii+440 pages. Price $9.00.

As in past volumes of this series, each chapter describes a separate reaction and assembles in one place the major portion of the knowledge regarding that particular reaction. A chemist interested in a reaction described in this volume may be saved many hours of library searching. Enough detailed procedures illustrating the reactions are described that a chemist will probably need only to make minor adjustments in order to adapt a procedure to his own problem. This book is a valuable addition to any chemical library.

The titles of the Chapters are: The Pechmann Reaction; The Skraup Synthesis of Quinolines; Carbon-Carbon Alkylations with Amines and Ammonium Salts; The Von Braun Cyanogen Bromide Reaction; Hydrogenolysis of Benzyl Groups Attached to Oxygen, Nitrogen, or Sulfur; The Nitrosation of Aliphatic Carbon Atoms; and Epoxidation and Hydroxylation of Ethylenic Compounds with Organic Peracids.

The general format of the chapters is essentially the same as in previous volumes, i.e., a brief description of the reaction, a discussion of the mechanism, scope and limitations, experimental conditions, and procedures with detailed examples, tables listing hundreds of examples, and a bibliography. All tables list the reactants and products, and give references for each; in addition, most tables list the expected yield of product, conditions, and promoting agent, if any.

KENNETH A. FREEMAN