TUESDAY—MORNING SESSION

REPORT ON VITAMINS

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

Vitamin A.—The recommendations of the Associate Referee are approved.

Vitamin B_1.—The recommendations of the Associate Referee are approved.

Vitamin B_2 (Riboflavin).—The recommendations of the Associate Referee on riboflavin are approved.

Vitamin C.—The recommendations of the Associate Referee are approved.

Vitamin D.—The recommendations of the Associate Referee are approved.

Nicotinic Acid.—The recommendation of the Associate Referee on nicotinic acid is approved.

Pantothenic Acid.—The Associate Referee made no report.

Folic Acid.—The extensive collaborative study conducted this year has tested the suitability of a number of methods for the determination of folic acid. Results of the study serve as a basis for selecting a single procedure for intensive study next year. The Referee recommends that during the coming year a group of well-experienced collaborators study the procedure considered by the Associate Referee to be the most suitable.

Carotene.—The recommendations of the Associate Referee on carotene are approved.

REPORT ON VITAMIN A

FURTHER COMPARISON OF THE SPECTROPHOTOMETRIC AND ANTIMONY TRICHLORIDE METHODS FOR VITAMIN A IN MARGARINE

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

Two years ago the results of a collaborative study of the determination of vitamin A in margarine were discussed before this Association.[1] The results of this study were not entirely satisfactory, but they indicated, as more recent work has, the nature of the principal faults of the method used. It was quite clear from these results that deterioration of vitamin A occurred at certain points in the procedure. It was suggested at that time that such deterioration might be prevented by proper use of vitamin A

[1] Wilkie, J. B., Report on Vitamin A, *This Journal*, **30**, 382 (1947).

stabilizers, and by increasing the speed of the chromatographing step to minimize the loss of vitamin A on the column.

Studies presented here are concerned with better use of the stabilizers normally present with vitamin A in natural sources. The use of diethyl ether in place of petroleum ether in the extraction step improves the extraction not only of vitamin A, but also of extraneous materials that may increase the stability of vitamin A. Absorption characteristics indicate that these consist chiefly of tocopherols. It is also clear from this study that the use of a smaller adsorption column is advantageous in eluting the vitamin A rapidly and with greater certainty.

The larger column previously recommended has been replaced by a smaller 1 cm diameter column, with a somewhat stronger mixture 1:1 of magnesium oxide and Celite. The solution prior to chromatographing is now evaporated partially to dryness in the presence of sodium hydrosulphite and then evaporated completely to dryness after decantation from the hydrosulphite. This more thorough evaporation eliminates extraneous volatile materials, some of which may also prove to be undesirable eluents. The material to be chromatographed is thus made more uniform from the standpoint of its behavior in the small column on which the vitamin A is more clearly defined and more sharply segregated.

With these changes in the procedure the green fluorescent band seen on the adsorption column, with the use of the weak ultraviolet lamp previously described,[2] is quite compact and its lower surface is sharply defined. The band can be well controlled and proceeds rapidly to the fritted exit disc of the column. The vitamin A band is then very rapidly eluted with 1 per cent of ethanol and the eluate is made to volume with petroleum ether. The density of this solution is measured at various wave lengths and the vitamin A strength is calculated from the E value obtained at 340 mμ.

A factor of 2375, which is in harmony with the 1894 U.S.P. factor, is now used to convert the $E^{1\%}_{1cm}$ values to units per gram.[3]

DISCUSSION

The summarized results in accompanying tables give the results obtained with this modified method. Two tables are given. Table 1 includes results from margarines labeled to contain 9,000 units of vitamin A per pound, and Table 2 from margarines labeled 15,000 units of vitamin A per pound. Values in these tables were calculated from the 325 mμ absorption, from the 340 mμ absorption, and from the antimony trichloride method as well. The tabulated coefficients of variation are es-

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[2] Wilkie, J. B. and De Witt, J. B., *This Journal*, **28**, 174 (1945), "Spectrophotometric Procedure of the E Estimation of the Vitamin A in Oleomargarine."

[3] More recently the figure 1900 has been recognized as a conversion factor in connection with the adoption of vitamin A acetate as the internationa lstandard. If this figure receives general recognition the value of 2375 should be changed to 2382.

pecially revealing. As shown in the third column the actual average unitage values from the two tables calculated from the 325 mμ and the 340 mμ absorptions check within 10 per cent and 6 per cent, respectively, and the 325 mμ values are nearly always higher, as one would expect. Thus, the values based upon 340 mμ value are but little different from 325 mμ values, but, as indicated previously, they lack the extraneous absorption

TABLE 1.—*Summary of vitamin A determinations from 9000 u/lb margarines*

	U/LB				SPEC RATIO		
	Spec 325 mμ basis	Spec 340 mμ basis	*Ratio* Spec 325 / Spec 340	$SbCl_3$ Method	E 290 / E 340	E 325 / E 340	E 350 / E 340
Av. of 16 Samples	10,556	9,668	1.10	8,680	3.56	1.35	0.68
CV*	19.5	10.7	5.0	36.3	20.4	2.7	4.9

* Coefficient of variation.

TABLE 2.—*Summary of vitamin A determinations from 15,000 u/lb margarines*

	U/LB				SPEC RATIO		
	Spec 325 mμ basis	Spec 340 mμ basis	*Ratio* Spec 325 / Spec 340	$SbCl_3$ Method	E 290 / E 340	E 325 / E 340	E 350 / E 340
Av. of 68 Samples	16,122	15,645	1.06	15,360	2.7	1.32	0.678
CV*	25.5	6.9	9.86	24.0	23.0	2.4	4.8

* Coefficient of variation.

which may make the values derived from the 325 mμ values about 8 per cent high, with the possibility of greater variation at this wave length. The coefficient of variation values calculated from these data substantiate the original judgment in this respect, 10.7 and 6.9 being the CV values from the 340 mμ data, against 19.5 and 25.5 from the 325 mμ data.

The manner in which the antimony trichloride values compare with the 340 mμ values is also of interest. The antimony trichloride unitage averages were only 10 per cent and 1½ per cent lower than the 340 mμ "spec" values, but the CV values were 36.3 and 24.0, against 10.7 and 6.9 values from the 340 mμ spectrophotometric data.

The ratio of the E_{290}/E_{340} values have a relative high CV of 23 compared

with low values of 2.4 for the E_{325}/E_{340} ratio and 4.8 for E_{350}/E_{340} ratios. This merely reflects the variability in 290 mμ region undoubtedly caused by the variable extraneous material present and emphasizes the lack of such a disturbing factor in the 325 mμ to 340 mμ region.

Thus the chromatographic spectrophotometric evaluation for vitamin A from readings at 340 mμ has greater precision than that obtained from readings at 325 mμ or from that determined by the antimony trichloride method.

It is believed that this superiority should warrant further collaborative work with the method as now modified.

PROCEDURE

(*A*) *Preparation of Test Solution by Saponification and Extraction.*—Weigh 10 g of oleomargarine into a 300 ml beaker and add 30 ml of boiling 95% ethanol. Stir until sample is completely disintegrated. Add 25 ml of 50% KOH soln. Stir continuously for 5 min. and allow to stand at room temp. for 15 min. (stirring occasionally).

Transfer soln to a 500 ml separatory funnel. Rinse beaker with 100 ml of distilled water in several portions, adding these rinsings to separatory funnel. Add 100 ml of U.S.P. ethyl ether. Shake vigorously, and allow to stand ca 2 minutes. Separate aqueous portion into another 500 ml separatory funnel. Likewise extract the aqueous fraction successively 4 times with 50 ml portions of ethyl ether, adding each in turn to the original extract. In case of slow separation add 2–5 ml of 95% ethanol and swirl gently.

Pour two 200-ml portions of distilled water thru combined ether extracts and discard each washing without shaking. Shake once vigorously with ca 10 ml dilute KOH soln (ca 0.02 *N*). Then pour distilled water with gentle agitation thru extract until it is free from alkali as shown by phenolphthalein. Allow to stand 10 min., discard the separated water, and filter with aid of vacuum thru 2.5 cm. of anhydrous Na_2SO_4 in a 2-cm fritted filter.

After adding ca 5 g of sodium hydrosulphite to the filtrate, evaporate this dry soln on steam bath in a 300-ml beaker to volume of 25–50 ml. Transfer the soln by decanting to 50 ml beaker with 6 vigorous washings of the $Na_2S_2O_4$ using 2–5 ml portions of diethyl ether to completely remove vitamin A from the residue.

Evaporate this soln to dryness on the steam bath. (Dryness is taken to mean cessation of boiling.) Heat for precisely 2 min. after the cessation of boiling, then add 5 ml of petroleum ether[4] and make to volume in a 10 ml glass-stoppered flask with petroleum ether.

(*B*) *Chromatography.*—Prepare column by using small diameter tube (1 cm. in diam.) and ca 10 cm long. (Bottom of tube should be fitted with sealed-in fritted disc of porosity sufficient to offer no significant[5] retardation to flow of solvent or eluate.)

Add 1:1 mixture of MgO (Westvaco #2641) and Celite to form column with height of 2–2.5 cm in the tube. (This material should be packed with blunt rod under 20″ of vacuum to the specified height. Add 15 cm of anhydrous Na_2SO_4 on top of this column.)

Pipet 5 ml of the 10 ml test soln into a 10 ml beaker. After wetting column with petroleum ether and just before surface is dry, pour this 5-ml soln rapidly onto the

[4] The petroleum ether when measured in a 1 cm quartz cell with an ultraviolet spectrophotometer against a no-cell blank should have a transmission of at least 85%. Better grades should have a transmission of 90%.

[5] If the disc will allow a height of 5 ml of water to pass in less than 20 sec. the disc will probably be satisfactory in this respect.

column. A vacuum of 20″ should be maintained on the column during chromatographic separation.

(The surface of the column should be kept wet with petroleum ether at all times. The segregation and progress of the vitamin A through the column is rapid and is easily followed at all times with the weak ultraviolet lamp[2] previously described.)

The lower surface of the A band progresses to the fritted disc with petroleum ether alone, using a 50 ml beaker in a micro-bell jar. The vacuum is then released and the eluate collected is discarded. Use a clean 50 ml beaker in the micro-bell jar to receive the eluted vitamin A. Elute this as rapidly as possible by the use of 1% of absolute ethanol in petroleum ether. The passage of the A band thru the column should be completed in less than 10 minutes.

Make the vitamin A eluate to volume of 10 ml with petroleum ether and make density determination with the spectrophotometer at 290, 325, 340, and 350 mμ, respectively. Calculate the $E^{1\%}_{1cm}$ value at 340 mμ $E^{1\%}_{1cm}$ (340 mμ) $\times 2375 =$ U.S.P. Vitamin A units/gm margarine.

ANTIMONY TRICHLORIDE CHECK METHOD

The 5 ml of test solution remaining after removal of the spectrophotometric sample is made to 10 ml with petroleum ether. One ml of this solution is used for the antimony trichloride determination. This procedure uses distilled chloroform, 5 ml of reagent, and increment method and direct reading photometry. Since adequate details of this method have been published elsewhere, the unimportant minor modifications used in this paper will not be elaborated upon.

RECOMMENDATIONS*

After the following minor corrections are made it is recommended that the present tentative method for vitamin A in fish liver oils be made official, first action.

In sec. **36.2,** page 599, the ether should be specified as "U.S.P. ethyl ether in ½-pound cans, anesthesia grade, free from peroxides."

In sec. **36.3,** second sentence, the "ground glass joint" should be changed to "glass joint."

In sec. **36.3,** seventh line, change the time from "2 minutes" to "allow the mixture to stand (about 2 minutes) until separation is visibly complete, as determined by the absence of refraction streaming and the presence of distinct layers."

Sec. **36.4,** page 600, "density of 0.4" should be changed to "0.398."

Sec. **36.6,** page 601, the density values given in the transmittancy-density table should be corrected to agree with the following:

T.	D.	T.	D.
0.7	2.16	29.5	0.530
7.0	1.16	32.5	0.488
11.0	0.959	55.5	0.256
26.5	0.577	63.0	0.201
27.5	0.561	71.5	0.146

* For report of Subcommittee A and action of the Association, see *This Journal*, **32, 47** (1949).

REPORT ON THIAMINE (VITAMIN B_1)

By O. L. KLINE (Division of Vitamins,* Food and Drug Administration, Federal Security Agency, Washington, D.C.), *Associate Referee*

In accordance with the request of the Committee on Methods the procedures for the determination of thiamine (vitamin B_1) contained in the Sixth Edition of Methods of Analysis have been reviewed in the interest of revising their status, where necessary, in preparation for the forthcoming edition of Methods of Analysis.

GROWTH METHOD

The growth method for vitamin B_1 as it appears in the Sixth Edition of Methods of Analysis was adopted as the tentative method in 1940 and was subjected to collaborative study during the following year. The method was used extensively for low potency food materials during the period of development of the chemical and microbiological assay procedures and served well as a means of checking the biological specificity of those shorter procedures. The growth method is not in wide use at the present time, but it is considered advisable to retain it for the purpose of checking biological specificity and to have it appear as an official method in the next edition of Methods of Analysis.

THE FERMENTATION METHOD

This method appears in the Sixth Edition of Methods of Analysis with status undesignated. The procedure is based upon the effect of thiamine as a stimulating agent for the rate of yeast fermentation and is finding wider use as more investigators become familiar with its easy application to a wide variety of assay materials. It has the advantage of being rapid while possessing the important characteristic of biological specificity. Yeast fermentation is stimulated by the pyrimidine of thiamine as well as by thiamine itself, necessitating the use of a sulfite blank step in which the true thiamine is inactivated in the presence of the bisulfite ion. This is of advantage in studies on stability of thiamine. In view of these advantages it is considered important therefore to retain the fermentation method for thiamine.

FLUOROMETRIC (THIOCHROME) METHOD

The thiochrome method was adopted in 1943 as tentative and following collaborative study last year (1947) was made official, first action. This method has now come to be used almost universally for the determination of thiamine in all types of materials. It has stood the test not only of extensive collaborative study but of wide use in many countries.

In the interest of widening the application of the official method as it

* E. M. Nelson, Chief.

appears in the Sixth Edition of Methods of Analysis certain minor definitive changes are needed.

RECOMMENDATIONS*

It is recommended—

(1) That the growth method for thiamine (vitamin B_1) be adopted as official, first action.

(2) That the fermentation method for the determination of thiamine be made official, first action.

(3) That sec. 36.25 (Preparation of assay solution) be changed as recommended in the report of the Associate Referee.†

REPORT ON RIBOFLAVIN (MICROBIOLOGICAL)

By HENRY W. LOY, Jr. (Food and Drug Administration, Federal Security Agency, Washington, D.C.), *Associate Referee*

The tentative microbiological method for the determination of riboflavin (*This Journal*, **30**, 79) was studied collaboratively (*This Journal*, **24**, 413; **25**, 459; **26**, 81; **28**, 560; **29**, 25; and **31**, 701). This method has been in use since such adoption and is regarded as suitable. Furthermore, this year's work on the chemical method for the determination of riboflavin included a comparison with the microbiological method. The results are included in that report (*This Journal*, page 461). More uniform results have been obtained in the later studies of the microbiological method than were obtained in the earlier studies, indicating that experience with the method is helpful in its proper application.

RECOMMENDATION‡

It is recommended that the tentative microbiological method for the determination of riboflavin, as revised, be made official, first action.

REPORT ON RIBOFLAVIN (CHEMICAL)

By HENRY W. LOY, Jr. (Food and Drug Administration, Federal Security Agency, Washington, D.C.), *Associate Referee*

Last year, it was recommended that further work be conducted on the fluorometric method (*This Journal*, **30**, 392; **31**, 701) that was the subject of that year's study. Although many of the collaborators for last year obtained results that were in good agreement with those obtained by the microbiological method, there was a wide divergence of some of the

* For report of Subcommittee A and action by the Association, see *This Journal*, **32**, 47 (1949).
† Details of the revision are given in *This Journal*, **32**, 104 (1949).
‡ For report of Subcommittee A and action of the Association, see *This Journal*, **32**, 48 (1949). Details of the revised method are given in *This Journal*, **32**, 105 (1949).

results. Therefore, the study did not lead to the recommendation for adoption of a fluorometric method, but it seemed desirable to determine if further experience with the method would lead to more uniform results.

The fluorometric method used in this year's study was the same as the one used in last year's study, except for a few minor modifications, related to more specific directions for some steps of the procedure. The collaborators were asked to submit results obtained by the specified fluorometric method, and, where possible, by the tentative microbiological method (*This Journal*, **30**, 79) and any other methods in which they might be interested, for 3 samples: No. 1, a solution of riboflavin[1]; No. 2, an enriched flour; and No. 3, a dried brewers' yeast.

Of the 9 collaborators who submitted results by the specified fluorometric method, 5 submitted results by the tentative microbiological method. These collaborators also submitted results in 4 cases by fluorometric methods that differed from the specified method, and in 1 case, by a microbiological method that differed from the tentative method.

TABLE 1.—*Results of collaborative study on Riboflavin*

COLLABORATOR NO.	FLUOROMETRIC METHODS		MICROBIOLOGICAL METHODS	
	COLLABORATIVE	OTHER	TENTATIVE	OTHER
	Sample No. 1—Riboflavin Solution (Mg. of riboflavin per ml. of sample)			
1	0.401			
2	0.39	0.39		
3	0.399		0.400	
4	0.40	0.40	0.40	
5	0.383		0.438	
6	0.381	0.373	0.396	
7	0.40			
8	0.390			
9	0.400		0.400	0.420
Av. ±S.E. of Av. for the 9 collaborators on the fluorometric method	0.394 ±0.003			
Av. ±S.E. of Av. for the 5 collaborators on both methods	0.393 ±0.004		0.407 ±0.008	
Student's t value for comparing both methods			=1.56	
P value			=0.16	
Comment	No significant difference			

[1] This sample was a 0.02 *N* acetic acid solution containing, per ml, 0.4 mg of U.S.P. Riboflavin Reference Standard, 100 mg of urea, and 5.0 mg of chlorobutanol.

TABLE 1—(*continued*)

COLLABORATOR NO.	FLUOROMETRIC METHODS		MICROBIOLOGICAL METHODS	
	COLLABORATIVE	OTHER	TENTATIVE	OTHER
	Sample No. 2—Enriched Flour (Mg. of riboflavin per lb. of sample)			
1	1.00			
2	0.96	1.15		
3	0.98		0.98	
4	1.03	1.01	1.09	
5	1.09		1.04	
6	1.01	1.00	1.08	
7	1.04			
8	1.10			
9	1.05		1.12	1.03
Av. ±S.E. of Av. for the 9 collaborators on the fluorometric method	1.03 ±0.02			
Av. ±S.E. of Av. for the 5 collaborators on both methods	1.03 ±0.02		1.06 ±0.02	
Student's t value for comparing both methods			=1.06	
P value			=0.32	
Comment	No significant difference			
	Sample No. 3—Dried Brewers' Yeast (Mg. of riboflavin per g. of sample)			
1	0.0399			
2	0.04	0.04		
3	0.0396		0.0397	
4	0.041	0.042	0.041	
5	0.041		0.041	
6	0.0431	0.0425	0.0390	
7	0.041	0.043		
8	0.0416			
9	0.0525		0.0413	0.0435
Av. ±S.E. of Av. for the 9 collaborators on the fluorometric method	0.0422 ±0.0013			
Av. ±S.E. of Av. for the 5 collaborators on both methods	0.0434 ±0.0023		0.0404 ±0.0004	
Student's t value for comparing both methods			=1.28	
P value			=0.24	
Comment	No significant difference			

The results are shown in Table 1. In only one case, collaborator 9, sample No. 3, in the specified fluorometric method, was there a wide divergence in the results. There is no explanation to offer for this wide divergence. Except for this one case, the results obtained by the specified fluorometric method are more uniform than those of last year's study and compare favorably with results obtained by the tentative microbiological method. From the results of the 5 collaborators who used both methods, the average results by the specified fluorometric method and the tentative microbiological method are, respectively, 0.393 and 0.407 mg. per ml., for sample No. 1; 1.03 and 1.06 mg. per lb., for sample No. 2; and 0.0434 and 0.0404 mg. per g., for sample No. 3. As can be seen from Table 1, in no instance is there a statistically significant difference between the results of the two methods.

This study indicates that experience with the fluorometric method is helpful in its proper application. This has been found true with the microbiological method as well (*This Journal*, **32**, 105).

COLLABORATORS

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Victor B. Williams and Laura M. Flynn, Univ. of Missouri, Columbia, Mo.

The Associate Referee wishes to express appreciation to the collaborators and to their organizations for their cooperation in this study.

RECOMMENDATION*

It is recommended that the fluorometric method† for the assay of riboflavin herein described be made official, first action.

REPORT ON FOLIC ACID

By Laura M. Flynn (Dept. of Agricultural Chemistry, University of Missouri, Columbia, Mo.), *Associate Referee*

The microbiological method for determining folic acid was subjected to collaborative assay under the auspices of the Association of Official Agricultural Chemists in 1947. Results showed fair agreement among the seventeen laboratories participating in the study. However, the findings in this first collaborative investigation of the vitamin (1) indicated the need for further study of its determination.

* For report of Subcommittee A and action of the Association, see *This Journal*, 32, 47 (1949).
† The details of the method are given in *This Journal*, 32, 108 (1949).

Both *Lactobacillus casei* and *Streptococcus faecalis* have been used as test organisms in the microbiological assay of folic acid. *S. faecalis* grows well in a semi-synthetic medium, is less dependent on unidentified growth stimulants and is often read turbidimetrically after 18 hours' incubation. It does not respond to folic acid conjugates. On the other hand, *L. casei* shows greater acid production, and is sensitive to the vitamin over a wider range and at a lower concentration. When turbidity is used as the criterion of growth dose-response curves for standards and for unknowns do not show parallelism until a long incubation period (40 hours or more) has elapsed, when *L. casei* is used. Comments on the 1947 assays indicated that peptone is not a satisfactory source of the unidentified growth substances known to be necessary when this organism is used in folic acid assays. *L. casei* was selected for the 1947 study, and since half of those replying to the questionnaire sent with the 1947 samples indicated a preference for this bacterium, collaborators were asked to try it again in the 1948 investigation.

Roberts and Snell (2) have recently proposed a medium for *L. casei* which might improve materially the assay for pteroylglutamic acid. Its content of trypsinized casein should insure rapid early growth of the organism, thus overcoming one of the main objections to the use of *L. casei*. For small laboratories, however, which often do occasional assays for several vitamins, it seems advantageous to use, whenever possible, only materials which are commercially available, constant in composition, and easily stocked and stored in quantity. This would cut to a minimum the time used by technicians in digestions, adsorptions, and the preparation of special supplements of limited use. The utilization of materials commercially available should also make possible greater uniformity between laboratories in a collaborative assay.

Several years ago Davis and Dubos (3) demonstrated that the early growth of tubercle bacilli in submerged culture in liquid media was facilitated by the addition of the commercial product "Tween 80" (polyoxyethylene sorbitan monooleate). Unpublished data complied in the vitamin assay laboratory of the Department of Agricultural Chemistry at the University of Missouri have proved that "Tween 80" markedly stimulates the early growth of *L. casei* also, in a semi-synthetic medium. Further studies in this laboratory showed that a modified Landy and Dicken medium containing "Tween 80" provides a very acceptable medium for use in folic acid assays. Since this medium had been used successfully for this purpose over a period of several months in the laboratory of the Associate Referee it was selected as the test medium for the 1948 study of the vitamin. This medium differs from the Teply and Elvehjem medium (4) which was used last year, as follows: Substitution of cysteine for cystine, omission of peptone and alanine, minor changes in the vitamin mixture, increase in the amount of manganous sulfate used, and the

addition of "Tween" and glutathione. The medium is easily prepared, is reproducible, and by altering the buffer it can be used successfully with either *L. casei* or *S. faecalis*. It gives much better results with *L. casei* than the original Landy and Dicken medium (5).

Detailed directions for the method were sent to all laboratories which had expressed willingness to participate. Chemists co-operating in the study were asked to assay the samples by any other methods they were using routinely, if time permitted. Five laboratories accustomed to making chick assays for folic acid agreed to assay the samples by chick methods.

The materials chosen for assay in 1948 were (1) dehydrated powdered mustard greens, (2) soy flour, (3) dehydrated powdered egg yolk, and (4) brewers' yeast. In order to insure as much uniformity as possible among the laboratories each collaborator was sent a desiccated culture of bacteria, crystalline pteroylglutamic acid for use as a standard, and sufficient desiccated chicken pancreas for the enzyme hydrolysis of the samples. It was requested that the culture be stored under refrigeration until used, then sub-cultured several times in a complete medium containing liver extract before its utilization as inocula or storage on agar.

At the present time no material is available which is recognized as a reference standard for folic acid, although steps looking toward the development of a reliable reference standard have been taken by the proper authorities. In the absence of an official standard the synthetic pteroylglutamic acid to be sent to collaborators (Folvite 7–7904) was checked in the laboratory of the Associate Referee. A Beckman spectrophotometer was used in the measurement of extinctions at wave lengths 255, 282.5, and 365 millimicrons. $E^{1\%}_{1cm}$ values at these wave lengths were, respectively, 555, 545, and 190. The vitamin was tested at *p*H 11.5, at a concentration of 0.002%. The data given here will serve to characterize the material used as a standard by collaborators. Physical constants for the compound, including extinction data, have been published by Parke, Davis and Company (6) and by the Lederle Laboratories Division of the American Cyanamid Company (7). From extinction data for crystalline folic acid, as published by Parke, Davis and Co., chemists, the material used by collaborators would be calculated to be about 90 per cent pure. On the basis of the extinction data for the crystalline compound as published by chemists at the Lederle Laboratories, the same material would be considered 98 per cent pure.

COLLABORATORS

The chemists co-operating in this study are listed below. Grateful acknowledgement is made of the generous and gracious help of all who took part in the endeavor.

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RESULTS

Assay results submitted by the co-operating laboratories are summarized in Tables 1–6. To facilitate comparisons, arithmetic means and median values are listed in the tables. In the calculation of these means and medians only one figure was used for each laboratory, an average of the several figures from each laboratory as shown in the tables. When a laboratory submitted several assay results the range of these is indicated in the tables. Frequently laboratories sent in only one estimate for each sample, but this estimate represented the average from several assays on the material. Several of the estimates listed, as is shown in the footnotes, were not included in the averages. In one case (Laboratory 12) the chemist sending in the data specified which one of his tests he considered best, giving the reasons, and only that figure was included. The Associate

* Assayed samples by both microbiological and chick methods.

TABLE 1.—*Results of 1948 A.O.A.C. collaborative study of folic acid*
(Assays with *Lactobacillus casei* in specified medium)

LAB. NO.	METHOD OF EVALUATION	SAMPLE I MUSTARD GREENS		SAMPLE II SOY FLOUR		SAMPLE III EGG YOLK		SAMPLE IV BREWERS' YEAST	
		AVE.*	RANGE	AVE.*	RANGE	AVE.*	RANGE	AVE.*	RANGE
		mmg/g	*mmg/g*	*mmg/g*	*mmg/g*	*mmg/g*	*mmg/g*	*mmg/g*	*mmg/g*
(1)	Acidimetric	8.76 (4)	4.98–16.16	3.49 (3)	2.62–4.76	0.828 (3)	0.69–1.00	16.10 (2)	14.50–17.70
(2)	Acidimetric	6.57 (1)		2.14 (1)		0.093 (1)†		18.1 (1)	
(3)	Acidimetric	10.38 (1)		4.12 (2)	3.96–4.29				
(3)	Turbidimetric	8.95 (2)	8.86– 9.03	4.27 (2)	4.02–4.27				
(5)	Turbidimetric	9.3 (1)		3.7 (1)		0.82 (1)		19.7 (1)	
(8)	Acidimetric	Free		Free		Free			
		3.0 (1)		1.1 (1)		0.37 (1)			
		Total		Total		Total		Total	
		9.4 (1)		4.8 (1)		0.48 (1)		21.7 (1)	
(9)	Acidimetric	7.32 (3)		5.34 (3)		0.666 (3)		15.01 (3)	
(10)	Turbidimetric	6.7 (1)		4.0 (1)		1.2 (1)		18.0 (1)	
(11)	Turbidimetric	9.9 (3)	9.3 –10.3	5.8 (3)	5.6 –6.1	1.9 (1)		24.5 (3)	22.6 –27.0
(12)	Acidimetric	6.87 (3)†	6.5 – 7.4	3.27 (3)†	3.0 –3.6	0.84 (3)†	0.65–1.00	17.3 (3)†	14.4 –19.9
	Acidimetric	7.4		3.6		1.0		19.9	
(13)	Acidimetric	9.29 (4)	8.27–9.94	3.48 (4)	3.30–3.75	0.722 (4)	0.471–0.953	16.18 (4)	15.29–16.80
(14)	Acidimetric	7.5 (1)				0.55 (1)			
(15)	Acidimetric	8.41 (1)†		3.11†		0.65†		13.2†	
Mean	Turbidimetric	8.71 (4)	6.7 – 9.9	4.25 (5)	3.49–5.8	1.19 (4)	0.82–1.90	19.33 (4)	16.10–24.50
	Acidimetric	8.45 (8)	6.57–10.38	3.91 (6)	2.14–5.34	0.72 (4)	0.48–1.00	18.18 (5)	15.01–21.70
Median	Turbidimetric	8.95–9.3 (4)	6.7 – 9.9	4.00 (5)	3.49–5.8	0.83 –1.20 (4)	0.82–1.90	18.00–19.7 (4)	16.10–24.50
	Acidimetric	7.50–8.76 (8)	6.57–10.38	3.60–4.12 (6)	2.14–5.34	0.67–0.72 (4)	0.48–1.00	18.10 (5)	15.01–21.70
Over-all Mean	Turbidimetric & Acidimetric	8.54 (12)	6.57–10.38	4.07 (11)	2.14–5.8	0.92 (8)	0.48–1.90	18.69 (9)	15.01–24.50
Over-all Median	Turbidimetric & Acidimetric	8.76–8.95 (12)	6.57–10.38	4.0 (11)	2.14–5.8	0.82 –0.83 (8)	0.48–1.90	18.10 (9)	15.01–24.50

* Numbers in parentheses indicate number of determinations included in average.
† Not included in calculating mean and median.

Referee did not include the data from Laboratory 15, using *L. casei*, in the averages because the blank was excessively high. To show the range of results in the hands of many technicians (of varying degrees of experience in microbiological assays of folic acid) it seems worthwhile, however, to present in the tables all the data submitted.

The fifteen laboratories submitting data from microbiological assays sent to the Associate Referee detailed information on at least two hundred microbiological determinations on the four samples tested. Assayists will appreciate the enormous amount of work involved in so large a number of microbiological tests, as well as the effort expended in the time- and labor-consuming chick assays made in five laboratories.

A comparison of results obtained when a laboratory carried out a microbiological assay by more than one method, or with more than one organism on the same day, demonstrates that the varied procedures gave (within experimental error) almost identical results. Occasionally this was not true.

Results compiled in Table 1 (from assays with *L. casei* in the medium specified for the assay) indicate remarkably good agreement between laboratories. This is particularly encouraging since the data on "free folic acid," for which we are indebted to Laboratory 8, prove that the larger portion of the total folic acid in each sample (with the possible exception of the egg yolk) is in the form of conjugates. It is very satisfying, also, to find that results from turbidimetric assays check closely with the results from assays evaluated by titration.

When *S. faecalis* was used as the test organism in the specified medium, there was again good agreement among the results from various laboratories and between acidimetric and turbidimetric determinations. Although fewer data were reported with *S. faecalis* than with *L. casei* in the medium specified for the collaborative test, results shown in Table 2 prove that estimates from assays with the two organisms were in harmony with one another.

Four laboratories contributed data showing comparison of results from assays with *L. casei* in the specified medium and in other commonly used media. These data are shown in Table 3. Determinations made in Laboratory 3 showed that assays with *L. casei* in the Roberts and Snell medium yielded results which paralleled those obtained with *L. casei* or *S. faecalis* in the medium used for the collaborative study. Data showing results from assays with *L. casei* in the Teply and Elvehjem medium are too few to justify comparison with results in the other media. In Laboratories 9 and 12 results from the several methods were in excellent agreement. In Laboratory 13 results from the Teply and Elvehjem medium were slightly lower than those from the A.O.A.C. medium. In general it is observed that assay results from assays with *L. casei* in the various media are in good agreement.

TABLE 2.—*Results of 1948 A.O.A.C. collaborative study on folic acid*
(Assays with *Streptococcus faecalis* in specified medium)

LAB. NO.	METHOD OF EVALUATION	SAMPLE I MUSTARD GREENS		SAMPLE II SOY FLOUR		SAMPLE III EGG YOLK		SAMPLE IV BREWERS' YEAST	
		AVE.*	RANGE	AVE.*	RANGE	AVE.*	RANGE	AVE.*	RANGE
		mmg/g	*mmg/g*	*mmg/g*	*mmg/g*	*mmg/g*	*mmg/g*	*mmg/g*	*mmg/g*
2	Turbidimetric	5.13 (2)		2.63 (1)		0.192 (1)		11.47 (1)	
3	Acidimetric	9.8 (1)		4.2 (1)		0.65 (1)		22.0 (1)	
3	Turbidimetric	11.10 (2)	10.62–11.57	5.30 (2)	5.0 –5.61	0.895 (2)	0.5 –1.29	22.54 (1)	
7	Acidimetric	8.14 (1)		4.07 (1)		1.26 (1)		19.60 (1)	
8	Acidimetric	Free		Free		Free		Free	
		1.47 (1)		0.50 (1)		0.44 (1)		0.54 (?) (1)	
		Total		Total		Total		Total	
		10.70 (1)		5.5 (1)		0.65 (1)		24.4 (1)	
9	Acidimetric	7.38 (1)		3.33 (1)		1.08 (1)		17.09 (1)	
13	Turbidimetric	9.38 (1)		5.03 (1)		0.526 (1)		24.15 (1)	
Mean	Turbidimetric	8.54 (3)	5.13–11.10	4.32 (3)	2.63–5.30	1.11 (3)	0.526–1.92	19.39 (3)	11.47–24.15
	Acidimetric	8.76 (4)	7.38–10.70	4.27 (4)	3.33–5.50	0.91 (4)	0.65– 1.26	20.77 (4)	17.09–24.4
Median	Turbidimetric	9.38 (3)	5.13–11.10	5.03 (3)	2.63–5.30	0.895 (3)	0.526–1.92	22.54 (3)	11.47–24.15
	Acidimetric	8.14–9.80 (4)	7.38–10.70	4.07–4.20	3.33–5.50	0.65–1.08 (4)	0.65 –1.26	19.6 –22.0 (4)	17.09–24.4
Overall Mean	Turbidimetric & Acidimetric	8.77 (7)	5.13–11.10	4.29 (7)	2.63–5.50	0.997 (7)	0.526–1.92	20.18 (7)	11.47–24.4
Overall Median	Turbidimetric & Acidimetric	9.38 (7)	5.13–11.10	4.20 (7)	2.63–5.50	0.895 (7)	0.526–1.92	22.0 (7)	11.47–24.4

* Numbers in parentheses indicate number of results included in average.

TABLE 3.—*Results of 1948 A.O.A.C. collaborative study on folic acid*
(Assays with *L. casei* in media other than that specified)

LAB. NO.	METHOD OF EVALUATION	MEDIUM	SAMPLE I MUSTARD GREENS		SAMPLE II SOY FLOUR		SAMPLE III EGG YOLK		SAMPLE IV BREWERS' YEAST	
			AVE.*	RANGE	AVE.*	RANGE	AVE.*	RANGE	AVE.*	RANGE
			mmg/g	*mmg/g*	*mmg/g*	*mmg/g*	*mmg/g*	*mmg/g*	*mmg/g*	*mmg/g*
3	Turbidimetric	Roberts and Snell	10.93 (1)		5.095 (1)		0.581 (1)		30.243 (1)	
	Acidimetric	Roberts and Snell	10.00 (1)		5.00 (1)		0.556 (1)		28.56 (1)	
9	Acidimetric	Modified Teply and Elvehjem	6.94 (1)		5.09 (1)		—		—	
12	Acidimetric	Teply and Elvehjem	6.5 (1)		3.2 (1)		0.65 (1)		17.7 (1)	
13	Acidimetric (?)	Teply and Elvehjem†	7.5 (1)		3.32 (1)		0.49 (1)		15.36 (1)	
		Teply and Elvehjem‡	7.96 (4)	7.50–8.72	3.30 (4)	3.10–3.67	0.697 (4)	0.539–0.870	15.52 (4)	14.12–17.09
Over-all Mean			8.31 (6)	6.50–10.93	4.17 (6)	3.20–5.095	0.595 (5)	0.490–0.697	21.45 (5)	15.36–30.24
Over-all Median			7.50–7.96 (6)	6.5 –10.93	3.32 –5.00 (6)	3.20–5.095	0.581 (5)	0.490–0.697	17.70 (5)	15.36–30.24

* Numbers in parentheses indicate number of results included in average.
† Hydrolyzed with hog kidney enzyme.
‡ Hydrolyzed with chicken pancreas enzyme.

TABLE 4.—*Results of 1948 A.O.A.C. collaborative study on folic acid*
(Assays with *S. faecalis* in media other than that specified)

LAB. NO.	METHOD OF EVALUATION	MEDIUM	SAMPLE I MUSTARD GREENS		SAMPLE II SOY FLOUR		SAMPLE III EGG YOLK		SAMPLE IV BREWERS' YEAST	
			AVE.*	RANGE	AVE.*	RANGE	AVE.*	RANGE	AVE.*	RANGE
			mmg/g	*mmg/g*	*mmg/g*	*mmg/g*	*mmg/g*	*mmg/g*	*mmg/g*	*mmg/g*
2	Turbidimetric	—	29.17†		16.82†		9.07†		31.04†	
4	?	?	4.8 (1)		3.4 (1)		0.55 (1)			
5	Turbidimetric	Modified Teply and Elvehjem	7.3 (1)†,‡		3.8 (1)†,‡		0.66 (1)†,‡		19.6 (1)†,‡	
6	?	Rabinowitz and Snell	6.8 (1)		3.6 (1)		0.66 (1)		13.3 (1)	
8	Turbidimetric	?	10.5 (1)		6.35 (1)		—		21.6 (1)	
11	Turbidimetric	Teply and Elvehjem	12.4 (1)		7.1 (1)		3.9 (1)		30.2 (1)	
14	?	Teply and Elvehjem	12.35 (2)		5.4 (2)		0.73 (2)		22.15 (2)	
Over-all Mean			9.21 (5)	4.80–12.40	5.17 (5)	3.40–7.10	1.46 (4)	0.55–3.90	21.81 (4)	13.30–30.2
Over-all Median			10.50 (5)	4.80–12.40	5.40 (5)	3.40–7.10	0.66–0.73 (4)	0.55–3.90	21.60–22.15 (4)	13.30–30.2

* Numbers in parentheses indicate the number of results included in average.
† Not included in calculation of mean and median.
‡ A different pteroylglutamic acid standard was used here.

The estimates of folic acid based on assays with *S. faecalis* in media other than that specified for collaboration are summarized in Table 4. These data are too few to warrant more than brief comment. Checking these results with other results from the same laboratories, however, reveals good agreement within each laboratory in tests with *S. faecalis*, regardless of the medium chosen.

Results from chick assays in several laboratories have been assembled in Table 5. The values listed for Laboratory A were averaged in the office of the Associate Referee from data submitted by the aforementioned laboratory, showing estimates at several dosage levels for the test materials. The range of the estimates averaged is indicated. The values listed for Laboratory B were calculated by these assayists as tentative estimates, pending a statistical evaluation of their data by the Method of Least Squares. The data shown in Table 5 indicate good agreement in the reports from the different laboratories. As pointed out by the chemist submitting the report for Laboratory C, "assays based on total cell count and hematocrit are in agreement with the microbiological results but are divergent from the weight assay data. The reasons for this are not known but may be due to an influence of the 'liver principle.'" The divergence between folic acid estimates based on chick weight and those based on blood tests or microbiological tests is particularly marked in the case of the dried egg yolk. This material may be a comparatively poor source of folic acid, but it apparently is an excellent source of unidentified growth stimulants for both chicks and bacteria.

Data showing averages from assays by various methods are compiled in Table 6, to enable comparison of results from the different methods. This summary reveals excellent agreement among folic acid estimates from assays by varied microbiological procedures. From these findings one may conclude that it is possible to obtain very good results from assays with either *L. casei* or *S. faecalis*, using the organisms in any one of several media.

INFORMATION FROM QUESTIONNAIRES

Standard cultures.—Collaborators were asked to submit specific data from acidimetric or turbidimetric tests, showing bacterial growth in standard and test cultures in folic acid assays. Almost without exception these data were excellent. When *L. casei* was used in the medium specified the blanks were low (usually one ml. or less of 0.1 *N* acid) and the acid production was high (10 to 14 ml. of 0.1 *N* acid at a dosage of one millimicron of folic acid). Several collaborators compared *L. casei* in the medium suggested by the Associate Referee with *L. casei* in the Teply and Elvehjem medium, and felt that the medium with "Tween" and glutathione gave lower blanks, better acid production, and more satisfactory results in general. It was pointed out last year by several collaborators that *L.*

TABLE 5.—*Results of 1948 A.O.A.C. collaborative study on folic acid*
(Assays by chick methods)

LAB. NO.	METHOD OF EVALUATION	SAMPLE I MUSTARD GREENS		SAMPLE II SOY FLOUR		SAMPLE III EGG YOLKS		SAMPLE V SPINACH	
		AVE.	RANGE	AVE.	RANGE	AVE.	RANGE	AVE.	RANGE
		mmg/g	*mmg/g*	*mmg/g*	*mmg/g*	*mmg/g*	*mmg/g*	*mmg/g*	*mmg/g*
A	Weight	13.3	12.0–15.5	2.9	2.0–5.5	4.3	—	15.3	14.6–16.1
B	Weight	14.4		5.5		2.8		18.6	
	Hematocrit	13.6		4.6		1.4		16.9	
	Average	14.0		5.0		2.1		17.8	
C	Chick Weight	14.6							
	Chick Thymus Weight	14.7							
	Total Cell Count	8.6							
	Hematocrit	10.0							
	Average	12.0							
D	Weight	—		1.8*				3.3*	
	Hemaglobin	7.0*		2.6*		1.7*		5.3*	
E	Weight	13†		4.42		1.43		14.8	

* Ration contained supplement of anti-pernicious anemia liver extract, to supply "animal protein factor."
† 13 assumed to be correct value for mustard greens.

TABLE 6.—*Results of 1948 A.O.A.C. collaborative study on folic acid*
(Comparison of results of assays by several methods)

METHOD OF ASSAY	SAMPLE I		SAMPLE II		SAMPLE III		SAMPLE IV	
	AVE.*	RANGE	AVE.*	RANGE	AVE.*	RANGE	AVE.*	RANGE
	mmg/g	*mmg/g*	*mmg/g*	*mmg/g*	*mmg/g*	*mmg/g*	*mmg/g*	*mmg/g*
1. *L. casei* in medium specified								
Mean	8.54 (12)	6.57–10.38	4.07 (11)	2.14–5.8	0.952 (8)	0.48 –1.90	18.69 (9)	15.01–24.50
Median	8.76–8.95 (12)	6.57–10.38	4.0 (11)	2.14–5.8	0.82–0.83 (8)	0.48 –1.90	18.10 (9)	15.01–24.50
2. *S. faecalis* in medium specified								
Mean	8.77 (7)	5.13–11.10	4.29 (7)	2.63–5.50	0.997 (7)	0.526–1.92	20.18 (7)	11.47–24.4
Median	9.38 (7)	5.13–11.10	4.20 (7)	2.63–5.50	0.895 (7)	0.526–1.92	22.0 (7)	11.47–24.4
3. *L. casei* in other media								
Mean	8.31 (6)	6.50–10.93	4.17 (6)	3.20–5.095	0.595 (5)	0.490–0.697	21.48 (5)	15.36–30.24
Median	7.5 –7.96 (6)	6.5 –10.93	3.32–5.00 (6)	3.20–5.095	0.581 (5)	0.490–0.697	17.70 (5)	15.36–30.24
4. *S. faecalis* in other media								
Mean	9.21 (5)	4.8 –12.4	5.17 (5)	3.4 –7.1	1.46 (4)	0.55 –3.9	21.81 (4)	13.3 –30.2
Median	10.5 (5)	4.8 –12.4	5.4 (5)	3.4 –7.1	0.66 (4)	0.55 –3.9	21.6–22.15 (4)	13.3 –30.2
5. Chick tests								
Laboratory A	13.3	12.0 –15.5	2.9	2.0 –5.5	4.3			
Laboratory B	14	13.6 –14.4	5.0	4.6 –5.5	2.1			
Laboratory C	12	8.6 –14.7						
Laboratory D†	7.0		2.6		1.7			
Laboratory E‡	13		4.42		1.43			

* Numbers in parentheses indicate number of results averaged.
† Ration contained supplement of anti-pernicious anemia liver extract, to supply "animal protein factor."
‡ 13 assumed to be correct value for mustard greens.

casei gives very acceptable results in a modified Teply and Elvehjem medium, without peptone, but using commercial enzyme-hydrolyzed casein as the source of both amino-acids and growth stimulants. *S. faecalis* also gave very acceptable results in the medium suggested by the Associate Referee, or in a modified Teply and Elvehjem medium.

Basal media.—Very few comments concerning media were made by collaborators. One suggested that the stock solutions specified for the collaborative medium were too dilute for easy handling; he preferred them about four times as strong. Another felt that the medium suggested was more complex than was justified by its advantages; he likes as a medium for *L. casei* the old Mitchell-Snell medium with pyridoxine increased and using Difco Casamino acids as a source of hydrolyzed casein (8). Four remarked that they liked the medium suggested by the Associate Referee for *L. casei*.

Choice of test organism.—Of the twelve collaborators expressing a preference eight prefer *S. faecalis* for folic acid assays, while four liked *L. casei* better. It is agreed that it is easier to get low blanks with *S. faecalis*, that it can be read turbidimetrically after a very short incubation period, and that its nutritive requirements are less fastidious. *L. casei*, however, gives markedly better acid production, shows greater sensitivity to the vitamin, and is sensitive to it at a lower level of potency. The following comments by three collaborators are typical of the opinions of others: Laboratory 5, "*S. faecalis* when used with the medium of Teply and Elvehjem but omitting peptone supplement gives more consistent and reproducible results. Also it is much easier to obtain proper blanks with *S. faecalis;*" Laboratory 8, "We like *S. faecalis*—we have had much greater experience with it and it is faster. Obviously on some determinations *L. casei* must be used." Laboratory 13, "We prefer *L. casei*. In our experience it has given more consistent results on day-to-day testing and in replicate cultures on the same day."

From the data submitted by many collaborators it seems obvious to the Associate Referee that either organism may be used with excellent results for folic acid tests.

Purification of casein hydrolysates.—Whenever it is wished to use *L. casei* in assays of folic acid it is essential that the hydrolyzed casein used in the medium shall be very low in the vitamin. From data submitted in this collaborative assay it is evident that *S. faecalis* responds quantitatively to the vitamin in the range 0.5 to 5 (or perhaps to 6 or 7) millimicrograms per 10 ml. of culture. *L. casei* is sensitive to the vitamin in the range 0.1 to 1 (or probably 2) millimicrograms per 10 ml. culture. Several of the chemists co-operating in this study commented on special adsorptions needed to make casein hydrolysates suitable for use with *L. casei* in folic acid tests. Laboratory 1 found it necessary to treat the hydrolysate

with Norite after an earlier treatment with Nuchar; Laboratory 6 observed that they obtained lower blanks with GBI vitamin-free casein after treating it with Norite; Laboratory 7 had difficulty with high blanks in tests with *L. casei*, and subsequent tests on various casein hydrolysates made it seem very possible that their earlier difficulties were caused by folic acid in the casein. Laboratory 3 finds that Darco is a suitable adsorbent for freeing Difco casamino acids from folic acid, for use in tests for the vitamin.

Extraction procedures.—Only one laboratory submitted data showing a comparison of findings when a variation was made in the extraction procedure. Data submitted by Laboratory 13 (listed in Table 3) show very little difference in results when hog kidney enzyme was substituted for chicken pancreas enzyme in the hydrolysis of samples. It is apparent that the suggested procedure for extraction is adequate, in the assay of the mustard greens, soy flour, brewers' yeast, and of the spinach sample assayed collaboratively last year. Very possibly the procedure for extraction of the egg yolk needs further study.

In the 1947 collaborative study of folic acid, reports on the microbiological assay of the spinach sample showed a mean value of 12.89 mmg./g., and median values of 12.9–13.7 mmg./g. using *L. casei* in the Teply and Elvehjem medium. Three laboratories, all experienced in folic acid assays, reported values of 14.4 to 17 mmg./g. Two laboratories reported results from chick assays, 11 and 16 mmg./g. Because of the divergence of these findings further study was made of the potency of the sample. The desiccated spinach was stored at $-40°$ and was submitted to chick assay again in 1948. Data on the spinach as listed in Table 5 are in harmony with the higher results from microbiological assays in the 1947 study.

In commenting on extraction procedures, attention should be called to data submitted by Laboratory 2, as shown in Table 4. The results listed were obtained in assays after the samples were submitted first to treatment with papain (Caroid) and takadiastase (Mylase P) before the addition of pancreas enzymes. Because these data are out of line with the results of other microbiological assays and of the chick tests they were not included in the averages.

Logarithmic plotting.—In reply to a question whether collaborators considered logarithmic plotting (as suggested by Wood (9)) an aid in evaluating, criticizing, and calculating data, differing opinions were expressed. Seven find logarithmic plotting an aid, three do not consider it helpful, and several made no comment. Observations typical of those expressed by others are quoted here: The chemist reporting from Laboratory 8 says, "It is of value in criticizing data but of little benefit for calculation. The characteristics of some turbidimeters place a break in the turbidity curves, even with logarithmic plotting." The person reporting for Labora-

tory 9 remarks, "Arithmetric grid paper gives satisfactory curves, is easy to work with, easier to read, and cheaper to buy. Perhaps a person thoroughly accustomed to working with logarithmic plotting might find it simpler but I do not." From graphs sent in by collaborators and from graphs plotted by the Associate Referee from the collaborator's data, it appears that acidimetric data, almost without exception, can be criticized easily when plotted on a log-log grid; turbidimetric data can very frequently be evaluated very easily when plotted on such coordinates. However, regardless of the type of graph paper used, the graphical representations sent in and the calculations made by the persons submitting results were without exception of unquestioned excellence and beyond criticism.

SUMMARY

The microbiological method for determining folic acid was subjected to collaborative assay in 1948. Chick tests on three of the four samples were made in five laboratories, as a check on the microbiological methods. Results showed excellent agreement among the laboratories.

Whether *L. casei* or *S. faecalis* was used in the medium suggested by the Associate Referee, and whether response of the test organism to the vitamin was measured acidimetrically or turbidimetrically, assay results were very acceptable.

The use of either of the bacteria in other media, measuring response either acidimetrically or turbidimetrically, gave results in harmony with those found in the medium suggested by the Associate Referee.

Folic acid values as determined by chick tests were higher than values obtained by microbiological assays. These findings emphasize again the need for further study of methods of extraction of the vitamin, and the possible influence of unidentified growth stimulants. New knowledge seems essential before results from microbiological assays will duplicate the results from chick tests.

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REPORT ON NICOTINIC ACID (NIACIN) OR NICOTINAMIDE (NIACINAMIDE)

MICROBIOLOGICAL METHOD

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

The 1945 U.S.P.—A.O.A.C. official, first action, method for nicotinic acid, as described in *This Journal*, **30**, 82 (1947), has been revised, and it is recommended† that this revised method be approved. The details of the method as revised are published in *This Journal*, **32**, 110 (1949).

REPORT ON VITAMIN C (ASCORBIC ACID)

By WALLACE L. HALL (Food and Drug Administration, Federal Security Agency, Washington, D.C.), *Associate Referee*

RECOMMENDATIONS‡

It is recommended—

That the official, first action, method for vitamin C (36.47, 36.48) be made official, final action, after the following minor corrections and additions are made:

(1) Add to the first sentence after "ferrous Fe," "stannous Sn and cuprous Cu."

(2) Change under REAGENTS (a), second line, "freshly pulverized stick HPO_3" to read, "glacial HPO_3, pellets, or freshly pulverized stick."

(3) Change "Note" to read as follows: "Products containing ferrous Fe, stannous Sn, and cuprous Cu give values in excess of their actual ascorbic acid content by this method."

Following are simple tests to ascertain whether these reducing ions are present in appreciable quantities to invalidate analysis: Add 2 drops of 0.05% H_2O soln of methylene blue to 10 ml of freshly prepared mixture of juice and the HPO_3-acetic acid reagent, mix. Disappearance of methylene blue color in 5–10 seconds indicates presence of interfering substances. Stannous Sn does not give the test and may be tested for by using another 10 ml sample soln to which 10 ml of 25% HCl is added, mix, then 5 drops of 0.05% H_2O soln of indigo carmine, mix. Disappearance of indigo carmine color in 5–10 seconds also indicates presence of interfering substance.

No report was given on vitamin D—poultry.

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* Appointed to succeed Frank M. Strong, resigned.
† For report of Subcommittee B and action of the Association, see *This Journal*, **32**, 48 (1949).
‡ For report of Subcommittee A and action of the Association, see *This Journal*, 32, 48 (1949).

REPORT ON VITAMIN D

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

VITAMIN D IN MILK

The method for vitamin D in milk was proposed in 1937, and after collaborative study, was adopted in 1938. The procedure as it appears in the Sixth Edition of *Methods of Analysis* has been found entirely satisfactory and it is generally used in the assay of vitamin D in both fluid and evaporated milk. It is important to retain this method in the next edition of *Methods of Analysis*.

RECOMMENDATION†

It is recommended that the method for determining vitamin D in milk, which appears in the Sixth Edition as a tentative procedure (**36.49–36.60,** p. 621–625), be made official, first action.

REPORT ON CAROTENE

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

Since the dropping of the phasic separation method for the determination of carotene in 1946, the Association has had no official method for the determination of carotene in foods and feeds. Two tentative methods have been introduced, one in 1946 and one in 1947. The objective this year was to compare these two tentative methods in collaborative study to determine whether one might be sufficiently satisfactory to be considered for adoption as an official method. A third method was also included for comparison. This method included two features which had been requested by several laboratories, namely, cold extraction and chromatography with a lower concentration of acetone.

Procedure I was the tentative procedure introduced in 1946, *This Journal*, **30,** 84 (1947).

Procedure II was the one introduced in 1947, *Ibid*, **31,** 111 (1948).

Procedure III was essentially the procedure of Silker, *et al.*, *Ind. Eng. Chem.* (*Anal. ed.*) **16,** 513 (1944). The details sent to collaborators were as follows:

Extraction.—Weigh accurately a 1–2 gram sample into an Erlenmeyer flask or sample bottle, add 60 ml of a mixture of 1 part acetone and 2 parts hexane (Skellysolve B). Shake mixture, stopper container tightly, and set in the dark for 16–

* E. M. Nelson, Chief.

† For report of Subcommittee A, and action of the Association, see *This Journal*, **32,** 48 (1949).

18 hours, usually overnight. Filter extract thru a Büchner funnel and wash the residue thoroly by decantation with several portions of hexane. Heat extract on a steam cone to drive off most of remaining acetone and to concentrate the soln to a volume of ca 40 ml.

This concentration is usually accomplished in 15 minutes. The entire solution is now chromatographed.

Chromatography.—Prepare a column and chromatograph the soln as in Procedure II, but use a 1:24 mixture of acetone and hexane instead of 1:9 for elution of the carotene. Collect the carotene eluate, make up to volume with 1:24 mixture of acetone and hexane, and determine the carotene concentration as directed under Procedure II.

Directions were sent out to a large number of laboratories and samples were mailed to each about two weeks later. Analyses were to be postmarked not later than ten days after the mailing date of the sample, and collaborators were instructed to hold samples at room temperature prior to analysis. Two samples of alfalfa meal were sent to each of the collaborators. One was a comparatively fresh dehydrated sample, the other had been in storage for a long period and had undergone considerable deterioration in carotene. The Referee is indebted to Herbert Schaefer, of the Ralston Purina Company, for packaging and distributing the samples.

COLLABORATORS

State and federal laboratories.—California, Indiana, Louisiana, Maine, Michigan, New Hampshire, North Dakota, Ohio, Oregon, South Dakota, Texas, Utah, Washington; Botany Laboratory, University of Chicago; Kansas State College; Utah State Agricultural College; Eastern, Southern, and Western Regional Laboratories; U. S. Food and Drug Administration, Washington, D. C.; Fish and Wild Life Service, Laurel, Maryland.

Industrial laboratories.—Wirthmore Research Laboratory, Malden, Mass.; The Best Foods, Inc., Bayonne, N. J.; Coop. G. L. F. Exchange, Inc., Buffalo, N. Y.; Eastern States Farmers' Exchange Incorporated, Buffalo, N. Y.; Central Mills, Inc., Dunbridge, Ohio; General Mills, Inc., (Larrowe Division), Rossford, Ohio; General Biochemicals, Inc., Chagrin Falls, Ohio; B. F. Goodrich Chemical Company, Akron, Ohio; M. F. A. Milling Company, Springfield, Mo.; Cerophyl Laboratories, Inc., Kansas City, Mo.; Ralston Purina Company, St. Louis, Mo.; National Alfalfa Dehydrating and Milling Company, Lamar, Colo.; Laucks Laboratories, Inc., Seattle, Wash.; Ayerst, McKenna and Harrison Limited, Montreal, Quebec.

SUMMARY OF SELECTED COMMENTS OF COLLABORATORS

The willing cooperation of the various collaborators is gratefully acknowledged. The individual comments were helpful in giving the full comparison of the procedures and it is regretted that space will not permit the full statement of each. Some of the most pertinent statements are quoted below.

Coll. No. 1.—In Procedure III it was necessary to increase the acetone concentration for satisfactory elution. A 2:23 acetone-hexane mixture was used.

Coll. No. 2.—A mixture of magnesium oxide and magnesium carbonate was used in Procedure I.

Coll. No. 5.—We believe that the presence of stereoisomers of *beta*-carotene should eventually require reinvestigation of the applicability of the absorption coefficient 196 at 435 millimicrons.

Coll. No. 8.—In Procedure II 50 ml. of acetone-hexane mixture was insufficient to wash the carotene through the absorbent. At least 100 ml was required.

Coll. No. 9.—Procedure II is susceptible to giving excessive values through too strong an eluting action.

Coll. No. 11.—We do not believe that any one method has all the features desirable in an official method. Each method has, we feel, one or more steps which need further study. As now written, however, our choice would be for Method No. 2.

Coll. No. 14.—No Magnesia #2642 available. Used #2641 in Procedures II and III.

Coll. No. 15.—Satisfactory separation of pigments could not be obtained by magnesium carbonate as adsorbent.

Coll. No. 16.—Procedure I should be abandoned for studies on dehydrated leaf material. It is far too laborious.

Coll. No. 23.—In Procedure III the 1:24 acetone-hexane would not elute carotene.

Coll. No. 25.—We had difficulty in elution of the carotene with 1:24 acetone-hexane.

Coll. No. 31.—Procedure II seems most adaptable to routine or control analyses. An overnight wait is undesirable in a control method and in this case gave lower results in our hands. Procedure I has many of the cumbersome separatory funnel operations of earlier procedures which it is desirable to avoid.

Coll. No. 32.—In Procedures II and III, using Westvaco 2642 magnesia 1:1 with Supercel, we find the carotenes pass through very slowly.

Coll. No. 34.—Certain lots of Hyflo Supercel will retain more than 5 per cent of carotene.

RESULTS AND DISCUSSION

The experimental results, received from thirty-five laboratories, are summarized in Table 1.

Procedure I gave the lowest average results. As pointed out by some collaborators, conditions used in this procedure favor isomerization. Isomers such as neo-*beta* carotene B with maxima shifted to lower wave lengths would predominate, thus resulting in low values when read at 450 millimicrons and calculated with the extinction coefficient of 2580. Actually, Collaborator 14 found the results to be 12 per cent higher when his solutions from Procedure I were read at 436. This evidences the high degree of isomerization in Procedure I. If 12 per cent were added to the average results of Beckman users for this procedure, the results would be 38 for the old sample and 131 for the fresh sample, which are probably nearly correct.

Procedure II gave the highest results. One collaborator (No. 9) reported that the acetone content of the solution during chromatographing was high enough to elute non-carotene pigments. However, studies in the Referee's laboratory do not substantiate this and other collaborators do

TABLE 1.—*Results of analysis of alfalfa meal samples*

COLL. NO.	INSTRUMENT	FRESH SAMPLE			OLD SAMPLE		
		PROCEDURE—			PROCEDURE—		
		I	II	III	I	II	III
1	Beckman-DU	37	34	32	122	123	123
2	"	31	51	37	115	133	124
3	"	32	36	30	115	128	117
4	"	33	39	21	119	115	101
5	"	35	38	35	119	129	130
6	"	39	36	36	122	118	125
7	"	26	47	30	106	140	115
8	"	29	38	36	117	131	131
9*	"	20	73	41	92	340	126
10	"	—	32	33	—	129	136
11	"	29	35	32	110	124	115
12	"	—	—	—	—	120	128
13	"	48	45	38	127	143	133
14	"	30	44	38	102	146	111
15	"	44	48	31	99	164	164
16	"	—	39	39	—	136	142
17	"	35	34	35	129	131	129
18	"	—	36	32	—	136	125
19	"	42	39	30	124	129	124
20	"	36	42	—	124	135	—
21	"	34	33	37	120	119	118
22	"	34	39	31	123	129	128
23	"	34	31	—	120	119	—
24	"	27	47	38	96	136	125
25	"	—	34	—	—	124	111
Averages, Beckman		34	39	34	117	131	125
26	Coleman-11	33	37	32	136	135	123
27	"	32	34	33	104	113	121
28	"	39	41	35	141	140	122
29	"	34	37	32	120	128	131
30	Evelyn	39	39	38	138	137	140
31	"	35	39	32	127	135	126
32	"	38	36	36	140	138	132
33	Cenco-Sheard	40	48	41	108	150	135
34	Cenco	37	34	34	132	124	119
35	Klett-Summerson	32	41	—	142	130	—
Averages, other instruments		36	39	35	129	133	128
Averages, all values		35	39	34	120	131	126

* Values not included in averages.

not seem to have experienced such difficulty. Some indicated preference for the use of a thimble in the extraction. In our experience this necessitates a much longer period of heating to effect complete extraction and results in a substantial increase in isomerization of the carotene.

Procedure III gave lower results than Procedure II in twenty cases, equal or higher results in eleven cases. Several collaborators expressed doubt on the stability of carotene solutions in the desk overnight. Four collaborators who obtained low values stated that the 4 per cent acetone solution did not elute all of the carotene. Three of these considered their results unreliable and did not report them.

To the question "Do you feel that any of the three procedures should be adopted by the A.O.A.C. as official?" twenty recommended that Procedure II be adopted, three others voted for either Procedure II or III, three expressed preference for Procedure II but did not recommend official adoption, one recommended Procedure III only, one preferred Procedure I, four recommended other procedures than those used in the collaborative study, and four offered no opinion. In view of the recommendation by a large majority it is felt that Procedure II should be adopted as official, first action, for hays and dried plants.

The instrument used for analysis of the carotene fraction following chromatography apparently had little influence on the result, since the averages of twenty-five values obtained with Beckman spectrophotometers agreed well with the averages of ten values obtained with other instruments, except in the case of Procedure I. This difference possibly reflects the error mentioned above, of measuring alkali-treated carotenes at 450 millimicrons. Further efforts should be made to work out analytical procedures which will not produce isomerization. Until such time as this is accomplished total carotenes must be measured. The high selectivity of the Beckman instrument is probably not advantageous for the routine measurement of total carotenes in chromatographed extracts from leafy materials. Less costly equipment should serve the purpose equally well.

RECOMMENDATIONS*

It is recommended—

(1) That Procedure II (*This Journal*, **31**, 111, 1948), which was adopted in 1947 as an alternative tentative method, be adopted as official, first action, for analysis of hays and dried plants.

(2) That Procedure I (*This Journal*, **30**, 84, 1947) be discontinued as a method for hays and dried plants, but that it be continued as a tentative method of analysis for other materials.

(3) That studies on analysis for carotene be continued.

No report was given on pantothenic acid.

* For report of Subcommittee A and action of the Association, see *This Journal*, 32, 48 (1949).

REPORT ON PRESERVATIVES AND ARTIFICIAL SWEETENERS

By MARGARETHE OAKLEY (State Department of Health, Baltimore 18, Md.), *Referee*

The chapter on Preservatives and Artificial Sweeteners got off to an auspicious start this year with requests for the assignment of ten topics for study and the final assignment of nine of these. No Associate Referee was obtained for the sweetener 1-propoxy 2-amino 4-nitrobenzene.

Saccharin.—Early in the year the Associate Referee on saccharin asked to be relieved of the assignment because of the pressure of routine work and requested that it be transferred to W. S. Cox of Atlanta, who is at present interested in both saccharin and dulcin but is not doing A.O.A.C. work on them.

Dulcin.—No report was received from the Associate Referee on dulcin. With the increase in sugar supplies the urgency of the problem has passed; however, it is deemed advisable to continue with this work, especially since the present method has been so roundly condemned by those called upon to refer to it.

It is therefore recommended that both dulcin and saccharin be reassigned for study during the coming year.

Quaternary Ammonium Compounds and Monochloracetic Acid.—Methods for determining quaternary ammonium compounds and monochloracetic acid were completed last year. The work for the current year was to extend the application of these methods to a greater variety of foods and to study further details of the methods collaboratively.

Dichloracetic Acid.—No report on dichloracetic acid was submitted, the Associate Referee being unable to find time this year to devote to the problem. This subject was covered very ably and thoroughly by Charles F. Bruening (*This Journal*, **28**, 620 (1945)). He presented a procedure for the quantitative determination of dichloracetic acid in food products involving its isolation by an ether extraction and the conversion of the chloride of the acid to the ionic form by ignition with sodium carbonate. The chloride thus formed was determined by the Volhard method. In the same article Mr. Bruening gave an identification test for dichloracetic acid involving the preparation of the p-toluide derivative of the acid which is characterized by a melting point determination.

With this work as background it was thought that a little collaborative work would be all that was necessary to complete the problem. However, since the methods for extraction and determination are the same for mono and dichloracetic acids and it has been submitted to collaborative study with monochloracetic acid, the identification test for dichloracetic acid is the only part of this problem remaining. In view of the extremely slight need for this identification test and since the work of Mr. Bruening

is available in *The Journal* for those who do need it; and considering the policy of pruning the *Methods of Analysis* to the very essentials, it is therefore recommended that the subject of dichloracetic acid be dropped for the present.

Thiourea.—The Associate Referee has studied a short oxidation method for thiourea before its reaction with Grote's reagent and has already submitted it to collaborative study. He is enthusiastic about the results of his study and recommends its substitution for the present tentative method.

Formaldehyde.—After a review of the many tests for formaldehyde in the Book of Methods, and elsewhere, the Associate Referee offered many recommendations including the deletion of three present methods. Two other present methods he recommends for collaborative study and to all of these he adds one modified and two new methods which he recommends for collaborative study. The Referee concurs and hopes that the final outcome of these endeavors will produce one or two satisfactory procedures to replace the present seven antiquated ones.

Esters of Benzoic and Vanillic Acids.—The Associate Referee worked on the recovery of ethyl vanillate from orange and tomato juices. He used an extraction method, determining the vanillic acid by actual weight. The method proved satisfactory in his hands but he does not recommend submitting it to collaborative study. Information received by him indicates that ethyl vanillate is not being manufactured commercially and under those circumstances he feels it cannot be a regulatory problem at present.

The questions with regard to this Associate Refereeship are: (1) Are there any hydroxy benzoates being sold in the United States at present? and (2) Are the methods for benzoates in the chapter on Preservatives in *Methods of Analysis* adequate for their determination?

Formic Acid.—There has been some discussion relative to the advisability of eliminating the formic acid method from the chapter on Preservatives and Artificial Sweeteners, putting in its place a reference to the formic acid determination along with the other volatile acids. The Referee recommends the appointment of an Associate Referee on formic acid to study the subject, to confer with Mr. Hillig concerning elimination of duplication in the *Methods of Analysis* of the formic acid determination, and also to investigate the colorimetric method for the detection of formic acid which appeared in *Analytical Chemistry,* **19,** 206–7 (1947).

Mold Inhibitors—Propionates.—The Associate Referee concentrated his efforts during the past year on determining the loss of propionates and diacetates during baking and subsequent air drying of bread. He recommends determination on the fresh basis and warns that acetic acid is present normally in bread in amounts that must be taken into consideration

in the detection of added acetic acid or sodium diacetate. He further recommends that an alternate chromatographic technic for the identification and estimation of volatile fatty acids in bakery products be undertaken.

RECOMMENDATIONS*

The Referee concurs in the recommendations of the Associate Referees on preservatives and artificial sweeteners which have been presented.

REPORT ON BENZOIC AND VANILLIC ACID ESTERS

By W. J. McCarthy (Food and Drug Administration, Federal Security Agency, Cincinnati 2, Ohio), *Associate Referee*

During the past year experimental work on the recovery of ethyl vanillate from orange juice and tomato juice was carried out on solutions of the juices made up to contain 0.1 per cent ethyl vanillate. For this work a stock solution of ethyl vanillate was prepared by dissolving the required amount in a slight excess of normal sodium hydroxide solution and then adding distilled water to desired volume.

The method of analysis used on these samples was the general outline as described in the previous report October 1947,** except that chloroform was used as solvent instead of ether, and any alcohol formed on hydrolysis was not removed. Recovery of ethyl vanillate from the orange juice preparation was 95.4 per cent, and from the tomato juice 94.6 per cent. The vanillic acid was determined by actual weight along with melting point.

In view of the fact that ethyl vanillate is not manufactured commercially and hence is not being used in food products, the above method has not been subjected to collaboration.

It was noted in last year's report that ethyl vanillate was not available commercially, and recent information from the Institute of Paper Chemistry, Appleton, Wisconsin, shows that the same situation exists today.

Since the use of ethyl vanillate as a preservative for food is not a current problem, and in view of the workable method herein described, it is recommended† that work on this subject be discontinued until a regulatory problem exists.

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* For report of Subcommittee C and action of the Association, see *This Journal*, **32**, 57 (1949).
** *This Journal*, **31**, 478 (1948).
† For report of Subcommittee C and action of the Association, see *This Journal*, **32**, 57 (1949).

REPORT ON QUATERNARY AMMONIUM COMPOUNDS IN FOODS

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

This year the Associate Referee made up samples of thirteen foods by adding quaternary ammonium compounds to them and submitted them to nine collaborators. The samples could not be sent out until the latter part of July because of difficulties encountered in obtaining some of the groceries which had been selected for study. So far, complete reports have been submitted by only three collaborators and partial reports from three others. The Associate Referee feels that more time should be allowed the collaborators and is postponing the report on these samples for another meeting of the Association. The foods used fall into five groups, as follows:

1. Beverages: Root beer, creme soda, imitation grape soda.
2. Dressings: Mayonnaise, French dressing, sandwich spread.
3. Milk: Two water solutions to be added to milk.
4. Relish: Chopped pickle relish with the addition of quaternary ammonium compounds at two levels.
5. Fruit Juices: Apple, tomato, orange.

It is hoped that the remaining collaborators will have an opportunity to complete the analyses during the coming year and have reports ready before the next meeting.

RECOMMENDATIONS*

It is recommended—

(1) That the ferricyanide method for quaternary ammonium compounds in commercial preservatives, *This Journal*, **31**, 105 (1948), be made official, final action.

(2) That the method for table sirup, *Ibid.*, **31**:108 (1948) be made official, final action.

(3) That the method for bottled beverages containing fruit juices, *Ibid.*, **31**, 106 (1948), be made official, final action.

(4) That the method for beer, *Ibid.*, **31**, 108 (1948), be made official, final action.

(5) That collaborative study be continued on the following methods:

- (a) Method for Fruit Juices, *Ibid.*, **29**, 318 (1946).
- (b) Shorter Method for Fruit Juices, *Ibid.*, **29**, 319 (1946).
- (c) Method for Bottled Sodas, *Ibid.*, **29**, 323 (1946), subject to increasing the volume of bromophenol blue reagent to 5–10 ml.
- (d) Method for Milk, *Ibid.*, **29**, 324 (1946), on samples containing preservative quantities of quaternary ammonium compounds.

* For report of Subcommittee C and action of the Association, see *This Journal*, **32**, 57 (1949).

(e) Method for Mayonnaise, Salad Dressings, and Sandwich Spreads, *Ibid.*, **29**, 323 (1946).

(f) Method for Pickles and Relishes, *Ibid.*, **29**, 326 (1946).

REPORT ON MONOCHLOROACETIC ACID

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

INTRODUCTION

Collaborative samples 5–10[1] were submitted to the collaborators listed, with the instruction given below.

Sample 5 was a commercial preservative.

Sample 6 consisted of canned orange juice to which a weighed amount of monochloroacetic acid was added and mixed in a five-gallon bottle. The mixture was filled into quart bottles which were then heated at 100°C. in an "Arnold Steam Sterilizer" for 30 minutes with the screw cap barely loose to permit the escape of air.

Samples 7, 8, and 9 consisted of Orange Soda, Imitation Strawberry Soda, and Beer, respectively. In these cases water solutions of monochloroacetic acid were prepared containing such quantities of the chemical that when 5 ml of the solution was pipetted into a bottle of the product it would impart the desired amount of the preservative. As these products were all carbonated and closed with crown caps, they were cooled in a freezing room for several hours. When cold the crown cap was removed, 5 ml of the appropriate solution added with a pipet, the bottle recapped at once, and mixed.

In the preparation of Sample 10 a weighed quantity of monochloroacetic acid was dissolved in 16 liters of red wine and after thorough mixing, filled into quart bottles closed with screw caps.

DIRECTIONS FOR COLLABORATORS (1948)

I. Efficiency of Extractors.

Before proceeding with the analysis, determine the efficiency of your extractors as follows:

Prepare a solution containing 1 gram of monochloroacetic acid per liter. Following the method, extract several aliquots (100 ml, 50 ml, 25 ml) diluted to 100 ml with water, for various lengths of time (2 hrs, 3 hrs, 4 hrs, etc.), hydrolyze and determine Cl in order to find the time needed to obtain a recovery of 95% or more.

II. Sample 5. Preservative.

(a) Pipet a portion of sample into a volumetric flask, make up to the mark with water and determine monochloroacetic acid in duplicate, in aliquots containing 1 to 2 ml of the original sample, by methods 1 and 2 below. Report as mg CH_2Cl COOH per 100 ml.

Method 1. Follow *This Journal*, **31**, 104 (1948).

Method 2. To 100 ml of sample add 3 ml of H_2SO_4 and shake in a separatory

[1] For last year's report, using 4 samples, see *This Journal*, **31**, 484 (1948).

funnel with three equal volumes of ether. Unite the ether extracts and wash by shaking with two 30 ml portions of 1 *N* NaOH. Hydrolyze for 2 hrs on the steam bath and determine Cl as in Method 1.

Barium Test

(b) Dilute 4–5 ml of sample to 100 ml, add 6 ml of H_2SO_4 (1+1), and extract with an equal volume of ether in separatory funnel. In cases where emulsions form, extract in a continuous extractor for 1 hour. Transfer the ether extract to a separatory funnel, add a few drops of phenolphthalein indicator, 5 ml of ± 0.1 *N* $Ba(OH)_2$, and shake for 30 seconds. If the water layer takes on the pink color of phenolphthalein, transfer thru a filter paper to a small beaker. Add ± 0.05 *N* acetic acid until colorless and evaporate to 1–2 ml on the steam bath. Allow the remaining liquid to evaporate spontaneously in the air and finally in a desiccator. If 5 ml of $Ba(OH)_2$ does not give a pink water layer, add 5 ml more before separating. Repeat the extraction with $Ba(OH)_2$ several times or until a pink soln is obtained, evaporating each barium soln in a separate beaker. Examine the crystals under the polarizing microscope.

Optical-Crystallographic Properties of Barium Monochloracetate

Barium monochloracetate monohydrate crystallizes from water in plates, many of which are hexagonal in habit and frequently forming in overlapping layers. Even in material that has been finely powdered for microscopic examination, the pointed terminations of the plates, often in pairs, can be observed. In parallel polarized light (crossed nicols), the extinction is parallel and the sign of elongation negative on the more elongated plates. The plates invariably extinguish sharply with crossed nicols and therefore interference figures were not observed in convergent polarized light (crossed nicols). In view of the fact that the plates persistently lie in one orientation the significant refractive indices were determined by the statistical method, measuring the lowest and highest indices, respectively, on plates showing the maximum amount of double refraction. These two indices are therefore arbitrarily designated as n_α (the minimum value) and n_γ (the maximum value). The two significant refractive indices are: $n_\alpha = 1.582$ and $n_\gamma = 1.611$, both ± 0.002, and frequently shown on the platy fragments.

Solutions containing dichloracetic acid yield a barium compound that produces a vitreous residue, which is hygroscopic. A crystalline barium trichloracetate is formed under the conditions set forth, but so far it has not been found suitable for microscopic identification although it crystallizes in a habit distinctly different from barium monochloracetate.[2]

(c) Barium-Indigo Test

REAGENTS

(1) *Anthranilic acid reagent.*—Dissolve 1 g anthranilic acid in 40 ml of H_2O, add 0.3 g NaOH, and make up to 50 ml.

(2) *Caustic soda soln.*—Dissolve 10 g NaOH in 10 ml H_2O and filter if necessary.

PROCEDURE

Dissolve 0.17 g of the barium salt in 5 ml H_2O in a 10 ml graduate, add 1.05 ml of 1.0 *N* H_2SO_4, make up to 10 ml, and mix. Let stand until the precipitate settles or filter if preferred. Pipet 3 ml of the clear liquid into a small beaker, add 2 ml anthranilic acid reagent and 30 mg Na_2CO_3 (weighed). Test with litmus paper.

[2] NOTE: Have a field chemist familiar with the procedure make the microscopic examination of the crystals (*This Journal*, **27**, 447 [1944]), and send any remaining crystals to the Microchemical Section for check analysis.

If acid, add one additional 30 mg of Na_2CO_3. Pour into test tube and heat in water bath for ½ hour. Place the test tube in an oven at 125° ±5°C. until only a moist residue remains. Remove from the oven, add 2 drops of caustic soda soln directly upon the residue. (If the residue is entirely dry, add 1–2 drops H_2O and let stand until absorbed by the residue before adding the strong NaOH), return to the oven until completely dry (at least 1 hour). Remove from the oven and heat the test tube at 310°–320°C.[3] until the contents assume an orange color. (This requires 15 seconds to 2 min., but must be carefully watched to remove from the heat as soon as the reaction is complete). Cool slightly, add 5–7 ml H_2O from a wash bottle, splashing the water to incorporate air into it. Warm over a flame and blow air thru the soln 1–2 min. using a pipet or glass tube. Heat to boiling over the flame and again blow air thru the soln. (As the oxidation progresses, the soln turns red if monochloroacetic acid is present, then green or blue or a combination of the two, and finally solid particles of indigo separate out. They have a tendency to rise to the surface at first.) Let the mixture stand about 10 min., then acidify slightly with HCl (1+1). After standing further for ½ hr. filter and wash the precipitated indigo with water to remove acid. Allow the paper to dry in the air and preserve as an exhibit.

Indigo Test

(d) Dilute 2 ml of sample to 100 ml, add 3 ml of H_2SO_4 and shake with 100 ml of ether. Add 3 ml of anthranilic acid reagent to the ether extract, evaporate at a low temp., filter off any insoluble matter and apply the indigo test as under (c) beginning "Test with litmus paper, etc."

Pyridine Test

(e) Extract 2 ml of sample as under (d) and treat the ether extract by the method in *This Journal*, **29**: 104 (1946) under the heading "Qualitative method applicable to Beverages and Fruit Juices" parts 1 and 2.

III. Samples 6 to 10, inclusive.

(a) Mark the contents level of the bottles containing samples 7, 8, and 9 so that they may be measured subsequently and report the contents measured.

(b) Determine monochloroacetic acid in duplicate in 100 ml of sample by methods 1 and 2 above, p. 489. Report as mg $CH_2Cl\ COOH$ per 100 ml, and in case of Samples 7, 8, and 9 also report as mg $CH_2Cl\ COOH$ per bottle.

PREPARATION OF SAMPLE

(c) Acidify two 100 ml portions with 3 ml of H_2SO_4 and extract, using either continuous extractors or separatory funnels (state which is used) and apply the indigo test to one extract and the pyridine test to the other.

1. Indigo test.—Add 3 ml of anthranilic acid reagent to the ether extract and evaporate at a low temp. If any insoluble matter (oily or solid) separates out, filter the remaining liquid thru a small wet filter paper into a 50 ml beaker. If no insolubles come out, transfer the residue to beaker. Now apply the test as described under II (c) "Barium-Indigo Test" beginning "Test with litmus paper, etc."

2. Pyridine test.—Treat the extract by the method in *This Journal* **29**: 104 (1946) under the heading "Qualitative Method applicable to Beverages and Fruit Juices," parts 1 and 2.

(d) In the case of Samples 9 and 10 apply the Mallory-Love procedure to 100 ml of sample. *Ind. Eng. Chem., Anal. Ed.*, **15**: 492 (1943).

[3] For the fusion at 310–320°C use a brass block having a well to contain the test tube and a second well to contain a thermometer. The block is wrapped with a coil of nicrome wire and the heat controlled by a variable voltage transformer. Analysts have used muffle furnaces, micro-burners, Wood's metal, or solder baths, etc., for the fusion with equal success.

The following persons collaborated in these tests: H. M. Bollinger, Los Angeles, Calif.; Harry W. Conroy, Kansas City, Kans.; Joyce Holberg, Minneapolis, Minn.; Gardner Kirsten, New York, N. Y.; D. W. McLaren, Buffalo, N. Y.; Angus J. Shingler, Atlanta, Ga.; John B. Wilson, Washington, D. C. All were members of the staff of the U. S. Food and Drug Administration, Federal Security Agency.

Since most of the collaborators reported the results obtained in the determination of the efficiency of their extractors, they have been brought together in Table 1.

TABLE 1.—*Efficiency of extractors used on monochloroacetic acid*

COLLABORATOR	TIME		
	PRESENT	2 HRS.	3 HRS.
	mg	*per cent*	*per cent*
H. M. Bollinger	25		97
	50	92	
	100	93	99
Harry W. Conroy			Over 95
Joyce Holberg	25	94	97
	50	92	94.5
	100	87.5	97
D. W. McLaren[1]	25	102.3	102.3
	100	99.7	99.7
Angus J. Shingler	25	94.0	99.2
	50	97.6	99.2
	50	98.6	99.2
	100	93.5	99.0
John B. Wilson	25	90.8	95.2
	50	94.6	99.2

[1] Extractors used by this collaborator are made of tubing 1 inch in diameter. They are about 47 inches in length, with the delivery tube 30 inches from the bottom. The 100 ml of sample was diluted with 100 ml of water for the extraction.

The results obtained with quantitative methods 1 and 2 (which differ as to mode of extraction) are given in Table 2. The average recoveries by both methods are well above 90 per cent for all samples except the orange juice.

QUALITATIVE TESTS

The collaborative results on the qualitative tests are given in Table 3.

These results warrant the adoption as official, first action, of the following qualitative tests:

TABLE 2.—*Monochloroacetic acid (Quantitative Methods 1 and 2)*

SAMPLE NO. PRODUCT	(5) PRESERVATIVE		(6) ORANGE JUICE		(7) ORANGE SODA		(8) STRAWBERRY SODA		(9) BEER		(10) WINE	
	g/100 ml	*g/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
Present	5.44	5.44	22.7	22.7	13.0	13.0	11.0	11.0	14.4	14.4	19.0	19.0
Found by Method	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
Collaborator:												
H. M. Bollinger	5.24	5.22	20.3	17.8	14.2	13.8	11.0	11.3	14.5	13.4	18.5	18.5
	5.22	5.20	19.0	19.5	14.2	13.5	11.0	10.9	14.0	14.0	18.2	18.3
Harry W. Conroy	5.28	5.27	18.4	16.5[1]	13.1	12.8	10.9	10.2	13.9	13.3	18.0	17.9
	5.27	5.27	18.0	16.0[1]	12.9	12.8	10.4	10.4	13.7	13.2	17.8	17.7
Joyce M. Holberg	5.15	5.08	24.0	17.3[1]	11.8	10.3	10.1	10.5	14.5	13.5	13.0[2]	20.4
	5.13	5.23	23.3	17.8	11.3	11.3	9.4	10.0	14.3	13.3	10.0[2]	21.0
Gardner Kirsten	5.30	5.21	19.1	—[1]	12.8	12.7	11.0	10.5	14.9	—[1]	18.7	—[1]
	5.30	5.22	18.6	—[1]	12.5	11.8	11.1	10.1	14.8	—[1]	18.4	—[1]
D. W. McLaren	5.42	5.39	16.3	14.4[1]	13.1	10.9	11.4	10.4	13.2	12.7[1]	16.7	17.5
	5.42	5.24	19.2	15.8[1]	12.8	11.1	10.6	9.9	11.1	13.2[1]	17.2	17.7
Angus J. Shingler	5.23	4.83	18.0	15.7[1]	12.7	12.4	11.4	10.6	13.9	15.0[1]	17.0	17.7
	5.26	4.49	18.0	18.5	12.2	12.4	11.4	10.6	14.2	13.1[1]	17.7	17.5
John B. Wilson	5.43	5.22	23.0	17.4[1]	12.9	12.3	11.2	10.6	14.0	13.4	19.0	18.5
	5.42	5.38	21.0	18.2[1]	12.3	11.8	10.8	10.1	14.3	14.0	18.5	17.9
Maximum	5.43	5.39	24.0	19.5	14.2	13.8	11.4	11.3	14.9	15.0	19.0	21.0
Minimum	5.13	4.49	16.3	14.4	11.3	10.3	9.4	9.9	11.1	12.7	10.0	17.5
Average	5.29	5.15	19.7	17.1	12.8	12.1	10.8	10.4	13.9	13.5	17.2	18.4
Average recovery, per cent	97.2	94.6	86.8	75.3	98.5	93.1	98.2	94.5	96.5	93.8	90.5	96.8

[1] Emulsions formed and interfered with the determination.
[2] If these two results are eliminated because of the obvious incomplete extraction, the minimum result becomes 16.7; the average 17.9; the average recovery, 94.2%.

A. For use on commercial preservatives:
   (1) The Barium Test, accompanied by microscopic identification of the crystals.
   (2) The Barium-Indigo Test.
   (3) The Pyridine Test.

B. For use on carbonated beverages, fruit juices, and beer:
   (1) The Indigo Test, as given in this report.
   (2) The Pyridine Test.

Several collaborators consider the Mallory-Love version of the indigo test too tedious and others lack confidence in it because of frequent fail-

TABLE 3.—*Monochloroacetic acid (qualitative tests)*

TEST	SAMPLE	B	C	G	H	K	McL	S	W
Barium	5	+[1]	+[1]		+[1]	+[2]	+[1]	+[1]	+[1]
Indigo Barium	5	+	+		+	+	+	+	+
Indigo	5	+	+		+	+[3]	+[4]	+[4]	+[3]
	6	+[3]	+[4]	+	+[3]	+	+	−	+
	7	+	+	+	+	+	+		+
	8	+	+	+	+	+	+	+	+
	9	−	+	+	+	+	+	−	+
	10	−	+	+	+	−	+	+	
Mallory-Love	9	−	+		+	+	+		+
	10	+	+		+	+	+		+
Pyridine	5	+	+		+	+	+	+	+
	6	+	+	+	+	+	+	−	+
	7	+	+	+	+	+	+	+	+
	8	+	+	+	+	+	+	+	+
	9	+	+	+	+	+	+	−	+
	10	+	+	+	+	+	+	+	+

[1] Crystals of barium monochloracetate were identified microscopically by W. G. Helsel, Microanalytical Section.
[2] Crystals of barium monochloracetate were also identified microscopically by C. A. Wood, New York, N. Y.
[3] Extractions for these tests made with a continuous extractor.
[4] Extractions for these tests made in separatory funnels.

ures to obtain the test when they felt certain that monochloroacetic acid was present in the sample being examined. However, the Associate Referee freely admits that no qualitative test for monochloroacetic acid has yet been devised which can be guaranteed against failure, so that he has reached the conclusion that while a positive test by any of the procedures studied is conclusive evidence of the presence of monochloroacetic acid, a negative test by any or all of them is no guarantee of the complete absence of monochloroacetic acid.

For instance, on frequent occasions it has been found that the barium monochloracetate crystals in the residues obtained from food samples were very much like the proverbial needle in a haystack. For this reason it has been decided to limit its use to commercial preservatives. There have been times, however, when the crystals could not be found but the Indigo test on the crystals was successful.

The Pyridine test has been subject to failure at times because of unknown impurities which interfere with crystallization of the betaine. However, most acid substances are eliminated if sufficient pyridine is added in the test. If more than 5 ml of normal sodium bicarbonate solution is required to extract the acids from the ether, as has happened frequently in this laboratory, there should be a corresponding increase in the quantity of pyridine added, in which case the acids distil off under diminished pressure with the pyridine.

The Indigo test is subject to failure because the dried mixture in the test tube may be so dark that the color changes during the fusion cannot be observed, and the analyst is not only deprived of seeing the color changes which show the presence of the chemical, but is even unable to tell when the requisite fusion period is over.

In the Mallory-Love version of this test there are three evaporations, (1) in a dish from ether and alcohol, (2) in a beaker from ether, and (3) in a beaker from benzene. Monochloroacetic acid may be lost at any of these points if the residue is allowed to go completely dry. The vessel must be removed from before the fan at just the right moment to have the test work perfectly.

Another danger point is in the extraction with benzene, since an excessive quantity of water at this point will cause enough loss of monochloroacetic acid in the water layer to vitiate the test.

Members of the Food and Drug Administration in Cincinnati, Chicago, and Buffalo have suggested procedures for purification of the ether extract by treatment with barium hydroxide and other means which the Associate Referee will investigate further for next year's work on the analysis of wine.

RECOMMENDATIONS*

It is recommended—

(1) That the method for the determination of monochloroacetic acid, *This Journal*, **31**,104 (1948), be adopted as official, final action, for carbonated beverages.

(2) That the method for the determination of monochloroacetic acid, *Ibid.*, **31**,104 (1948), be adopted as official, first action, for beer and wine.

(3) That the following parenthetical expression be added to the method for monochloroacetic acid in carbonated beverages, beer, and wine (*Ibid.*,

* For report of Subcommittee C and action of the Association, see *This Journal*, 32, 57 (1949).

p. 105) after the sentence ending "extract with ether 2–3 hours." "(Use the length of time found necessary for a recovery of at least 95 per cent when known quantities of monochloroacetic acid are extracted in the apparatus.)" (Official, first action.)

(4) That the following paragraph be added to the method for monochloroacetic acid: (*Ibid.* 104 (1948): "The following equally efficient means of extraction may be used:

'To 100 ml of sample add 3 ml of H_2SO_4 and shake in a separatory funnel with three equal volumes of ether. Unite the ether extracts and wash by shaking with two 30 ml portions of 1 *N* NaOH. Unite the two NaOH solutions and digest as above.'" Official, final action.

(5) That the following method be adopted as official, first action.

Determination of Monochloroacetic Acid in Commercial Preservatives

Prepare a dilution of the sample that will permit the measurement of a convenient aliquot containing 50–100 mg of monochloroacetic acid and determine in such aliquot as directed under the method for carbonated beverages.

(6) That the following qualitative tests for monochloroacetic acid in commercial preservatives be adopted as official, first action, as given above under "II. Sample 5." Barium Test; Barium-Indigo Test; Indigo Test; Pyridine Test.

(7) That the following qualitative tests for monochloroacetic acid in carbonated beverages, orange juice, beer and wine be adopted as official, first action, as given above under "III. Samples 6–10, inclusive." Indigo Test; Pyridine Test.

(8) That further work be done on the determination of monochloroacetic in fruit juices including orange juice.

(9) That further work be done on qualitative tests for monochloroacetic acid.

REPORT ON MOLD INHIBITORS

By Lewis H. McRoberts (Food and Drug Administration, Federal Security Agency, San Francisco, Calif.), *Associate Referee*

LOSSES OF PROPIONIC AND ACETIC ACIDS IN THE BAKING AND AIR DRYING OF BREAD

The previous report (1) outlined procedures for the detection of added propionates in bakery products. Samples of air dried white bread prepared with sodium propionate were submitted to collaborators for analysis by the method now adopted as "tentative" (2). These experiments disclosed a loss of propionate that apparently took place during baking and/or air drying in preparation of samples. The present investigation has been directed to determining the source or sources and the magnitude of this loss. The experiments were expanded to include the addition of acetic acid by means of the product "sodium diacetate" and to both white and

whole wheat breads. The fresh bread was analyzed to detect baking loss and the air dried bread to determine total loss by that method of sample preparation. The total loss less the apparent baking loss was then attributed to air drying.

BREAD PREPARATION

The bread for the experiments described in this report was baked in the cereal section laboratory of the U. S. Food and Drug Administration in Washington, D.C., by V. E. Munsey, and was forwarded by air express to this destination. The following described formula was used for both white and whole wheat breads:

Flour	315 gms.	Lard	10 gms.
Water	198 gms.	Sugar	12 gms.
Yeast	8 gms.	Salt	7 gms.
Dry Skim Milk	12 gms.		

The first baking consisted of nine one-pound, white loaves of which three were for control, three contained added "sodium diacetate" and three contained added sodium propionate. The inhibitors were added to the flour in the proportion of 0.25 per cent diacetate and 0.20 per cent propionate, or, respectively, 0.16 per cent and 0.12 per cent in the bread. This series of samples was then duplicated in whole wheat bread with the exceptions that the inhibitors were added to the dough for each loaf in amounts of 0.16 per cent of propionate or diacetate on the finished bread basis. The loaves were weighed one hour after baking and upon receipt at destination. Weight losses in transit were found to be 3–5 per cent. Analyses were started on the fresh bread in from one to two days following baking. The white bread was air dried for four days and the whole wheat bread for seven days at 23–25°C previous to grinding to 20 mesh in a steel burr mill.

Samples of the propionate-flour mixture, diacetate-flour mixture, wheat flour, sodium propionate, and "sodium diacetate" used in preparing the breads were analyzed to check the mixing of flour and inhibitor, the amount added, and the purity of the inhibitors as to propionic or acetic acids available.

SAMPLE PREPARATION

The three loaves of each batch were sliced to give about eighteen slices per loaf and divided into two composites by alternate slices. The composites were then weighed and designated as one portion to be analyzed fresh and one portion for air drying. From this point, subsequent preparation was as previously described (2).

METHOD

The tentative method for volatile fatty acids in bakery products (2) was used in the analysis of all samples. Flour mixtures with added inhibitors were treated the same as air dried bread. Sodium propionate or sodium diacetate were distilled directly on the basis of 500 mgs of the original inhibitor in the distillation flask.

RESULTS

(1) *Sodium Propionate.*—The propionate added in the preparation of the bread was labeled sodium propionate. In that the calcium salt is often used for the same purpose, tests were made for calcium with negative results. The *p*H of a 0.5 per cent solution was found to be 6.9. When distilled (3) from a solution made acid to Congo Red with sulphuric acid, propionic acid was identified by the standard "C" ratio for that acid, and by the formation of characteristic mercurous propionate crystals. No other volatile acids were detected. The purity as sodium propionate was calculated to be 98.5 per cent.

(2) *"Sodium Diacetate."*—The chemical sold under this name has been described as a "solid form of acetic acid." The powder is acid to litmus and has a faint odor of acetic acid. The *p*H of a 0.5 per cent solution was found to be 4.7. Free acid was determined by titration to phenolphthalein end-point. When distilled (3), acetic acid was identified as the only volatile acid present. Total titrations on the basis of 250 ml distilled were calculated to this acid. The following results were obtained:

	Per cent
Free acid calculated as acetic	34.02
Total volatile acids as acetic	76.81
Combined acid (By difference)	42.79
Combined acid (Calc. as anh. sodium acetate)	58.45
Total Calculated	92.47

The figure of 76.8 per cent total available acetic acid was used in calculating recoveries of added "sodium diacetate."

(3) *White flour—sodium propionate mixture.*

Added——————————0.20% sodium propionate

Determined:—Formic Acid— 1.3, 1.3 } Av. 1.3 mgs./100 gms.

Acetic Acid—None detected

Propionic Acid— 168.0, 165.0 } Av. 166.5 mgs./100 gms.

Calculated:— Sodium Propionate— 216.5 mgs./100 gms. (0.22%)

(4) *White flour—sodium diacetate mixture.*

Added——————————0.25% sodium diacetate

Determined:—Formic Acid— 1.3, 1.5 } Av. 1.4 mgs./100 gms.

Acetic Acid— 204.1, 200.8 } Av. 202.5 mgs./100 gms.

Propionic Acid—None detected

Calculated:—Sodium Diacetate— 263.7 mgs./100 gms. (0.26%)

(5) *Flour.*

(a) *White flour.* Previous analyses of starch and white flour by the volatile acid procedure (2) indicated trace amounts of acetic acid (less than 0.01%).

(b) *Whole wheat flour.* The whole wheat flour used in the preparation of the wheat bread was analyzed (2) with the following results:

Formic Acid 3.8, 3.4 } Av. 3.6 mgs./100 gms.

Acetic Acid 7.0, 7.5 } Av. 7.3 mgs./100 gms.

Propionic Acid None

[The above described analyses show that the inhibitors were thoroly mixed with the white flour and that the method gave efficient recoveries when applied to these mixtures. Recovery experiments were previously reported wherein propionic acid was added to air dried bread (1)].

(6) *White and Whole Wheat Breads—Propionate and Diacetate Recovery.*

The results of analysis for volatile fatty acids on control breads and breads containing added inhibitors are listed in Table 1. All of the results on fresh bread and on air dried samples have been calculated to the original fresh basis—one hour after baking.

DISCUSSION

Substantial losses of both propionate and diacetate have been found to take place during baking and for the most part additional greater losses during the air drying of bread in sample preparation. Bread is slightly acid and it would be expected that losses of the volatile acids would occur at the relatively high temperature of baking. The time required for the air drying of bread to about 10 per cent moisture depends on the relative humidity and circulation of air. From two to four days are allowed at this location. Apparently there is a gradual loss of propionic and acetic acids during the period of drying. Greater air drying losses are observed in the wheat bread than in the white bread. The *p*H of both types of the samples analyzed is about the same and does not afford any basis for conclusion as to the reason for this difference. The difference may have been due to the longer period of drying for the wheat bread.

Where propionates are used in amounts considered general commercial practice (0.2–0.25% in the flour or 0.12–0.16% in the fresh bread) they may be detected in the analysis of air dried bread by the volatile acid procedure described in the previous report. However, the results of the present experiments indicate the fresh sample basis to be preferable even though it is necessary to air dry for reserve and subsequent analysis.

No propionic acid was calculated in the control samples or in samples with added diacetate.

All previous and present analyses made by the Associate Referee in the determination of volatile fatty acids in bread show that acetic acid is normal in bread and in amounts that must be taken into account in the detection of added acetic acid whether from vinegar or sodium diacetate.

ACKNOWLEDGMENT

The Associate Referee acknowledges with appreciation the assistance of V. E. Munsey (U. S. Food and Drug Administration, Washington, D. C.) in the preparation of the bread samples that are the basis of the present report.

It is recommended* that investigation of an alternative chromatographic technique (4) for the identification and estimation of volatile acids in bakery products be undertaken.

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* For report of Subcommittee C and action of the Association, see *This Journal*, 32, 57 (1949).

TABLE 1.—*Losses of sodium propionate or sodium diacetate in baking and air drying calculated basis volatile acid determinations*
Results (mgs/100 gms fresh basis)

BREAD PRODUCTS	VOLATILE FATTY ACIDS					
	FRESH BREAD			AIR DRIED BREAD		
	FORMIC	ACETIC	PROPIONIC	FORMIC	ACETIC	PROPIONIC
White Control	2.0	56		1.4	44	
White Control	1.8	59		—	—	
White Control (Av.)	1.9	58	None	1.4	44	None
White+Diacetate	2.6	164		2.8	122	
White+Diacetate	2.8	166		2.1	117	
White+Diacetate (Av.)	2.7	165	None	2.5	120	None
White+Propionate	2.6	59	80	2.4	36	68
White+Propionate	2.8	70	66	—	47	58
White+Propionate	—	61	81	—	—	—
White+Propionate	—	66	76	—	—	—
White+Propionate (Av.)	2.7	64	76	2.4	42	63
Whole Wheat Control	8.4	50		7.0	33	
Whole Wheat Control	—	—		—	35	
Whole Wheat Control (Av.)	8.4	50	None	7.0	34	None
Whole Wheat+Diacetate	9.2	158		10.3	100	
Whole Wheat+Diacetate	9.0	155		—	100	
Whole Wheat+Diacetate (Av.)	9.1	157	None	10.3	100	None
Whole Wheat+Propionate	9.0	44	101	8.8	32	70
Whole Wheat+Propionate	8.6	35	105	—	30	72
Whole Wheat+Propionate (Av.)	8.8	40	103	8.8	31	71

TABLE 1.—*Continued*

BREAD PRODUCTS	SODIUM PROPIONATE						SODIUM DIACETATE					
	ADDED	RECOVERED		LOSSES			ADDED	RECOVERED		LOSSES		
		FRESH	AIR DRY	BAKING	AIR DRYING	TOTAL		FRESH	AIR DRY	BAKING	AIR DRYING	TOTAL
				per cent	*per cent*	*per cent*				*per cent*	*per cent*	*per cent*
White Control												
White Control												
White Control (Av.)	None	None	None	—	—	—	None	76[1]	57[1]	—	—	—
White + Diacetate												
White + Diacetate												
White + Diacetate (Av.)	None	None	None	—	—	—	157	215	156	8[2]	25[2]	33[2]
White + Propionate												
White + Propionate												
White + Propionate												
White + Propionate												
White + Propionate (Av.)	124	99	82	20	14	34	None	82[1]	54[1]	—	—	—
Whole Wheat Control												
Whole Wheat Control												
Whole Wheat Control (Av.)	None	None	None	—	—	—	None	65[1]	44[1]	—	—	—
Whole Wheat + Diacetate												
Whole Wheat + Diacetate												
Whole Wheat + Diacetate (Av.)	None	None	None	—	—	—	157	204	130	8[2]	33[2]	41[2]
Whole Wheat + Propionate												
Whole Wheat + Propionate												
Whole Wheat + Propionate (Av.)	155	134	92	14	27	41	None	52[1]	40[1]	—	—	—

[1] Normal acetic calculated to sodium diacetate.
[2] Per cent loss of total acetic acid (added plus normal).

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REPORT ON THIOUREA IN FOODS

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

In last year's report, two methods were proposed for the determination of thiourea; one for oranges or orange juice and the other for frozen dessert peaches. These methods were proposed as tentative. The one for orange juice employed amyl alcohol for the extraction of thiourea from the concentrated sample and the removal of the thiourea from the amyl alcohol by a water extraction. This procedure was followed in order to separate the thiourea from a substance in the juice which inhibited the reaction with a hydrated form of sodium cyano ferrate (modified Grote's reagent).

It was stated in last year's report that the inhibitor appeared to be a reducing agent. Experiments this year have shown that ascorbic acid exerts a similar inhibitory effect on the reaction between thiourea and the modified Grote's reagent. The ascorbic acid in the juice therefore is evidently one substance which inhibits the above reaction. Inasmuch as the reagent apparently oxidizes[1] the thiourea first to formamidine disulfide before the coupling reaction, it seems possible that the effect of the inhibitor (ascorbic acid) might be removed by a preliminary oxidation of the sample. At the same time, the thiourea (being easily oxidized) would be oxidized to the disulfide stage and could be determined if conditions were such that it remained stable during the course of the determination.

The disulfide has been shown to form rather stable salts with some strong acids. However, the reaction with the cyano ferrates does not take place readily in too strong acid solutions.

In view of these findings a direct and rapid method was devised which gave excellent results, in the hands of the Associate Referee, on samples of fresh orange juice containing known quantities of added thiourea. Briefly, the method consists of the clarification of the strained orange juice, oxidation with excess of standard iodine solution in strong acid, reduction of acidity with sodium acetate, and determination of thiourea by use of sodium cyano ferrate reagent (modified Grote's reagent) and a photometer. Some results obtained by the Associate Referee are given in Table 1.

In the opinion of the Associate Referee, the results obtained are excellent.

[1] *This Journal*, 31, 476 (1948); *Analyst*, **71**, 562 (1946).

A sample of orange juice was prepared, to which was added 30 p.p.m. of thiourea, and after thorough mixing was placed in pint bottles having plastic caps. The bottles were loosely capped and placed in an oven at 100°C for 30 minutes to sterilize the sample. The bottles were then tightly capped and allowed to cool. After cooling and standing, the samples appeared dark, with a rather brownish cast.

One bottle of the sample was sent to each of a number of collaborators, with the request that they determine thiourea both by the tentative method proposed last year and by the new rapid oxidation method. The results obtained by collaborators and the Associate Referee were disap-

TABLE 1.—*Results of thiourea determination on fresh orange juice, by the rapid oxidation method*

THIOUREA ADDED	THIOUREA FOUND	DIFFERENCE
p.p.m.	*p.p.m.*	*p.p.m.*
9.1	9.2	+0.1
18.2	18.2	+0.0
30	29.0	−1.0
27.3	27.4	+0.1

pointing. Those obtained by the rapid oxidation method varied from 20.7 to 24.9 p.p.m., and results by the tentative method varied from 16.5 to 26.0 p.p.m.

Immediately after making the determination on the A.O.A.C. sample, the Associate Referee prepared a sample of fresh orange juice to which were added 30 p.p.m. of thiourea; no heat treatment was applied. Determinations of thiourea were made on this sample by the rapid oxidation method and recoveries of 29.0 and 29.1 p.p.m. were obtained.

It is the opinion of the Associate Referee, therefore, that the heat treatment for sterilization of the A.O.A.C. sample was too drastic and destroyed a portion of the added thiourea and partially caramelized some of the sugars, as shown by the dark color. No doubt this accounts for the low, rather erratic results obtained by collaborators. Comments by collaborators indicated that the rapid oxidation method was much preferred to the tentative method. The rapid method is proposed as a tentative method until further collaborative work can be done. Details of the rapid oxidation method are given in the February number of *This Journal*, on page 100, under "Changes in Methods of Analysis."

RECOMMENDATIONS*

It is recommended—

(1) That the rapid oxidation method for thiourea in oranges or orange

* For report of Subcommittee C and action of the Association, see *This Journal*, **32**, 58 (1949).

juice, given in this report, be adopted as tentative, and that the present tentative method for thiourea in orange juice be dropped.

(2) That work on the detection and determination of thiourea in foods be continued.

REPORT ON FORMALDEHYDE

By HOWARD P. BENNETT (Food and Drug Administration, Federal Security Agency, New Orleans, La.), *Associate Referee*

Methods for the detection of the toxic preservative, formaldehyde, have not been extensively studied by the Association for many years. Work done by Oakley (1) pointed up a need for restudy of this topic. Two recent references in the literature (2, 3) show that the use of this compound is not exactly a dead issue and there is an ever present temptation to apply it to food material.

The Association has indicated that present unused and out-dated methods should be deleted from the *Methods of Analysis* and newer methods for the detection of formaldehyde studied. The study of Oakley, mentioned above, shows well the strength and weakness of present methods. The writer undertook the study of some new methods; and on the basis of these studies, the following recommendations are made.

RECOMMENDATIONS*

It is recommended that the Hehner Test (**32.21**) for testing milk be studied as modified according to directions of Fulton (4), as follows:

HEHNER-FULTON TEST FOR FORMALDEHYDE IN MILK

Dilute 8 ml of concd. sulfuric acid with 5 ml of water, cool, and put 4 ml of this diluted acid in a test tube. Add 1 ml of the milk to the sulfuric acid slowly and mix with cooling. A clear and practically colorless soln results unless a large proportion of formaldehyde is present. Prepare a bromine oxidizing soln by mixing equal volumes of concd. sulfuric acid and saturated bromine water, and cooling. Add ca 0.5 ml of this oxidizing soln to the sulfuric acid milk soln and shake. In the presence of formaldehyde a violet color develops at once, a color ranging to light purplish pink for very small amounts of formaldehyde. Make a blank determination for comparison.

It is further recommended that, the two following methods be subjected to collaborative study at an early date.

TANNIC ACID TEST FOR FORMALDEHYDE

Reagent.—Dissolve 50 mg of tannic acid in 100 ml of concd. sulfuric acid.

THE TEST

To 5 ml of the reagent in a small casserole add 1 ml of the distillate (**32.19**). Heat on steam bath for 5 min. The appearance of a green or blue-green color indi-

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* For report of Subcommittee C and action of the Association, see *This Journal*, **32**, 57 (1949).

cates formaldehyde. Make a blank determination for comparison. (As little as 2 micrograms of formaldehyde can be detected.)

CHROMOTROPIC ACID TEST FOR FORMALDEHYDE (5)

Reagent.—Make a saturated soln of purified chromotropic acid (ca 500 mg/100 ml) in ca 72% sulfuric acid.

THE TEST

Place 5 ml of the reagent in a test tube, and add with mixing 1 ml of the distillate (**32.19**). Place in a boiling water bath for 15 min. The presence of formaldehyde is indicated by a light to deep purple color, depending upon the amount present. (As little as 4 micrograms may be detected, and the test is very specific.)

LITERATURE CITED

(1) OAKLEY, MARGARETHE, *This Journal*, **28**, 296 (1945).
(2) *Chemical Abstracts*, **38**, 4706 (1944).
(3) *Ibid.*, **42**, 2687 (1948).
(4) FULTON, C. C., *Ind. Eng. Chem., Anal. Ed.*, **3**, 199 (1931).
(5) EEGRIWE, EDWIN, *Z. Anal. Chem.*, **110**, 22 (1937).

No report was given on saccharin, dichloroacetic acid, dulcin, or 1-propoxy, 2-amino, 4-nitro benzene.

No report was given on fill of container methods (food, drugs, and cosmetics), or on coffee and tea.

REPORT ON EGGS AND EGG PRODUCTS

By FRANKLIN J. MCNALL (Food and Drug Administration) Federal Security Agency, Cincinnati, Ohio), *Referee*

ADDED GLYCEROL

During the past year the Associate Referee, George Keppel, has devised a periodate method for the determination of added glycerol. It is recommended that this work be continued and the method be submitted for collaborative study.

ACIDITY OF FAT

No report on acidity of fat was given by the Associate Referee. It is recommended that this study be continued.

RECOMMENDATIONS*

In line with the Report of Committee on Classification of Methods, the following recommendations are made:

(1) That an Associate Referee be appointed to study the present tentative method for Fat by Acid Hydrolysis (**23.8**). This method was adopted

* For report of Subcommittee C and action of the Association, see *This Journal*, **32**, 53 (1949).

as tentative in 1933 but was never made official because of the lack of collaborative study.

(2) That the tentative Qualitative Method for Glycerol **(23.25)** be made official, first action. This test was given some collaborative study in 1932 and 1933 and it should be made official.

(3) That the tentative Rapid Method for Acidity of Ether Extract **(23.31)** be made official, first action. The 1938 report shows method gives results in close agreement to present official method **(23.29)**.

(4) That an Associate Referee be appointed to study the tentative Method for Ammonia Nitrogen **(23.33)**. It is our understanding that several states use this method or a similar one for the interpretation of cases involving the age of shell eggs.

(5) That an Associate Referee be appointed for Succinic Acid in Eggs and that the method be studied collaboratively next year.

REPORT ON ADDED GLYCEROL IN EGGS AND EGG PRODUCTS

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

Studies were continued with special reference to the problem of isolating glycerol from interfering compounds normally occurring in egg yolk and in whole egg. The method used for examining the isolated glycerol is based on a modification of the periodate oxidation procedure by Newburger and Bruening.[1] To obtain a sharper end point in titrating the formic acid formed, the excess periodate is reduced, and bromcresol purple is substituted for methyl red as the indicator.

EXPERIMENTAL

Samples of fresh egg yolk containing no added glycerol were mixed with water and clarified by treatment with sodium tungstate solution and dilute sulfuric acid. This is essentially the method of Folin and Wu, commonly used in blood analysis for the preparation of protein-free blood filtrate. Aliquots of the egg filtrate, analyzed by the modified periodate method, show blanks of 0.2–0.3 per cent apparent glycerol. A number of other clarification reagents gave poorer results. Lead acetate, for example, introduces a buffer effect that interferes with the subsequent neutralization and titration. Lead nitrate-sodium hydroxide gives excellent clarification but the excess lead reacts with periodate and must be removed by separate steps. The same is true of other precipitants such as copper, zinc, or

[1] Newburger, S. H., and Bruening, C. F., *This Journal*, 30, 651 (1947).

mercury salts. Phosphotungstic acid yields clear filtrates, but titration end points are not as sharp as with tungstate.

The nature of the interfering substances is not definitely known, but it appears that it may consist chiefly of dextrose, small amounts of which are normally present in eggs. Dextrose is one substance which would not be removed by the above clarification methods and which will yield acid by periodate oxidation.

As the result of a number of experiments designed to eliminate or reduce dextrose interference, a technique was devised which is effective in reducing blank values to the range of 0.05–0.08% apparent glycerol. An aliquot of the filtrate is made alkaline and heated to boiling. This treatment

TABLE 1.—*Effect of alkaline treatment on dextrose*

DEXTROSE	APPARENT GLYCEROL	APPARENT GLYCEROL AFTER ALKALINE TREATMENT	REDUCTION IN APPARENT GLYCEROL
mg	*mg*	*mg*	*per cent*
5	3.96	1.34	66.2
10	7.92	2.78	64.9
25	19.80	7.80	60.8
50	39.6	15.07	61.9
100	79.2	25.30	68.1

is based on the instability of reducing sugar solutions when heated in the presence of alkali. The solutions become yellow to brown in color, and various products of an acid nature are among the substances formed. According to Browne and Zerban[2] lactic acid is produced in considerable amount by the action of alkalies upon many reducing sugars in the presence of air or oxidizing agents. Among the other oxidation products, formic acetic and oxalic acid have been found.

Results on dilute dextrose solutions treated with alkali and heated are shown in Table 1. In each case the dextrose solution was diluted to 25 ml. in an Erlenmeyer flask, 2 ml. of 10% NaOH added, mixed, heated to boiling, and boiled 15 seconds. To eliminate carbonate which interferes with the formic acid titration, the alkaline mixture is acidified, again boiled and cooled. It is then neutralized and the glycerol determination made.

The results show that not all of the dextrose interference can be removed by alkaline treatment. However, in the case of quantities of sugar comparable to those found in normal eggs, the interference can be reduced to a relatively small value.

On the basis of the experimental work described, a tentative method was devised to be used on egg products containing no added sugars.

[2] Browne, C. A., and Zerban, F. W., "Physical and Chemical Methods of Sugar Analysis," p. 653, Third Edition (1941).

METHOD

REAGENTS

Sodium tungstate soln.—Dissolve 10 g of reagent grade sodium tungstate ($Na_2WO_4 \cdot 2H_2O$) in water and dilute to 100 ml.

Potassium periodate, .02 M.—Dissolve 4.6 g of C.P. KIO_4 in ca 500 ml of hot water. Dilute to about 900 ml with water, cool to room temp., and make to 1 liter.

Sodium hydroxide, .02 N.—Dilute 100 ml 0.1 *N* NaOH to 500 ml with CO_2–free water.

Bromcresol purple indicator.—Dissolve 0.1 g of indicator in 100 ml of alcohol.

Propylene glycol.—Use a pure product containing no free acidity or liberating acidic substances on oxidation.

DETERMINATION

Weigh accurately by difference ca 2 g of well mixed sample **23.1(a)** or **(b)** into 100 ml volumetric flask containing 50–75 ml of H_2O. Mix and add 2 ml of the sodium tungstate soln. Add slowly with continuous mixing 2 ml of 1 *N* H_2SO_4. Dilute to mark with H_2O, mix well, and filter (18.5 cm folded filter).

Transfer 20 ml of filtrate containing not more than 40 mg of glycerol to a 300 ml Erlenmeyer flask. Add 2 ml of 10% NaOH, heat to boiling, and boil for 30 sec. Cool slightly, add 2 drops of bromcresol purple indicator, neutralize with 1 *N* H_2SO_4 and add 2–3 drops excess. Boil 1 min. and cool to room temp. Neutralize with 0.02 *N* NaOH, titrating to light purple shade.

Add ml 0.02 *M* KIO_4, mix, wash down sides of flask with H_2O, and allow to stand 30 min. Add 10 drops of propylene glycol to the oxidized mixture, mix and let stand for 10 min. Add 3 drops of bromcresol purple indicator and titrate with 0.02 *N* NaOH.

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

To correct for error due to volume occupied by insoluble matter, repeat determination, weighing same quantity of sample into 200 ml volumetric flask, and proceed as directed above, except that a 40 ml aliquot is used for the determination instead of 20 ml. To obtain amount of glycerol subtract % glycerol obtained in 100 ml dilution from twice % obtained in 200 ml dilution determination.

Results on a variety of egg products containing no added glycerol are shown in Table 2, indicating the blank range and substances which interfere.

TABLE 2.—*Results on egg products containing no added glycerol*

PRODUCT	APPARENT GLYCEROL
	per cent
Whole egg	0.05
Egg yolk	0.05
Egg white	0.08
Frozen whole egg (decomposed)	0.07
Dried whole egg	0.06
Whole egg containing 10% salt	0.06
Whole egg containing 10% sucrose	0.63
Whole egg containing 3.53% dextrose	0.67

As shown, sucrose and dextrose interfere appreciably. Salt has no apparent effect. The frozen whole egg sample was a commercial product classified as decomposed when originally sampled. After sampling, it had been kept under cold storage for about 1 year. The product had a strong disagreeable acid odor.

Results on recovery of glycerol added to whole egg or egg yolk are tabulated under Table 3. Glycerol used was USP grade glycerin, and its strength was checked by periodate oxidation, by dichromate oxidation, and by specific gravity. The various batches were prepared by weighing glycerol and fresh egg, laboratory separated, into glass-stoppered flasks. Contents were mixed by shaking the closed flask and allowed to stand overnight in a refrigerator to insure uniformity.

TABLE 3.—*Application of method to eggs containing known amounts of glycerol*

PRODUCT	GLYCEROL		RECOVERY
	PRESENT	FOUND	
	per cent	*per cent*	*per cent*
Egg yolk	9.74	9.85	101.1
		9.91	101.7
Egg yolk	11.44	11.56	101.0
		11.60	101.4
Egg yolk	7.36	7.44	101.1
		7.42	100.8
Whole egg	10.40	10.41	100.1
		10.35	99.5
		10.39	99.9

These results are uncorrected for volume occupied by insoluble material. Table 4 gives results on a series corrected by the method of double dilution and a comparison with the uncorrected results.

TABLE 4.—*Glycerol results of double dilution method*

PRODUCT	GLYCEROL PRESENT	GLYCEROL FOUND		RECOVERIES	
		UNCORRECTED	BY DOUBLE DILUTION	UNCORRECTED	DOUBLE DILUTION
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Egg yolk	7.36	7.43	7.38	101.0	100.3
Whole egg	7.68	7.71	7.69	100.4	100.1
Whole egg	10.40	10.38	10.34	99.8	99.4

An unsuccessful attempt was made to obtain samples of commercial

egg preparations containing added glycerol for examination by the above method. It appears that very little of this type product is being manufactured at the present time because of the current high price of glycerol.

Because of lack of time no comparisons have been made with the present tentative method for glycerol, and no collaborative work has been done.

It is recommended* that study on methods for isolating glycerol from egg mixtures be continued.

No report was given for acidity of fat (in egg products).

A contributed paper, by F. Hillig, on "Water-insoluble Acids in Dried Eggs," was published in *This Journal*, **31**, 731 (1948).

No report was given on microbiological methods for canned fishery products, canned meats, canned acid foods, canned vegetables, eggs and egg products, nuts and nut products, frozen fruits and vegetables, and sugar.

REPORT ON DECOMPOSITION IN FOODS

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

RECOMMENDATIONS†

Recent progress in the chemical detection of cow manure in milk suggests that the subject of filth in foods might be included under a title still to be selected in the 1949 A.O.A.C. list of subjects. In the Referee's opinion, the simplest way is to combine it with decomposition, one possible title being "Chemical Indices of Filth and Decomposition in Foods." It is recommended that the scope of the subject "Decomposition in Foods" be broadened to include chemical methods for detecting filth in foods.

The Referee concurs in the recommendations of the Associate Referees for decomposition in foods, as follows:

(1) For decomposition in dairy products, that the method for water insoluble acids in butter and cream be adopted as official first action, and that the study of other chemical methods for decomposition in dairy products be conducted.

(2) For decomposition in fish, that the revised method for volatile acids in fish be studied collaboratively.

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* For report of Subcommittee C and action of the Association, see *This Journal*, **32**, 53 (1949).
† For report of Subcommittee C and action of the Association, see *This Journal*, **32**, 54 (1949).

(3) For decomposition in fruit and fruit products, that the study of chemical tests for "blackheart" in pineapple be developed further, and that the study of other possible chemical means of detecting decomposition in fruit products be pursued.

REPORT ON DECOMPOSITION IN FRUITS AND FRUIT PRODUCTS

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

INTRODUCTION

The first report on decomposition in fruit products was made last year by T. H. Harris.[1] That report dealt mainly with the problem of detecting the use of rotten fruit in the preparation of apple juice or apple butter. After considerable research, three products of decomposition, probably from the pectin, were found. These were an alcohol soluble non-dialyzable substance, alcohol soluble furfural-yielding substances, probably pentoses, and D galacturonic acid. Of these products Dr. Harris concluded that the D galacturonic acid offered more promise as a criteria of rot than the other substances studied. A method was proposed for the determination of D galacturonic acid, which was a modification of the Deichman and Dierker method[2] using napthoresorcinol. It was shown by the method that some of the galacturonic acid was present in sound, ripe fruit, but the amount in rotten fruit greatly exceeded (20 times or more) that in the sound fruit. However, the reagent used is not specific for galacturonic acid, producing small quantities of color with various other acids. A study of the conditions of the reaction may further improve the specificity of the reagent.

Determinations of galacturonic acid in sound and rotten apples have been made by the writer, using the proposed method, but the results showed somewhat less difference in galacturonic acid content of these substances than was reported by Dr. Harris. A part of this reduction in the ratio of rotten to sound fruit may be due to the greater age of the materials used. Further study of the reaction conditions should render the method more valuable.

The writer has approached the problem more from the biochemical standpoint than as a strictly organic chemistry problem. Nearly all food decomposition is the result of the biological action of microorganisms, the chemical changes which they initiate being the result of the action of enzymes which these organisms secrete. The problem is therefore closely associated with the metabolism of the various microorganisms. The products resulting from the metabolic action are different in different groups

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[1] *This Journal*, **31**, 501 (1948).
[2] *J. Biol. Chem.*, **163**, 753 (1946).

but probably similar in related species. The decomposition of most fruits is brought about by the action of molds or yeasts. Bacterial action causing rot in fruit is rather rare.

Many fruits and, no doubt, other foods are attacked generally by a particular group of microorganisms. Rot in apples is usually caused by the molds *Penicillia expansum* or the *Mucors*, particularly *Mucor racemosus*. In oranges or citrus fruits we usually have *P. digetatum* and *P. italcum*.[3] *Rhizopus nigricans* is important in the spoilage of strawberries and stored potatoes,[3] etc.

The biochemistry of the molds has been studied systematically and very extensively by Raistrick[4] and his co-workers in England. These investigators have cultured many of the fungi, have made an intensive study of the media, and have isolated many of the products of mold metabolism. They have isolated and studied numerous compounds resulting from the biological processes of the various species of these organisms. They developed a rather ingenious scheme of analysis for determining the presence of metabolic products in the culture filtrate. The writer believes it would be profitable to follow a course similar to the one they have outlined.

Among the rather common compounds which have been isolated as products of mold metabolism are: ethyl alcohol, acetic acid, formic acid, oxalic acid, citric acid, succinic acid, fumaric acid, mannitol, butanol acetaldehyde, glycerol, acetone, fatty acids, and galacturonic acid. Among the products which are more particularly characteristic of mold metabolism are kojic, furoic, aspergillic, penicillic, carolic, carolinic, carlic, carlosic, aconitic, gentisic, fulvic, luteic, glycolic, and tetronic acids; citrinin, citromycetin, boletol, catenarin, and other anthroquinone derivatives; sterols, glycerides, and some polysaccharides. Ethyl acetate is also a rare product of some molds. Numerous other substances have also been found as products of mold metabolism.

The compounds citrinin and citromycetin are produced by the molds *Penicillium citrinum* and *Citromyces* and appear to be characteristic of these organisms. The determination of these constituents would be effective in establishing the use of moldy, decomposed fruit.

Time did not permit of a search for many of the products enumerated, but a limited search was made for the presence of mannitol, kojic acid, glycerides, sterols, and polysaccharides in rotten and sound apples. The presence of mannitol was investigated by two polarimetric methods based on rotation as a result of complex formation of the mannitol with borax or molybdate. These methods gave indication of the presence of mannitol in rotten apples in significant amounts, but also showed lesser and vary-

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[3] *J. Soc. Chem. Ind., London*, 581 (1936).
[4] *Trans. Roy. Soc. (London)*, B 220, 1–367 (1931), and *Biochem. J.*, 26, 1441 (1902), 1907 (1932), to 1947.

ing amounts in different varieties of sound apples. It is thought probable that other substances may affect the determination, and more sensitive and specific methods for mannitol will be sought.

Tests for kojic acid, in the rotten apple extracts tested, proved negative.

Tests on glycerides and sterols were not conclusive, an account of the difficulty of removing the natural waxes; but indications of the presence of sterols were obtained and these substances will be further investigated.

A study of the constituents of rotten apple tissue revealed the presence of a substance which appears to be a polysaccharide but not a starch or a pentosan. The material is of a mucilaginous character and was present in considerable quantity, approaching 0.8%, in rotten apple juice, but in very small quantity in the sound apple juice. The identity of this material has not been determined but will be investigated, and its use as a criterion of rot, particularly in apples, will be ascertained.

"BLACKHEART" IN PINEAPPLE

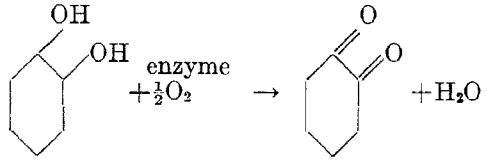
At the beginning of the work on decomposition of fruits, the problem of detecting the condition called "blackheart" in pineapple which had been frozen in the fresh condition was assigned to the writer. There appears to be some confusion in the minds of the public regarding the terms "blackheart," "black rot," etc. As far as can be determined by the writer, the condition called "black rot" is due to the fungus *Thielaviopsis paradoxa*. This organism soon disintegrates the fruit to a watery consistency. The macrospores of the fungus form on exposed tissues and on decayed tissues near the core of the fruit, turning it black.[5]

In the condition known as "blackheart" and also termed "internal breakdown," fungal growth or spores are not apparent. The flesh becomes dark near the core and spreads outward toward the surface. The discolored flesh often appears firm but is flat and insipid to the taste. Examination of a considerable number of these fruits (containing so-called "blackheart"), by the Microbiological Division of the Food and Drug Administration, failed to reveal the presence of the spores or hyphae of fungus growth. Also, samples sent to the Agricultural Research Center at Beltsville, Maryland, failed to disclose the presence of microorganisms. The decay or breakdown appears to be brought about as the result of an abnormal physiological development and is stimulated or accelerated by adverse conditions of moisture and temperature. The problem was attacked from this point of view. In cooperation with W. I. Patterson, the Referee, it was determined that the darkened color in the fruit was no doubt a melanin type compound probably resulting from the enzymatic oxidation of the amino acid tyrosine.

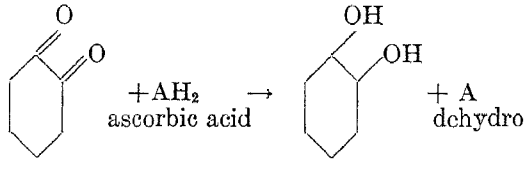
A search of the literature was necessary to obtain a satisfactory method for tyrosinase (catecholase) activity. The method finally used is a modifi-

[5] U.S.D.A. Circular No. 511.

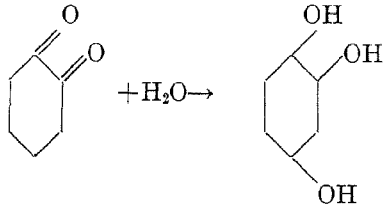
cation of the chronometric method of Dawson and Nelson,[6] and is based on the catecholase activity of the material. Results by the method showed a very striking difference between the sound and the blackheart samples. Of the samples so far tested, blackheart samples showed comparatively strong catecholase activity, while sound, just-ripe samples of pineapple showed only a trace or no activity. Sound, rather overripe samples showed only a weak activity. Although the catecholase activity test has not been proven specific for blackheart, it has been found to be capable of detecting the presence of blackheart in the authentic samples examined. Therefore, the procedure is proposed as a method for the detection of blackheart or of other types of decomposition in samples of fresh frozen pineapple which may show catecholase activity. The reactions involved in the determination are as follows:

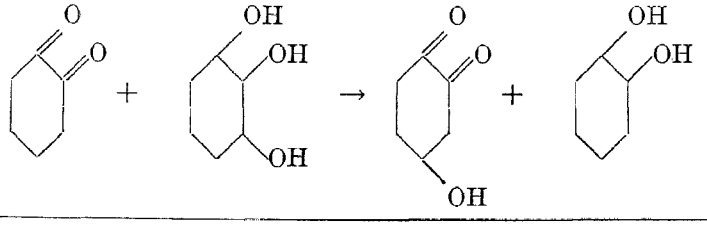


When ascorbic acid is present:

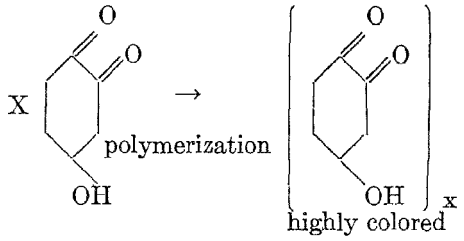


When no ascorbic acid is present or it has been used up, the reaction of the o-benzoquinone continues as follows to form the colored product.





[6] *J. Amer. Chem. Soc.*, 63, 3375 (1941).



METHOD FOR THE DETECTION OF BLACKHEART IN FROZEN PINEAPPLE

REAGENTS

(a) *Buffer soln.*—0.2 *M* citric acid.—0.4 *M* secondary sodium phosphate.
(b) *Pyrocatechol soln.*—0.2%.—200 mg catechol in 100 ml.
(c) *Sulfuric acid pyrogallol.*—2 *M* sulfuric acid containing 1% pyrogallol prepared fresh each day.
(d) *Potassium iodide.*—10% soln of KI freshly prepared each day.
(e) *Starch indicator*—1% soln of soluble starch.
(f) *Ascorbic acid soln stock (strong).* 100 mg ascorbic acid U.S.P. in 100 ml of 0.1% metaphosphoric acid.—Keep at 40°F.
(g) *Ascorbic acid (weak).* 10 mg per 100 ml.—Place 10 ml of soln (f) (stock) in a 100 ml flask and make to volume with 0.1 per cent metaphosphoric acid (HPO_3). Prepare fresh from reagent (f) each day.

PREPARATION OF SAMPLE

Cut the frozen material in the opened package with a knife completely across from side to side, cutting first in half, then in quarters, eighths, sixteenths, etc., until the contents are well chopped. Pour the chopped material into a large beaker or casserole, chop up any remaining lumps, and mix by stirring well.

Weigh 100 grams of chopped sample into a tared beaker (400–600 ml). Then add 100–110 grams of acetone and stir with a glass rod to mix contents. Decant mixture into a Waring blendor and rinse in any material in the beaker with 60% acetone (volume). Thoroly disintegrate the material by blending for 1 min.

Pour out the blended mixture into original beaker, allow to drain ca 20–30 seconds, and rinse blendor well with 60% acetone. Filter mixture on a rapid fluted filter (E & D No. 195 is suitable) 18½ to 24 cm in diam. Allow the filtrate to run thru until there is only a slow dropping, then rinse the beaker and filter with ca 100 ml of 60% acetone. When almost all the liquid has passed thru and there is only a slow drip of filtrate, remove filter and precipitate to a wide-mouthed short-stemmed funnel set in an 800 ml beaker. Puncture filter and wash precipitate from the filter into the beaker with 60% acetone from a wash bottle. Stir to disperse the solid. Filter mixture on a Büchner funnel (11 cm. diam.) using two No. 54 or 41 H Whatman filter papers with aid of *gentle* suction. Rinse beaker and transfer any residue quantitatively to funnel with 60% acetone. When most of liquid has passed thru and residue has formed a rather firm cake on the filter (filtrate is only a trickle or dropping), wash residue with another 75–80 ml of the 60% acetone wash. Allow the residue to suck dry until no more filtrate passes thru the filter and the residue becomes a firm hard cake. Discard the filtrate.

Strip the filter from cake of pineapple residue and transfer the latter quantitatively to a dry Waring blendor. Remove any solid from sides of funnel with a spatula. Add 195 ml (graduate) of water to the blendor and blend for 1 min. Pour the blended material into a 250-ml centrifuge bottle, stopper and shake for 30 seconds,

then centrifuge for 12–14 min. at ca 1800 r.p.m. Remove the bottle and filter the supernatant liquid thru a rapid filter (E and D No. 195 is suitable). Use portions of filtrate (X) for determination of catecholase activity.

DETERMINATION OF CATECHOLASE (TYROSINASE) ACTIVITY

(*A*) *By oxidation of ascorbic acid*—

Place a 300–500 ml round-bottomed three-neck flask in a bath held at 25°C. ±0.5°. Insert a glass tube having a spray bulb at end, fitted in a stopper in the one side-neck so that the tube extends to near bottom of flask. (This tube is to carry a current of air of ca 0.35 liter per min. from an air hose.) In the other side-neck, fit a capillary (1 mm bore) siphon tube the inside end of which will extend about two-thirds of the way below the surface of a quantity of 100 ml of liquid in the flask. The outside end of the tube is longer, extending below bottom of flask. Below the end of this tube is placed a 250-ml white casserole as a receiver.

Place 10 ml of buffer soln (reagent (a)) in the three-neck flask. In each of two 250 ml white porcelain casseroles place 25 ml of reagent (c), 25 ml of reagent (d), and 5 ml of reagent (e), and stir with glass rods to mix. Place one of the casseroles as a receiver under siphon tube. (Measuring of these reagents into casseroles should be done immediately before time to add other reagents to flask.)

Measure 30 ml of the sample filtrate (X, above) into a graduate and 50 ml of water into a second (50 ml) graduate, and measure also 10 ml of reagent (b), catechol, into a 10 ml graduate. Pour ca 25 ml of the water from graduate into flask, then pour in the 30 ml of sample soln. Now add 1 ml reagent (g) (0.1 mg ascorbic acid*). Rinse the cylinder which contained the sample soln with remaining water in the graduate and pour into flask, rinsing down the sides of the latter. Rotate to mix contents, place in position, and immediately start the air bubbling thru the spray tube. (The rate should have been previously adjusted to 1 liter every 3 min.) Finally, pour in the 10 ml of catechol soln (b) and start a stopwatch when this is added. Rotate the flask once or twice to bring all of the last soln into the body of the liquid.

Now (within 15 seconds after addition of catechol) start the siphon by momentarily closing the center neck of the flask with a stopper. Gently stir the liquid in the receiver as the liquid drops in from the siphon tube. Note the time required to develop a pronounced pink color, and the time to produce a purple color in the receiving soln. Compare the color in the receiver with the liquid in the other casserole. A definite or pronounced pink color should not develop in less than 5* min. with sound pineapple samples, nor a purple color in less than 8* min. If a sample gives an almost immediate pink or purple color at the entrance of the drops, the activity can be more closely measured by using a larger quantity (0.4–0.5 mg) of ascorbic acid on another portion of sample soln (X) A strong catecholase activity indicates blackheart.

(*B*) *By the development of color in the aerated solution*—

Place 10 ml of buffer soln (a) in a 300 ml round-bottomed flask. Measure 30 ml of the prepared sample soln (X) into a graduate and 50 ml of water into a second graduate. Add a portion of the water to the flask, pour in the sample soln (X) and rinse the graduate with the remaining water. Finally, add 10 ml of reagent (b) (catechol). Rotate the flask a few times to mix the contents. Aerate by passing a stream of air thru the spray tube as in determination (*A*), at the rate of ca 0.35 liter per min. for a period of one hour. Remove the air tube, pour the soln into an Erlenmeyer flask, and call this soln "P" (phenol catechol treated).

Determine the increase of color and turbidity in the treated aerated soln P (phenol treated) over that of the original sample by obtaining the following color and turbidity index:

* These figures are subject to revision.

$I_{c+t} = (1 - Tgp)$

where

I_{c+t} = color and turbidity index.

Tgp = transmittancy in the green (520 $M\mu$) of soln P compared to soln S

where

S is given a transmittancy of 1.

Dilute some of the original sample soln (X) with the same relative amounts of buffer and water as used in the aerated catechol-treated portion, but omit the addition of the catechol soln and substitute water in its place. Designate this prepared standard soln as "S." In a photoelectric photometer or spectrophotometer cell, place some prepared soln S, above, and using a wave length of 520 millimicrons set the instrument so that a reading of 100 (100% transmission) is obtained with this soln. In a second matched cell place some of the soln P and obtain the per cent transmission of this soln.

Obtain the color and turbidity index by the formula above. Repeat the readings after the solns "P" and "S" have stood overnight.

The index I_{c+t} on sound samples of frozen pineapple should not be above 0.1* for the immediate reading, or more than 0.2* for readings after standing overnight.

Increase in color may be determined and referred to standard soln of caramel in glycerol if this is found more desirable.

DISCUSSION

Since the oxidation of a known quantity of ascorbic acid is used in one part of the method as a measure of the o-benzoquinone produced by the enzyme, it was reasoned that, if an analogous reaction occurred with the tyrosine in the sample, the ascorbic acid would be destroyed in a similar manner and no melanin compounds would be formed until all the ascorbic acid had been oxidized. Determinations of ascorbic acid, together with catecholase activity, were therefore made on samples of sound, fully ripe, just-ripe, or slightly underripe pineapple, and on samples of pineapple containing blackheart. Results of the determination of catecholase activity and ascorbic acid are given in Table 1.

Examination of the data show that no ascorbic acid remained in the samples of blackheart pineapple, and as previously stated, these samples gave relatively strong tyrosinase activity as measured by oxidation of catechol. Samples containing 80 or 90 per cent of sound pineapple and 20 or 10 per cent of blackheart pineapple, respectively, showed a rather readily distinguishable increase in tyrosinase activity.

RECOMMENDATIONS*

It is recommended—

(1) That work on the detection of decomposition in fruits be continued.

(2) That the study of the method for galacturonic acid as a criterion of rot in apple products be continued.

(3) That the study of the polysaccharide, described in this report as a criterion of rot by mold, be continued.

* For report of Subcommittee C and action of the Association, see *This Journal*, 32, 54 (1949).

TABLE 1.—*Analysis of various types of pineapple samples for tyrosinase activity and some chemical constituents*

SAMPLE NO.	DESCRIPTION	TYROSINASE ACTIVITY			VITAMIN C	ACIDITY AS CITRIC	SUGAR AS INVERT AFTER INVERT	SUGARS AS INVERT BEFORE INVERT	MILLONS REACTION FOR TYROSINE
		A[1] ASCORBIC ACID OXIDATION	CD[2] COLOR DEVELOPED OVERNIGHT BY OXIDATION OF CATECHOL	I_{c+t}					
					mg/100 gm	*per cent*	*per cent*	*per cent*	
5048 7A	Sound, fully ripe crushed pineapple	0.1 mg Asc pk in 6½ min.	±Grey	.195					
Sub A4	Sound, fully ripe crushed pineapple	0.2 mg Asc no purp	+Yel Br		3.9	.55	13.95		
Sub A2	Sound, fully ripe crushed pineapple	0.1 mg Asc no pk in 6 min. light pk in 8½ min.	+Yel						positive
Sub A5	Sound, fully ripe crushed pineapple	0.2 mg Asc pk in 4½ min. no purp	+Br Yel		2.8	.49	12.2		
Sub B1	Sound, fully ripe crushed pineapple, with 1 part added sugars to 5 parts fruit	0.14 mg Asc pk in 6 min. no purp	+Yel		3.7	.49	33.76	9.06	positive
Sub B2	Sound, fully ripe crushed pineapple, with 1 part added sugars to 5 parts fruit	0.1 mg Asc no color in indicator	+Yel or Br Yel		2.2				
Sub C3	Sound, barely ripe, or slightly underripe crushed pineapple	0.15 mg Asc no color	Nil same as blank with no catechol		8.5	.76	7.98		positive
Sub C4	Sound, barely ripe, or slightly underripe crushed pineapple	0.1 mg Asc slight pk			9.2	.96		7.13	

Sub C5	Sound, barely ripe, or slightly underripe crushed pineapple			.088					
Sub D	Sound, barely ripe or slightly underripe crushed pineapple with 1 part sugar to 5 parts fruit	0.2 mg Asc very faint color in 10 min.	No developed color		7.6	.65	28.52		positive
Sub H1	Straight crushed pineapple containing blackheart	0.4 mg Asc purp in 4½ min.	+++++ Black		nil				
Sub H2	Straight crushed pineapple containing blackheart		+++++		nil				
Sub H4	Straight crushed pineapple containing blackheart	0.2 mg Asc pk in 50 sec. purp in 3½ min.	Br Bk ++++ Dk Br to Black		nil	.46	9.8		
Sub H5	Straight crushed pineapple containing blackheart	0.4 mg Asc purp in 4 min. 40 sec.	+++++ Black		nil	.43	8.8	8.9	
Sub K6	Ground pineapple containing 10% blackheart		± + Light Br		8.0	.87			positive
Sub L1	Ground pineapple containing 22% blackheart by weight	0.1 mg Asc purp in 5 min.	++ Brown						
Sub L2	Ground pineapple containing 22% blackheart by weight		+++ Dk Br		3.6				

[1] The method used was essentially that of Miller and Dawson (*J. Amer. Chem. Soc.*, 63, 3375 (1941)), but using lower quantities of ascorbic acid (0.1 to 0.4), as described herein. After the addition of the catechol the time of aeration was measured with a stopwatch to the appearance of a pink or purple color. After several minutes a pink color was found to gradually develop in the indicator solution even though very little or no catacholase activity was present. After a few determinations, this difficulty was overcome by preparing reagents fresh each day.

Abbreviations used are: Ascorbic acid = Asc; pk for pink, and purp for purple.

[2] Most of the determinations recorded here were made before it was decided to measure the "darkening" quantitatively as proposed in the method (b) herein. After a time the color intensity (darkening) was estimated visually and indicated by the following signs ,"+" showed some or a small amount of color, and "++," "+++," etc., indicated increasing color intensity or darkening.

Abbreviations used in column, Dk = dark; Br, brown; Bk, black.

(4) That the determination of mannitol as an index of mold decomposition in fruits be continued.

(5) That other compounds elaborated by microorganisms, as indicated in this report, be investigated.

(6) That the method for detection of blackheart in pineapple by tyrosinase activity, given in this report, be studied further.

REPORT ON DECOMPOSITION IN DAIRY PRODUCTS

WATER-INSOLUBLE FATTY ACIDS IN CREAM AND BUTTER

By FRED HILLIG (Food and Drug Administration, Federal Security Agency, Washington 25, D. C.), *Associate Referee*

In a previous paper[1] a method for the determination of water-insoluble fatty acids in cream and butter was proposed. The method was submitted to the New Orleans, Cincinnati, and St. Louis Stations of the Food and

TABLE 1.—*WIA in butter—collaborative results*

COLLABORATOR	W.I.A.	COLLABORATOR	W.I.A.
	mg/100 grams		*mg/100 grams*
	Cincinnati Station		*St. Louis Station*
	796	4	2484
1	762		2574
	773		
	763	5	2640
			2668
	843		
2	790		*New Orleans Station*
	852		*Sample 1*
	862	6	356
			343
	830	7	354
3	788		376
	783		
	813		*Sample 2*
		6	344
			349
		7	364
			334

Drug Administration for trial. The results obtained by these stations on samples of butter obtained in their respective cities are given in Table 1.

[1] *This Journal*, 30, 575 (1947).

Duplicate determinations were satisfactory and the analysts at the respective stations closely checked each other. No comparison of results between stations is possible, since each station had different butter samples on which to work.

Four samples of butter were next submitted to collaborators in Cincinnati, St. Louis, New Orleans, and Washington. The results given in Table 2 are satisfactory.

TABLE 2.—*WIA in butter—all collaborative results: same sample*
mg/100 g

COLLABORATOR	SAMPLE 1	SAMPLE 2	SAMPLE 3	SAMPLE 4
3	178	245	248	420
	176	240	242	437
2	168	232	250	432
	165	224	250	447
4	161	207	180	421
	164	200	178	413
8	163	200	237	429
	159	210	247	432
9	158	218	250	428
	159	212	249	424
10	157		195	424
	165	268	186	432

It is recommended that the method for the determination of water-insoluble fatty acids in cream and butter be adopted as official first action. It is further recommended that the study of chemical methods for the detection of decomposition in dairy products be continued.

The Associate Referee wishes to thank the following members of the Food and Drug Administration who collaborated in this work: F. M. Garfield and M. A. Braun, St. Louis Station; S. D. Fine, F. J. McNall, and H. C. Van Dame, Cincinnati Station; George McClellan, R. E. Duggan, and H. P. Bennett, New Orleans Station; and Dorothy Montgomery, Food Division, Washington, D. C.

REPORT ON DECOMPOSITION IN FISH PRODUCTS

By FRED HILLIG (Food and Drug Administration, Federal Security Agency, Washington, D. C.), *Associate Referee*

Subsequent to the work which led to the adoption of the method for volatile acids as official,[1] a method appeared in which a mixture of volatile acids was separated on a chromatographic column and the amount of each acid from the column determined by titration.[2] Numerous analyses on fish have shown this procedure to be satisfactory, once the volatile acids are isolated from the fish. This is accomplished by a simplified steam distillation in which the carefully controlled conditions of the former method are unnecessary. The equations used in the former method for calculating the results are not needed in the chromatographic procedure, although a simple calculation based on the percentage of each acid which distils in 200 ml of distillate is used. Any standard 500 ml distilling flask with side arm midway of the neck and assembled as previously described[1] is satisfactory.

It is recommended that the simplified distillation procedure, followed by a chromatographic separation of the isolated volatile acids, be studied collaboratively.

Preliminary work on water-insoluble fatty acids in fish and fish products indicates that this may be another method for the detection of the use of decomposed material. Likewise, succinic acid has been found to offer similar possibilities in fish.

No reports were given for decomposition in shellfish, or in apple products.

A contributed paper, entitled "Water-insoluble Fatty Acids in Cream and Butter," by Fred Hillig and S. W. Ahlmann, was published in *This Journal*, **31**, 739 (1948); and a paper entitled "Volatile Acids in Cream and Butter," in two sections, "Part I, The Development of Butyric Acid During the Progressive Decomposition of Cream," by Fred Hillig, and "Part II, Butyric Acid in Commercial Creams and Butters," by Fred Hillig and Dorothy Montgomery, was published in *This Journal*, **31**, 750 (1948).

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[1] *Methods of Analysis, A.O.A.C.*, 6th Ed., p. 361 (1945).
[2] *This Journal*, **28**, 644 (1945).

REPORT ON GELATIN, DESSERT PREPARATIONS, AND MIXES

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

Last year your General Referee recommended that collaborative study be undertaken of the jelly strength methods for plain gelatine and gelatine dessert powders in paragraphs **9.6** and **9.12.** The Associate Referee on jelly strength is Paul A. Kind of the Kind-Knox Gelatine Company, Camden, New Jersey, assisted by Dr. D. Tourtellotte, of that firm. During the year, the Association was fortunate in securing the services as Associate Referee on Gelatin and Gelatin Desserts (Constituents) of Dr. Joseph H. Cohen, General Manager, Atlantic Gelatin Division, General Foods Corporation.

No report on jelly strength was received in time for this meeting. The recommendation* of last year is repeated, that the methods for jelly strength (*This Journal*, **31**, 74 (1948) and paragraph **9.12**) be studied collaboratively with a view to making them official. In addition, it is recommended* that the methods for sucrose, dextrose, and starch (paragraphs **9.13, 9.15,** and **9.21**) be studied collaboratively with a view to making them official for the 1950 Edition of *Methods of Analysis*.

No report was given on jelly strength.

REPORT ON GUMS IN FOODS

By F. Leslie Hart (Food and Drug Administration, Federal Security Agency, Los Angeles, California), *Referee*

Last year the Association recommended that further work be done on detection of gums in curd cheeses, cacao products, frozen desserts, and salad dressings. Only one formal report was received by the Referee, that of Associate Referee Coulter on detection of gums in mayonnaise and French dressing. The Associate Referee has shown that the amended method, **33.5,** adopted as tentative in 1945, and published in *Methods of Analysis*, Sixth Edition, is applicable to the detection of gums in these products, and he recommends its adoption as official after minor changes in wording. Your Referee concurs in this recommendation.

Associate referees assigned to the subjects of cacao products, and soft curd cheeses have both done work during the year, but they have not progressed sufficiently to render a formal report. No work was done on frozen desserts during the year.

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* For report of Subcommittee C and action of the Association, see *This Journal*, 32, 54 (1949).

RECOMMENDATIONS*

It is recommended—

(1) That the method for detection of gums in mayonnaise and French dressing, given in *Methods of Analysis*, Sixth Ed. **33.57,** be amended by substituting "50 ml" for "1.5 oz." in line 3, paragraph 2, and that the method, as amended, be adopted as official, first action.

(2) That studies be continued on the detection of gums in soft curd cheeses.

(3) That studies be continued on the detection of gums in cacao products.

(4) That studies be continued on the detection of gums and other stabilizers in frozen desserts.

(5) That an Associate Referee be appointed to study detection of gums in catsup and related tomato products.

REPORT ON GUMS IN MAYONNAISE AND FRENCH DRESSING

By E. W. COULTER (Food and Drug Administration, Federal Security Agency, Chicago 7), *Associate Referee*

The Associate Referee on Gums in Mayonnaise and French Dressing recommended, in 1945, that "the amended method be adopted as tentative and that studies on the detection of gums in Mayonnaise and French Dressing be discontinued." The amendment referred to was a change in the wording which would aid the analyst in differentiating between the precipitate due to added gums and that due to spices. Since no collaborative work was done following this change in wording, Subcommittee C in 1947 recommended that Gums in Mayonnaise and French Dressing be studied.

Collaborative work completed this year is confined to the detection of added gum in mayonnaise. Four different brands of mayonnaise were examined for gums by method **33.57**, page 548, *Methods of Analysis*, 6th edition. All four were negative for added gum, but varying amounts of precipitate due to spices were obtained. The brand giving the largest amount was used in preparing samples for collaborative study.

These samples were prepared by adding to the mayonnaise 0.2 per cent by weight of the dry powdered gum. The powder was incorporated into the mayonnaise by thorough hand stirring with a broad spatula. Each batch consisted of sufficient mayonnaise to yield six 225 gram portions. Three gums were used—tragacanth, guar, and carob bean. Each collaborator received four 225 gram subs (one without added gum) and was instructed to test each for gums by Method **33.57.**

* For report of Subcommittee C and action of the Association, see *This Journal*, 32 ,55, (1949).

The following table gives the results obtained by six analysts on four samples:

TABLE 1.—*Collaborative results*

SUB	1	2	3	4
Type of added gum.	Trag.	None	Guar	Carob
Conclusions:				
Analyst: S. H. Perlmutter	Present	Absent	Present	Present
H. D. Silverberg	"	"	"	"
H. W. Conroy	"	"	"	"
F. H. Collins	"	"	"	"
E. C. Deal	"	"	"	"
J. H. Bornman	"	"	"	"

ANALYSTS' COMMENTS

S.H.P.—"Sample 2 was difficult to judge, there was a voluminous precipitate in the alcohol, but the Benedicts test was negative and the Molisch test doubtful."

H.D.S.—"Copper reduction test. Voluminous red precipitate in subs 1, 3, and 4 No precipitate in sub. 2."

H.W.C.—"Sample 2 produced a fairly heavy alcohol insoluble precipitate. The confirmatory test with Benedicts was negative and the tests with napthol and thymol were also negative."

F.H.C.—"Sub 2 with Molisch test gave a positive indication after standing a while. It is suggested that the metric system be used in the 3rd line of the 2nd paragraph of the method. Since the 1.5 oz. figure used is more or less arbitrary the maximum might be satisfactorily changed to 50 ml."

E.C.D.—"Subs 1, 3 and 4 gave a considerable amount of flocullent precipitate and sub 2 a small amount of alcohol insoluble material. A very faint positive Molisch test was obtained on sub 2, but the copper reduction test was negative."

J.H.B.—"No difficulty was experienced with the method. The only question in my mind was 'how much is a slight precipitate?' The slight precipitate obtained in sub. 2 gave negative results."

The above results show that all six analysts agree as to the presence or absence of added gum. One analyst reported for Sub 2 a doubtful Molisch test and one a positive Molisch after standing, but these results did not lead to erroneous conclusions.

RECOMMENDATIONS*

It is recommended—

(1) That the wording of the method be changed by substituting "50 ml." for the "1.5 oz." in the third line of the second paragraph.

(2) That the method, with the recommended change in wording be adopted as official, first action, and that the subject be closed.

No reports were given on gums in cheese, frozen desserts, or cacao products.

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* For report of Subcommittee C and action of the Association, see *This Journal*, 32, 55 (1949).

REPORT ON MEAT AND MEAT PRODUCTS

By R. M. MEHURIN (Bureau of Animal Industry, Meat Inspection Division, Washington 25, D. C.), *Referee*

Since the last meeting, the Associate Referee on Soybean Flour in Sausage and Similar Products has continued work on the method presented last year for the determination of soybean flour in sausage. However, because of press of other work, samples for analysis were sent to collaborators too late to secure results in time for a report at this meeting. A report will be given on this subject at the next meeting.

Preliminary work on the method of Eggleton, *et al.*,[1] referred to last year as possibly suitable for the direct determination of creatine in meat extracts, has given promising results. The method consists in the addition of diacetyl and alphanaphtol to a suitably diluted extract. The solution is allowed to stand about 15 minutes and the absorption at 525 millimicrons wave length read in a photoelectric colorimeter or spectrophotometer. The concentration is read from a standard curve prepared by treating solutions of known creatine content in the same manner. The method is rapid and appears to give good reproducibility. It is now being compared with the official method. Results to date indicate that further study of the method is warranted, with a view to providing a direct method for the determination of creatine in lieu of or in addition to the present method in which creatine is determined by difference. Credit for this preliminary work should go to John M. McCoy, Meat Inspection Division, Department of Agriculture, Washington, D. C.

It is recommended*—

(1) That collaborative work be continued by the Associate Referee on the determination of soybean flour in sausage and similar products.

(2) That an Associate Referee be appointed to continue work on methods for the determination of creatine and creatinine in meat and meat products.

No reports were given on dried skim milk, soybean flour, or creatin in meat products.

No report was given on naval stores.

No report was given on radio activity, including quantum counter.

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[1] *Biochem. Journal*, **37**, 526 (1943).

* For report of Subcommittee C and action of the Association, see *This Journal*, **32**, 55 (1949).

REPORT ON NUTS AND NUT PRODUCTS

By A. M. HENRY (Food and Drug Administration, Federal Security Agency, 416 Federal Annex, Atlanta 3, Ga.), *Referee*

Several mechanical devices for preparing nuts and nut products, some of which may be patented, were investigated during the year. While some of them were of value for special products, none were an improvement on an ordinary food chopper for preparing a variety of nuts and nut products.

Further investigations were made of the methods for moisture determinations reported on last year. This work was done by Phyllis B. Rokita and Beulah V. McMullen. The Toluene Distillation Method was modified by washing the apparatus with an aerosol solution and by adding a small amount of aerosol solution to the distillation and receiving flasks in order to prevent the formations of droplets of water. This modification was not satisfactory as droplets of water still formed. This method does not seem to be adaptable to nuts and nut products in general.

A considerable amount of work was done to ascertain the minimum drying time for various nut products. Most nut meats will come to an equilibrium after two hours drying, although some nut products, such as sweetened grated coconut, require five hours. Additional drying of ten to fifteen hours does not make any appreciable difference unless decomposition takes place. The per cent of loss is different under different conditions. For instance, a sample of raw peanuts dried in the vacuum oven for five hours gave a range of 6.92 to 7.01 per cent for several separate determinations, while when dried for ten hours, the range was 6.97 to 7.00 per cent and fifteen hours 7.01 to 7.04 per cent. When this same sample was dried in a mechanical convection oven at 100°C. the loss on drying was 6.61 per cent at two hours, 6.63 per cent at three hours, 6.57 per cent at four hours, 6.63 per cent at five hours, and 6.58 per cent at six hours.

In products with low moisture content, as most nuts have, the use of a covered dish is not necessary. Typical results with covered aluminum dishes and sintered glass extraction tubes as containers on peanuts and pecans are as follows:

TABLE 1.—*Typical results on peanuts and pecans*

SAMPLE NO.	COVERED ALUMINUM DISHES	SINTERED GLASS TUBES
	PER CENT MOISTURE	PER CENT MOISTURE
1	6.99	6.96
2	7.02	6.98
3	7.04	6.99
4	7.00	6.99
5	6.98	6.99
6	6.96	6.98

The drying of nuts at 100° in air, 100° in nitrogen, and 100° in vacuum was compared. The following results on a sample of peanuts is typical of this investigation:

At 100° in air, 6.70 per cent.
At 100° in nitrogen, 6.70 per cent.
At 100° in vacuum, 6.92 per cent.

Further investigation was made by Mrs. Rokita and Miss McMullen on the methods for fat determination reported on last year. The methods for fat in chocolate liquor, as given in **19.23** and **19.24** were further studied. These methods do not seem to be applicable to most nut materials, as the fat is not completely extracted from such materials without first extracting nearly to completeness and then regrinding the material and final extraction with several portions of ether.

The method for fat as given in **30.6** was modified by making the chloroform extraction up to a definite volume and taking an aliquot. This modification was an improvement over the original method. This modification does not give satisfactory results on products with very high fat content as in many nut meats.

The Soxhlet method **27.24** and **27.25** gave very good results. Determination on one sample of peanuts gave the following results for fat: 44.33, 44.39, 44.31, 44.27, and 44.13, average 44.30. Most peanut meats gave some trouble with this method as fine starch comes through most filtering mediums. This can be prevented by care in selecting and preparing the filtering mediums.

All the methods for Nuts and Nut Products were adopted as "tentative" in 1935 following the Referee's recommendation in 1934. These methods were an adaptation of methods used for other products that had appeared in chemical literature and of special methods developed by individuals for particular adulterations in certain products. No further investigational or collaborative work has been done on them. In 1947 the Referee made a preliminary investigation on the methods for moisture and ether extract, which indicated that such methods should be of a broad general application, as nuts vary greatly in the percentage of moisture and ether extract and the oils in the ether extracts vary greatly in the characteristics of the oils from pecans to cocoanuts.

Not only were the "tenative" and other methods discussed with Federal and State Control Officials and Federal and State Chemists doing research work, but they were also discussed with chemists interested in the examination of nuts and nut products in "industry."

As a result of these conferences and discussions, and the suggestion of the Committee on Classification of Methods, a careful study was made of the whole chapter. The chapter has been rewritten.[1] Methods of Preservation and Preparation of Samples are added. A number of methods

[1] Details of some of the tentative methods proposed are published in *This Journal*, 32, 96 (1949).

for determinations of general type which have been studied and adopted for many other food products are applicable to the analysis of nuts; and several methods have been listed for study, which are believed to be desirable for the chapter, including a sorting method, a method for added starch in nut butters and pastes, and added glycerol and glycols.

RECOMMENDATIONS*

It is recommended—

(1) That methods for preservation of samples and preparation of samples be adopted.

(2) That methods for moisture, crude fat, crude protein, crude fiber ash, reducing sugars, sucrose, sdoium chloride, and water-insoluble inorganic residue be adopted as official, first action.

(3) That methods for added coloring matters; metals, other elements, and residues; and preservatives and artificial sweeteners, be adopted by reference to the appropriate chapters.

(4) That sorting methods for moisture and fat, and methods for added starch in nut butters and pastes, and added glycerol and glycols be studied.

REPORT ON VEGETABLE DRUGS AND THEIR DERIVATIVES

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

RECOMMENDATIONS†

(1) *Chemical Methods for Ergot Alkaloids.*—No report was received. It is recommended that the subject be continued.

(2) *Physostigmine in Ointments.*—The Associate Referee recommends that the proposed method be adopted as tentative. The Referee recommends that the method be adopted as official, first action.

(3) *Theobromine and Phenobarbital*—The Associate Reference submitted a report and recommended that the subject be continued. The Referee concurs in this recommendation for the purpose of developing the spectrophotometric method.

(4) *Aminopyrine, Ephedrine, and Phenobarbital.*—No report was received. It is recommended that the subject be continued.

(5) *Quinine.*—The Associate Referee recommended that the subject be continued. The Referee concurs in this recommendation for the purpose of submitting the Herd procedure to collaborative study to determine if it should be made official.

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* For report of Subcommittee C and action of the Association, see *This Journal*, 32, 56 (1949).
† For report of Subcommittee B and action of the Association, see *This Journal*, 32, 49 (1949).

(6) *Chemical Methods for Penicillin.*—No report was received. The Referee recommends that the subject be continued.

(7) *Rutin in Tablets.*—No report was received. The Referee recommends that the subject be continued.

(8) *Ethylmorphine in Syrups.*—The Associate Referee recommends that the usual alkaloidal assay procedure be used to determine ethylmorphine in syrups in the absence of other alkaloids, and that this procedure be submitted to collaborative study. The Referee concurs in this recommendation.

(9) *Arecoline Hydrobromide.*—The Referee recommends that method 39.73 be made official, first action.

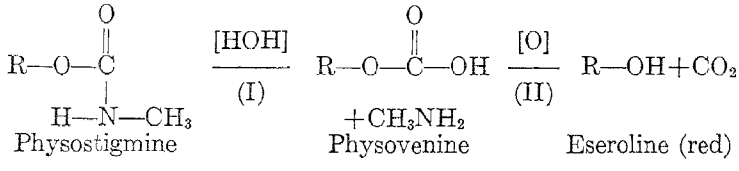
REPORT ON PHYSOSTIGMINE IN OINTMENTS

By MATTHEW L. DOW (Food and Drug Administration, Federal Security Agency, St. Louis, Mo.), *Associate Referee*

The work of the previous year indicated that the official A.O.A.C. titration method (1) for physostigmine salicylate might apply also to ointment preparations if some method for completely extracting the alkaloid from the base could be found. Accordingly, several variations of a direct acid extraction procedure were tested, using as controls carefully weighed amounts of the pure alkaloid added to a 1–9 lanolin-petrolatum base. An alternate melting and chilling operation in an Erlenmeyer flask containing a glass stirring rod for mixing and transfer purposes was found to give consistent recoveries of 95–98 per cent when the extracted alkaloid was titrated by 39.99. Despite the most careful technic it was never possible to recover 100 per cent of the alkaloid except by the direct extraction of an aqueous solution of the pure salt.

A very sensitive colorimetric procedure suggested by Shupe (2) based on the formation of a blue compound with nitrous acid and strong potassium hydroxide, was investigated, in the hope that better results would be obtained. This method proved to be valuable in the study of the stability of physostigmine in solution, but it could not be used to determine the original amount of alkaloid in a preparation.

Apparently physostigmine decomposes in solution as follows:



Step (I) was found to take place slowly in warm 2% sulfuric acid. By means of controls, prepared immediately before extraction, it was determined that from 5 to 15 per cent of the alkaloid salt added to the oint-

ment hydrolyzed during the proposed extraction procedure. In dilute alkaline sodium bicarbonate solution, both the hydrolysis and oxidation steps were found to proceed rapidly, unless the solution was kept cold (less than 15°C).

Since the blue color is formed only with secondary alkyl urethanes, only the unchanged physostigmine in the extract can be measured by this colorimetric method. On the other hand, both combined and free methylamine can be titrated by 39.99. Therefore, since methylamine is extracted completely along with the alkaloidal salt, the original amount of physostigmine added can be determined in this way.

Since the titration method determines the unchanged physostigmine plus any methylamine resulting from hydrolysis, either before or during extraction, and since the colorimetric procedure determines only the unchanged alkaloid in the extracted solution, which has already undergone some hydrolysis, it is not possible by the methods now at hand to determine exactly the amount of unchanged physostigmine in an ointment. The Associate Referee now has on hand several ointments made up during the past few years. Analysis of these ointments by both methods should show whether there is appreciable hydrolysis of the alkaloid on aging, and whether the resultant methylamine leaves the ointment.

Two ointments prepared in 1944 and 1947, respectively, and stored at room temperature until examination, were analyzed by the proposed titration method and found to have lost about 10 per cent in the case of the former, and approximately 5 per cent in the case of the latter, of the added amounts of physostigmine salicylate. Apparently some methylamine escapes from the ointment. It is intended to repeat the work by both methods, to determine the extent of the hydrolysis.

Table 1 gives the results of a series of recovery experiments from freshly prepared controls in which a carefully weighed amount of physostigmine salicylate was thoroughly mixed with approximately 5 grams of ointment base and immediately extracted and titrated by the proposed method.

TABLE 1.

DETERMINATION	% RECOVERY
1	97.1
2	96.9
3	97.5
4	96.7
5	95.2

A carefully prepared ointment containing 0.25 per cent of physostigmine salicylate was submitted for collaborative study.[1] Results are shown in the table below.

[1] Details of the proposed method are published in *This Journal*, 32, 113 (1949).

TABLE 2.—*Collaborative results*

COLLABORATOR	PHYSOSTIGMINE SALICYLATE FOUND	RECOVERY
	per cent	*per cent*
	0.19	76.0
1	0.20	80.0
	0.22	88.0
	0.24	96.0
2	0.25	100.0
	0.24	96.0
	0.24	96.0
3	0.25	100.0
	0.24	96.0
4	0.24	96.0
	0.25	100.0
	0.25	100.0
5	0.25	100.0
	0.25	100.0

COMMENTS OF COLLABORATORS

Rupert Hyatt, Cincinnati, Ohio.

Accurate weighing is not so necessary with a small proportion of physostigmine. I suggest that a beaker be listed as optional because it is hard to put the ointment in a flask without smearing the neck, unless a piece of glassine paper is used. The test for complete extraction could be eliminated by requiring an additional shakeout.

Henry R. Bond, Kansas City, Mo.

No operational difficulties encountered. The titration is so small (1 ml) that there is a difference of 5% in recovery per drop of N/50 acid or alkali. Perhaps more accurate results would be obtained if .005 N solutions were used.

The Associate Referee is indebted to the following members of the Food and Drug Administration for their valuable comments and participation in this work: H. R. Bond, Kansas City, Mo.; Rupert Hyatt, Cincinnati, Ohio; G. S. Keppel, Minneapolis, Minn.; and Mary McEniry, St. Louis, Mo.

DISCUSSION

The results obtained by four of the five collaborators were excellent, both from the standpoint of recoveries obtained and replicability. The recoveries of the fifth collaborator were not only low as compared to the others but also showed considerable variance.

It is recommended* that the proposed method be adopted as tentative.

* For report of Subcommittee B and action of the Association, see *This Journal*, 32, 49 (1949).

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(1) *Methods of Analysis*, 6th Ed., 39.99.
(2) I. W. SHUPE, *Cosmetics and Color*, 9, 12 (1941).
J. E. SAUL, *Pharm. J.* (i.i.i.), 17, 642 (1887).
(3) H. W. HIND and F. M. GAYAN, *J. Am. Pharm. Assn.*, 36, 33 (1947).

REPORT ON THEOBROMINE AND PHENOBARBITAL

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

In studying methods for the analysis of mixtures containing theobromine and phenobarbital Deal showed[1] that the two drugs could be separated by the use of ether and dilute acid as immiscible solvents. Later Richardson and Campbell described[2] a procedure for the determination of theobromine by precipitation as the phosphotungstate from acid solution, during prolonged digestion on the steam bath. It has been found that the heating period can be materially reduced, with the production of larger crystals, by boiling the acidified theobromine-phosphotungstate mixture.

To test the efficiency of the shake-out separation, and the accuracy of the modified method for the alkaloid, a prepared mixture consisting of theobromine (20.44%), phenobarbital (7.34%), and starch was subjected to collaborative study. Instructions for procedure and results of analysis are shown below.

METHOD

Transfer a portion of the well-mixed sample containing at least 100 mg phenobarbital to 125 ml separatory funnel, add 10 ml of 5% NaOH and extract with three 30-ml portions of $CHCl_3$. Wash the $CHCl_3$ layers successively with 5 ml of 5% NaOH in a second separator. Reject the chloroformic extracts.

Add 30 ml of H_2SO_4 (1+4) to the alkaline mixture in the first separator, cool, and shake vigorously with 50 ml of ether. Transfer the aqueous layer containing dissolved theobromine to the second separator, cool, and shake with 35 ml of ether. Remove the lower phase to a third separator and wash with another 35 ml of ether. Repeat the extracion thru the three separators using two 40 ml portions of H_2SO_4 (1+4), and three 20 ml portions of water. Collect the aqueous layers in 250 ml volumetric flask, dilute to the mark with water and mix.

Theobromine.—Transfer an aliquot containing 100–200 mg of theobromine to a 250 ml beaker, dilute to 100 ml with water, and stir in 10 ml H_2SO_4 (1+1), and 10 ml of a clear 20% soln of phosphotungstic acid crystals in water. Cover the beaker with watch glass and partially immerse in glycerine bath. (A 600-ml beaker half-filled with glycerine is a convenient vessel.) Heat to gentle simmering by maintaining a bath temperature of 115–130°, and stir frequently until the precipitate subsides as a lemon-yellow crystalline mass and the supernatant liquid becomes clear (usually 20 min.). Digest at boiling point an additional hour with occasional stirring. Filter hot with suction thru a tared Gooch crucible. Transfer precipitate to the crucible quantitatively with the aid of rubber policeman and ten 10 ml portions of cold HCl

[1] Deal, E. C., *This Journal*, 24, 818–20 (1941).
[2] Richardson, A. G., and Campbell, Y. C., *J. Am. Pharm. A.*, 31, 24–26 (1942).

(1+9). Aspirate several minutes, dry to constant weight at 120°, cool and weigh. The weight so obtained×0.1563 is the weight of theobromine in the aliquot. Potassium iodide interferes.

Phenobarbital.—Filter the ethereal soln thru a pledget of cotton into a tared beaker, washing the three separators and the filter successively with three 5 ml portions of ether. Evaporate to dryness on steam bath with aid of a current of air, heat to constant weight at 110°C. and weigh as phenobarbital. In the presence of stearic acid, proceed as directed in *Methods of Analysis*, 1945, Sec. 39.49.

TABLE 1.—*Recoveries of theobromine and phenobarbital*

COLLABORATOR	THEOBROMINE		PHENOBARBITAL	
	FOUND	RECOVERY	FOUND	RECOVERY
	per cent	*per cent*	*per cent*	*per cent*
A. G. Buell	19.91	97.8	7.43	101.2
	19.81	96.9	7.45	101.5
A. W. Steers	19.86	97.2	7.44	101.4
	19.83	97.0	7.45	101.5
C. R. Joiner	19.45	95.2	7.10	96.7
	19.43	95.1	7.09	96.6
D. Banes	20.15	98.6	7.31	99.6
	20.20	98.8	7.36	100.3
R. D. Stanley	19.77	96.7	7.19	98.1
	19.69	96.3	7.22	98.5
H. Isacoff	20.5	100.2	7.4	101.0
	20.5	100.2	7.3	99.6
Average	19.93	97.5	7.31	99.7

DISCUSSION AND RECOMMENDATIONS

While recoveries of theobromine are somewhat low, due, perhaps, to the solubility of its phosphotungstate in dilute acid, the method appears to be promising. Results for phenobarbital are particularly encouraging

Richardson and Campbell state[2] that potassium iodide interferes in the gravimetric method for theobromine. Since both that alkaloid and phenobarbital absorb ultra-violet light, a spectrophotometric procedure was devised in an attempt to obviate the difficulty. Collaborative results indicated weaknesses in the method, and it has not been included in this report.

It is recommended* that the problem be subjected to further study.

* For report of Subcommittee B and action of the Association, see *This Journal*, 32, 49 (1949.)

REPORT ON QUININE

By DAVID J. MILLER (Food and Drug Administration, Federal Security Agency, Buffalo, New York), *Associate Referee*

The acidimetric titration of quinine by the A.O.A.C. method (1) has been criticized because of difficulty in determining the end point. Herd (2) ascribed this difficulty to the buffering action of the monoacidic salt first formed, and to overcome this difficulty titrated quinine in glacial acetic acid solution with standard perchloric acid in glacial acetic acid, following the theory developed by Conant, Hall, and Werner, and Nadeau and Braucher. In addition to the perchloric acid titration Herd outlined a procedure for the separation of quinine and strychnine using dichloracetic acid in chloroform. It is the purpose of this preliminary report to compare the Herd perchloric acid titration with the method now official in the *Methods of Analysis* using pure quinine alkaloid only. At the same time there is presented a third procedure, developed by the Associate Referree, in which quinine is titrated with standard sulfuric acid using the blue fluorescence which appears after the formation of the normal salt and which is easily apparent under ultra violet light, to indicate the end point.

EXPERIMENTAL

REAGENTS

The quinine used in the study was a Mallinckrodt U.S.P. XI Product. Since the purpose of the study was to compare simplicity of titration and recoveries, rather than to determine exact quinine content, no attempt was made to purify the alkaloid. The quinine was passed through a 30-mesh screen and dried at 105°C before use. The melting point (uncorrected) was 175–176°C.

0.1 *N* and 0.02 *N* sulfuric acid, **43.14**
0.1 *N* and 0.02 *N* sodium hydroxide, **43.2**

0.1 *N* and 0.02 *N* perchloric acid in glacial acetic acid was prepared as outlined by Herd (*loc. cit.*) and standardized against anyydrous sodium acetate, recrystallized α-naphthylamine, and an especially purified quinine obtained from Chemical Section, Medical Division, U. S. Food and Drug Administration, Washington, D. C.

α-naphtholbenzein indicator, 0.2 g/100 ml glacial acetic acid
Bromocresol purple indicator, **39.11**
Glacial acetic acid, ACS.

METHODS

Method I is the present A.O.A.C. method. The sample is dissolved in 5 ml neutral alcohol and titrated to a yellow end point using bromocresol purple indicator, the alcohol evaporated and the solution further titrated if there is any change from the yellow. Approximately 0.3 ml of 0.02 *N* acid is consumed from the first color change to a final yellow.

Where 0.02 *N* acid is used to titrate approximately 0.1 g quinine, it is

possible to follow the directions in *Methods of Analysis* but the end point is somewhat difficult to detect. However, the end point can be improved by adding a slight excess of acid, evaporating the alcohol, cooling, filtering, washing, and back titrating with 0.02 *N* alkali, using the first change from yellow as the end point. Where 0.1 *N* sulfuric acid is used to titrate

TABLE 1.—*Per cent quinine in sample by titration with 0.02 N solutions*

	METHOD I		METHOD II	METHOD III	
	DIRECT* TITRATION	BACK† TITRATION	DIRECT TITRATION	DIRECT TITRATION	BACK TITRATION
	99.9	100.4	100.2	97.5	99.3
	100.3	99.8	99.6	97.6	97.2
	98.8	100.2	99.9		98.3
	99.5	99.7	99.7		99.2
	100.3	100.9	99.6		99.2
	98.9	100.7	100.1		98.0
Av.	99.6	100.3	99.9	97.6	98.5

* A.O.A.C. method exactly.
† Excess acid added to same sample and back titrated as described under "Methods."

TABLE 2.—*Per cent quinine in sample by titration with 0.1 N solutions*

	METHOD I		METHOD II	METHOD III	
	DIRECT* TITRATION	BACK† TITRATION	DIRECT TITRATION	DIRECT TITRATION	BACK TITRATION
	100.5		100.5	99.6	99.1
	98.8		100.4	99.4	99.3
	99.8	100.1	100.0	98.1	99.3
	101.7	100.3	100.1	100.1	99.1
		99.6	100.4		98.2
		99.2	100.5		
Av.	100.5	99.8	100.3	99.3	99.0

* A.O.A.C. method plus addition of 15 ml H_2O.
† Excess acid added and back titrated as described under "Methods."

approximately 0.5 g of quinine the voluminous precipitate of the sulfate makes an accurate titration almost impossible if the A.O.A.C. directions are followed exactly; however, a direct titration can be made if approximately 15 ml water is added and the solution is heated. Approximately 0.25 ml 0.1 *N* acid is used from the first color change to a final yellow. Here, too, it is preferable to add an excess of acid, evaporate the alcohol, etc., and back titrate. Tables 1 and 2 include the results obtained by using the official method exactly and the back titration procedure.

Method II is the Herd perchloric acid titration method. The sample is

dissolved in 20 ml glacial acetic acid and titrated with standard perchloric acid in glacial acetic acid to a green end point using α-naphtholbenzein indicator. It is necessary to determine the temperature of the standard solution and make a correction (approximately one part in a thousand for every degree C difference) if the temperature is different from the one at which it was standardized. Approximately 0.25 ml of 0.02 *N* acid titrating 0.1 g quinine or 0.20 ml of 0.1 *N* acid titrating 0.5 g quinine is used from the first color change to a final green.

Method III consists of the titration of quinine dissolved in 5 ml neutral alcohol with standard sulfuric acid, using as the end point the appearance (or, if back titrating, the disappearance) of a blue fluorescence under ultra violet light. This point corresponds to the formation of the normal sulfate $(C_{20}H_{24}N_2O_2)_2 \cdot H_2SO_4$. It is necessary to make the titration in the dark since the fluorescence is not sufficiently marked, even under ultra violet, if much daylight is present. At the end point there is an interval of approximately 0.15 ml of acid, either 0.1 *N* or 0.02 *N*, where there is some question as to the presence of fluorescence. Although the change both by direct titration and back titration is marked it appeared somewhat easier to back titrate, noting the change from fluorescence to absence of fluorescence. Halides and acetophenetidin interfere with this titration.

Results are shown in Tables 1 and 2.

CONCLUSIONS

On the basis of this preliminary study it is concluded that the recoveries of quinine are about equal by the Herd perchloric acid titration and the A.O.A.C. method. The Herd procedure is somewhat superior to the A.O.A.C. method in that with a direct titration the end point is sharper. However, this superiority is not so marked that it outweighs the disadvantage of having to keep prepared still another standard solution which is somewhat unpleasant to use and whose temperature correction is much larger than for aqueous solutions. The titration of quinine, using its fluorescence in sulfuric acid solution as an indicator, is a simple and interesting method of titration, but with pure quinine it offers no particular advantage over either of the other two methods. With impure, colored residues its usefulness may be enlarged.

It is proposed to study the Herd procedure for the separation of quinine and strychnine and it is recommended* that the subject be continued.

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(1) *Methods of Analysis*, 6th Ed. (1934), sec. **39.13**, p. 669.
(2) HERD, R. L., *J. Am. Pharm. Assoc.*, **XXXI**, 1, 9 (1942).

* For report of Subcommittee B and action of the Association, see *This Journal*, 32, 49 (1949).

REPORT ON ETHYLMORPHINE IN SIRUPS

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

In accordance with the recommendation of Subcommittee B of the Association (*This Journal*, **31**, 46) a preliminary study was made for the determination of ethylmorphine in sirups.

Ethylmorphine hydrochloride ($C_{19}H_{23}O_3N \cdot HCl \cdot 2H_2O$), a synthetic alkaloid salt commonly known as Dionin, is used internally in a sirup vehicle, for the relief of excessive cough and pain in the chest. A review of cough sirups on the market containing ethylmorphine showed that, in addition to ethylmorphine, many contained other alkaloid-bearing drugs, such as ephedra, lobelia, ipecac, cocillana, and sanguinaria.

A review of the literature indicated that very little work had been done on the quantitative determination of ethylmorphine. Only one reference was found relative to the separation of ethylmorphine in the presence of other alkaloids. This article "Chromatographic Analysis of Alkaloidal Salts," is reported by F. Reimers and K. R. Gottlieb, in *Chem. Zentr.* II, 1387 (1943).

Ethylmorphine in a simple sirup in the absence of other alkaloids may easily be determined by the usual alkaline chloroform shake out. 30 mg. added to a simple sirup was extracted with a recovery of 98.7 per cent. Sodium hydroxide, which is used to separate morphine from other alkaloids, was tried with a mixture of codeine and ethylmorphine without success.

Alkaloids found in cough sirups other than ethylmorphine are usually present in very small amounts, and as such would not appreciably affect the quantitative estimation of ethylmorphine.

It is recommended* that the usual alkaloidal assay method be submitted for collaborative study for the determination of ethylmorphine in sirups in the absence of other alkaloids.

No reports were given on chemical methods for ergot alkaloids, aminopyrine, ephedrine and phenobarbital, chemical methods for penicillin, or rutin in tablets.

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* For report of Subcommittee B and action of the Association, see *This Journal*, 32, 49 (1949).

REPORT ON SYNTHETIC DRUGS

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

RECOMMENDATIONS*

(1) *Butacaine Sulfate.*—A preliminary report was received by the Referee. Some exploratory work was performed and it was recommended that the subject be continued. The Referee concurs.

(2) *Propadrine Hydrochloride.*—No report. The Referee recommends that the subject be continued.

(3) *Pyribenzamine and Benadryl.*—No report. The Referee recommends that the subject be continued.

(4) *Carbromal.*—No formal report was received but the Associate Referee in correspondence indicated that he has worked on two methods involving bromine determination. These appear satisfactory and he is ready to submit samples for collaborative study. The Referee recommends that the study be continued.

(5) *Methylene Blue.*—A report was received describing procedures for the determination of methylene blue in compound tablets. The Associate Referee recommended that a collaborative study be made. The Referee concurs.

(6) *Synthetic Estrogens.*—No report. The Referee recommends that the subject be continued.

(7) *Propyl-Thiouracil.*—No report. The Associate Referee has resigned from the Food and Drug Administration. Some preliminary work was performed in an attempt to apply the method for thiouracil recommended for adoption last year. This was not successful. The Referee recommends that the subject be continued.

(8) *Spectrophotometric methods.*—No report. The Referee recommends that the subject be continued.

(9) *Phenolphthalein in Chocolate Preparations.*—No report was submitted by the Associate Referee; it is recommended that the subject be continued.

(10) *Sulfanilamide Derivatives.*—No report. The Referee recommends that the subject be continued.

(11) *Trichloroethylene.*—The Associate Referee has submitted a report and recommends that the method which was studied collaboratively be adopted as official, first action, and the subject closed. The Referee concurs.

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* For report of Subcommittee B and action of the Association, see *This Journal*, 32, 49 (1949).

REPORT ON METHYLENE BLUE IN COMPOUND TABLETS

By HARRY O. MORAW (Food and Drug Administration, Federal Security Agency, Chicago 7, Ill.), *Associate Referee*

The 1948 work on the subject was a continuation of unreported investigational work of 1944, 1945, and 1946, on the problems of separation and determination in coated tablets of complex mixtures of essential oils, resins, balsams, powdered plant materials, plant extracts, excipients, and lime carbonate, sugar, and color in the coating.

The present A.O.A.C. dichlorhydrin extraction method was developed for application of a suitable volumetric method of determination. It does not provide for complete separation of water-insoluble material and may permit small amounts of dichlorhydrin, carbon tetrachloride, and water-soluble material to be carried over. The line of separation between layers of dichlorhydrin and water in the beginning of extractions is indistinguishable. The supply of dichlorhydrin available at the time of this recent work tended to dissolve or remain suspended in the water layer to an objectionable extent. Therefore, extractions by this method unless further treated are not suitable for application of a gravimetric method, or a volumetric method in which the contaminants might enter into the reaction.

In view of the above, it was necessary to experiment with reagents and solvents for dissolving or separating which would not interfere with the determination. Hence, concurrent trials of both method of dissolving and methods of determination were made on unmixed known methylene blue and authentic tablet mixtures.

A major difficulty in dissolving the methylene blue in such mixtures as the above is that of knowing when it is completely dissolved. The intense blue color of its solutions prevents observation of solid material. Moreover, the methylene blue may be embedded in or absorbed by some, or a combination, of the tablet ingredients during the manufacturing or pulverizing for analysis.

Because of this difficulty in observing lines of separation between water and solvent layers, the possibility of reducing methylene blue to the leuco base and extracting the latter was considered. It was found that the methylene blue in a commercial sample of tablets containing the ingredients mentioned above could be reduced in 10 to 15 minutes with alkaline hydroxylamine and apparently completely extracted with 4 or 5 50-ml portions of ether. Completeness of extraction can be shown by absence of blue on acidifying a few ml of the reducing mixtures. It would appear that if the reduced compound could be restored to methylene blue by an acid shake-out from the ether, leaving oils and similar materials in the ether, a most desirable quick and direct method of separation would be available. Some further work on this appears to be justified. Since the methods thus far tried for separating or dissolving methylene blue in such mixtures were not satisfactory, attempts were next made to develop a

direct solution method utilizing the extreme solubility of the methylene blue in methanol. It was found that most of the color could be separated from the solid material by triturating repeatedly with methanol. The undissolved solid material was then further extracted and disintegrated by grinding in a mortar after treatment with dilute hydrochloric acid and methanol until practically colorless. From this investigation it appeared to be practical to extract a sample of the ground tablet mixture containing 0.2 to 0.25 g of methylene blue using 20 ml of methanol and 40 ml of 5% hydrochloric acid and making to a volume of 200 ml or equivalent to 10% methanol and 1% hydrochloric acid (gas). After filtering, this solution could be used for determinations by the gravimetric perchlorate and spectrophotometric methods. It was found by trials on known straight methylene blue that these amounts of the reagents did not affect the accuracy of the determinations by these methods. Results are given in Table 1.

The following two solution methods were next tried on authentic tablet mixtures:

(1) Methanol—HCl-water treatment on ground tablets (without previous extraction).

(2) Methanol—HCl-water treatment after dry ether extraction of the oils, resins, etc.

Using solution method (1) there was a tendency for lumps to form in some instances. This appeared to be due to too rapid addition of the hydrochloric acid and methanol. Also the methylene blue crystallized out after making up to volume. Notwithstanding some instances of complete recovery, the low results in other determinations are believed due to incomplete solution. These difficulties were not encountered with solution method (2). This is based on more logical practices, but requires more care in manipulation to avoid losses.

The authentic tablet mixtures were prepared by adding approximately 2 grams of tablet mass to an accurately weighed amount of methylene blue (0.2 to 0.25 g) of known purity and moisture content, and mixing thoroughly. The table mass consisted of the following ingredients in the approximate proportions used in this type of tablet:

Extract kava, powdered cubeb, powdered nutmeg, copaiba, oil of santal, oil of cinnamon, starch, lactose, sucrose, calcium carbonate, talc.

Results on these experiments are given in Table 2.

Methods of determination studied were as follows:

(1) *Volumetric Silver Nitrate*[1]—based on det. of Cl displaced when methylene blue perchlorate is formed.

(2) *Volumetric Dichromate*[2]—based on precipitation by excess dichromate and titration of the excess by thiosulphate.

(3) *Modified U.S.P.* gravimetric perchlorate.

(4) *Spectrophotometric.*

[1] Maurina and Deahl, *J. Am. Pharm. Assoc., Sci. Ed.*, XXXII, 11, 301 (1943).
[2] Ferrey, G. J. W., *Quart. J. Pharmacol.*, 16, 208 (1943).

TABLE 1.—*Determinations on known methylene blue (unmixed)*
Modified perchlorate and spectrophotometric methods

DET. NO.	METHYLENE BLUE ANHYDROUS 100% BASIS					VARIATIONS TRIED:	
	USED	FOUND				CRUCIBLES PREWASHED	REAGENTS PRESENT DURING PRECIPITATION; TIME FOR PPTN.
		BY PERCHLORATE		BY SPECTROPEOTOMETRIC			
	g	*g*	*per cent*	*g*	*per cent*		
113-1	.0912	.0921	101.0			No	pptn. in 10% methanol 1% HCl. 10 min. for pptn.
113-2	.0932	.0937	100.5			No	do. 10 min. for pptn.
113-3	.0849	.0866	102.0			No	No methanol or HCl used. 10 min. for pptn.
114-1	.1145	.1159	101.2			No	pptn. in 10% methanol 1% HCl stood 30 min. for pptn.
114-2	.1279	.1291	100.9			No	do. stood 45 min. for pptn.
114-3	.1149	.1162	101.6			No	do. stood 50 min. for pptn.
114-4	.1810	.1814	100.2	.188	103.8	No	do. heated 1 hr. on steam bath with HCl CH_3OH before pptn.
115-1	.0980	.0981	100.1			Yes	do. 10 min. for pptn.
115-2	.0856	.0848	99.1			Yes	do. 75 min. for pptn.
115-3	.1024	.1030	100.6			Yes	do. 30 min. for pptn.
115-4	.0865	.0870	100.6			Yes	No methanol or HCl used. Stood 10 min. for pptn.
152-1	.2000	.1996	99.6	.199	99.5	No	10% methanol 1% HCl
118-1	.2284	.2274	99.6	.2254	98.7	Yes	do.
118-2	.2079	.2076	99.8	.207	99.6	Yes	do.
118-3	.1016	.1018	100.2			Yes	do.
118-3	.1016	.1004	98.8			Yes	10% methanol 1.5% HCl
118-3	.1016	.1011	99.5			Yes	10% methanol 1% HCl
118-4	.2038	.2054	100.8	.205	100.6	Yes	do.

TABLE 2.—*Determinations on authentic tablet mixtures*

DET. NO.	METHYLENE BLUE ANHYDROUS 100% BASIS					
	USED	FOUND BY METHODS				METHOD OF SOLUTION
		BY MODIFIED PERCHLORATE	% RECOVERY	BY SPECTROPHOTOMETRIC	% RECOVERY	
	g	g		g		
153-1	.2000	.1862	93.1	.186	93.0	Method (1)—Methanol-HCl-oils not removed by ether-reagents added rapidly.
153-2	.1694	.1696	100.1	.170	100.4	Method (1)—do. Reagents added fractionally.
154-3	.2000	.1862	93.1	.188	94.0	Method (1)—CH_3OH-HCl mixed and added piecemeal.
154-4	.1710	.1672	97.7	.168	98.3	Method (1) reagents added slowly. soln. stood 2 days. M.B. crystallized out; redissolved by warming.
155-5	.1700	.1692	99.5	.168	98.8	Method (1)—Methanol and 5% HCl added alternately in small portions.
155-6	.1728	.1680	97.2	.173	100.0	Method (1)—Heated 30 min. at 80–90°C. with methanol and HCl.
156-7	.1945	.1908	98.1	.193	99.2	Method (1) methanol and HCl added piecemeal; lumps formed during solution. Stood overnight; M.B. crystallized out; redissolved.
157-1	.2000	.1944	97.4	.187	93.5	Method (2)—ether soluble removed before dissolving in methanol-HCl.
166-8	.1840	.1804	98.0	.184	100.0	Method (2)—ether solubles removed by dry extraction. Color exhausted with methanol followed by HCl-methanol.
166-9	.2019	.2004	99.3	.198	98.1	do. do.
167-10	.2008	.1980	98.6	.198	98.6	do. do.
167-11	.1999	.1960	98.1	.197	98.5	do. do.
167-12	.1974	.1938	98.2	.195	98.8	do. do.

Method (1) yielded good results on purified samples of straight methylene blue but somewhat high results on unpurified samples. With solutions obtained from mixtures the end point with Ferric indicator was unsatisfactory. The amount of sample required for determination (0.5 g) would be impractical to handle in mixtures. Method (2) yielded such favorable results on known straight methylene blue samples of 0.1 to 0.5 g that considerable work was done to check its accuracy. When applied to smaller samples, *i.e.*, 45 to 60 mg. low results were obtained. Use of more acid in the flask for the reaction with iodide at first seemed to solve the difficulty. Occasional discordant results suggested substitution of 0.4 to 1% hydrochloric acid for acetic acid for the precipitation with dichromate, This improved the character of the precipitate, but occasional discordant results occurred which indicated that the method was not sufficiently perfected. Moreover, the possible oxidizing action of dichromate on contaminants in extracts from mixtures weighed against further work.

Method (3), the U.S.P. gravimetric perchlorate method, yielded high results for the author on straight methylene blue of known purity. This method provides for the use of 100 ml of methylene blue perchlorate test solution for washing the precipitate. A number of batches of this T.S. have formed additional perceptible but scarcely noticeable precipitates on standing after the original filtration. If not refiltered before use a variable error might be introduced. Some variation in the composition of this T.S. may occur because of the difficulty of recognizing when "a slight permanent turbidity results" when preparing it according to the U.S.P. thus allowing larger excess of methylene blue solution to be added to the perchlorate solution. Recent experience indicates the advisability of specifying the approximate volume of the former to add. Another factor is the absorption by the asbestos of the crucibles of a weighable amount of a blue compound from the 100 ml of the T.S. It was found to average 0.0007 g from freshly filtered T.S. Prewashing of the crucibles and using freshly filtered T.S. are therefore recommended when applying the method to straight methylene blue or solutions, extracts, etc., in which other inherent potential errors are negligible.

Since the U.S.P. method provides for precipitation with potassium perchlorate in water solution, and the solution method reported herein prepares a solution of methylene blue in (1+10) 10% hydrochloric acid-water and 10% methanol, it was necessary to try the perchlorate method with these modifications on methylene blue of known purity. Results of these determinations are reported in Table 1 as well as checks by the spectrophotometric method.

SPECTROPHOTOMETRIC DETERMINATIONS

The solution method developed for methylene blue in compound tablet mixtures requires the use of (1+10) 10% hydrochloric acid-water and

(1+10) methanol-water in preparing the original solution. Hence amounts in the diluted aliquots for the colorimetric determinations vary from 0.003 to 0.01 *N* in hydrochloric acid and 2 to 35 mg methanol per 100 ml. No investigation was made to determine whether ageing of solutions of methylene blue in these reagents affects the colorimetric determination. However, peak absorption values relative to blanks of the respective solvents were determined on solutions of 0.3 mg of 100% anhydrous methylene blue in water in 0.1 *N* hydrochloric acid, in 0.05 *N* hydrochloric acid and in 5 ml of 5% hydrochloric acid (approx. 0.15 *N*) plus 5 ml methanol per 100 ml.

This was found to be approximately 667 mμ in each case. A plot of concentrations corresponding to E values was made for amounts varying from 0.01 to 0.4 mg per 100 ml in the same solutions using a slit width of 0.06. The absorption curve and the graph of the concentration corresponding to E value at 667 mμ for the compound in approximately 0.1 *N* hydrochloric acid appear in Figs. 1 and 2. Concentrations above 0.35 mg and below 0.08 mg per 100 ml generally did not fall on a straight line, indicating a slight deviation from the Beers-Lambert Law. However, the graph was used for the colorimetric determinations in Tables 1 and 2 and appears to be practical.

Some further work should be done on this to determine whether a more favorable reagent strength can be found.

PURIFIED SAMPLE TESTS

The sample used for control tests in this work was prepared by dissolving U.S.P. methylene blue in water, filtering and recrystallizing by evaporating and long standing. Crystals were washed with small portions of water and alcohol and allowed to stand in air several days. They were ground to pass a No. 50 sieve and stored in tight bottles. A more rapid method of crystallization tried on another sample was suggested by Martin, Neuhaus, and Reuter[3] utilizing alcohol containing 5% methanol.

Nitrogen was determined by the A.O.A.C. method using both copper sulfate and mercuric oxide catalysts. The results by three determinations as anhydrous methylene blue were 84.28%, 84.47%, 84.60%, average 84.45%. Moisture was determined by heating in vacuo at 100°C. for 3 to 4 hour periods to constant weight. Heating for shorter periods, *i.e.*, 1 hour, caused no change but subsequent heating of the same samples for longer periods caused appreciable changes. Placing of fresh samples in the oven with previously dried samples apparently caused the latter to gain weight. Moisture found in the purified sample averaged 15.37% by three determinations. Keeping air oven temperatures constant at 110°C. as required by the U.S.P. was found impractical. Temperature

[3] *Analyst*, 71, 29 (1946).

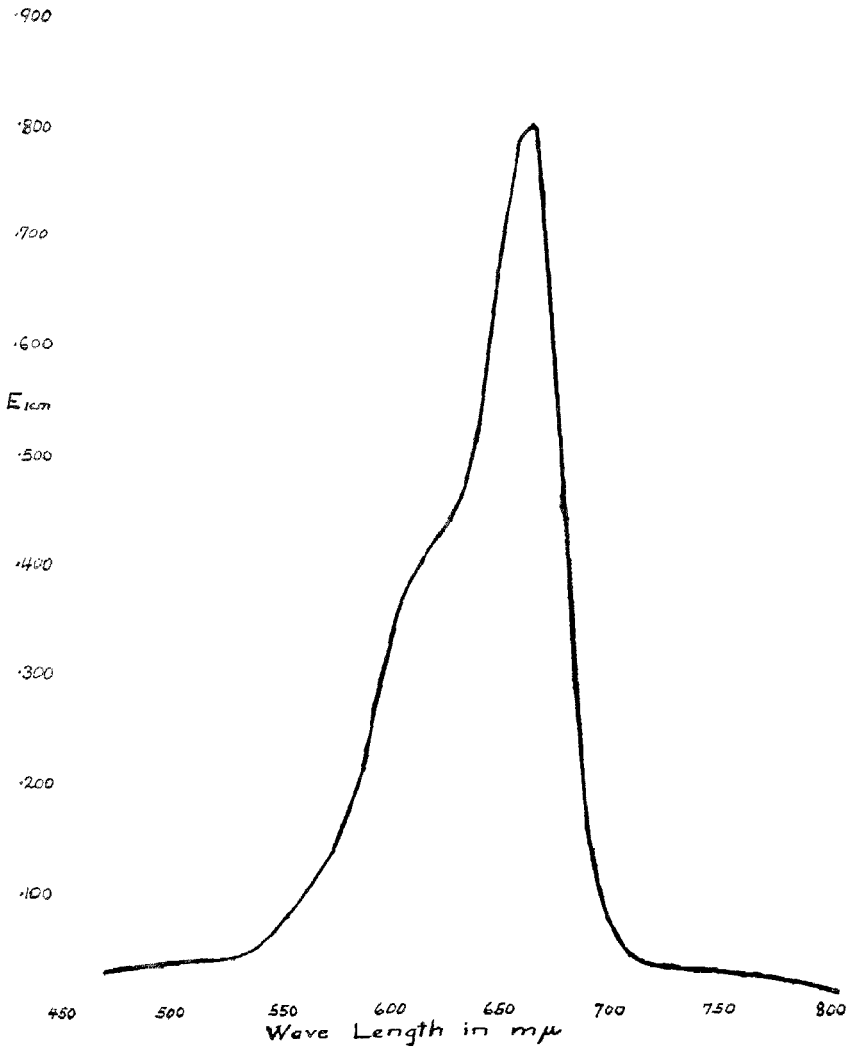

FIG. 1.—The Absorption Spectrum of Methylene Blue in 0.1 *N* HCl.

variations in the vacuum oven method do not influence the results to the extent they do in the air oven.

The hydrogen peroxide digestion method for nitrogen recommended by Maurina and Deahl[1] in place of sulfuric acid appears to have advantages in saving of time but was not tried. For samples to be used for collaborative study later it is expected to make use of this method if it is found satisfactory.

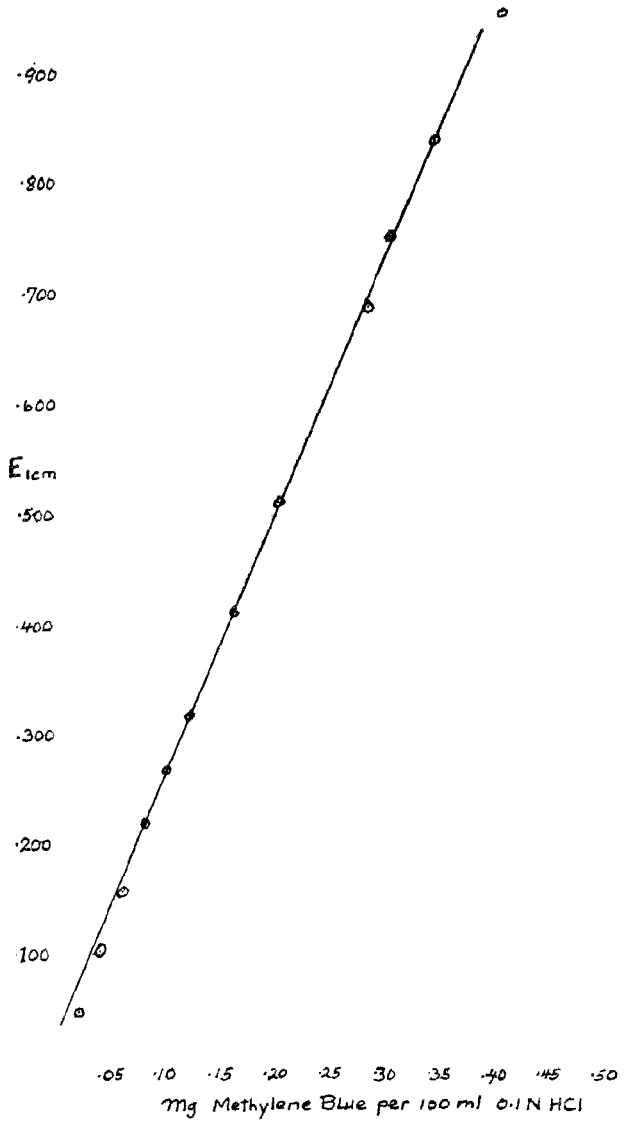

FIG. 2.—Plot of E against Concentration at 667 Mμ.

RECOMMENDATIONS*

It is recommended—

(1) That further work be done to determine if there is a more favorable

* For report of Subcommittee B and action of the Association, see *This Journal*, 32, 49 (1949).

concentration of hydrochloric acid and methanol for determining the E values corresponding to different concentrations of methylene blue.

(2) That details of solution Method 2 be submitted to collaborators for trials on authentic tablet mixtures and on a sample of commercial tablets of the approximate composition referred to in paragraph 1 of this report. It is also recommended that details of the modified perchlorate and the spectrophotometric methods referred to herein be prepared and tried by collaborators on the authentic tablet mixtures and authentic methylene blue of known purity.

ACKNOWLEDGMENTS

I wish to acknowledge help and suggestions on the colorimetric work received from my associates, Daniel Banes and R. D. Stanley.

No report was made on phenolphthalein in chocolate preparations.

REPORT ON BUTACAINE SULFATE

By Llewellyn H. Welsh (Chemical Section, Medical Division, Food and Drug Administration, Federal Security Agency, Washington, D. C.), *Associate Referee*

Butacaine, or butyn, is 1-(*p*-aminobenzoxy)-3-dibutylaminopropane, and is used in the form of its neutral sulfate. It appears commercially as the pure salt and in the following dosage forms (N. N. R.): aqueous solution; tablets with and without epinephrine; ophthalmic ointment, one per cent (plain) and two per cent (with metaphen).

From exploratory work in this laboratory, butacaine base appears to be amorphous at room temperature. It is not significantly volatile at 105°C., and may be extracted easily with chloroform from alkaline aqueous systems and weighed. Acid solutions of the substance may be assayed by titrating with bromide-bromate solution which introduces two atoms of bromine into the molecule. The neutral equivalent may be determined by titration with acid to a methyl red end point.

At the present stage of the work, it appears that preparations which will yield sufficient drug, without causing complications due to the nature of the vehicle, may be analyzed by extracting out the base, determining it gravimetrically, and checking the identity of the residue by acid titration followed by bromination of an aliquot of the titrated solution. Products not suited to such a procedure might be analyzed by separating the butacaine by extraction procedures, brominating in acid solution, and identifying the bromination product after isolating it and converting to the hydrochloride or hydrobromide.

This report is of a preliminary nature. In the coming year an effort

will be made to develop a procedure applicable to all dosage forms and to conduct a collaborative study.

It is recommended* that the subject be continued.

REPORT ON TRICHLOROETHYLENE

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

One of the methods devised by Rauscher[1] for determining organic halogen compounds has been applied this year to trichloroethylene. The central feature of this method consists of heating the sample in a closed tube with monoethanolamine. This converts the chlorine to a form in which it can be precipitated from aqueous solution by silver nitrate. The heating medium used by Rauscher was a bath of boiling diethanolamine, which held the temperature constant at about 268°C. In this work on trichloroethylene a mineral oil bath has been substituted, with the object of greater safety and simplicity. To compensate for the lack of any close automatic temperature control, a rather wide range of temperature is permitted, above the necessary minimum.

It was found that heating at 210°C. or above for 1 hour would give recoveries close to 100%. No attempt was made to determine closely the minimum time and temperature required. One half hour at 200°–220°C. appeared to be insufficient, giving recoveries in the vicinity of 96 or 97 per cent. Heating one hour in the steam bath gave a recovery of only 34 per cent.

The sample used in working out the method was prepared by distilling a commercial product three times, discarding end fractions. The resulting liquid had a density of 1.4562 20°/4° and a refractive index of 1.4774 at 20°. These constants as given in the *Handbook of Chemistry and Physics* for trichloroethylene are, respectively, 1.4556 and 1.4777. Thus the material was regarded as practically 100 per cent trichloroethylene.

Volumetric determination of chloride in the reaction product was first tried, by the usual silver nitrate-thiocyanate method. However, the reaction product has some color, which seems to increase near the end point, making the latter difficult. Results ranged from 98.5 to 99.6 per cent. Gravimetrically, weighing the silver chloride, the recoveries obtained were 99.8, 100.3, 100.1, and 99.6 per cent. One collaborator, Arthur Kramer of the New York Station, also obtained recoveries close to 100 per cent by this method. It was decided to submit the gravimetric method only to collaborative study.

For this purpose, a sample of U.S.P. trichloroethylene was made up,

* For report of Subcommittee B and action of the Association, see *This Journal*, **32**, 49 (1949).

[1] *Ind. Eng. Chem., Anal. Ed.*, **9**, 296 (1937).

since it is such a substance that would be assayed in practice; 198.6 g of the pure distillate were mixed with 1.4 g of absolute ethanol; thus the theoretical percentage of trichloroethylene in the sample was 99.3.

All the collaborators were members of the Food and Drug Administration.

Details of the method are given in *This Journal*, **32**, 113 (1949).

TABLE 1.—*Collaborative results*

COLLABORATOR	PER CENT TRICHLORO-ETHYLENE	COLLABORATOR	PER CENT TRICHLORO-ETHYLENE
C. F. Buening, Baltimore	99.6	L. W. Ferris, Buffalo	94.5
	99.3		99.6
	99.4		99.5
			99.6
A. Kramer, New York	99.4		
	99.6	H. P. Eiduson, Buffalo	99.0
			99.5
A. G. Buell, San Francisco	98.4		99.3
	98.0		
		L. H. Welsh, Washington	99.9
A. W. Steers, San Francisco	99.4		99.4
	99.1		
	99.4	G. Smith, New York	98.9
			99.2
			99.1

Most of the results are in close agreement. Of a total of 22 determinations, 17 are in the range 99.0% to 99.6%. The average of these is 99.4%. The average of 21 determinations, omitting the single widely divergent one, is 99.3%.

There was little comment by collaborators. Four saw the need of a filtration step after the reaction product is washed out of the tube, to remove any broken glass. This step has been inserted in the method with the proviso "if necessary." One station reported that several tubes cracked without explosion while being heated.

The method seems accurate enough for practical use. With some study of details it probably could be made more precise and proof against variation.

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

No reports were given on sulfanilamide derivatives, propadrine hydrochloride, carbromal, spectrophotometric methods, thiouracil, pyribenzamine and benadryl, or synthetic estrogens.

* For report of Subcommittee B and action of the Association, see *This Journal*, 32, 49 (1949).

REPORT ON MISCELLANEOUS DRUGS

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

Iodine.—Mr. Fine submitted for collaborative study a modification of the official method for iodine (*Methods of Analysis*, 6th Ed. **39.202**). The results show conclusively the necessity for having present sufficient organic material for complete recovery of iodine. On the basis of the results the present official method should be reworded as described in the Associate Referee's report, adopted as official, first action, and the subject closed.

Calcium, Phosphorus, and Iron in Vitamin Preparations.—Mr. Banes has submitted a report which includes a collaborative study. The results are in excellent agreement and are of an accuracy and precision to warrant that the method with the addendum be adopted as official, first action, and the subject be closed.

Separation of Bromides, Chlorides, and Iodides.—Mr. Stewart made a progress report and recommends that the subject be continued.

Mercury Compounds.—Mr. Green reports that preliminary work with Rotondaro method for small amounts of mercury compounds in creams is not applicable and recommends that the subject be continued.

Methyl Alcohol.—Mr. Guymon was appointed last year as Associate Referee to study the procedures for the determination of methyl alcohol which appear in Sec. **16.25** and Secs. **39.161–2** of the *Methods of Analysis*, 6th Ed., with the view to any needed revision and unification of directions.

Mr. Guymon has submitted a report covering the effect of temperature upon color development and recommends that the study be continued.

RECOMMENDATIONS*

It is recommended—

(1) That the official method for iodine (**39.202**) be reworded as recommended by the Associate Referee and be adopted as official, final action.

(2) That the method for the assay for calcium, phosphorous, and iron be adopted as official, first action.

(3) That the studies of the separation of bromides, chlorides, and iodides be continued.

(4) That the study of mercury compounds be continued.

(5) That the study of methyl alcohol be continued.

(6) That the following topics on which no reports have been received be continued for another year:

Alkali Metals

Glycols and Related Compounds

Preservatives and Bacteriostatic Agents in Ampul Solutions

* For report of Subcommittee B and action of the Association, see *This Journal*, 32, 49 (1949).

Microscopic Tests for Alkaloids and Synthetics
Estrone and Estradiol
Chromatographic Separation of Drugs.

REPORT ON ASSAY OF MERCURY COMPOUNDS

By MELVIN W. GREEN (American Pharmaceutical Association Laboratory, Washington, D. C.), *Associate Referee*

At the start of the refereeship on the determination of mercury in phenylmercuric acetate and iodide, it appeared that the portion of the problem needing solution most urgently was the microdetermination of mercury in ointments and creams.

Members of the laboratory staff of the American Pharmaceutical Association had previously determined mercury in all of the official drugs and dosage forms containing mercury by the Rotandaro procedure or some modification of it (1, 2). In this method the mercury is reduced to the metallic state by refluxing with a mixture of ethanolamine, butyric alcohol, and zinc dust. Since the method was essentially satisfactory our attention was immediately focused on an attempt to use the same basic reduction procedure on creams containing less than 1 per cent of phenylmercuric acetate. All attempts to modify such a procedure for small amounts of mercury ended in complete failure.

Due to the impossibility of freeing the small amount of mercury from such a large mass of organic matter, mostly lipoids, attention was turned to the procedure of Laug and Nelson (3), a procedure which frees the mercury by digestion with nitric and sulfuric acids.

After much painstaking work, conditions were found which gave recovery of 97 to 100 per cent of the mercury from such mixtures. Attention was then turned to the preparation of a cream containing small amounts of phenylmercuric acetate homogeneously distributed. This was found to be possible by adding the mercurial in a finely divided state to the molten lipoid phase and building the emulsion around it. The emulsified cream had the following formula:

	gram
Phenylmercuric acetate	0.090
Triethanolamine	0.110
Glycerin	3.750
Glycerylmonostearate	5.250
Stearic acid	18.000
Water, qs. ad	150.000

Two creams were prepared according to this formula for the collaborative assays. One (A-2) contained 0.087 g of phenylmercuric acetate per 150 g and the other (B) 0.093 g per 150 g. Both creams were assayed in our laboratory for uniformity by the proposed method to be used by the

collaborators. Cream A-2 yielded a recovery of 98.8 to 99.3 per cent of the known value and Cream B yielded 97.4 to 98.0 per cent of the calculated value.

Since most of the collaborators reported the results in per cent of mercury recovered, this method of reporting will be used in the tabulation of results which are as follows:

TABLE 1.—*Collaborative results*

COLLABORATOR	CALCULATED Hg		RECOVERY OF Hg	
	A2	B	A2	B
	per cent		*per cent*	
	0.034	0.037		
Collaborator A			0.0338	0.0360
			0.0336	0.0362
Collaborator B			0.0313	0.0204
			0.0319	0.0218
Collaborator C			0.056	0.049
			0.058	0.048
			0.058	0.051
Collaborator D			0.060	0.063
			0.068	—

COMMENTS BY COLLABORATORS

Collaborator B: Soft glass separatory funnels from our special lead-free stock were used because pyrex funnels were not available.

The curve obtained was a straight line which passed thru every one of 5 points. A Beckman spectrophotometer was used to measure absorption at 490 mμ.

Collaborator C: The directions for addition of hydrogen peroxide until no more brown fumes pass off was somewhat confusing. The brown fumes produced during digestion may be removed with hydrogen peroxide but on heating further more brown fumes form. It may not be necessary to have complete removal of nitrates so I only added hydrogen peroxide for the removal of brown fumes produced during the first part of the digestion. While adding hydrogen peroxide the heat was lowered.

If the sulfuric acid used in preparing standards contains sulfur dioxide this may be destroyed by adding a dilute solution of potassium permanganate until a slight excess remains for a few minutes.

Alcohol used as a preservative for the chloroform should contain no aldehydes.

The mercury dithizonate extracted appears somewhat unstable when put in the spectrophotometer but the lowest per cent T obtainable was used. For 10 micrograms of mercury the readings vary from 57.0 per cent to 61.0 per cent T during three minutes.

Mercury is not completely extracted by the extraction procedure and as larger

amounts of mercury (over 8 micrograms) are present smaller percentage of mercury are extracted. By comparing the procedure with a curve made by extracting mercury from a solution of mercuric sulfate in (1+9) sulfuric acid with dithizone (5.5 mg./liter) 78 per cent of 10 micrograms of mercury and 83 per cent of 8 micrograms of mercury are present. Ag should give negative interference when present in quantities which will ppt. as AgCl. Pb and Bi should give negative interference when present in quantities which will ppt. as sulfates. Copper should offer little interference. Iodine should give negative interference. Bromine may give negative interference.

The method should give reproducible results if the above elements are not present in interfering amounts and if portions of the same extracting solutions are used for both the standards and sample.

Collaborator D: This collaborator is familiar with this method, having used the Laug and Nelson method for mercury, *This Journal*, **25**, 399 (1942), on several occasions. It is my opinion that the painstaking precautions and time required cast doubt on the usefulness of this method for an occasional determination of mercury.

Because of the very minute amounts of mercury in the final determination and consequent danger of contamination at any point, the additional precaution of rinsing all glassware with 1+1 HNO_3 followed by water was taken. A Coleman Junior Model 6A Spectrophotometer was used. With the largest size tubes (25 mm. dia.), the 10 ml. of dithizone-mercury extract prescribed was insufficient for obtaining readings. Smaller tubes would have given too narrow a range of readings.

The readings were observed to drift appreciably in the direction of increased transmission very shortly after placing sample in the spectrophotometer, which is contrary to the Laug and Nelson observation that this does not occur with the Coleman instrument. The immediate or lowest reading was the one recorded.

Some adaptation of this method which would permit the determination of large quantities of mercury would also minimize the need for purifying all of the reagents and extreme precautions required, and result in a more practical method. In this connection I call to your attention the mercury method by W. O. Winkler (*Methods of Analysis A.O.A.C.*, 6th ed., p. 470). The final determination by dithizone titration is much simpler than might appear at first reading and I believe more readily applicable to an occasional determination. It seems that adaptation of the HNO_3-H_2SO_4 preparation of sample in the collaborative method to the Winkler determination would offer no great difficulties.

Although many of the results are rather far from the expected results, they are sufficiently uniform to warrant further study. It is recommended* that modified procedures be developed and further samples be assayed in the hope of arriving at an adequate method of assay.

REFERENCES

(1) Rotondaro, F. A., *J. Am. Pharm. Assoc.*, **33**, 353 (1944).
(2) Green, N., Green, M. W., and Powers, J. L., *Bull. National Formulary Comm.* **15**, 92 (1947).
(3) Laug, E. P., and Nelson, K. W., *This Journal*, **25**, 399 (1942).

* For report of Subcommittee B and action of the Association, see *This Journal*, **32**, 49 (1949).

REPORT ON SEPARATION OF BROMIDES, CHLORIDES, AND IODIDES

By VINCENT E. STEWART (Food and Drug Laboratory, State of Florida, Department of Agriculture, Tallahassee, Florida), *Associate Referee*

A considerable amount of published and unpublished work on the problem has been done by the previous Associate Referee, N. E. Freeman. The investigation was transferred to this Associate Referee because of the retirement of Mr. Freeman.

Little progress has been made during the year because of the necessity of examining the voluminous literature dealing with the problem. The laboratory investigations were confined largely to a study of the method recommended by Freeman[1] for the determination of chloride in the presence of large amounts of bromide and/or iodide.

This method has been investigated by the Associate Referee and a few samples were submitted to collaborators.

Some of the collaborators were unable to complete the analyses in the short time which was allowed and the results are too incomplete to justify a report at this time. It is obvious that still further investigation is necessary before the procedure can be recommended as a tentative method.

RECOMMENDATIONS*

It is recommended—

(1) That the revised acetone method for the determination of chloride in the presence of large amounts of bromide and/or iodide be investigated further and then subjected to additional collaborative study.

(2) That the volumetric cyanide method for the determination of iodide and bromide in the presence of chloride[1] be compared with the aeration absorption method and that these methods be submitted to collaborators.

REPORT ON IODINE

By SAM D. FINE (Food and Drug Administration, Federal Security Agency, Cincinnati, Ohio), *Associate Referee*

Last year a preliminary report was made on the need for revision of the present official method for iodine. To summarize that report briefly, it was found that the low results obtained on samples containing little or no organic material could be corrected by the addition of organic material before charring, as directed in the present method. It was recommended that the proposed modification be subjected to collaborative study.

The Associate Referee found it impossible to prepare a mixture of

[1] Freeman, N. E., and Beulah V. McMullen, *This Journal*, 31, 550 (1948).

* For report of Subcommittee B and action of the Association, see *This Journal*, 32, 49 (1949).

iodide and other salts which was homogeneous. Accordingly, a mixture of salts of iron, magnesium, sodium, and calcium was prepared and a solution of potassium iodide, stabilized by the addition of alkali, was prepared separately. Collaborators were sent the mixture of inorganic salts and the solution of potassium iodide and asked to add identical aliquots of the

TABLE 1.—*Comparative results*

COLLABORATOR	METHOD 39.202		MODIFICATION OF 39.202	
	MG KI/10 ML	REC.	MG KI/10 ML	REC.
		per cent		*per cent*
1	33.8	67.6	49.6	99.2
	34.8	69.6	49.8	99.6
2	25.3	50.6	49.5	99.0
	41.5	83.0	49.9	99.8
	—	—	49.8	99.6
3	41.8	83.6	49.9	99.8
	41.2	82.4	49.8	99.6
4	33.0	66.0	50.0	100.0
	34.0	68.0	50.0	100.0
5	35.7	71.4	49.7	99.4
	35.7	71.4	49.6	99.2
6	35.4	70.8	50.2	100.4
	36.0	72.0	50.3	100.6
7	35.3	70.6	49.6	99.2
	36.5	73.0	49.8	99.6
Ave.	35.7	71.4	49.8	99.6

iodide solution to identical weights of the inorganic mixture. It was directed that determinations be made in duplicate by the present method and by a modification which consisted essentially in the addition of one gram of starch before the addition of the solid potassium hydroxide. A further modification consisted of the use of 1% ammonium chloride as a wash in place of the water specified by the present method.

The results obtained by the collaborators are shown in Table 1.

DISCUSSION

The Associate Referee noted trouble occasionally with colloidal manganese dioxide running through the filter paper. In such instances the filtrate was returned to the steam bath and allowed to digest until the

manganese dioxide had become flocculent and then a second filtration was made. Rupert Hyatt, of Cincinnati Station, suggested the use of 1% ammonium chloride as a wash and this suggestion was incorporated in the instructions to all collaborators after it had been tried by the Associate Referee and found beneficial in most instances. D. M. Taylor, of the Denver Station, made the following suggestion:

"I have found, I believe, a wash water that works much better than the ammonium chloride solution that you have recommended. This wash water is prepared by running a blank on all reagents in the usual manner and then adding an equal volume of water to the filtered blank. In the nine or ten determinations that I have made in the last year, I have not had any trace of manganese dioxide washing thru into the final solution using this procedure."

The occasions when manganese dioxide have been noted to pass through the filter paper are so seldom that it is felt scarcely necessary by the Associate Referee to adopt Mr. Taylor's suggestion. Redigestion has been found in every instance to produce a clear filtrate and this is believed preferable to the preparation of the wash solution suggested by Taylor.

One of the collaborators at the Cincinnati Station obtained low results on his first set of determinations by the proposed modification. He was asked to repeat the determinations and all steps were closely observed by the Associate Referee. The difficulty was readily apparent in that insufficient permanganate was added to completely oxidize the iodide to iodate. The present method directs:

" . . . Heat to boiling and add saturated $KMnO_4$ soln slowly until $KMnO_4$ color remains after several minutes boiling. Then add ca 0.5 ml in excess, continue boiling ca 5 min., and allow to cool. . . . "

The collaborator had failed to add sufficient permanganate to maintain the characteristic coloration for the period specified. A collaborator at another laboratory had the same difficulty. After directing his attention to the necessity of adding permanganate sufficient to maintain the characteristic coloration for the period specified, excellent results were obtained. The same collaborator pointed out that there are no specific instructions for thoroughly mixing the potassium hydroxide with the sample and suggested the use of a stirring rod, allowing it to remain in the crucible during the charring.

The possibilities of failure to mix the alkali thoroughly with the iodide and of failure to add sufficient permanganate to oxidize completely to iodate had not occurred to the Associate Referee. However, the collaborators' comments and results indicate a definite need for cautionary statements relative to these two steps in the procedure.

REWORDING OF THE OFFICIAL METHOD

The rewording of the official method is intended to incorporate the modification previously suggested for samples low in organic material and

also the cautionary statements that are indicated as a result of collaborative study. The details of the method as modified are given in *This Journal*, **32**, 115 (1949).

ACKNOWLEDGMENT

Acknowledgment is gratefully extended to the following collaborators from the Food and Drug Administration who participated in this study: D. M. Taylor, Denver Station; C. R. Joiner, St. Louis Station; Sidney Williams, Rupert Hyatt, F. J. McNall, and H. C. Van Dame, Cincinnati Station.

It is recommended*—

That the present official method for iodine be reworded as described in this report.

REPORT ON CALCIUM, PHOSPHORUS, AND IRON IN VITAMIN PREPARATIONS

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

Calcium, phosphorus, and iron are among the elements indispensable for normal metabolism, and they are included in many special dietary and vitamin preparations. There are already present in the A.O.A.C. *Methods of Analysis* several excellent methods describing the assay of organic materials for these substances. This study was therefore limited to selecting that procedure deemed most readily applicable to vitamin preparations.

The volumetric method for calcium as the oxalate, employing potassium permanganate solution, is rapid and accurate, even in the presence of phosphorus, iron, aluminum, and magnesium (1). Phosphorus in the orthophosphate form is easily estimated by alkaline titration of precipitated ammonium phosphomolybdate. (Pyrophosphates, to which the method is inapplicable, are often encountered, but they are quantitatively converted to the ortho- state by heating with concentrated hydrochloric acid.) The colorimetric method for iron using α-α dipyridyl or *o*-phenanthroline requires only small amounts of the metal, and it is reliable. All three procedures are applicable to dilute acid solutions of ashed materials.

Significant losses of iron during dry ashing have been reported (2, 3), presumably due to the volatilization of ferric chloride. To test the extent of this loss a series of powdered samples (a–d), each containing 5 mg of iron were analyzed. (a) was mixed with 5 ml of normal sodium hydroxide; (b) was mixed with 5 ml of normal sodium chloride; (c) was mixed with 3 ml of normal sodium chloride and 2 ml of concentrated hydrochloric acid; (d) was untreated. The four mixtures after drying and ashing

* For report of Subcommittee B and action of the Association, see *This Journal*, **32**, 50 (1949).

yielded congruous results within the limits of error, indicating that the volatilization of iron is negligible in quantities of the order of 1–10 mg of iron per sample. Munsey (4) previously reported similar data for cereal products.

RESULTS OF ANALYSIS

Two samples were prepared for collaborative study. Sample A was a commercial vitamin product consisting of tablets which were ground together and passed through a 150-mesh sieve four times. Sample B was a synthetic mixture prepared from purified starch, standard ferrous ammonium sulfate, thrice recrystallized potassium acid phosphate, and calcium carbonate derived from precipitated calcium oxalate. It contained 35.8 mg of calcium, 10.9 mg of phosphorus, and 8.16 mg of iron per gram.* The results are shown in Table 1.

TABLE 1.—*Calcium, phosphorus, and iron in vitamin preparations*

COLLABORATOR	SAMPLE A			SAMPLE B		
	Ca	P	Fe	Ca	P	Fe
	mg/gm	*mg/gm*	*mg/gm*	*mg/gm*	*mg/gm*	*mg/gm*
H. F. O'Keefe	124.3	96.7	8.7	35.1	10.7	8.2
	124.1	98.0	8.7	35.8	10.9	8.2
D. Banes	122.5	97.8	8.7	35.3	10.8	8.0
	124.6	97.2	8.7	35.3	10.8	8.0
R. Hyatt	122.4	96.1	8.7	35.1	11.1	8.5
	122.9	96.0	8.7	35.4	11.1	8.3
S. D. Fine	123.3	97.4	—	35.9	11.2	—
	123.5	97.2	—	35.9	11.2	—
G. E. Keppel	122.3	94.2	8.5	35.2	10.6	8.5
	122.6	94.5	8.5	34.8	10.7	8.7
H. R. Bond	120.8	96.9	8.8	34.4	10.7	8.0
	121.6	97.3	9.0	34.7	10.9	8.3
Average	122.9	96.6	8.7	35.3	10.9	8.2
Average Recovery %	—	—	—	98.6	100.0	101.3

COMMENTS OF COLLABORATORS

Rupert Hyatt.—"The method should be broadened to accommodate analyses for other elements, such as magnesium. This could be accomplished by adding at the end of the calcium method: . . . Reserve the filtrate for the determination of magnesium as in *Methods of Analysis*, 1945, **26.21–4.**"

* Details of the method are given in *This Journal*, **32**, 114 (1949).

DISCUSSION AND RECOMMENDATION

The data reported indicate that the proposed methods are of an accuracy and precision suitable for the analysis of vitamin preparations. It is recommended* that the method, with the addendum suggested, be adopted as official, first action, and that the subject be closed.

REFERENCES

(1) MITCHELL, J. H., *This Journal*, **4**, 391 (1921).
(2) HOFFMAN, C., SCHWEITZER, T. R., and DALBY, G., *Ind. Eng. Chem., Anal. Ed.*, **12**, 454 (1940).
(3) GRAY, P. P., and STONE, I. M., *ibid.*, **10**, 415 (1948).
(4) MUNSEY, V. E., *This Journal*, **27**, 398 (1944).

No reports were given on microscopic tests for alkaloids and synthetics, alkali metals, glycols and related compounds, preservatives and bacteriostatic agents in ampul solutions, estrone and estradiol, or chromatographic separation of drugs.

For report of methyl alcohol, see "Methanol in Distilled Spirits," by J. F. Guymon, *This Journal*, **32**, 163 (1949).

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* For report of Subcommittee B and action of the Association, see *This Journal*, **32**, 50 (1949).

TUESDAY—AFTERNOON SESSION

REPORT ON STANDARDIZATION OF MICROCHEMICAL METHODS. CARBON, HYDROGEN, AND NITROGEN

C. O. WILLITS, *Referee*, and C. L. OGG, *Associate Referee*, Eastern Regional Research Laboratory,* United States Department of Agriculture, Philadelphia 18, Pennsylvania

This year's studies, following the recommendations set forth in last year's report, have been devoted to the determination of carbon and hydrogen and nitrogen by the Kjeldahl and Dumas procedures. The selection was based on the results of a questionnaire, which indicated that in the opinion of a majority of micro analysts these determinations should be studied first.

Two samples, nicotinic acid and benzyl-iso-thiourea hydrochloride, were sent to two groups of collaborators. One group was asked to analyze the samples for carbon and hydrogen; the other was asked to determine nitrogen both by the Kjeldahl and Dumas procedures. These compounds were chosen for this work because both are stable and nonhygroscopic and because they differ considerably in constitution and ease of decomposition.

Although a statement of purity did not accompany the samples, the collaborators were informed that they were relatively pure. They were asked to report all the numerical values obtained for each sample, regardless of whether or not the data appeared to be correct.

The collaborators for carbon and hydrogen were requested to analyze the two samples by their own methods only, since there is no one method in common use today. The collaborators for nitrogen were asked to analyze the two samples by their own Kjeldahl and Dumas methods and by the A.O.A.C. Tentative Microkjeldahl Method.

Questionnaires accompanying the samples asked for details of the apparatus, procedure, and laboratory conditions under which the analyses were conducted. From the information so obtained and the accompanying analytical results, a method will be adopted or devised for each determination, and each will be given rigorous collaborative study before it is proposed as a tenative method. Since so many methods were used in obtaining the data for each determination and consequently the number of variables was so large, the data have been analyzed statistically in an attempt to determine which variations in procedure and apparatus are important. In the statistical comparisons of these data influenced by a large number of variables, the assumption was made that all variables other than the one being evaluated cancelled themselves. The conclusions drawn are based on the available data, and although there is a possibility

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

that they may be erroneous because of the assumption on which they are based, they will be used as guides for future studies until more data are available. Since some data were received after the analysis of the data was started, the total number of analyses is in some cases greater than that used in the statistical treatment.

CARBON AND HYDROGEN DETERMINATION

Twenty-five analysts from 19 laboratories reported 111 carbon and hydrogen analyses for sample 1 (nicotinic acid) and 92 analyses for

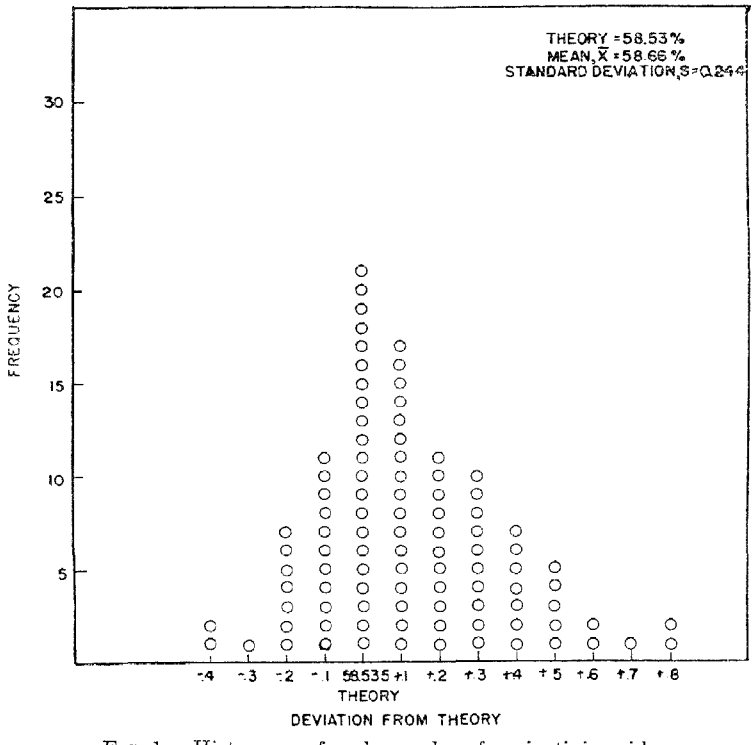

FIG. 1.—Histogram of carbon values for nicotinic acid.

sample 2 (benzyl-iso-thiourea hydrochloride). The number of analyses reported from different laboratories ranged from 2 to 20.

Carbon.—The histogram (Fig. 1) shows the frequency distribution of the carbon values obtained for nicotinic acid. Although all points in the figure are located on the class marks, they show the number of analyses which fell within the class boundaries (class mark $\pm 0.05\%$). Three results are not shown and are not included in the subsequent treatment. The chi square distribution test for measuring goodness of fit of frequency distribution showed that these three values fell outside the representative

population of the carbon values for this compound. This test has shown that the values retained in the histogram are a representative sample of a normal population, and therefore they can be treated statistically.

Figure 1 shows that a majority of the values are higher than the theoretical value (58.53%). The mean ($\bar{X}$ or average) of the values is 58.66%, and the standard deviation (S, the variation about a mean) is 0.244, indicating that 67% of the carbon values for this sample should fall within ± 0.244 or 95% within ± 0.488 of the mean value.

Although the mean is 0.13% higher than the theoretical value, the mode (class which contains the largest number of values) falls on theory, indicating that there is a good possibility of finding among the methods one which will give high accuracy as well as good precision. Inspection shows that even though there is a preponderance of values above the theoretical value, the skewness of the histogram toward the theoretical value and the mode indicate that there is a tendency to obtain theoretical values.

All carbon methods followed the same general procedure, which consisted in the catalytic combustion of a weighed sample to carbon dioxide, followed by the absorption and weighing of the combustion product. Although no two methods were identical, each step in any one method was in general similar to the corresponding step in several other methods. Therefore, the carbon values for nicotinic acid were divided into two groups, one representing the results obtained by a certain operation for one step in the determination, and the other a second operation for the same step. These two groups were treated statistically to determine whether or not there was a significant difference in the results obtained by the two operations for the step or condition in question. By this procedure, the following comparisons were made: (1) electrical *vs.* gas sample burner, (2) mechanical *vs.* hand-operated sample burner, (3) semimicro (10–30 mg) *vs.* micro (2–10 mg) sample weight, (4) air-conditioned *vs.* nonair-conditioned laboratories, (5) balance in an air-conditioned balance room *vs.* balance adjacent to the furnace. The only case in which there was a significant difference in the carbon results for nicotinic acid was semimicro *vs.* micro samples. The significance level used throughout this study was 5%, which means that if the experiment was repeated a number of times the differences obtained should be at least as large as the one found in 95% of the cases. The theoretical frequency distribution curves for these two methods are shown in Figure 2. The means ($\bar{X}$) for the two procedures were 58.52 and 58.69, whereas the standard deviations (S) were 0.198 and 0.239, respectively. Since the difference in the two means was significant, and the semimicro mean was closer to the theoretical value, better values can be obtained by the semimicro than the micro method and, similarly, since the standard deviation is less, the precision of the semimicro method is greater.

The data for sample 2, benzyl-iso-thiourea hydrochloride, were treated in the same manner as those for nicotinic acid. Figure 3 shows the histogram of the values obtained for this material. The chi square distribution test showed that the values obtained should all be included in the

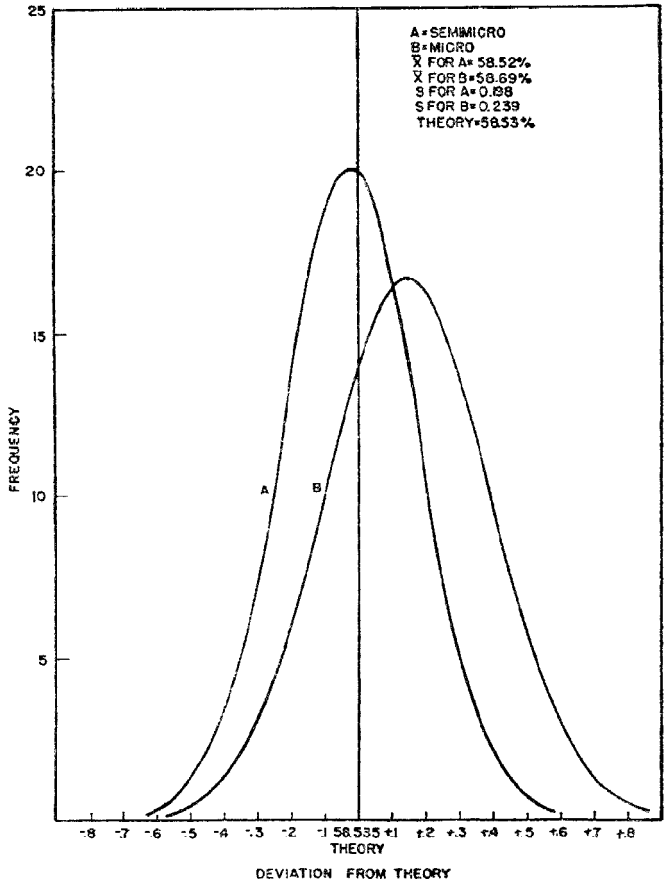

FIG. 2.—Theoretical distribution curves for micro and semimicro carbon values. Nicotinic acid.

statistical treatment. The mean ($\overline{X}$) of the values is 47.51% or 0.11% above theory, with a standard deviation (S) of 0.184. Since the mean is nearer the theoretical value for this sample than that for sample 1 (nicotinic acid), and the standard deviations is less, the methods used gave slightly better carbon values for sample 2. These differences must be due to an inherent difference in the two compounds, since the methods and conditions were the same for the analyses of the two samples. The same comparisons were made for the values for benzyl-iso-thiourea hydro-

chloride as were made for nicotinic acid. There is again a significant difference between the values for the semimicro *vs.* micro procedures and, as for sample 1, the difference is in favor of the semimicro method. Figure 4 shows the theoretical frequency distribution curves for the two methods. The means are 47.38% and 47.53%, and the standard deviations 0.078 and 0.189 for the semimicro and micro methods, respectively.

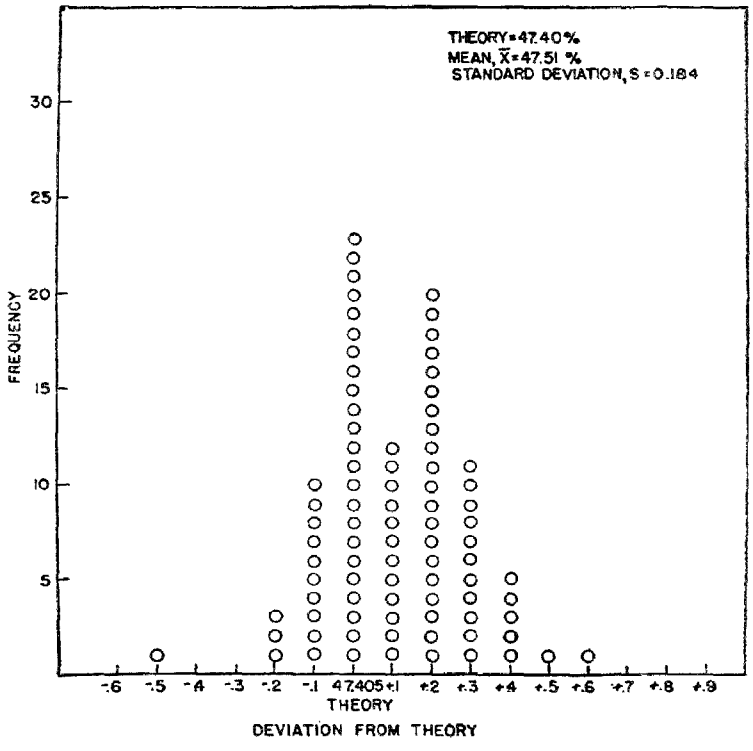

FIG. 3.—Histogram of carbon values for benzyl-iso-thiourea hydrochloride.

The results of the carbon analyses for the two samples are similar, in that the mode in both cases falls on theory, the means are above theory, and the standard deviations for the two are comparable. Semimicro methods were significantly better than micro methods for both samples, as is shown in Figures 2 and 4. None of the other comparisons showed any significant difference which would lead one to recommend the preferential use or adaptation of one procedure over another. Table 1, however, shows slight trends which favor certain operations, and these may serve as guides in planning future studies.

Hydrogen.—The number of hydrogen values received and the number of

collaboratores were the same as for carbon, since the two determinations were made simultaneously. Histograms of the hydrogen values for nicotinic acid and benzyl-iso-thiourea hydrochloride are shown in Figures 5 and 6, respectively.

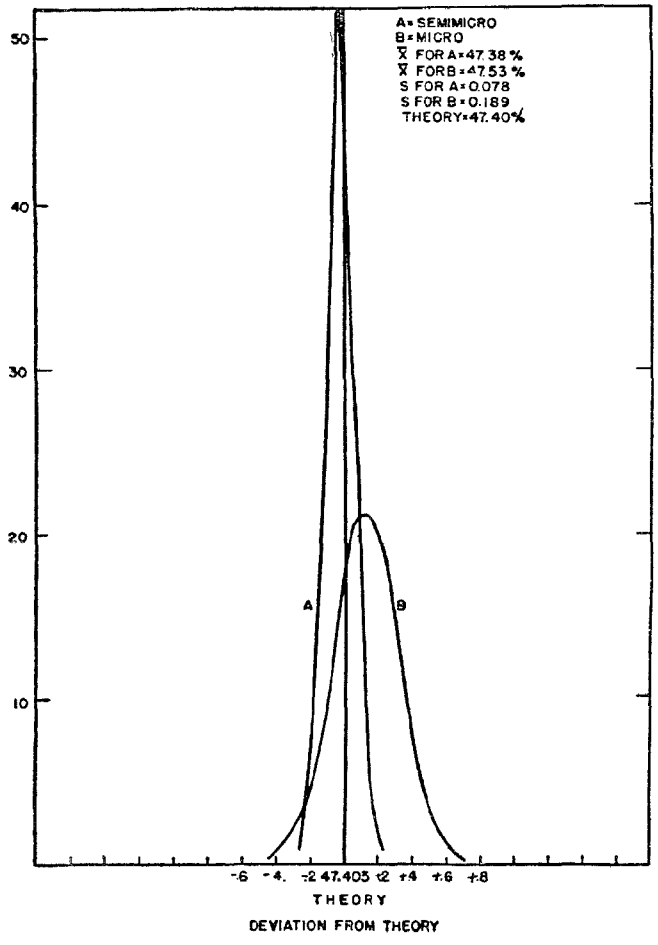

FIG. 4.—Theoretical frequency distribution curves for semimicro and micro carbon values. Benzyl-iso-thiourea hydrochloride.

A marked similarity in the data for the two samples can be seen. The means are 0.10 and 0.06% above the theoretical value, and the two standard deviations are 0.208 and 0.200. In both cases the mode, class with the highest frequency, is 0.10% above theory.

The same operations as listed previously for carbon were compared to determine whether or not they were significantly different. The values

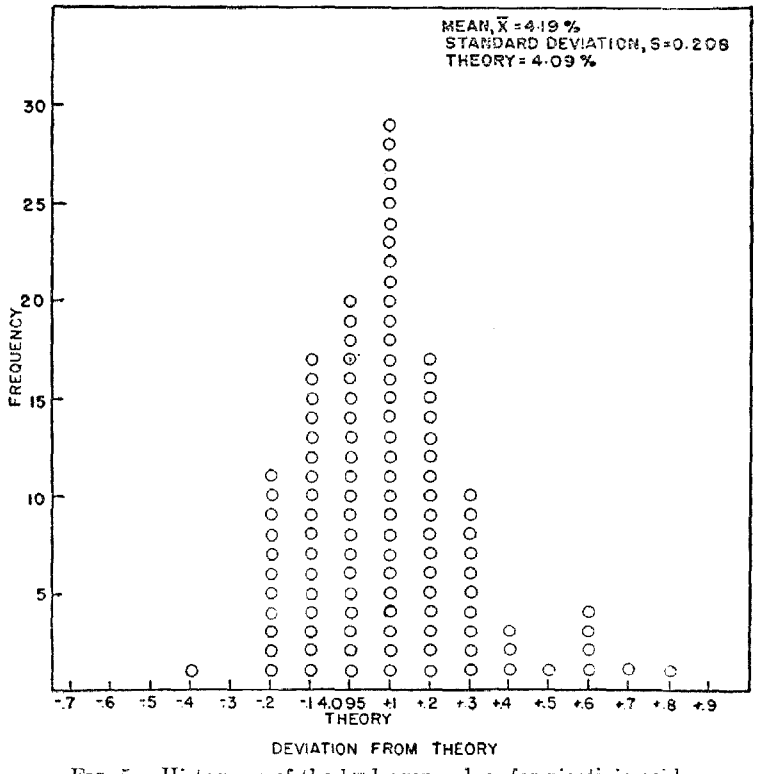

FIG. 5.—Histogram of the hydrogen values for nicotinic acid.

TABLE 1.—*Carbon data obtained for samples 1 and 2 by catalytic combustion methods*

	NICOTINIC ACID (1)			BENZYL-ISO-THIOUREA HYDROCHLORIDE (2)		
	NUMBER OF SAMPLES	$\bar{X}$	S	NUMBER OF SAMPLES	$\bar{X}$	S
		per cent			*per cent*	
Semimicro method	16	58.52	0.198	14	47.38	0.078
Micro method	81	58.69	0.239	76	47.53	0.189
Electric sample burner	50	58.70	0.268	45	47.51	0.148
Gas burner	47	58.62	0.205	44	47.51	0.195
Mechanical burner	41	58.67	0.276	41	47.49	0.143
Hand-operated sample burner	56	58.65	0.209	49	47.53	0.212
Balance in air-conditioned balance room	42	58.72	0.286	34	47.54	0.161
Balance adjacent to furnace	34	58.61	0.221	43	47.47	0.200
Air-conditioned laboratory	59	58.65	0.241	52	47.51	0.176
Non-air-conditioned laboratory	38	58.67	0.179	38	47.52	0.200
Total samples	97	58.66	0.244	90	47.51	0.184
Theoretical values		58.53			47.40	

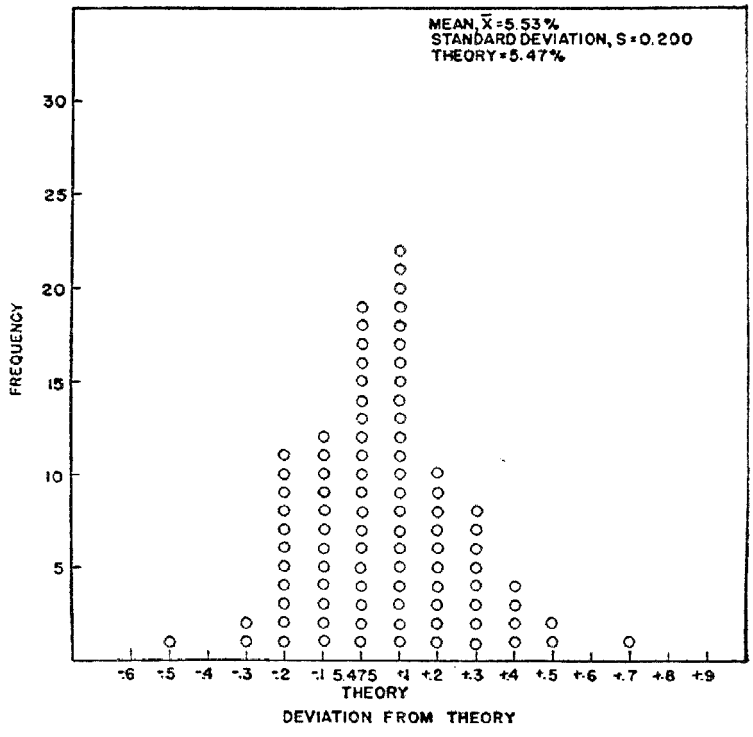

FIG. 6.—Histogram of hydrogen values for benzyl-iso-thiourea hydrochloride.

TABLE 2.—*Hydrogen data obtained for samples 1 and 2 by catalytic combustion methods*

	NICOTINIC ACID (1)			BENZYL-ISO-THIOUREA HYDROCHLORIDE (2)		
	NUMBER OF SAMPLES	$\bar{X}$	S	NUMBER OF SAMPLES	$\bar{X}$	S
		per cent			*per cent*	
Semimicro method	18	4.20	0.161	12	5.57	0.109
Micro method	87	4.21	0.214	80	5.53	0.207
Electrical burner	63	4.15	0.175	47	5.51	0.190
Gas burner	52	4.23	0.235	45	5.56	0.205
Mechanical burner	58	4.14	0.195	43	5.48	0.179
Hand-operated burner	57	4.24	0.229	48	5.58	0.210
Balance in air-conditioned balance room	41	4.17	0.176	34	5.50	0.207
Balance adjacent to furnace	44	4.22	0.261	40	5.55	0.219
Air-conditioned	62	4.16	0.178	53	5.49	0.182
Non-air-conditioned laboratory	53	4.23	0.237	39	5.59	0.205
Total samples	115	4.19	0.208	92	5.53	0.200
Theoretical values		4.09			5.47	

shown in Table 2 for mechanically operated sample burners are significantly better than those for hand-operated burners. Figures 7 and 8 show the theoretical frequency distribution curves for mechanical *vs.* hand-operated furnaces for samples 1 and 2, respectively. Only one other comparison, air-conditioning *vs.* non-air-conditioning, proved to be significant. The theoretical frequency distributions in Figure 9 show that

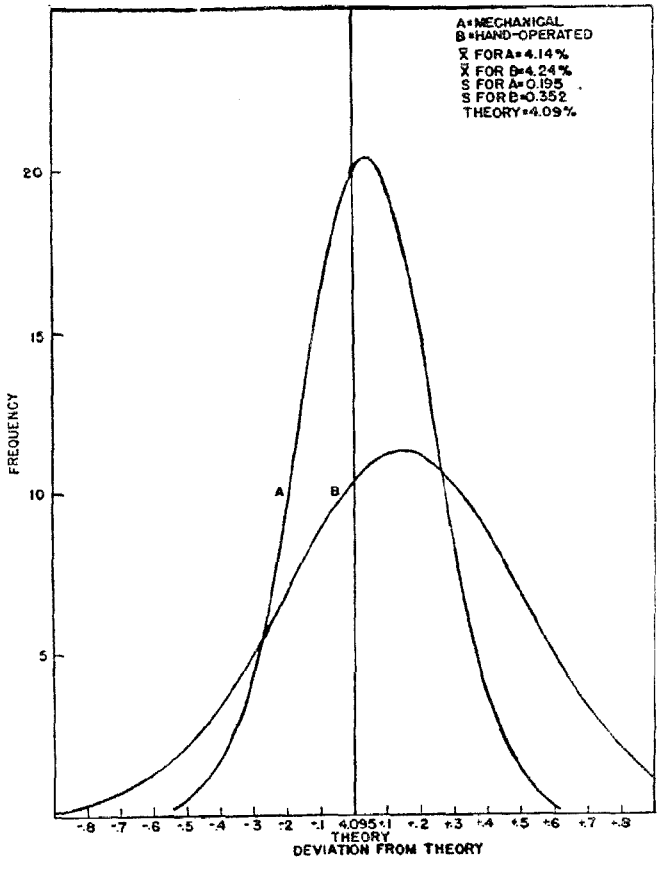

FIG. 7.—Theoretical frequency distribution curves for hydrogen values from mechanical and hand-operated furnaces. Nicotinic acid.

air-conditioned laboratories were superior for the hydrogen analysis of benzyl-iso-thiourea hydrochloride. While the mean is nearer theory and the S value smaller for hydrogen values from air-conditioned laboratories for nicotinic acid, the difference was not significant.

Summary for carbon and hydrogen.—A comparison of the data for hydrogen with those for carbon shows that the means, $\overline{X}$, for hydrogen

are slightly closer to theory than those for carbon, that in both analyses the means are above theory, and that standard deviations or precisions for hydrogen and carbon for both samples are similar.

The means for the carbon and hydrogen values obtained in air-conditioned laboratories are closer to the theoretical value than those

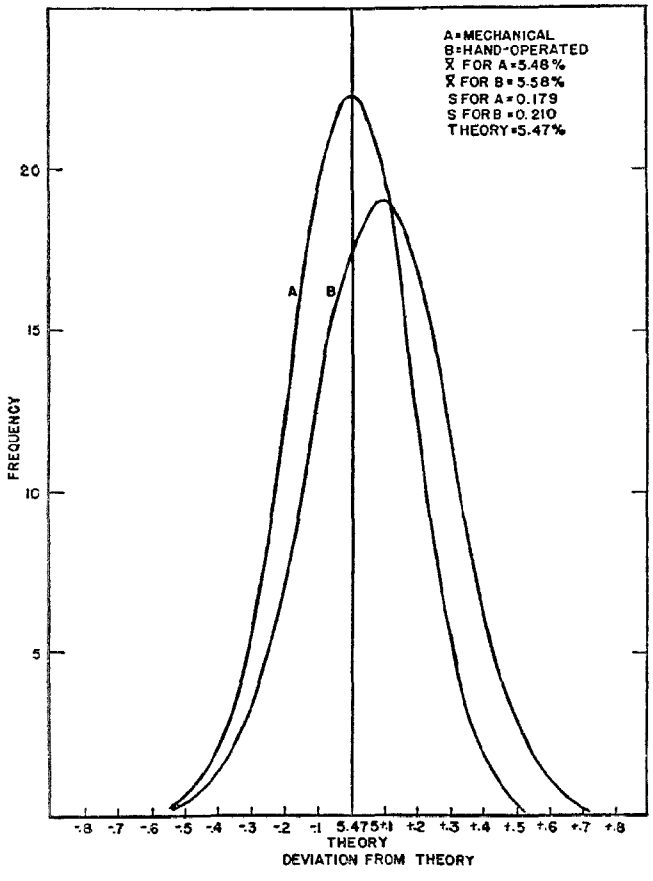

FIG. 8.—Theoretical frequency distribution curves for hydrogen values from mechanical and hand-operated furnaces. Benzyl-iso-thiourea hydrochloride.

made in non-air-conditioned laboratories, but the difference is significant only in the hydrogen values of sample 2. In three of the four determinations, the standard deviation (S) is lower for air-conditioned laboratories.

Comparison of the results obtained by mechanical *vs.* hand-operated furnaces showed that in three of the four determinations, mechanically operated furnaces gave means, $\overline{X}$, closer to the theoretical value and in two cases the difference was significant. In general, the means for electrical

burning furnaces are nearer the theoretical value than those for gas burners, and the standard deviations are less, but in no case is the difference significant.

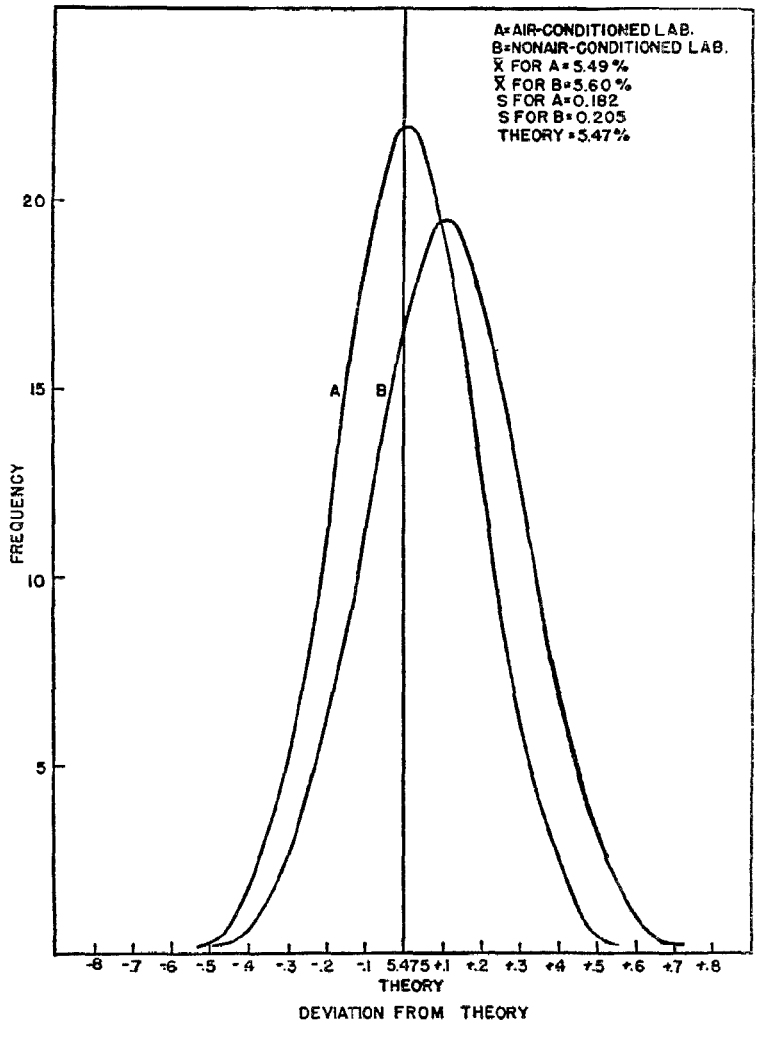

FIG. 9.—Theoretical frequency distribution curves for hydrogen values from air-conditioned and non-air-conditioned laboratories. Benzyl-iso-thiourea hydrochloride.

Perhaps the most significant comparison is that of the values obtained by semimicro and micro procedures for carbon. Here the semimicro procedures are superior, provided that the variables other than size of sample

can be neglected. As for the hydrogen values, the standard deviation also favors the semimicro method, and the average values are nearly identical.

Comparison of values obtained when the balance was in an air-conditioned balance room *vs.* those obtained when the balance was adjacent to the furnace shows that the means are nearer the theoretical value and the standard deviations are lower for carbon values when the latter method

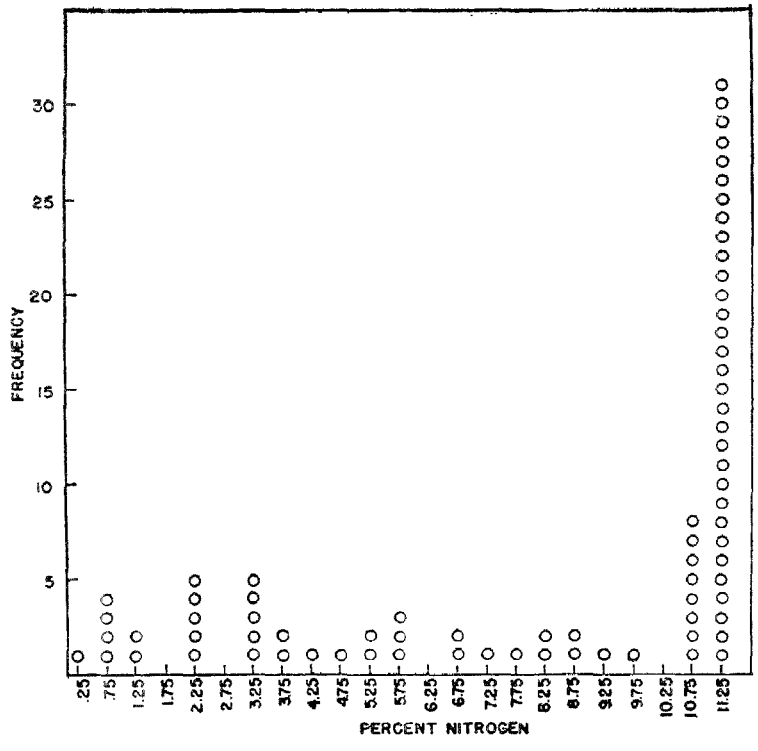

FIG. 10.—Histogram of Kjeldahl nitrogen values for nicotinic acid. Theory = 11.38.

was used, but the reverse is true for the hydrogen values. In no case, however, is the difference between the two procedures statistically significant. More comparisons of the different phases of the analysis, including tube fillings, absorbents, rate of gas flow and the like, are in progress and must be completed before a trial method can be recommended.

KJELDAHL NITROGEN DETERMINATION

The same two compounds used in the carbon and hydrogen studies were sent to a second group of collaborators, who were asked to determine the nitrogen in the two samples by both the Kjeldahl and Dumas procedures.

They were also asked to make the Kjeldahl analyses by their own method and by the A.O.A.C. (tentative) Microkjeldahl Method, a copy of which was enclosed.

One of the reasons for choosing nicotinic acid was that it contains a ring nitrogen, which is difficult to obtain by the Kjeldahl method. Replies to the questionnaire which accompanied the samples indicated that some

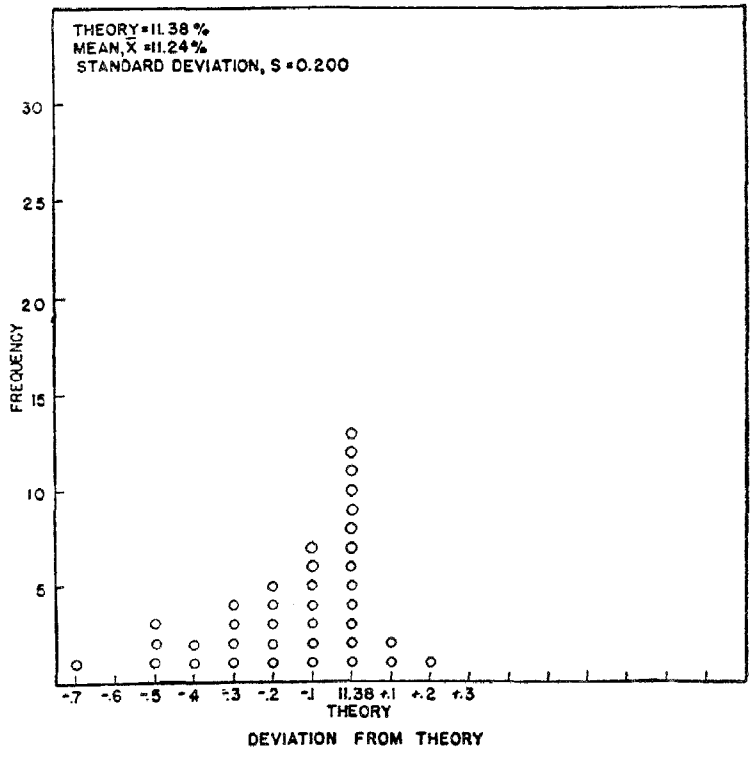

FIG. 11. Histogram of the Kjeldahl nitrogen values for nicotinic acid from the two upper classes in Fig. 10.

micro analysts do not attempt to determine nitrogen in such compounds by the Kjeldahl method but others do it regularly. Therefore, the nitrogen values obtained by the A.O.A.C. method should be a good test of its reliability.

The histogram of the population of the 75 values from twelve collaborators is shown in Figure 10. To present all the data in one histogram, the class intervals had to be in units of 0.5%, since the reported nitrogen values ranged from less than 0.5 to 11.5%.

It is obvious from the histogram that the data as a whole can not be treated statistically but that there is a sharp division of the values, about half being near the theoretical value and the remainder low and erratic.

The collaborators who reported results from 0 to 10% obtained no values higher than 11%, whereas those who reported values about 11% had no values below 10.5%. A histogram (Fig. 11) with class limits of 0.1% was made of the data obtained by those methods which gave one or more values above 11%. Although the mode falls on the theoretical value,

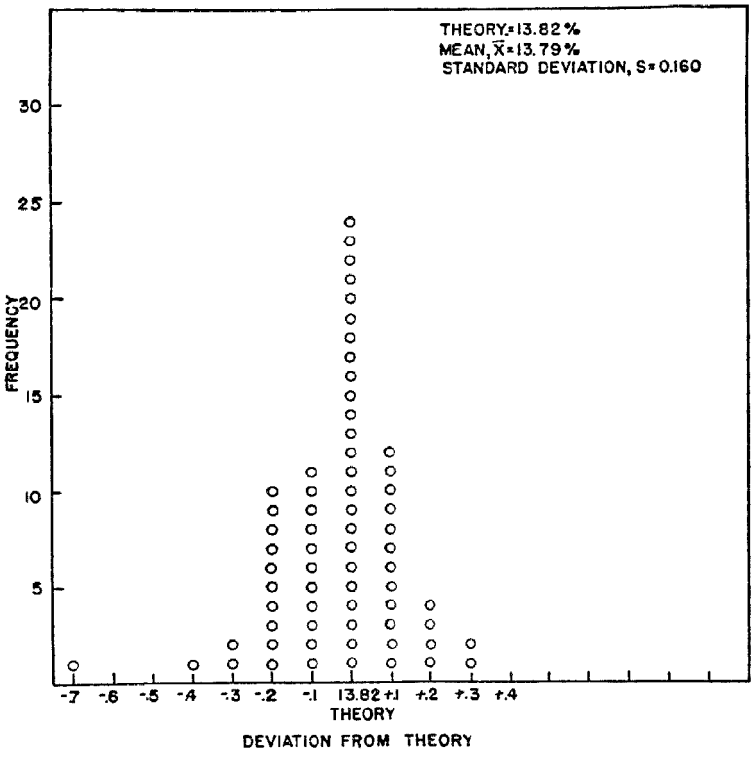

FIG. 12.—Histogram of Kjeldahl nitrogen values for benzyl-iso-thiourea hydrochloride.

the mean is considerably below this value, indicating much skewness in the data. The chi square test showed that the data are not a representative sample of a normal population, and therefore statistical comparisons can not be made. Nevertheless, the means were determined for those values obtained when mercury and mercury plus selenium were used as catalysts, and the mean was closer to the theoretical value when only mercury was used. The many values which are in agreement with the theoretical value indicate that a satisfactory method can be found.

The analysis of benzyl-iso-thiourea hydrochloride (sample 2) for nitrogen by the Kjeldahl method proved to be a much simpler task than

determining the nitrogen in nicotinic acid. Figure 12, the histogram of the values, shows that there is a fairly normal distribution of the values around the mode which falls on the theoretical value. The chi square distribution test indicated that the 67 values received are a representative sample and all should be included in the studies. The mean is 13.79, only 0.03% less than theory, and the standard deviation is 0.160.

TABLE 3.—*Nitrogen data obtained for sample 2 by the Kjeldahl method*

	BENZYL-ISO-THIOUREA HYDROCHLORIDE		
	NUMBER OF SAMPLES	$\bar{X}$	S
		per cent	
Semimicro methods	18	13.76	0.212
Micro methods	49	13.81	0.134
Mixed indicator	31	13.77	0.190
Single indicator	36	13.81	0.126
Digestion aid	35	13.80	0.118
No digestion aid	32	13.78	0.195
Electrical digestion	11	13.75	0.224
Gas digestion	56	13.80	0.141
Mercury catalyst	45	13.77	0.167
Mercury plus selenium catalyst	22	13.83	0.134
Parnas-Wargner apparatus	33	13.76	0.167
Other distillation apparatus	34	13.83	0.148
One-half hour digestion	10	13.73	0.228
One hour digestion	57	13.80	0.145
Silver or tin condenser tubes	34	13.75	0.170
Pyrex condenser tubes	33	13.84	0.134
Total samples	67	13.79	0.160
Theoretical values		13.82	

Twenty-three of the values were obtained by the A.O.A.C. procedure, and their mean is 13.795 with a standard deviation of 0.122. The remaining 44 values by various micro and semimicro procedures have a mean of 13.791 with a standard deviation of 0.176. The difference between the means is far from significant, but slightly better precision (smaller S value), was obtained with the A.O.A.C. procedure than with the sum of the other methods.

The data were used to make 8 other comparisons of variations in the Kjeldahl procedure. The comparisons and the mean and standard deviation of each are shown in Table 3. There was no significant difference between the means for any of the comparisons except for silver or tin versus Pyrex glass condenser tubes, the Pyrex glass being favored. The difference between the means for one-half and one hour digestions was nearly significant and in favor of the longer digestion.

The difference between the means for the Parnas-Wagner and other distillation apparatus is considerable, but there is so much duplication of the values obtained by this apparatus and the silver or tin condenser that without further study it is not possible to say whether the lower values are due to the apparatus, the condenser, or both.

Summary for Kjeldahl nitrogen.—The data for nicotinic acid indicate that the A.O.A.C. (tentative) Microkjeldahl method is unsatisfactory for many compounds with ring nitrogen, but it does show that a satisfactory method can probably be established, since four collaborators using four different methods obtained values in agreement with the theoretical values. For the less refractory material, sample 2, the A.O.A.C. method gave values with an excellent mean and with better precision than the sum of the other Kjeldahl methods used. The results indicate that the A.O.A.C. method will apply to compounds with ring nitrogen if the catalyst and digestion time are both increased by amounts yet to be determined.

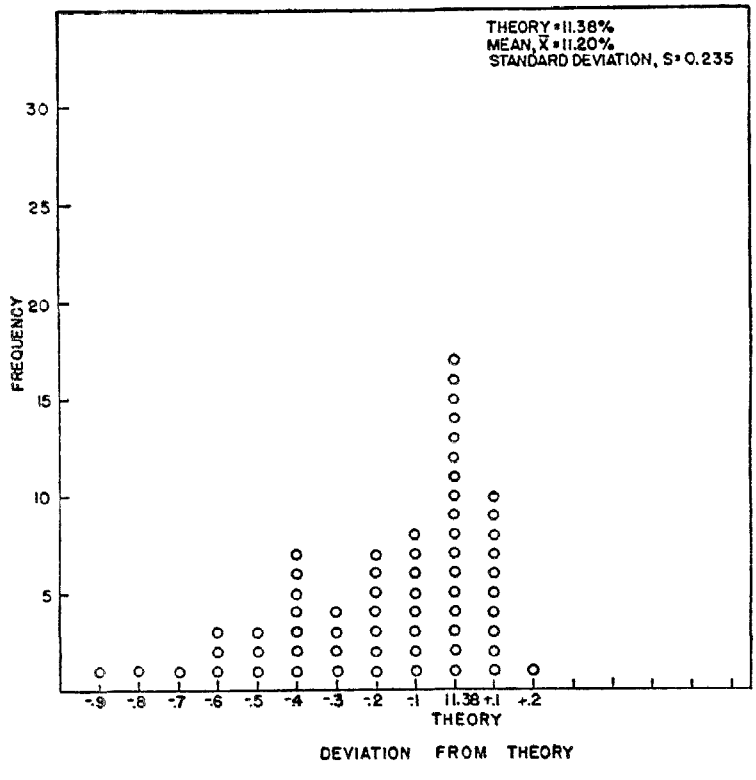

FIG. 13.—Histogram of Dumas nitrogen values for nicotinic acid.

DUMAS NITROGEN DETERMINATION

The same collaborators participated in this determination as in the Kjeldahl studies, and they used the same two samples. Histograms of the values obtained by the 13 collaborators for samples 1 and 2 are shown in Figures 13 and 14, respectively. The values for neither compound were representative samples of normal populations, as shown by the chi square test. Inspection of the histograms shows too many values in the classes

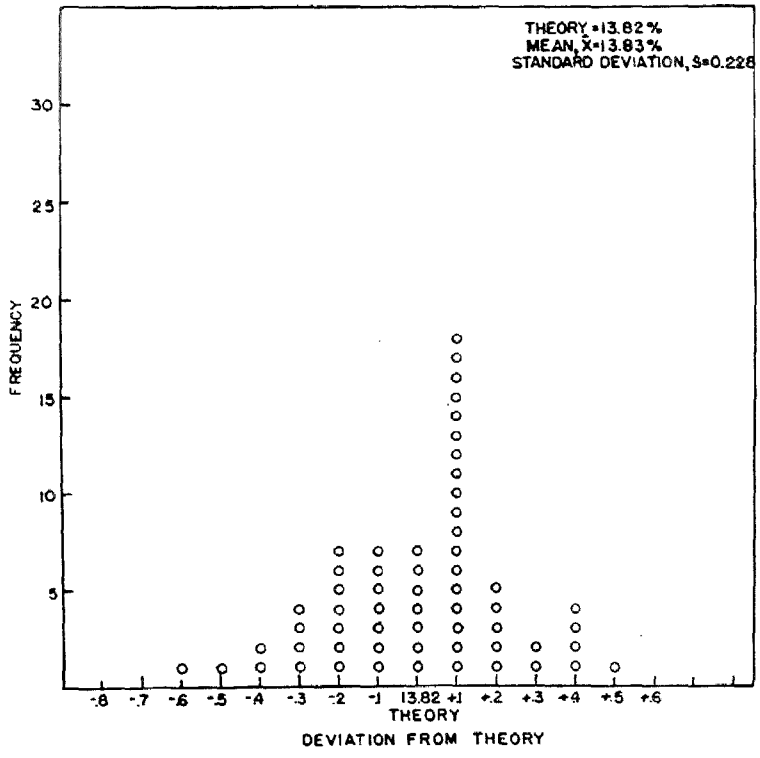

FIG. 14.—Histogram of Dumas nitrogen values for benzyl-iso-thiourea hydrochloride.

below theory, and nicotinic acid has a second mode 0.4% below the theoretical value. The questionnaires indicated that most of the lower values were obtained by methods which used temperatures below 650° C. Consequently, the nitrogen values for nicotinic acid obtained by methods with temperatures above 650°C. were tested and found to be representative of a normal population. Although the data for sample 2 were not as skewed as for sample 1, they were treated in the same manner and with the same result.

The data shown in Table 4 were obtained by methods in which a temperature above 650°C. was used. No comparisons can be made, since the number of representative analyses is too small and there is too much overlapping of values.

The data for sample 1 indicate that the better methods would use, in addition to temperatures above 650°C., a gas sample burner with two burnings, a gasometer, and 1.1% gas volume correction, and would take the temperature of the air as that of the gas in the nitrometer. The data for sample 2 indicate that the methods which use the alternates of these

TABLE 4.—*Nitrogen data obtained for samples 1 and 2 by the Dumas method*

	NICOTINIC ACID (1)			BENZYL-ISO-THIOUREA HYDROCHLORIDE (2)		
	NUMBER OF SAMPLES	$\bar{X}$	S	NUMBER OF SAMPLES	$\bar{X}$	S
		per cent			*per cent*	
Gas burner	18	11.41	0.055	16	13.97	0.152
Electrical burner	20	11.32	0.134	23	13.90	0.190
Sample burned twice	18	11.41	0.055	16	13.97	0.152
Sample burned once	20	11.32	0.134	23	13.90	0.190
Gasometer	17	11.41	0.063	17	13.97	0.145
No gasometer	21	11.33	0.134	22	13.90	0.197
1.1% gas volume correction	15	11.41	0.071	15	13.97	0.152
2% gas volume correction	12	11.29	0.158	12	13.78	0.155
Temperature measured in air	12	11.43	0.067	21	14.03	0.145
Temperature measured in liquid	26	11.33	0.121	18	13.81	0.145
Total samples	38	11.36	0.118	39	13.93	0.179
Theoretical values		11.38			13.82	

operations would be the better. This reversal of suitability of methods is improbable, and can no doubt be accounted for. Of these operations, the only arbitrary means of increasing or decreasing the per cent nitrogen is the correction applied to the gas volume. The mean nitrogen value for sample 2 after a 1.1% volume correction had been made, was too high by 0.15%. It seems unlikely that any of the other variations listed would cause this high value. Therefore, a 2% correction, which would give a mean value only 0.04% low, is apparently the more nearly correct and perhaps should be used in all cases. The reason that the 1.1% correction gave values nearer the theoretical value for nicotinic acid may be that this smaller correction compensated for some nitrogen not recovered from this refractory material.

Summary for Dumas nitrogen.—The study has shown that for satisfactory results by the Dumas method, the minimum temperature is 650°C.

A 2% volume correction appears to be required instead of the often-used 1.1% correction, but further work is necessary to prove this point and to establish preferences for other variations in the procedure.

The values submitted by the various collaborators for carbon, hydrogen, and nitrogen are presented graphically in Figures 15 to 19. No attempt has been made to analyze the values obtained from each laboratory separately.

The collaborators in these studies are listed below.

Collaborators on nitrogen analysis:

Alicino, J. F., Squibb Institute for Medical Research
Jones, G. A., E. I. du Pont de Nemours and Company
Brunner, A. H., Ansco
Ketchum, D. E., Eastman Kodak Company
Powers, D. A., Celanese Corporation of America
Ogg, C. L., Eastern Regional Research Laboratory
Hegeman, B., The Texas Company
Dutton, C. D., Picatinny Arsenal
Grodsky, J., Ortho Research Foundation
Milner, R. T., Northern Regional Research Laboratory
Blackman, S. W., The Wellcome Research Laboratories
Sundberg, O. E., Calco Chemical Division, American Cyanamid Company
Wagner, E. C., University of Pennsylvania

Collaborators on carbon and hydrogen analysis:

Kuck, J. A., American Cyanamid Company
Owens, J. K., E. I. du Pont de Nemours and Company
Sievers, D. C., Tennessee Eastman Corporation
Rachele, J. R., Cornell University Medical College
Paulson, R. A., National Bureau of Standards
Brown, L. E., Southern Regional Research Laboratory
Huffman, E. W. D., Huffman Microanalytical Laboratories
Conard, V. A., Oakwold Laboratories
Feldman, J. R., General Foods Corporation
Hallett, L. T., General Analine and Film Corporation
Hynes, W. A., Fordham University
Clark, H. S., Illinois State Geological Survey
Aluise, V. A., Hercules Powder Company
Butler, A. Q., Mallinckrodt Chemical Works
Shreve, L. S., Smith, Kline and French Laboratories
Streeter, K. B., Sharp and Dohme, Inc.
Steyermark, Al, Hoffman-La Roche, Inc.
Means, J. A., Charles Pfizer and Company, Inc.

RECOMMENDATIONS*

It is recommended that studies be continued on methods for the micro determination of carbon and hydrogen, and for nitrogen, by the Kjeldahl and Dumas procedures. Proposals based on the results of this year's work will be made concerning the procedures to be studied in the next year.

* For report of Subcommittee C and action of the Association, see *This Journal*, 32, 56 (1949).

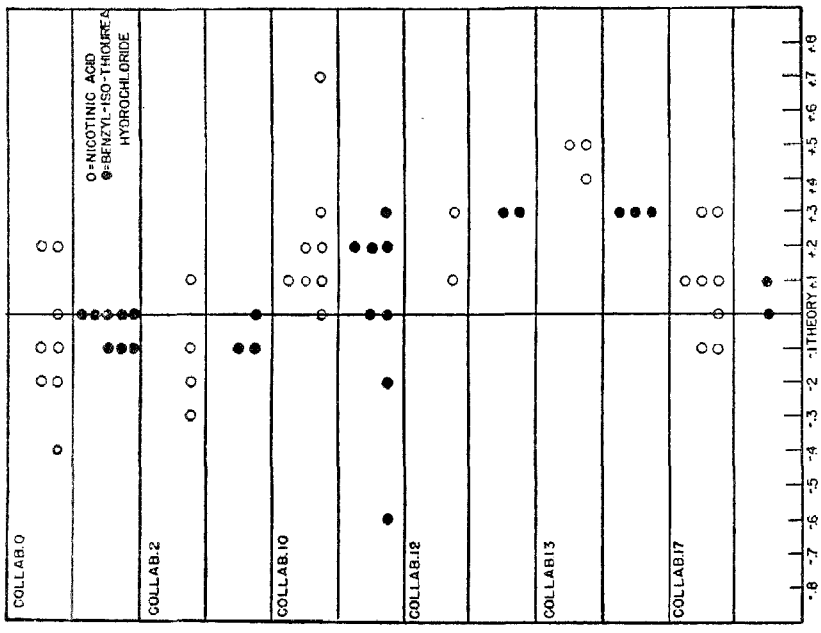

FIG. 15.—Distribution of carbon values from the different collaborators.

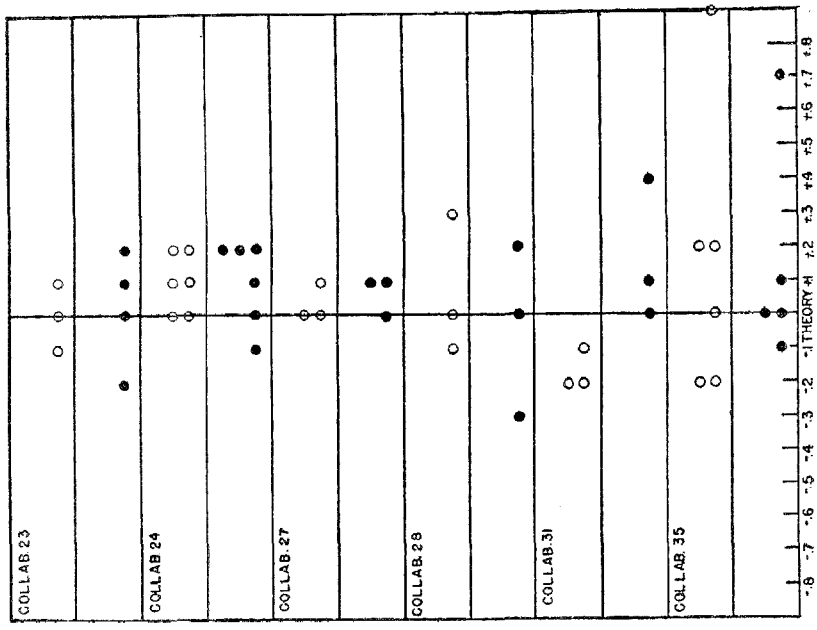

FIG. 15.—Continued.

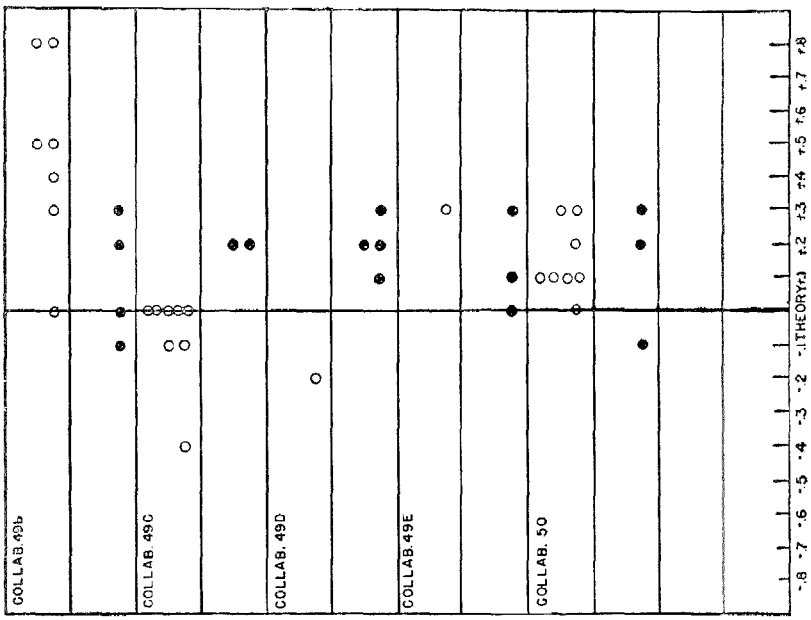

FIG. 15.—Continued.

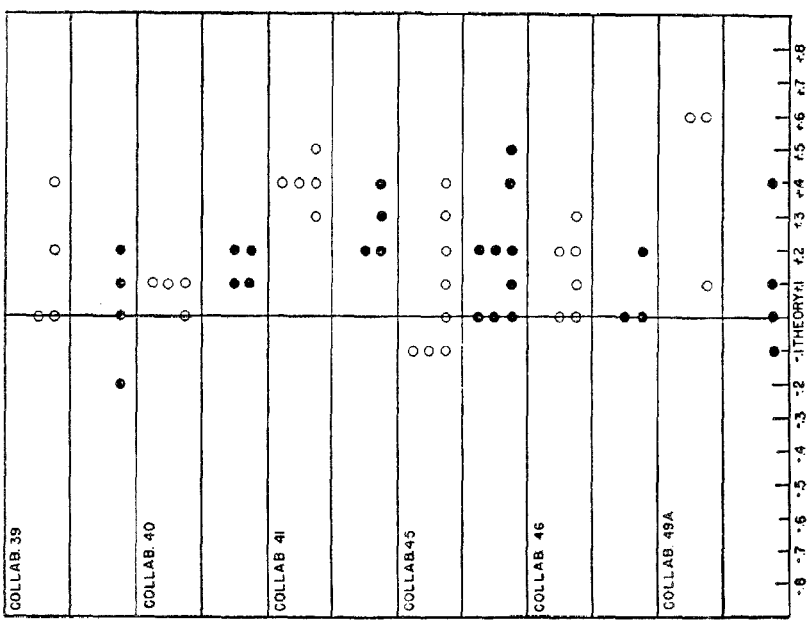

FIG. 15.—Continued.

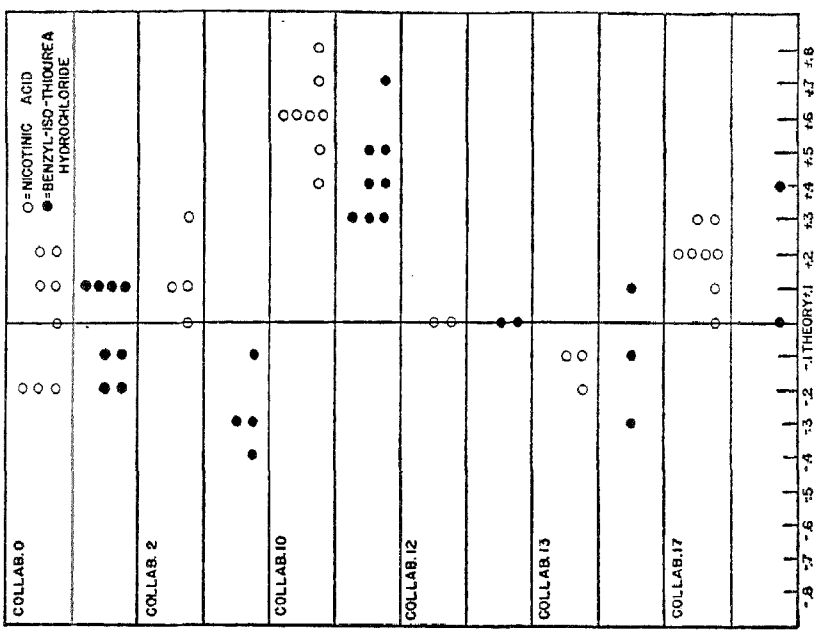

FIG. 16.—Distribution of hydrogen values from the different collaborators.

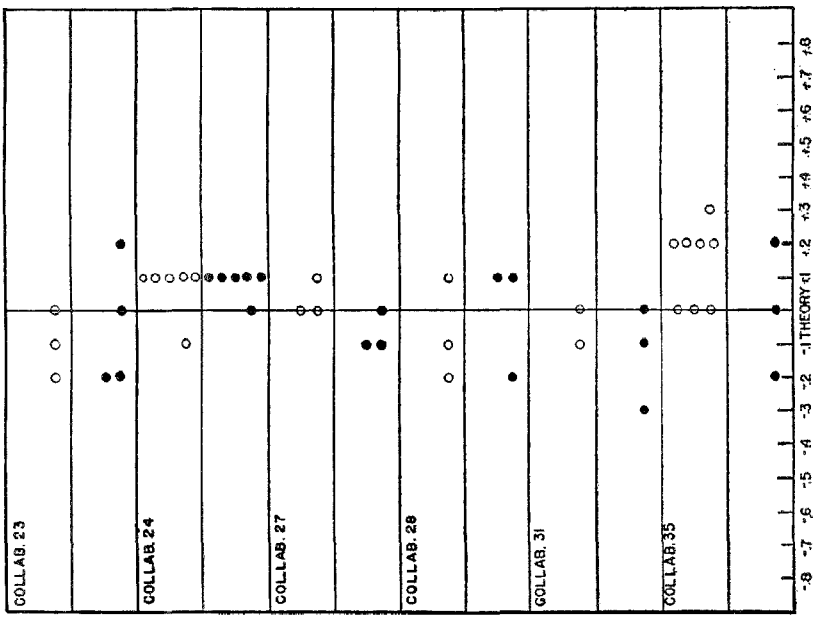

FIG. 16.—Continued.

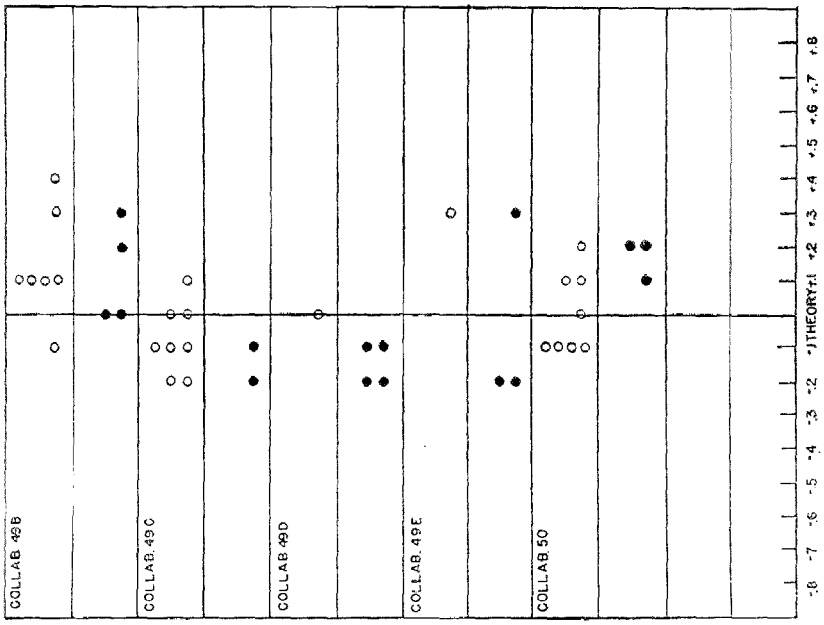

FIG. 16.—Continued.

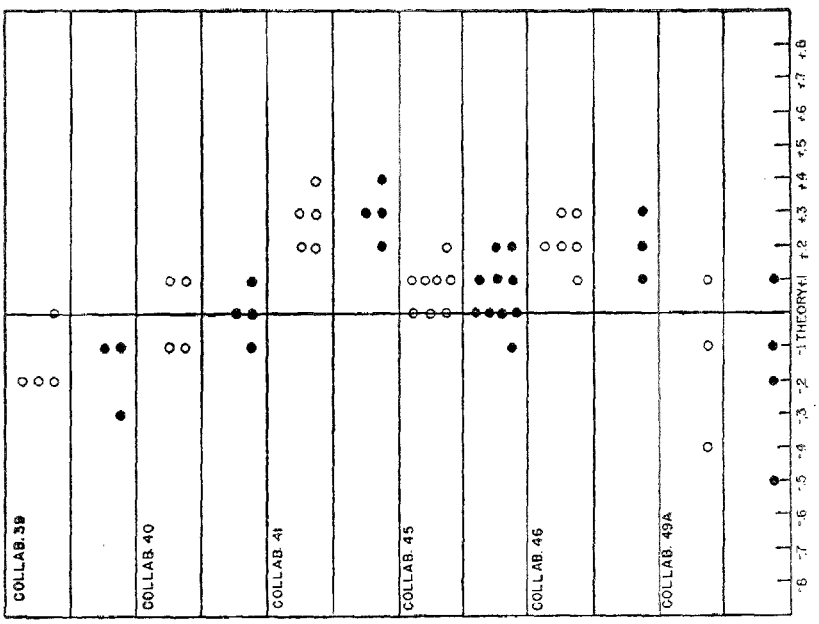

FIG. 16.—Continued.

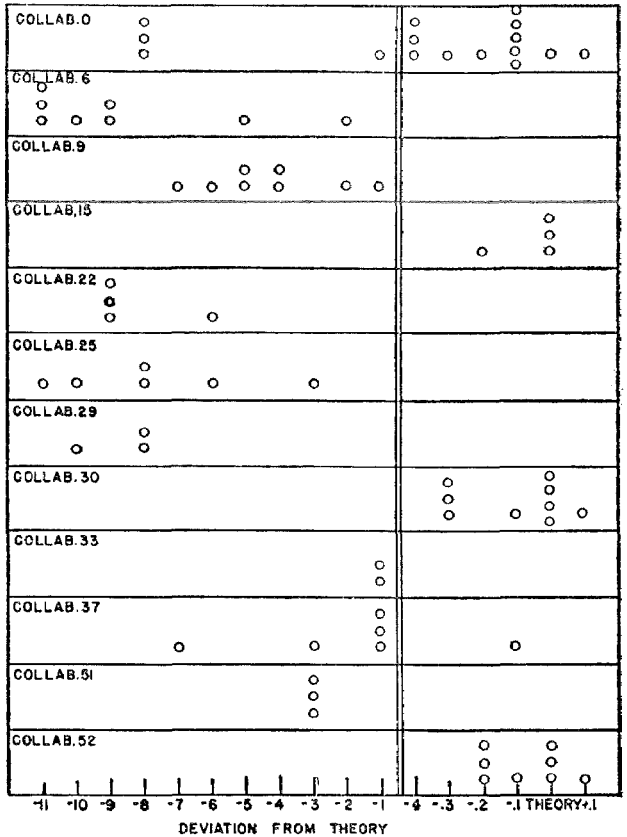

FIG. 17.—Distribution of Kjeldahl nitrogen values for nicotinic acid from the different collaborators.

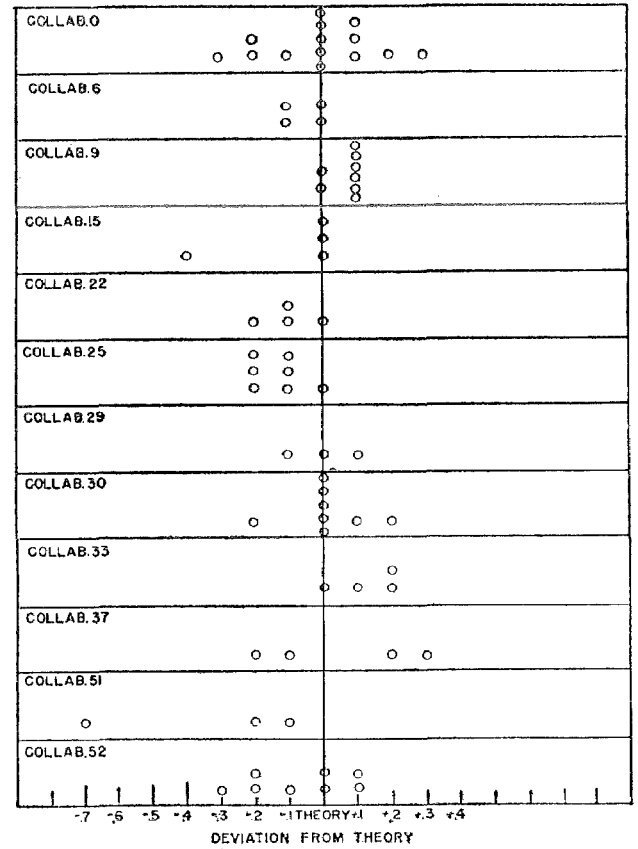

FIG. 18.—Distribution of Kjeldahl nitrogen values for benzyl-iso-thiourea hydrochloride from the different collaborators.

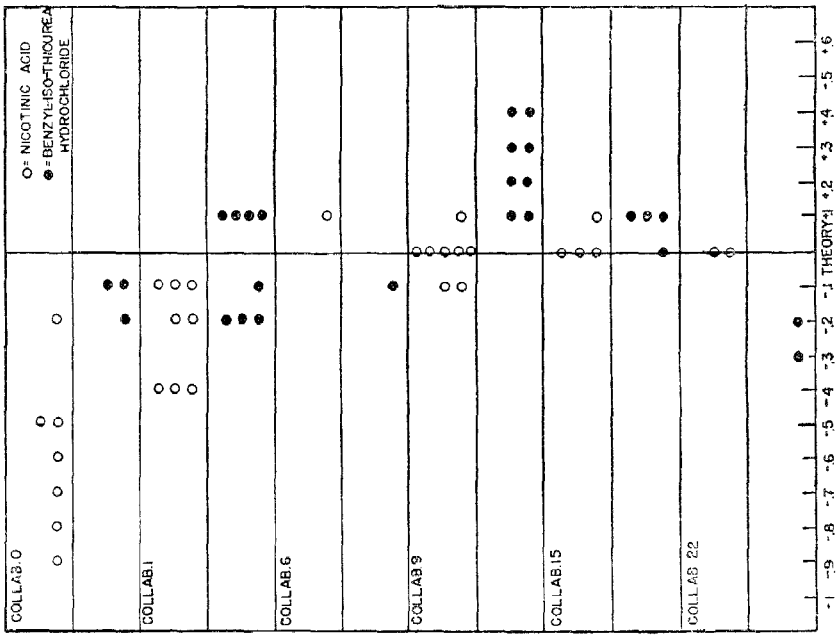

FIG. 19.—Distribution of Dumas nitrogen values from the different collaborators.

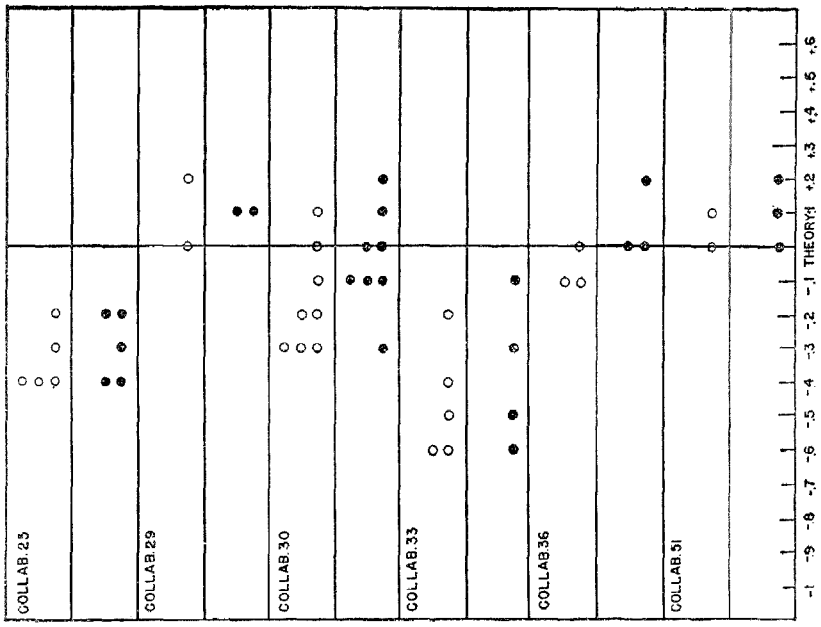

FIG. 19.—Continued.

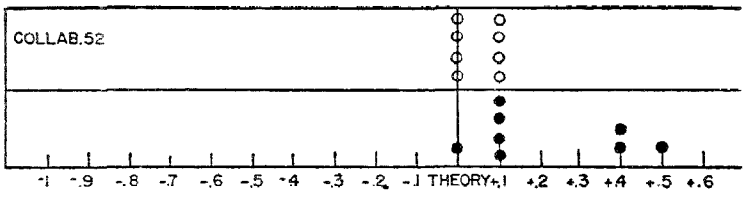

FIG. 19.—Continued.

The paper entitled "Kjeldahl Determination of Nitrogen in Refractory Materials," by C. O. Willits, M. R. Coe, and C. L. Ogg, is published in *This Journal*, **32**, 118 (1949).

REPORT ON STANDARD SOLUTIONS

By H. G. UNDERWOOD (Food and Drug Administration, Federal Security Agency, Chicago, Ill.), *Referee*

RECOMMENDATIONS*

Titanium Trichloride Solutions.—A report was received covering collaborative study of the three procedures of standardization reported on last year. The Associate Referee recommends that Method II, titanium trichloride solutions, **21.37,** substituting potassium dichromate for potassium permanganate, be made official, first action. The Referee concurs.

Potassium Dichromate Solutions.—The Associate Referee compared the oxidemetric strength of five commercial potassium dichromates with the Bureau of Standards product. He concludes that the findings may justify the preparation of standard solutions from assayed stocks of commercial potassium dichromate rather than from laboratory recrystallized potassium dichromate. The Referee suggests that the Associate Referee consider at least one recrystallization to insure uniformity. It is recommended that the subject be continued.

Standard Buffer Solutions.—No report was received. It is recommended that the subject be continued.

Hydrochloric Acid Solutions.—The method for the standardization of hydrochloric acid with standard sodium hydroxide was adopted as official, first action, at the 1938 meeting of the Association. It is recommended that the method be adopted as official, final action.

Sulfuric Acid Solutions.—The method for the standardization of sulfuric acid by the standard borax method was adopted as tentative at the 1939 meeting of the Association. Although the method was studied

* For report of Subcommittee A and action of the Association, see *This Journal*, **32**, 46 (1949).

collaboratively in 1940, and the Associate Referee recommended that the method be adopted as official, first action, the Referee failed to discuss this recommendation in his report. It is recommended that the method be adopted as official, first action.

REPORT ON STANDARD POTASSIUM DICHROMATE SOLUTIONS

By GEORGE MCCLELLAN (Food and Drug Administration, Federal Security Agency, New Orleans, La.), *Associate Referee*

The Sixth Edition of *Methods of Analysis, A.O.A.C.*, (*43.29*, p. 810) specifies that tenth-normal solution of sodium thiosulfate be standardized against potassium dichromate that has been thrice recrystallized and dried at 200°C. Since publication of the Sixth Edition, the Bureau of Standards has made available a standard preparation of potassium dichromate.

A study was undertaken to determine how many recrystallizations of analytical grade commercial potassium dichromate would be necessary to bring it to a strength equal to that of the Bureau of Standards product. Portions of five different brands of analytical grade potassium dichromate were secured from various government laboratories in the New Orleans Custom House. Each portion was dried for one hour in a weighing bottle at 100 degrees C. The Bureau of Standards potassium dichromate to be used for comparison was dried at 200°C. for two hours. A solution of approximately 0.1 *N* sodium thiosulfate was made up according to sec. 43.28 of *Methods of Analysis*. Its normality was then ascertained to five places by titration against the freshly dried Bureau of Standards potassium dichromate. Naturally, it is impossible to determine normality to five places using an ordinary 50-ml straight tube buret and titrating a 0.20 to 0.23 g portion of potassium dichromate as directed in 43.29. A far more sensitive standardization was devised as follows:

PROCEDURE

Weigh into a glass-stoppered 500-ml Erlenmeyer flask enough $K_2Cr_2O_7$ to have a titre of between 100.5 and 102.0 ml of the standard thiosulfate (0.4929–0.5002 g for a 0.1 *N* soln). Completely dissolve in 100 ml of H_2O. Add 4.0 g KI and swirl until dissolved. Add 4.0 ml of HCl, stopper flask, mix by swirling, and allow to set in the dark for 10 min. Cool flask for about a min. in ice-water. While constantly swirling flask, add with a 100-ml pipet exactly 100 ml of the standard thiosulfate. Add 5 ml of 0.5% starch soln, and complete the titration with more of the standard thiosulfate added from a 10-ml micro-buret. End point is from a bluish green to a clear green. The change takes place within 0.01 ml. Record titre to the nearest hundredth of a ml.

EXPERIMENTAL

Three standardizations of the thiosulfate against the Bureau of Standards potassium dichromate gave normalities of 0.10439, 0.10438, and

0.10438, respectively. A value of 0.10438 was taken as the true normality of the solution, and the Bureau of Standards product was taken to be 100.00% pure potassium dichromate. This same thiosulfate solution was then standardized in the same manner against each of the five commercial brands of dichromate being tested. Purity of each commercial dichromate was calculated as follows:

$$\frac{\text{Normality of } Na_2S_2O_3 \text{ by B. of S. } K_2Cr_2O_7 \times 100}{\text{Normality of } Na_2S_2O_3 \text{ by } K_2Cr_2O_7 \text{ being tested}} \text{ equals \% purity of the } K_2Cr_2O_7 \text{ being tested.}$$

Results were as shown below:

BRAND[1] $K_2Cr_2O_7$	DETN. NO.	GRAMS $K_2Cr_2O_7$ TITRATED	TITRE IN ML STD. $Na_2S_2O_3$	APPARENT NORMALITY OF $Na_2S_2O_3$	PURITY OF $K_2Cr_2O_7$ AS COMPARED WITH B. OF S. PRODUCT
					per cent
B of S	1	0.5212	101.81	0.10439	—
	2	0.5182	101.23	0.10438	—
	3	0.5165	100.90	0.10438	—
A	1	0.5175	101.10	0.10438	100.00
	2	0.5172	101.04	0.10438	100.00
B	1	0.5165	100.84	0.10444	99.94
	2	0.5174	100.98	0.10448	99.90
C	1	0.5173	101.10	0.10434	100.04
	2	0.5164	100.88	0.10438	100.00
	3	0.5171	101.01	0.10439	99.99
D	1	0.5172	101.08	0.10434	100.04
	2	0.5170	101.01	0.10437	100.01
E	1	0.5170	100.98	0.10440	99.98
	2	0.5170	100.98	0.10440	99.98

[1] Brands A, B, C, D, and E are Baker, Conray, Elk, Gennert, and Mallinckrodt, although not in that order.

CONCLUSIONS

It appears that four out of five of the commercial dichromates tested are already so pure that any improvement by recrystallization could probably not be determined by volumetric assay. Even the comparatively impure "Brand B" has an effective strength of 99.90 per cent or better.

The procedure outlined above can be used to determine the oxidimetric strength of any given batch of potassium dichromate as compared with that of Bureau of Standards potassium dichromate. From the data given, it appears that the analytical error will not exceed one part in two thousand.

RECOMMENDATIONS*

It is recommended that the procedure given above be utilized in a collaborative study to determine the oxidimetric strengths of two or more commercial "analytical grade" potassium dichromates in terms of the Bureau of Standards product. If collaborators agree within 0.05 per cent, then it will later be recommended that a procedure for assaying stocks of laboratory potassium dichromate, rather than a routine procedure of recrystallization, be incorporated in the *Methods of Analysis*.

REPORT ON STANDARDIZATION OF TITANIUM TRICHLORIDE

By JUANITA E. BREIT (Food and Drug Administration, Cincinnati, Ohio), *Associate Referee*

Methods for the preparation of solutions and for standardizing titanium trichloride have been previously described.[1] Briefly, Method I is the A.O.A.C. method using potassium permanganate, ferrous ammonium sulfate, and ammonium thiocyanate; Method II is the same, but substituting potassium dichromate for potassium permanganate; Method III uses potassium dichromate and diphenylamine indicator.

In view of the unstable nature of titanium trichloride, it was considered inadvisable to send out solutions of the chemical for standardization; instead, at each station doing collaborative work, one chemist prepared the titanium solution which was then standardized by all collaborators, each using the three methods as outlined, and each preparing his own potassium permanganate and potassium dichromate solutions.

LIST OF COLLABORATORS

Station 1—Food and Drug Administration, Color Certification Section, Washington, D. C.

Alice B. Caemmerer; Meyer Dolinsky; S. S. Forrest; Nathan Gordon; Charles Graichen; L. S. Harrow.

Station 2—Food and Drug Administration, Chicago, Ill.

Daniel Banes; Harold F. O'Keefe; Robert Stanley.

COMMENTS OF COLLABORATORS

1. Method III gave decidedly higher results than the others. The results are also more erratic. Methods I and II give better checks.

2. Method III gives a higher titre than the other two and the end point requires some patience to ascertain accurately; otherwise it is no more erratic than potassium permanganate.

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* For report of Subcommittee A and action of the Association, see *This Journal*, 32, 46 (1949).

[1] *This Journal*, 31, 573 (1948).

TABLE 1.—*Results of Collaborative Work—Stations 1 and 2*

COLLABORATOR	METHOD I		METHOD II		METHOD III	
	NORMALITY OF $TiCl_3$	AVE.	NORMALITY OF $TiCl_3$	AVE.	NORMALITY OF $TiCl_3$	AVE.
Station 1						
1	0.0941		0.0943		0.0948	
1	0.0941		0.0941		0.0950	
1	0.0940	0.0941	0.0939	0.0941	0.0955	0.0951
2	0.0946		0.0946		0.0955	
2	0.0950		0.0946		0.0955	
2	0.0943	0.0946	0.0941	0.0944	0.0952	0.0954
3	0.0941		0.0942		0.0949	
3	0.0941		0.0942		0.0950	
3	0.0939	0.0940	0.0942	0.0942	0.0949	0.0949
4	0.0937		0.0942		0.0948	
4	0.0938		0.0947		0.0948	
4	0.0942	0.0939	0.0943	0.0944	0.0947	0.0948
5	0.0944		0.0949		0.0952	
5	0.0942		0.0943		0.0948	
5	0.0941	0.0942	0.0945	0.0946	0.0948	0.0949
6	0.0937		0.0940		0.0957	
6	0.0940		0.0939		0.0957	
6	0.0938	0.0938	0.0937	0.0939	0.0963	0.0959
Average	0.0941		0.0943		0.0952	
Range	0.0937–0.0950		0.0937–0.0949		0.0947–0.0963	
Standard Deviation	0.00026		0.00023		0.00038	
Station 2						
7	0.1233		0.1237		0.1238	
7	0.1232		0.1237		0.1240	
7	0.1232	0.1232	0.1237	0.1237	0.1239	0.1239
8	0.1240		0.1238		0.1239	
8	0.1238		0.1236		0.1239	
8	0.1239	0.1239	0.1238	0.1237	0.1240	0.1239
9	0.1238		0.1237		0.1238	
9	0.1240		0.1237		0.1239	
9	0.1241	0.1240	0.1237	0.1237	0.1238	0.1238
Average	0.1237		0.1237		0.1239	
Range	0.1232–0.1241		0.1236–0.1238		0.1238–0.1240	
Standard Deviation	0.00035		0		0	

3. If the sulfuric acid and water have any blanks due to dissolved oxygen it cannot be determined in Method III, as the indicator will give no color unless chro-

mate is added. Any correction for a blank in Method III would result in a higher normality and a wider deviation from the first two methods.

4. The end point in Method III is difficult to determine since the purple color returns. Method II appears to be preferable.

5. Method III appears to give somewhat higher values than the two standard methods. It has the advantage of not requiring a blank determination, otherwise it is essentially the same as Method II.

6. Method III affords some difficulty. The diphenylamine indicator doesn't give a permanent end point; it has a tendency to fade out. Somewhat higher results are indicated using the diphenylamine indicator.

7. Prefer potassium dichromate titration with ferrous ammonium sulfate indicator because of ease of handling and relative stability of the potassium dichromate. Diphenylamine indicator seems to shift the stoichiometric point and gives a higher titanium trichloride factor. Diphenylamine titration must be run slowly to eliminate possibility of indicator precipitation.

8. Prefer Methods II and III to I.

It is recommended*—

That Method II be adopted as official, first action.

No report was given on buffer solutions.

REPORT ON COSMETICS

By G. Robert Clark (Cosmetic Division, Food and Drug Administration, Federal Security Agency, Washington, D. C.), *Referee*

RECOMMENDATIONS†

The Referee recommends—

(1) That the following topics on which no reports were received be continued:

Cosmetic Creams
Deodorants and Anti-perspirants
Depilatories
Hair Dyes and Rinses
Moisture in Cosmetics

(2) That the following topics be discontinued:

Alkalies in Cuticle Removers

Mercury Salts in Cosmetics.—It is believed that the methods described or reported as being studied under the topic "Miscellaneous Drugs" can be applied without modification to cosmetics.

Hair Straighteners

The Referee concurs in the following recommendations of Associate Referees.

(1) That the topic "Mascaras, Eyebrow Pencils, and Eye Shadows" be continued.

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* For report of Subcommittee A and action of the Association, see *This Journal*, 32, 46 (1949).
† For report of Subcommittee B and action of the Association, see *This Journal*, 32, 50 (1949).

(2) That the topic "Cosmetic Skin Lotions" be continued.

(3) That the proposed methods for "Pyrogallol in Hair Dyes" be adopted as official, first action, and the topic closed.

(4) That the proposed methods for the "Analysis of Face Powder" be adopted as official, first action, with the following changes:

(a) That the methods be adopted individually, as methods for the various constituents of face powder rather than as an entire method for the analysis of face powder. This will provide for the addition of methods for other constituents, not included in the study to date, should the necessity for them appear.

(b) The proposed method for stearate be designated "Fats and Fatty Acids as Stearic Acid."

The Referee further recommends reassignment of this topic, since the present Associate Referee has so requested.

(5) That the topic "Sun Tan Preparations" be made the subject of study by an Associate Referee.

REPORT ON PYROGALLOL IN HAIR DYES

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

A colorimetric method for the determination of pyrogallol in liquid hair dyes has been adopted as tentative (1, 2). In a later report (3) the writer discussed some of the problems involved in the extraction of pyrogallol from henna mixtures and recommended that further work be done on the method.

The method as previously outlined involved (1) extracting the pyrogallol from henna mixtures with ethyl acetate and evaporating the extract to dryness; (2) taking up the residue with water, adding alumina cream and filtering; and, (3) determining the pyrogallol in the filtrate by the tentative colorimetric method. The main objection to this method was the high and variable blanks obtained with it from henna powder. Several variations of this method were discussed.

Some recent work has shown that the use of alumina cream in clarifying the aqueous solution of the extracted material affected the slope of the curve. Extracting the pyrogallol from the clarified filtrate with ether prior to its colorimetric determination served to reduce the blank materially and to eliminate the effect of alumina cream on the development of color.

In order to integrate this method with the tentative one for liquid dyes, it was desirable, and in some cases necessary, to rewrite portions of the latter. The method as finally submitted to collaborative study is given below:

PYROGALLOL IN HAIR DYES

Qualitative Test

Add 5–10 ml of sample to separatory funnel containing ca 0.5 g of $NaHSO_3$ and extract with 2 or 3 successive 30-ml volumes of ether. Filter ether extracts thru cotton and evaporate to dryness on steam bath. Dry in oven at 100°C for 30–60 min. Pulverize residue, mix well, and take melting point. If it does not melt between 131° and 134°C, sublime and again take melting point, which should fall within above range. Mix small portion of residue with equal quantity of sublimed pyrogallol and determine the melting point; it should not change.

Quantitative Determination

REAGENTS

Ferrous Tartrate Reagent.—Dissolve 1.00 g of sodium potassium tartrate (Rochelle salt) and 0.200 g of $FeSO_4 \cdot 7\ H_2O$ in water and dilute to 100 ml in a volumetric flask. PREPARE FRESH DAILY.

Sodium Acetate Solution.—Dissolve 15.00 g $NaC_2H_3O_2 \cdot 3H_2O$ in water, bring to room temp. and dilute to 100 ml.

Alumina Cream.—**34.19(b).**

Standardization.—To six 100 ml volumetric flasks add from buret 2.50, 5.00, 7.50, 10.00, 12.50, and 15.00 ml of standard pyrogallol solution (Reagent Grade, 0.2000 g/500 ml). Develop color as follows on not more than three standards at a time, and make readings within 15 min. after color is developed. Pipet into flasks 10 ml each of Na acetate soln and ferrous tartrate reagent, dilute to volume and mix. Using 1 cm or $\frac{1}{2}$ inch cells, measure optical density of solutions with photometer or spectrophotometer at 540 mμ. With neutral wedge filter photometer, filter designated No. 56 (5.8 mm of Corning didymium #512, 2.0 mm of Jena VG 3, 2.0 mm of Jena BG 18, and 4.5 mm of Corning yellow shade yellow #351) is more suitable than filter No. 54. With filter photometers obtain zero point by reading "blank" soln containing 10 ml of Na acetate and ferrous tartrate reagents in 100 ml. Draw standard curve, plotting concentration of pyrogallol against photometer readings, on large scale graph paper so that pyrogallol can be read to 0.01 mg. A straight line should be obtained between concentrations of 1 mg and 6 mg per 100 ml. With spectrophotometer use freshly prepared "blank" as reference soln. Draw standard curve as directed above, or, if straight line passing thru origin is obtained, the average value of k may be calculated from the formula

$$k = \frac{D}{c}$$

where k is extinction coefficient
D is measured optical density of solution
c is concentration of pyrogallol in mg per 100 ml

This value of k may be used to calculate the concentration of unknowns directly from optical density readings.

Liquid Dyes

Extract a convenient aliquot of sample (usually 10 ml are sufficient) by one of the following methods. In handling sample give it a minimum of exposure to air, as pyrogallol is readily oxidized.

1. *Continuous Extraction.*—Pipet sample aliquot into suitable continuous extractor containing ca 0.3 g of $NaHSO_3$. Extract with ether until pyrogallol is completely removed (3–7 hours, depending upon efficiency of extractor). Determine time

required for each extractor under a certain set of conditions by extracting an aqueous soln of known pyrogallol content or by testing for complete extraction as follows: After the extraction is thought to be complete, remove flask containing ether and replace it with one containing fresh volume of ether and continue extraction for 30–60 min. Treat this extract as directed below and, use 50 ml aliquot of filtrate to develop color. Evaporate ether extract on steam bath to volume of 8–10 ml and continue evaporation at temp. not exceeding 40° until odor of ether is completely gone. Dissolve residue in 20 ml of water and wash completely into 100 ml volumetric flask. Dilute to volume and mix. (If the liquid sample contained chlorophyll treat residue from ether extraction as described for residue obtained from evaporation of ethyl acetate extract in method for henna powder dyes, beginning, "Add ca 10 ml water to beaker and loosen the residue. . . . "). Filter thru dry paper and discard first 20 ml of filtrate. (If determination cannot be completed same day extraction is made, let ether extract stand overnight, preferably in refrigerator, before ether is evaporated. Do not let aqueous soln stand overnight.) Use suitable aliquots of filtrate to develop color as directed under "Standardization," beginning "Pipet into flasks. . . . " If 5 ml aliquot contains more than 6 mg of pyrogallol, make suitable dilution in volumetric flask and use aliquots of diluted soln to develop color. For final calculation use average of results obtained on at least two aliquots of different sizes, preferably containing between 2 mg and 5.5 mg of pyrogallol. Calculate to g/100 ml in original sample.

2. *Extraction in Separatory Funnels.*—Pipet sample into 125 ml separatory funnel containing ca 0.3 g of $NaHSO_3$ and extract 6 times with ether. For each extraction use volume of ether equal to 3 or 4 times volume of sample and shake vigorously for one min. Filter ether extracts successively thru cotton wet with ether. (Six extractions carefully made will completely remove the pyrogallol. If desired, a 7th may be made and used to test for complete extraction as described under "Continuous Extraction.") Evaporate the combined ether extracts as directed under "Continuous Extraction."

Henna Powder Mixture

Weigh 0.9 to 1.1 g of thoroughly mixed sample in paper extraction thimble. Cover sample with small piece of cotton and place thimble in Soxhlet extractor. If temperature and humidity conditions are such that water will condense on condenser, connect tube containing drying agent to outlet of condenser. Extract 5 hours with ethyl acetate having minimum purity of 99% (N.F. VIII or better quality). Boil at such rate that solvent siphons off 15 to 20 times per hour. If ethyl acetate extract is clear, evaporate to dryness as directed below. If extract contains any sediment, evaporate to ca 75 ml if necessary, cool to room temperature, and completely transfer to 110 ml glass-stoppered volumetric flask. Dilute to volume and mix. Filter thru dry paper, taking precautions to prevent evaporation of solvent. Pipet 100 ml of filtrate into 250 ml beaker and evaporate to volume of ca 5 ml on hot plate or steam bath. Continue evaporation to complete dryness at temperature not exceeding 40°. Add 10 ml water and loosen residue with stirring rod. Pour into 50 ml volumetric flask. Rinse beaker 4 or 5 times with small volumes of water and add rinsings to flask. Add 1.2 ml alumina cream, dilute to volume, mix and filter thru dry paper. Extract 25 ml by one of methods given under "Liquid Dyes." (If extraction cannot be started immediately add ca 0.4 g of $NaHSO_3$ to filtrate and hold no longer than overnight.) Calculate to per cent pyrogallol in original sample.

EXPERIMENTAL

Recovery Experiments.—Approximately one gram samples of henna were weighed in paper extraction thimbles, and accurately weighed

quantities of pyrogallol ranging from about 40 to 150 milligrams were added to the thimbles. Three different lots of henna were used. By preparing the samples in this manner, the problem involving oxidation of pyrogallol when mixed with henna powder was entirely avoided. The results are given in Table 1. On thirteen determinations an average recovery of 99.3 per cent was obtained, with a range from 97.9 to 100.2 per cent.

TABLE 1.—*Recovery experiments*

PYROGALLOL ADDED	PYROGALLOL RECOVERED	
milligrams	*milligrams*	*per cent*
42.0	41.3	98.3
47.8	47.1	98.5
56.0	55.7	99.5
53.1	53.2	100.2
60.7	60.4	99.5
50.2	49.9	99.4
65.0	65.1	100.2
106.0	104.4	98.5
149.3	148.7	99.6
52.7	51.6	97.9
72.0	71.7	99.6
63.4*	63.5	100.2
123.8*	122.9	99.3
	Average.....	99.3

* No henna powder was added to these two samples.

Blanks.—Eight blank determinations were run on the three lots of henna used in the above recovery experiments. These results ranged from 0.073 to 0.118 per cent, with an average of 0.107 per cent, calculated as pyrogallol. Theoretically, with a blank of this magnitude, the recoveries listed in Table 1 should range from 0.7 to 2.5 per cent high. Since there was no tendency to get high results, either there must be a compensating loss of pyrogallol, or the presence of pyrogallol alters the blank values of the henna powder.

Storage Experiments.—Pyrogallol recoveries obtained on samples after 12 to 38 months' storage are given in Table 2. Filter cel was used for two of the samples in order to compare the stability of pyrogallol when mixed with an inert material with its stability in henna mixtures. Lawsone, 2-hydroxy-1, 4-naphthoquinone, the active constituent of henna for hair-dyeing purposes (4), was added to two of the samples to see what effect, if any, it would have on the rate of oxidation of pyrogallol. In both cases the filter cel samples (II and IV) showed about five times as much loss of pyrogallol as the corresponding henna samples (I and III). The loss of pyrogallol in the two samples containing lawsone (I and II) was considerably less than that in the corresponding samples with no added law-

sone (III and IV). Continuing the ethyl acetate extraction of the samples for two to three hours beyond the five-hour period designated in the method resulted in the recovery of no more pyrogallol.

TABLE 2.—*Storage experiments*

SAMPLE NO.	COMPOSITION OF SAMPLES	APPROX. AGE WHEN ANALYZED	PYROGALLOL FOUND	RECOVERY
		months	*per cent*	*per cent*
I	Egyptian Henna*	13	4.93	89.5
	Lawsone 1.0%			
	Pyrogallol 5.51%			
II	Filter cel	13	2.25	40.5
	Lawsone 1.0%			
	Pyrogallol 5.55%			
III	Egyptian Henna	13	4.66	84.1
	Pyrogallol 5.54%			
IV	Filter cel	13	1.57	28.4
	Pyrogallol 5.52%			
V	Bronzing Henna*	12	4.91	88.8
	Pyrogallol 5.53%		4.85	87.7
VI	Egyptian Henna	12	4.82	87.3
	Copper Sulfate 5%			
	Burnt Sienna 4%			
	Pyrogallol 5.52%			
5	Henna†	38	3.97	75.8
	Pyrogallol 5.24%			

* These two lots of henna were purchased from a national distributor of beauty shop supplies. A representative of the firm stated that both were Egyptian henna, but that the bronzing henna had been treated so that it would color the hair a different shade. The method of treatment was not known.
† This lot was purchased from another distributor and was labeled, "Egyptian Henna."

An absorption curve obtained with a Beckman spectrophotometer was similar to that of Mattil and Filer (5) for the color produced by gallic acid and the ferrous tartrate reagent. The curve had a very broad absorption band with a maximum at 540 millimicrons. However, with a neutral wedge photometer (6), filter No. 56 was superior to No. 54. The No. 56 filter gave greater absorption values, and the field was brighter and was easier to match than with filter No. 54. The measurements reported here were made with the spectrophotometer using a spectral band width of about 1.3 millimicrons.

COLLABORATIVE WORK

Three samples for collaborative study were prepared as follows:

No. 1

Pyrogallol	9.36 per cent
Burnt sienna	4.5 per cent
Copper sulfate	5.2 per cent

Egyptian Henna

No. 2

Pyrogallol	5.14 per cent

Bronzing Henna

No. 3

Pyrogallol	20.500 grams
Sodium bisulfite	10 grams

Distilled water to make one liter.

All ingredients for samples 1 and 2 were ground to pass a 60-mesh sieve. The samples were mixed in large jars on a revolving mixer for about ten hours. Each sample was divided into ten parts, and each part was placed in a small bottle. The writer analyzed portions from two bottles selected at random from both of the samples before they were sent out for collaborative study. The collaborators were asked to analyze the samples in

TABLE 3.—*Collaborators' results*

ANALYST	DATE ANALYZED	SAMPLE 1		SAMPLE 2		SAMPLE 3	
		PYROGALLOL FOUND	RECOVERY	PYROGALLOL FOUND	RECOVERY	PYROGALLOL FOUND	RECOVERY
		per cent	*per cent*	*per cent*	*per cent*	*g/100 ml*	*per cent*
C. R. Joiner	3/11/48	9.43	100.8	5.16	100.4	2.02	98.5
		9.36	100.0	5.19	101.0	2.02	98.5
1	4/ 1/48	9.30	99.4	5.08	98.8	2.00	97.6
		9.26	98.9	5.11	99.4	2.01	98.0
2	4/ 5/48	9.24	98.7	5.12	99.6	2.11	102.9
		9.26	98.9	5.09	99.0	2.11	102.9
						2.10	102.4
						2.11	102.9
3	4/13/48	9.13	97.5	4.89	95.1	2.09	102.0
		9.09	97.1	5.00	97.3	2.07	101.0
4	4/18/48	9.03	96.5	5.11	99.4	2.00	97.6
		9.04	96.6	5.00	97.3	2.01	98.0
5	5/11/48	8.94*	95.5	4.74*	92.2	2.00	97.6
		8.96*	95.7	4.86*	94.6	2.00	97.6
C. R. Joiner	5/19/48	8.63*	92.2	4.69*	91.2	—	—
C. R. Joiner	7/12/48	7.98*	85.3	4.47*	87.0	—	—
Averages		9.21	98.4	5.08	98.8	2.05	100.0
Standard Deviations		±0.13%		±0.084%		±0.046 g	
Range		0.40%		0.30%		0.11 g/100 ml	

* These results are not included in the averages. See explanation under "Discussion."

duplicate within a month if possible. The results are listed in Table 3. Samples 1 and 2 are similar in composition to commercial henna dyes. Samples similar to No. 3 have been subjected to collaborative study twice before, while this is the first such study for henna samples.

DISCUSSION

No significant comments were made by the collaborators. Apparently none of them had any difficulty in following the directions.

The results obtained by the collaborators are good when consideration is given to the nature of pyrogallol and the difficulties involved in isolating it from plant material. The recoveries on sample No. 1 show a steady decrease with increasing age of sample, and those on sample No. 2, with two exceptions, do the same. The samples were about five and one-half weeks old when analyst 4 made his determinations, and they were nine weeks old when analyst 5 made his. The determinations made by the Associate Referee one week later furnish ample justification for not including the results of analyst 5 in the averages used for evaluating the method. The apparent rate of loss of pyrogallol from these samples is considerably greater than that in any of the henna samples reported in Table 2. From these considerations it is probable that if all determinations had been made within two or three weeks, the results would have been better.

The discussion regarding loss of pyrogallol on aging does not apply to sample 3. The results obtained on this sample are approximately the same as those reported for similar samples in the past collaborative studies.

RECOMMENDATIONS*

It is recommended that the method presented in this report be adopted as official, first action, and that work on this subject be discontinued.

ACKNOWLEDGMENT

The writer wishes to express his appreciation to the following staff members of the field laboratories of the Food and Drug Administration who took part in this work as collaborators: S. H. Perlmutter and Gloria Getchell, Minneapolis, Minnesota; H. C. Van Dame, Cincinnati, Ohio; H. W. Conroy, Kansas City, Missouri; D. Banes, Chicago, Illinois; and F. M. Garfield, St. Louis, Missouri.

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* For report of Subcommittee B and action of the Association, see *This Journal*, **32**, 50 (1949).

REPORT ON MASCARAS, EYEBROW PENCILS, AND EYE SHADOWS

By PAUL W. JEWEL (Max Factor & Co., 1666 North Highland Avenue, Hollywood, Calif.), *Associate Referee*

II. THE ANALYSIS OF MASCARA

A method for the analysis of mascara reported earlier[1] has been improved to such an extent as to make its submission for collaborative study desirable. Some changes in procedure have been made but the experimental material has not been changed, since this type of mascara is rather universally used in the cosmetic industry.

THE METHOD

Total Base

Weigh sample of mascara, ca 3.0000 g, wrap in filter paper (Munktell 00) turning ends in so as to make a seal, and tie with thread. Place this wrapped sample in a double thickness extraction thimble, plug opening with cotton, and extract with chloroform in a Soxhlet Extractor at such a rate that the siphon dumps about once every 10 min. Continue extraction for 8 hours. When the extraction has been completed, evaporate solvent on steam bath, dry in oven for 30 min., cool, and weigh. Repeat the drying in the oven until all odor of chloroform has been removed and weight remains constant. Report result as per cent total base. (When most of chloroform has been removed by evaporation, it will be found that the addition of a few ml of absolute alcohol will facilitate removal of the last traces.)

Triethanolamine

To total base contained in extraction flask, add 10 ml of 95% ethyl alcohol, and heat until base is dissolved or dispersed. Add 10.00 ml of 0.50 *N* sulfuric acid, heat to boiling, add 50 ml distilled water, heat until fats melt, and chill thoroly in the ice box. When mixture is thoroly chilled, filter thru paper into titration flask. Wash waxes several times with water and titrate filtrate with 0.50 *N* aqueous sodium hydroxide using methyl red as an indicator. Divide the net titration expressed as ml of *N* acid by 7.0 to get g of triethanolamine found. Express results in terms of per cent of triethanolamine in original sample.

Total Acid as Stearic Acid

Return filter paper from the determination of triethanolamine to the extraction flask, add 50 ml absolute alcohol, heat to dissolve and titrate with alcoholic potassium hydroxide 0.50 *N*, using phenolphthalein as indicator. Calculate total acid found as stearic acid, using 208 as the acid number for stearic acid, and report the result as per cent stearic acid.

In order to test the reliability of this method in the hands of other chemists, it was submitted to collaborative study. Samples were sent to six laboratories, five of which responded. Table 1 gives the results obtained by these collaborators.

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[1] "I. The Analysis of Mascara" was published in *This Journal*, **29**, 32 (1946).

TABLE 1.—*Collaborative results*

COLLABORATOR	TOTAL BASE	TRIETHANOLAMINE	TOTAL ACID AS STEARIC ACID
	per cent	*per cent*	*per cent*
1	73.50	11.41	24.60
2	72.20	11.70	24.30
3	72.64	10.65	18.15
4	73.55	12.50	26.25
5	73.53	11.12	24.80
Av.	73.08	11.48	23.62
Theory	75.09	11.99	31.04

COMMENTS OF COLLABORATORS

#3. "It would appear that eight hours is not sufficient for complete extraction of the mascara base."

This collaborator then proceeded to continue the extraction for 10 and 12 hour periods. The results of this additional extraction appear in Table 2, together with a 24 hr. extraction made by the Associate Referee.

TABLE 2.—*Additional extraction*

	TOTAL BASE	TRIETHANOLAMINE	TOTAL ACID AS STEARIC ACID
	per cent	*per cent*	*per cent*
10 hours	73.54	11.22	24.68
12 "	74.05	11.79	26.54
24 "	73.94	11.26	26.50

The results for total base and for triethanolamine are not too precise from an analytical standpoint, but for mixtures of this sort they are fairly good. The results for total acid are too far from correct and indicate that some further treatment of the extracted base may be necessary. The time of extraction is important, and in order to make sure that this will be complete under any and all conditions, it is recommended that the extraction time be extended to 24 hours.

It is difficult to explain the lack of precision in results obtained by collaborators. The base, before pigments are added, is completely soluble in warm chloroform. The method described gives theoretical results for triethanolamine and for total acid when applied to the base. It is only after the base has been heated and milled with bone black that the method begins to give less than theoretical results.

It is recommended* that further work be done to improve the accuracy of this method, and that work be continued with eyebrow pencils and eye shadows.

* For report of Subcommittee B and action of the Association, see *This Journal*, 32, 50 (1949).

The Associate Referee wishes to acknowledge the kind cooperation of the following collaborators: H. Kohnstamm & Co., W. C. Bainbridge; Kolmar Laboratories, H. Heinrich; Avon Allied Products, Inc., J. M. Williams; Max Factor & Co., London, Frank Atkins; Max Factor & Co., John Hall.

REPORT ON COSMETIC POWDERS

By GEORGE MCCLELLAN (Food and Drug Administration, Federal Security Agency, New Orleans, La.), *Associate Referee*

A face powder mixture of known constitution was subjected to collaborative study. The method of analysis specified was essentially that published by the author in *This Journal*, **25**, 909 (1942), but with so many revisions that, for purposes of clarity, complete reproduction seems necessary.

CONSTITUENTS OF FACE POWDER

Fats and Fatty Acids as Stearic Acid

Weigh about 2 g of the powder into glass-stoppered 250-ml Erlenmeyer flask. Add 30 ml of benzene and swirl to mix thoroly. Add 10 ml of HCl, and swirl, removing stopper frequently to allow escape of CO_2 from carbonates. When pressure has spent itself, add 50 ml of petroleum ether, and shake cautiously with periodic removal of stopper until pressure again subsides. Then shake vigorously about 50 times. Decant ether layer thru a pledget of cotton into flask containing a few glass beads, that has been weighed with a similar flask as a counterpoise. (This decantation involves no danger of loss, for the particles of powder are tenaciously retained in the acid layer.) Again add 50 ml of petroleum ether and repeat shaking and decantation. Repeat with a third 50-ml portion of petroleum ether. Evaporate to dryness on steam bath under hood. Place in a draft oven at 100 degrees C. for one hour, heating flask used as a counterpoise at the same time. Remove flasks, cool, and weigh as stearic acid.

Total Zinc

REAGENTS

(a) *Wulfing's precipitant.*—Dissolve 80 g of finely ground ammonium carbonate in a mixture of 90 ml of NH_4OH and 375 ml of H_2O, and add 475 ml of 95% ethyl alcohol, which may or may not cause precipitation, depending on the temperature. Let any precipitate settle, and use supernatant soln.

(b) *Wash soln.*—Mix equal volumes of Wulfing's precipitant and 95% ethyl alcohol.

DETERMINATION

Weigh ca 2 g of the powder into a platinum dish and ignite to light gray ash at 600–650°C. Do not heat longer than necessary. With the aid of a wide-mouth funnel, transfer ash to a 500-ml glass-stoppered Erlenmeyer flask. Add 100 ml of Wulfing's precipitant in such manner as to wash down funnel. Stopper flask, and shake vigorously for 1 min., pausing from time to time to remove stopper and relieve pressure. Let sit overnight. Filter contents thru 12½ cm medium quantitative paper. With wash soln from a wash bottle, wash out flask, pouring washings thru filter; but make no attempt completely to transfer residue. Reserve flask for later deter-

mination of acid-soluble constituents. Wash residue on paper thoroly with wash soln. Determine zinc in filtrate as follows: Exactly neutralize to methyl red with HCl, add 200 ml of H_2O, and bring nearly to boiling on hot plate. Add 60 ml of 10% $(NH_4)_2HPO_4$, and continue to heat at just below boiling for 30 min. Remove and allow to cool slowly to room temp. Filter thru Gooch crucible that has been tared after ignition for 10 min. at full heat of Fisher burner. Wash with freshly prepared 1% soln of $(NH_4)_2HPO_4$, and finally with 50 ml of 50% alcohol. Discard filtrate. Place Gooch crucible in porcelain crucible of suitable size, and dry over low flame. Increase temp. and ignite at full heat to constant weight.

$Zn_2P_2O_7 \times 0.534$ equals ZnO.

(Talcum powders often contain boric acid. When present, it will accompany ZnO, and must be separated before precipitation of the zinc.)

Acid-Soluble Calcium

Place paper containing residue from the zinc separation in a platinum dish and burn off paper at below 650°C. Transfer to a 250-ml beaker. Use 100 ml of (1+9) HCl to wash residue out of flask used in zinc separation, adding washings to beaker. (If some residue still clings to inside of flask, tilt up at steep angle over beaker, and wash out with stream of water from wash bottle.) Stir thoroly, allow to sit for 10 min. and filter thru medium quantitative paper. Disregard turbidity in filtrate, since this will be recovered in the next step. Wash residue on paper three times with distilled water. Place in a platinum dish not less than 6 cm in diam. nor less than 2 cm high, and hold pending addition of recovered acid-soluble Fe, Al, and $BaSO_4$. With NH_4OH, nearly neutralize the filtrate to methyl red. Add 200 mg of $(NH_4)_2SO_4$ and enough Br_2 water to destroy indicator and distinctly color soln. Boil free of bromine, add more methyl red, and while still nearly boiling add NH_4OH dropwise to the first distinct yellow, avoiding any excess. Let sit for ca 3 min., and filter thru a medium quantitative paper. Wash with hot 2% soln of NH_4Cl. Transfer paper and residue to the platinum dish containing acid-insoluble constituents. Determine calcium in filtrate according to **6.48**, beginning with "heat to boiling. . . . "

Acid-Soluble Magnesium

Determine in filtrate from acid-soluble calcium as directed in **37.64** (p. 642).

$Mg_2P_2O_7 \times 0.3621$ equals MgO

Decomposition of Silicates; Solution of Titanium; Estimation of Barium Sulfate

APPARATUS

Air bath.—On a tripod over Fisher burner place clay triangle having a per side length of about 3 inches. In this triangle set a nickel or iron crucible of about 125 ml capacity, and on top of the crucible set a second clay triangle having a per side length of about 2½ inches. Purpose is to supply controlled radiated heat to platinum dish resting on the top triangle.

METHOD

Ash residues reserved in platinum dish (acid-insoluble portion and materials recovered prior to calcium precipitation) at below 650°C. Pulverize ash with flattened glass rod, and moisten with 4 ml of water. Add 4 ml of H_2SO_4, place under a hood, and fill dish to ca one-fourth of its depth with HF (48%). Evaporate on the air bath, swirling occasionally to mix contents, until only the H_2SO_4 appears to remain; then cautiously heat over the low flame of a Fisher burner to a pasty consistency. (Do not take to complete dryness.) Add 15 g of pulverized potassium pyrosulfate, and heat to melting. Continue heating, gradually raising temp. until a clear melt is ob-

tained. This will be achieved only when the dish glows red-hot and the melt orange-red. Too rapid heating will cause spattering. Foaming will occur but is not to be feared. At the completion of fusion, the clarity of the melt may be marred by bubbles and possibly by a few flakes of K_2SO_4 produced by the high temp., but these may be disregarded if the melt is generally clear. Set dish aside on an asbestos board and allow to cool. Melt will normally crack away from dish during cooling. Dislodge melt into a 600-ml beaker, wash dish with successive portions of hot (1+19) H_2SO_4 until a volume of about 150 ml is obtained, and boil until the melt goes into soln. If present, $BaSO_4$ comes down at this point. In this event, let digest on a steam bath for one hour, allow to cool, dilute to about 400 ml, stir well, and allow to sit for at least two more hours. Filter thru the finest available quantitative paper, catching filtrate in a 500-ml volumetric flask. Wash thoroly three times with water. Transfer residue to a tared porcelain crucible, burn off paper at low temp., and ignite at dull red heat. Weigh as $BaSO_4$. (Residues amounting to less than 0.5% should not be counted as $BaSO_4$. Where they occur, they represent HF-resistant silicate or quartz originally present in the talc or kaolin.)

Total Titanium and Iron

APPARATUS

Jones reductor.—Take a 50-ml pinchcock buret (without pinchcock attachment), and with a long glass rod ram down into its constricted lower end a pledget of glass wool. Fill buret to about the 15 ml mark with 20- or 30-mesh amalgamated Zn. (Zn may be amalgamated by letting fall into 200 ml of H_2O containing 4 g of dissolved $HgCl_2$ and 10 ml of H_2SO_4. It should be washed several times with distilled H_2O by decantation before being put into buret.) Fit constricted lower end of buret with a 4-inch piece of thick-walled rubber tubing bearing a screw-clamp about the middle and terminating in a glass tube thrust thru a one-hole #7 rubber stopper. The stopper should be fitted to a 500-ml vacuum flask and the glass tube should be of such length as to reach within about 2 inches of the bottom of the flask. When not in use, the Jones reductor should be kept filled with distilled water.

DETERMINATION

Make filtrate from $BaSO_4$ to volume, pipet into a beaker an aliquot of 100 ml, and add with stirring 5 ml of H_2SO_4. Place in vacuum flask 10 ml of 10% ferric alum (free of ferrous Fe and other substances reducing $KMnO_4$). Fit flask to reductor, apply vacuum, and open screw clamp enough to permit controlled passage of liquid into flask. When meniscus in buret has sunk nearly to level of zinc, add more soln. (It is preferable never to expose amalgamated zinc to the air.) When all of soln has been added, add in the same manner about 100 ml of distilled H_2O. Close screw clamp just before meniscus of last washing reaches level of zinc, release vacuum, and disconnect flask. Transfer contents to 300-ml tall-form beaker and add 3 ml of syrupy phosphoric acid. Using a 10 ml microburet, titrate over a white surface with 0.1 *N* $KMnO_4$ to the first pink. Make up a blank containing 3 g of potassium pyrosulfate and 6.5 ml of H_2SO_4 in 100 ml of distilled H_2O. Put this thru identically the same treatment the sample received, finally titrating to the same shade of pink. Subtract titre of blank from that of sample. Corrected titre $\times .008$ equals total (TiO_2 plus Fe_2O_3) (these have practically the same equivalent weight).

Total Iron

REAGENT

Titanium trichloride, 0.05 *N* $TiCl_3$.—Make up according to directions in **21.36** (p. 290), but containing only half as much $TiCl_3$ as required for the 0.1 *N* soln.

Standardize according to either of the methods listed under **21.37** except that standardization should be conducted using an ordinary micro-buret and titrating into an open beaker. The soln should be kept in an ordinary glass-stoppered bottle and restandardized immediately before each set of determinations.

DETERMINATION

Pipet an aliquot of 100 ml from volumetric flask into a 150-ml beaker. Add 1 g NH_4CNS. Slowly and with thoro stirring, titrate with 0.05 *N* $TiCl_3$ from a microburet to disappearance of the red color. Run a blank on 3 g of potassium pyrosulfate and 6.5 ml H_2SO_4 in 100 ml of distilled H_2O. (Blank is often nil.) Corrected titer $\times.004$ equals Fe_2O_3.

Total Titanium

Per cent total (TiO_2 plus Fe_2O_3)—per cent total Fe_2O_3 equals per cent total TiO_2.

Total Oxides of Iron, Titanium, and Aluminum

Pipet an aliquot of 250 ml from volumetric flask into a 600-ml beaker. Add a few drops of methyl red indicator and 5 g of NH_4Cl, and bring to boil. Neutralize by adding NH_4OH dropwise just to the first distinct yellow. Let sit for about 3 min., and filter thru a 12½ cm medium quantitative paper. Wash several times with hot 2% NH_4Cl. Place paper in a tared crucible and dry in an oven or an air bath. Transfer to a muffle furnace at room temp., and raise heat to about 1100 degrees. Ignite to constant weight. Result is total (Al_2O_3 plus Fe_2O_3 plus TiO_2).

Total Aluminum

Per cent total (Al_2O_3 plus Fe_2O_3 plus TiO_2)—per cent total (Fe_2O_3 plus TiO_2) equals per cent total Al_2O_3.

Acid-Insoluble Calcium

Determine calcium in the filtrate from the ammonium hydroxide precipitate according to directions in **6.48** (p. 66), beginning with "heat to boiling. . . ."

Acid-Insoluble Magnesium

Determine in filtrate from acid-insoluble calcium by **37.64** (p. 642). $Mg_2P_2O_7$ $\times 0.3621$ equals MgO.

Silica

Weigh about 1 g of the powder into a 250-ml beaker. Moisten with alcohol and add 100 ml of (1+9) HCl. Stir, and allow to stand for 10 min. Filter thru 12½ cm medium quantitative paper. Wash the residue 3 times with H_2O. Transfer paper to a platinum crucible and ash at below 650°C. Cool, and pulverize ash with a flattened glass rod. Add 6 g of Na_2CO_3 a portion at a time, intimately mixing with the same glass rod between additions. Use the last of the Na_2CO_3 to sprinkle over the top of the mixture. Place in a muffle furnace at below 800°C., and raise temp. to bring contents into fusion. Heat at ca 1000°C. for 15 min. Remove the crucible and let cool. Dislodge the melt into a dry 500-ml beaker. (Dislodging the melt is not always easy. It often helps to return the crucible to the hot furnace for ½ min., then remove it and immediately dip about two-thirds of its length in a beaker of H_2O. If repeated a sufficient number of times, this treatment causes the melt to crack away from the platinum so that it can be removed by simply upending the crucible over the beaker.) In a graduate mix 15 ml of HNO_3 with 5 ml of H_2O, and wash the crucible with small successive portions of the mixture, adding washings to the beaker. If soln of the melt becomes slow, hasten its disintegration by gentle pressure with a glass rod.

When the Na_2CO_3 in the melt has dissolved, place the beaker under a hood and add, in the order named, 5 g of NH_4Cl and 25 ml of $HClO_4$ (60%). Cover the beaker with a watch glass, and boil over a moderate flame until oxides of nitrogen have passed off and the $HClO_4$ refluxes down the sides of the beaker. Cool the mixture slightly, add 150 ml of very hot water, stir, and let sit until silica settles to the bottom. Decant supernatant liquid thru a 12½ cm medium quantitative paper, and transfer residue to paper using hot water and policing out beaker. Wash thoroly five times with hot water. Transfer to a platinum dish, burn off paper, and ignite to constant weight at about 1100°C. Weigh as crude silica. To the residue in the dish add ca 2 ml of (1+9) H_2SO_4 and enough HF (48%) to cover the silica. Heat on a steam bath under the hood until silica and excess HF have passed off. Cautiously heat over the non-reducing flame of a Fisher burner until fumes of SO_3 have ceased to be evolved, and then heat strongly for several min. Cool and reweigh. The difference between this weight and the weight of crude silica is weight of SiO_2.

Starch

Weigh ca 5 g of the powder into a 500-ml Florence flask (preferably standard taper). Moisten with 10 ml of alcohol. Acid-wash according to directions in **17.20** (p. 212), hydrolyze starch as directed under **27.33** (but filter hydrolyzed mixture before and not after making to volume), and determine dextrose by **34.39** and **34.40** (p. 572).

COLLABORATIVE

A collaborative face powder mixture was prepared from nine components, all previously assayed for all constituents except carbon dioxide and water. The Associate Referee was able to secure only two collaborators—Sylvan H. Newburger and Charles Graichen, both of the Cosmetic Division of the Food and Drug Administration, Washington, D. C. These gentlemen deserve much credit for bringing to a successful conclusion a most involved and time-consuming piece of analysis.

TABLE 1.—*Collaborative results*
CO_2 and H_2O not determined

CONSTITUENT	PRESENT	GRAICHEN		MCCLELLAN		NEWBURGER	
		1	2	1	2	1	2
Stearate as Stearic Acid	4.5	4.6	5.0	4.6	4.6	4.5	4.5
Total ZnO	10.0	9.9	9.9	10.1	9.9	9.8	9.9
Acid-soluble CaO	3.1	3.2	3.2	3.0	3.0	3.0	3.0
Acid-soluble MgO	2.5	2.4	2.4	2.5	2.5	2.5	2.5
$BaSO_4$	9.9	9.8	9.9	9.6	9.7	9.7	9.7
Total Fe_2O_3	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Total TiO_2	5.0	4.7	5.0	4.8	4.9	4.8	4.7
Total Al_2O_3	8.0	7.9	7.8	8.3	8.0	8.4	8.3
Acid-insoluble MgO	8.0	8.2	8.2	8.3	8.2	8.2	8.2
Acid-insoluble CaO	2.1	2.0	2.1	1.8	1.8	2.0	2.0
SiO_2	26.8	25.8	25.6	26.6	27.1	26.1	26.1
Starch	8.8	9.3	9.3	8.9	8.9	9.5	9.4
Total	88.9	88.0	88.6	88.7	88.8	88.7	88.5

Component	*Per cent by weight in mixture*
Talc	30
Kaolin	20
Zinc oxide	10
Starch	10
Barium sulfate	10
Titanium dioxide	5
Calcium carbonate	5
Magnesium carbonate	5
Magnesium stearate	5

RECOMMENDATIONS*

It is recommended—

(1) That the above method for face powders be adopted as official, first action.

(2) That this subject be continued.

REPORT ON COSMETIC SKIN LOTIONS

By HENRY R. BOND (Food and Drug Administration, Federal Security Agency, Kansas City, Mo.), *Associate Referee*

DETERMINATION OF GLYCEROL, ETHYLENE GLYCOL, AND PROPYLENE GLYCOL IN A CLEAR TYPE SKIN LOTION

As one phase of the study of cosmetic skin lotions, the clear type lotion was selected for the purpose of devising suitable assays for as many as possible of the common ingredients involved.

Glycerol, in particular, and two of the glycols, ethylene and propylene, are relatively common constituents of many skin lotions. It was decided to utilize a method for determining quantitatively each of the three in a mixture. Such a method had been devised by Irwin S. Shupe[1] under the title "Periodate Reaction Applied to Cosmetic Ingredients."

A slightly modified form of this method was employed for the assay of the samples prepared for collaborative study. Collaborators were also requested to determine the glycerol content of the samples by the method of Newburger and Bruening[2] for the purpose of comparing results obtained through the use of brom-cresol-purple indicator as opposed to methyl red.

Since the periodate volumetric method employed was semi-micro in nature, the collaborative samples contained only small amounts (1 gram or less) of the constituents to be determined. The solutions were slightly acetic and the two samples sent to each collaborator were identical in composition, in that each was made from one and the same stock solution

* For report of Subcommittee B and action of the Association, see *This Journal*, 32, 50 (1949).
[1] *This Journal*, 26, 249 (1943).
[2] *Ibid.*, 30, 651 (1947).

with the same degree of dilution. Therefore, results obtained from the two samples should have been practically identical.

The results of collaborative study are tabulated below in an abridged form, merely stating the average percentage recovery obtained by each collaborator.

TABLE 1.—*Collaborative results*

CONSTITUENT	MILLIGRAMS OF CONSTITUENT PER 20 ML OF SAMPLE	COLLABORATORS' PER CENT RECOVERY			
		A	B	C	D
(1) Glycerol (using methyl red)	18.65	97.59	94.91	—	108.71
(2) Glycerol (using brom-cresol-purple)		99.81	91.02	97.05	—
Propylene glycol	16.28	98.43	94.60	100.12	85.78
Ethylene glycol	13.00				
Calc. using (1) above.		100.54	107.67	—	99.32
Calc. using (2) above		97.56	109.27	98.85	—

While collaborators A and C obtained results which could be considered excellent, it is believed that the method should be subjected to further collaborative study before its acceptance is recommended. A comparison of recoveries of glycerol, using two different indicators, indicates brom-cresol-purple as the better.

RECOMMENDATION*

It is recommended that a new series of samples be subjected to more extensive collaborative study in order to determine the suitability of the method.

ACKNOWLEDGMENT

The Associate Referee wishes to express his appreciation of the efforts of the following members of the Food and Drug Administration: H. P. Bennett, New Orleans, and F. E. Yarnall, Kansas City, for aid in preliminary work; and G. E. Keppel, Minneapolis; S. T. Colamaria, Boston; W. S. Cox, Atlanta, and D. Banes, Chicago, as collaborators.

No reports were given on alkalies in cuticle removers, hair straighteners, cosmetic creams, deodorants and anti-perspirants, depilatories, hair dyes and rinses, mercury salts in cosmetics, or moisture in cosmetics.

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* For report of Subcommittee B and action of the Association, see *This Journal*, 32, 50 (1949).

The paper entitled "Analysis of Castor Oil in Lipstick," by S. H. Newburger, is published in *This Journal*, p. 658.

REPORT ON COAL-TAR COLORS

By G. Robert Clark (Cosmetic Division, Food and Drug Administration, Federal Security Agency, Washington, D. C.), *Referee*

RECOMMENDATIONS*

Acetates, Carbonates, Halides, and Sulfates in Coal-Tar Colors.—The Referee concurs in the recommendation for continuation of this topic. The Referee further recommends reassignment of this topic, as requested by the Associate Referee.

Buffers and Solvents in Titanium Trichloride Titration.—The Referee recommends continuation of this topic.

Ether Extract in Coal-Tar Colors.—The Referee recommends continuation of this topic.

Halogens in Halogenated Fluoresceins.—The Referee concurs in the recommendation that the proposed methods for chlorine and for chlorine and bromine, when both are present, be studied.

Identification of Coal-Tar Colors.—The Referee recommends continuation of this topic, and that it be reassigned.

Volatile Amine Intermediates.—The Referee concurs in the recommendation for continuation of this topic.

Non-volatile Unsulfonated Amine Intermediates in Coal-Tar Colors.—The Referee concurs in the recommendation for continuation of this topic.

Sulfonated Amine Intermediates in Coal-Tar Colors.—The Referee recommends continuation of this topic.

Unsulfonated Phenolic Intermediates in Coal-Tar Colors.—The Referee concurs in the recommendation that the topic be continued.

Sulfonated Phenolic Intermediates in Coal-Tar Colors.—The Referee recommends continuation of this topic.

Intermediates Derived from Phthalic Acid.—The Referee recommends continuation of this topic.

Mixtures of Coal-Tar Colors for Drug and Cosmetic Use.—The Referee recommends continuation of this topic.

Pure Dye in Lakes and Pigments.—The Referee concurs in the recommendation—

(1) That the proposed method for the determination of pure dye in D&C Reds Nos. 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 31, and 34, and Ext. D&C Red No. 2, be adopted as official, first action.

(2) That the topic be continued.

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* For report of Subcommittee B and action of the Association, see *This Journal*, **32**, 50 (1949).

The Referee further recommends that the topic be reassigned.

Spectrophotometric Testing of Coal-Tar Colors.—The Referee concurs in the recommendation that the topic be continued.

The Referee further recommends that the topic be reassigned.

Subsidiary Dyes in D&C Colors.—The Referee recommends continuation of this topic.

Subsidiary Dyes in FD&C Colors.—The Referee concurs in the recommendation that the topic be continued.

Hygroscopic Properties of Coal-Tar Colors.—The Referee recommends continuation of this topic.

Lead in Coal-Tar Colors.—The Referee concurs in the recommendation that the method, described by N. Ettelstein in a contributed paper (*This Journal*, **30**, 552 (1947) and supported by collaborative results, presented at the 1947 meeting of the Association, be adopted as official, first action, for the determination of lead in all straight coal-tar colors listed as certifiable under the regulations promulgated in accordance with the Federal Food, Drug, and Cosmetic Act, except those containing calcium, barium, or strontium.

Lead in Lakes of Coal-Tar Colors.—The Referee recommends that the methods described by the Associate Referee be adopted as official, first action, based upon the collaborative results presented in the report for this year and the recovery experiments presented at the 1947 meeting. The Referee further recommends the addition of the following topics for study:

The Determination of Arsenic in Coal-Tar Colors.

The Determination of Heavy Metals in Coal-Tar Colors.

The Boiling Range of Pseudocumidine and Xylidine in Certified Coal-Tar Colors.

REPORT ON HALOGENS IN HALOGENATED FLUORESCEINS

By N. GORDON (Cosmetic Division, Food and Drug Administration, Federal Security Agency, Washington, D. C.), *Associate Referee*

CHLORINE

The work covered by this report consists primarily of a collaborative study of the determination of chlorine and bromine by the method proposed by Clark and Jones (1). The method for determining bromine, **21.53**, is official, first action (*Methods of Analysis*, 6th Ed.). A previous collaborative study (2) of the method as applied to samples containing both bromine and chlorine did not give completely satisfactory results.

It was felt that the inconsistent results obtained in last year's study may have been due in part to difficulties in the decomposition step in the

analysis. It was decided, therefore, to submit samples that would permit an evaluation of each part of the procedure.

A sample of purified potassium bromide was submitted as a further check on the bromine titration and to familiarize the collaborators with the titration.

A solution containing potassium bromide, sodium chloride, and hydrazine sulfate was submitted to check the accuracy of the steps subsequent to the decomposition of the sample. The contents of this solution were not disclosed to the collaborators.

As a check on the reliability of the decomposition step a sample of a commercial preparation of D&C Red No. 24 was submitted for analysis.

Reports containing analytical data were received from the following collaborators:

Ansbacher-Siegle Corporation—H. Holtzman reporting.
Max Factor, Inc.—H. R. Cohen reporting.
H. Kohnstamm and Company, Inc.—Mrs. V. Schmuckli reporting.
National Aniline Division, Allied Chemical & Dye Corp.—A. T. Schramm reporting.
Cosmetic Division, Food and Drug Administration—J. H. Jones and L. S. Harrow reporting.

One collaborator reported considerable difficulty in obtaining reproducible results in the bromine determination.

Another collaborator suggests the rate at which the sodium thiosulfate solution is added during the titration may be a factor in the accuracy of the results; low results were attributed to a slow rate of addition in one instance and a rapid rate was recommended.

Difficulty was experienced with the digestion by one collaborator who reported that "There were sudden ebullitions that might account for the wide discrepancy in the findings for the chlorine determination."

All reported results are shown in the order that they were received, in Tables 1–3.

With one exception, the results of the collaborators on the purified potassium bromide are in good agreement with the theoretical value. The average results for these collaborators is 99.8 per cent of the theory; the highest, 100.1; and the lowest, 98.5.

The results of six of the seven collaborators on the solution containing both bromine and chlorine are in good agreement with one another and the calculated values. The maximum error in the values obtained by these collaborators is less than 1 per cent for either the bromine or chlorine determination. The other collaborator reported inconsistent results on the potassium bromide sample. It would appear, therefore, that the proposed method is capable of giving accurate results on solutions containing both bromine and chlorine.

The results for chlorine in D&C Red No. 24 are somewhat variable. The

TABLE 1.—*Collaborative results for bromine in purified KBr*

COLLABORATOR	BROMINE	COLLABORATOR	BROMINE
	per cent		*per cent*
Associate Referee	67.0	5	66.15
	66.2		66.15
	66.3		66.35
	66.4		66.73
	66.7		
		6	66.8
2	67.07		66.8
	66.96		
	67.19	7	67.0
3	57.0–77.0*	Average.....	66.8
4	67.03	Average Deviation..	0.23
	67.23		
	67.23	Theoretical.....	67.15

* Not included in average.

TABLE 2.—*Collaborative results for bromine and chlorine in a solution of purified KBr and NaCl*

COLLABORATOR	BROMINE	CHLORINE
	grams per 10 ml	*grams per 10 ml*
Associate Referee	0.0439	0.0214
	0.0439	0.0213
	0.0439	0.0213
2	0.04416	0.02142
	0.04426	0.02132
	0.04416	0.02135
3	0.0954	0.0232
	0.0949	0.0234
4	0.0443	0.0212
	0.0443	0.0212
	0.0443	
5	0.0440	0.0212
	0.0444	0.0214
	0.0440	0.0210
	0.0444	
6	0.0437	0.0215
	0.0438	0.0216
	0.0438	
	0.0439	
7	0.0439	0.0212
Calculated	0.0440	0.0214

TABLE 3.—*Collaborative results for chlorine in D&C Red No. 24**

COLLABORATOR	CHLORINE	COLLABORATOR	CHLORINE
	per cent		*per cent*
Associate Referee	29.2	5	31.14
	29.2		30.24
			29.96
2	29.35		
	29.35	6	29.5
	29.26		29.4
			29.5
3	28.6		
	28.7	7	28.7
			28.7
4	29.15		28.9
	29.02		
	29.19	Average	29.29
	29.05		
		Average Deviation	0.4 or 1.4% of the average

* The sample submitted was a commercial sample; a pure sample of D&C Red No. 24 would contain 30.2% Cl.

average deviation from the mean for the seven collaborators is 1.4 per cent. If the set of high results is omitted, the average deviation from the mean is less than 1 per cent. It would appear from these data that the decomposition procedure is capable of giving consistent results.

It seems reasonable to assume, therefore, that the proposed methods for bromine and/or chlorine in halogenated fluoresceins will give satisfactory results if the directions are carefully followed.

RECOMMENDATIONS*

It is recommended—

(1) That the chlorine method be adopted as official.

(2) That the method for bromine and chlorine, when both are present, be adopted as official.

(3) That the topic be continued, to study more rapid methods and to provide an official method for iodine.

REFERENCES

(1) CLARK, G. R., and JONES, J. H., "Determination of Chlorine and Bromine," *This Journal*, **26**, 433 (1943).

(2) GORDON, N., "Report on Halogens in Halogenated Fluoresceins," *This Journal*, **31**, 589 (1948).

* For report of Subcommittee B and action of the Association, see *This Journal*, 32, 50 (1949).

VOLATILE AMINE INTERMEDIATES IN COAL-TAR COLORS

By Alice B. Caemmerer (Cosmetic Division, Food and Drug Administration, Federal Security Agency, Washington, D. C.), *Associate Referee*

At the annual meeting of the A.O.A.C. in October 1947, a method[1] for the determination of volatile amine intermediates in coal-tar colors was reported. This method involved the isolation of the amine by steam distillation, diazotizing the amine and coupling with 1-(4 sulfophenyl)-3-methyl-5-pyrazolone. The resulting dye was then titrated with standard titanium trichloride solution and the weight of the amine calculated. This study has been extended to the determination of *p*-toluidine and *o*-toluidine in various certifiable colors. The results obtained are shown in Table 1.

Table 1.—*Recoveries of added intermediates in analyses by Associate Referee*

COLOR	RECOVERY
	per cent
Para-toluidine	
D&C Violet No. 2	96.5
D&C Blue No. 5	90.0
D&C Green No. 5	97.8
D&C Green No. 6	96.0
Average	95.0
Ortho-toluidine	
FD&C Orange No. 2	96.8

Since the recovery of *p*-toluidine and *o*-toluidine was good, it was decided to submit samples containing known amounts of each intermediate to collaborative study. Accordingly, samples of D&C Green No. 5 and FD&C Orange No. 2, each containing 20 mg of *p*-toluidine and *o*-toluidine, respectively, were submitted to various collaborators. The results are shown in Table 2.

From an inspection of Table 2 it is obvious that either the method is unworkable or that some difficulty was encountered with the samples. Since the Associate Referee has obtained good recoveries on known amounts of intermediate, it is thought probable that the difficulty lies in the sample. These samples were sealed in long tubes, hence it may be that all of the sample was not transferred to the distillation apparatus. Therefore, it is proposed that a new sampling procedure be devised and that the method be resubmitted to collaborative study.

[1] *This Journal*, 31, 592 (1948).

TABLE 2.—*Collaborative studies of recovery of o-toluidine and p-toluidine* 0.2% added to each sample

COLLABORATOR	D&C GREEN NO. 5 p-TOLUIDINE	FD&C ORANGE NO. 2 o-TOLUIDINE
	per cent	*per cent*
1	0.141	0.160
	0.144	0.150
2	0.166	0.148
	0.875	0.193
3	0.1051	0.1994
		0.1956
4	0.19	0.16
	0.18	0.15

The Associate Referee wishes to thank the following collaborators for their assistance in this work:

Ansbacher-Siegle Corporation—H. Holtzman reporting.
H. Kohnstamm and Company, Inc.—Louis Koch reporting.
Wm. J. Stange Company—W. H. Kretlow reporting.

It is recommended* that the topic "Volatile Amine Intermediates in Coal-Tar Colors" be continued.

REPORT ON ACETATES, CARBONATES, HALIDES, AND SULPHATES IN CERTIFIED COAL-TAR COLORS

SODIUM ACETATE IN FD&C BLUE NO. 1

By J. SCHIFFERLI and A. T. SCHRAMM (National Aniline Division, Allied Chemical and Dye Corporation, Buffalo, N. Y.), *Associate Referee*

The Coal-Tar Color Regulations stipulate that the sodium acetate content of FD&C Blue No. 1 must not exceed 3.0 per cent.

The tentative A.O.A.C. method[1] for the determination of this impurity in FD&C Blue No. 1 has been found to be time-consuming and productive of results lacking in precision and accuracy. Sclar and Clark described a method[2] based on Freudenberg's method[3] for the determination of acetyl groups. This method, however, did not progress to the desired state of collaborative work. The principle of the method of Sclar and Clark involves esterification of sodium acetate to ethyl acetate in the presence of

* For report of Subcommittee C and action of the Association, see *This Journal*, 32, 51 (1949).
[1] *Methods of Analysis, A.O.A.C.*, 1945, 286.
[2] Sclar, R. N., and Clark, G. R., *This Journal*, 27, 472 (1944).
[3] Freudenberg, K., and Harder, M., *Ann.*, 433, 230 (1923); 494, 68 (1932).

p-toluenesulfonic acid and silver toluenesulfonate, distillation, and saponification of the distilled ester with a measured excess of standard sodium hydroxide.

The present authors' investigation revealed that, although the method of Sclar and Clark is satisfactory in principle, it is subject to improvement by more careful definition of procedural detail. To effect the desired improvement, the following modifications have been made:

1. Sample weight increased from 2 grams to 5 grams;
2. Meta-cresol purple substituted for phenolphthalein as indicator to obtain the true equivalence point;
3. Hydrolysis of the ester protected with a tube containing carbon dioxide absorbing material;
4. Distillation time reduced by wrapping the Kjeldahl trap with insulating material;
5. Distillation time further reduced by addition of anti-bumping agent to the flask and use of an Allihn condenser;
6. Anhydrous *p*-toluenesulfonic acid used to favor the ester formation;
7. Sequence of addition of reagents changed to facilitate wetting of the dye and the *p*-toluenesulfonic acid.

METHOD

REAGENTS

p-Toluenesulfonic acid.—Dry *p*-toluenesulfonic acid monohydrate overnight at 110°C., cool, and grind to a powder.

Silver toluenesulfonate.—Dissolve reagent silver oxide or carbonate in about 10% excess of *p*-toluenesulfonic acid solution, evaporate to dryness, and dry at 135°C. for 8 hours.

APPARATUS

The apparatus can be assembled from stock items. The distilling flask has a capacity of 100–125 ml and is provided with a Kjeldahl trap which is wrapped with asbestos rope or other heat insulator to increase the distillation rate. The assembled apparatus is shown in Figure 1.

PROCEDURE

To a 500 ml Erlenmeyer flask add 100 ml of distilled water, a drop of *m*-cresol purple indicator soln (0.5 g of solid indicator triturated with 13 ml of 0.1 *N* NaOH and diluted with distilled water to make 100 ml.) and sufficient 0.1 *N* NaOH or 0.1 *N* HCl to turn the color of the soln just yellow. Place the flask and contents under the condenser.

Transfer 30 ml of anhydrous alcohol to the distillation flask and add 5.00 g of the sample, 5 g of *p*-toluene-sulfonic acid, and 1 g of silver toluenesulfonate thru a powder funnel.

Add 3–4 pieces of alundum or other anti-bumping agent and mark the level of the liquid in the distillation flask.. Wash the funnel and neck of the flask with 25 ml of anhydrous alcohol. Shake the flask to mix the contents thoroly and attach it to the condenser.

Immerse the distillation flask as far as possible in a beaker of hot water and heat the water to boiling. After about 25 ml of distillate has collected, remove the heat

source and slowly add 25 ml of anhydrous alcohol to the distillation flask. When it again begins to distill quietly replace the heat source until a second 25 ml of distillate has collected. Make a third addition and distillation in similar manner. Finally boil until the distillation rate is slow (about one-half hour total distillation time figured from the beginning of the first distillation).

Wash down the condenser with 50 ml. of distilled water into the receiver, and add to the receiver contents 50.0 ml of 0.1 *N* NaOH. Add 3-4 pieces of alundum and

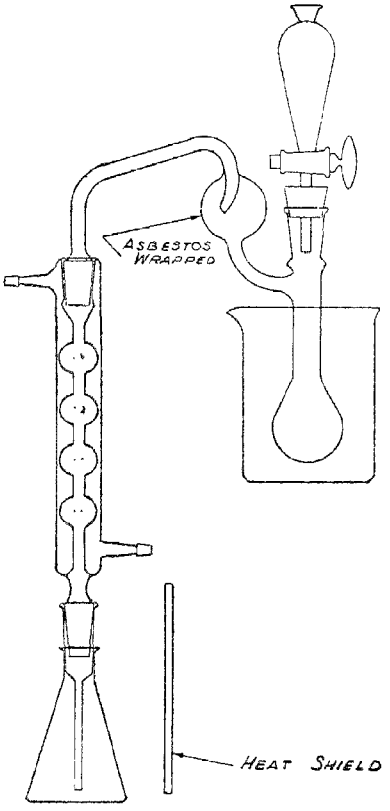

FIG. 1

connect to a reflux condenser fitted with an absorption tube containing ascarite or other carbon dioxide absorbing material.

Reflux for 10 min. Cool to room temp., add a few drops of *m*-cresol purple indicator, and titrate with 0.1 *N* HCl to the yellow-green color which does not change in hue on further addition of acid.

Determine the blank by duplicating the procedure with the omission of the sample.

Calculate the sodium acetate from the net volume of standard NaOH soln required.

1 ml of 0.1 *N* NaOH = 0.0082 g of $C_2H_3O_2Na$

Results by this method are shown in Table 1. Several determinations of the blank were run on the reagents used, including various supplies of anhydrous alcohol. The blank ranged from 0.3 to 0.5 ml of 0.1 *N* HCl corresponding to 0.05 to 0.08 per cent of sodium acetate in a 5 gram sample.

TABLE 1.—*Determination of sodium acetate in FD&C Blue No. 1*

BATCH	ADDED	FOUND*	CALCULATED TOTAL	DIFFERENCE
	per cent	*per cent*	*per cent*	*per cent*
A	0	0.08	—	+0.01†
A	0	0.05	—	−0.02†
B	0	1.62	—	+0.01†
B	0	1.59	—	−0.02†
C	0	2.95	—	−0.01†
C	0	2.96	—	0.00†
C	0	2.96	—	0.00†
A	2.81	2.95	2.88	+0.07
A	2.81	2.92	2.88	+0.04
A	2.81	2.94	2.88	+0.06
A	4.00	4.04	4.07	−0.03
A	4.00	4.08	4.07	+0.01
A	6.00	6.01	6.07	−0.06
A	10.00	9.93	10.07	−0.14

* Deductions for reagent blanks were made.
† This figure is the difference from the average of the replicate determinations on the batch.

An average difference of 0.01 per cent between a single determination and the average of replicate determinations was obtained in seven determinations involving three batches of FD&C Blue No. 1. An average difference of −0.007 per cent was obtained between the found and calculated values in seven determinations of the recovery of added sodium acetate.

SUMMARY

A method for determining sodium acetate in FD&C Blue No. 1 has been described. Typical results are given.

REPORT ON UNSULPHONATED PHENOLIC INTERMEDIATES IN COAL-TAR COLORS

BETA-NAPHTHOL IN D&C RED NO. 35

By H. HOLTZMAN (*Associate Referee*) and H. GRAHAM (Ansbacher-Siegle Corporation, Rosebank, Staten Island, New York)

This is the initial report of a series on the determination of unsulphonated phenolic intermediates in coal-tar colors.

The colors subject to this specification may be grouped into the following classifications, which will require similar extraction procedures, (except for Group IV):

I—Soluble Azo:	—FD&C Orange #1[b],	D&C Orange #4[a],
	Ext. D&C Red #8[b],	Ext. D&C Yellow #4[c].
II—Insoluble Azo:	—FD&C Orange #2[a],	FD&C Red #32[a],
	D&C Green #6[f],	D&C Orange #17[a],
	D&C Red #17[a],	D&C Red #18[a],
	D&C Red #35[a],	D&C Red #36[a],
	D&C Red #38[d].	
III—Difficulty Soluble Azo:	—D&C Red #8[a],	D&C Red #9[a],
	D&C Red #10[a],	D&C Red #11[a],
	D&C Red #12[a],	D&C Red #13[a],
	D&C Red #14[a],	D&C Red #15[a],
	D&C Red #16[a].	
IV—Miscellaneous:	—D&C Red #19[e],	D&C Red #20[e],
	D&C Red #37[e].	

The superscript refers to the particular phenolic intermediate to be determined: (a) beta-naphthol; (b) alpha-naphthol; (c) para-nitrophenol; (d) 3-hydroxy-N-(m-nitro phenyl)-2-naphthamide; (e) di-ethyl-meta-amino-phenol; (f) 1,4 di-hydroxy anthraquinone.

The determination of beta-naphthol is most often required. The initial study selected was, therefore, that of beta naphthol in D&C Red No. 35 of Group II.

The tentative method of analysis, issued by the Food and Drug Administration, Cosmetic Division, in 1939, and which has not so far been subjected to collaborative study, called for an aqueous acid extraction of the color followed successively by benzene and aqueous caustic soda extractions. The final extract is then coupled with diazotized sulfanilic acid and determined colorimetrically against graded standards.

A literature search revealed a number of methods for determination of beta naphthol. Most of these involve coupling to an azo dyestuff, followed by a colorimetric determination (1, 2, 3). Many qualitative tests are listed, some of which probably could be adapted for quantitative use (4, 5). An iodometric method for phenolic compounds (6), is listed, as well as a number of derivatives which could be applied to quantitative determinations.

In view of the ease with which coupling occurs to form an azo dye, which can be readily identified, it was decided to adhere to the dye coupling procedure for the determination. A titanous chloride reduction of the dye was adopted for the final evaluation. The extraction procedure has been simplified in accordance with the nature of the dyestuff group.

EXPERIMENTAL

Pure beta-naphthol was obtained by recrystallization from toluene and the crystals washed with petroleum ether (7).

A purified D&C Red No. 35 was prepared by successively washing with dilute caustic soda, followed by dilute acid and water washes (8).

The following method of analysis was evolved:

PROCEDURE

Transfer a 10 g sample to a 250 ml beaker. Add 50 ml of 1:10 HCl. Heat to 50°. Stir on automatic agitator (ca 120 r.p.m.), for 10 min. Filter. Wash with ca 50 ml 50° 1:10 HCl. Return the filter paper and residue to original beaker. Add another 50 ml of 1:10 HCl. Repeat the heating, stirring, and filtering. Wash with 50° 1:10 HCl. The total filtrates (ca 200 ml) are transferred to a 500 ml wide-mouth Erlenmeyer flask. The *p*H is adjusted to neutrality with dilute NaOH (using phenolpthalein indicator). Add 10 gm Na Acetate. Cool to 5° in ice bath.

PREPARATION OF DIAZO

Make a soln of ca 0.05 *N* sulfanilic acid by dissolving 4.779 g of sulfanilic acid in 500 ml of H_2O, to which has been added 5 ml of 12 *N* HCl. Make a soln of ca 0.05 *N* $NaNO_2$ by dissolving 1.04 gm $NaNO_2$ in 300 ml H_2O. Place 40 ml of the sulfanilic acid in a 100 ml vol. flask. When cooled to 5° C, add 44 ml of the $NaNO_2$ soln and allow to diazotize. Test for excess nitrous acid and destroy excess with a few mg of sulfamic acid. Dilute to volume.

Add slowly 20 ml of diazo soln. Stir 5 min. and test for excess diazo with alk. beta naphthol soln. If not positive, add additional diazo until a positive test is obtained. Let stand for one hour.

Heat on water bath for ½ hour to decompose excess diazo. Add 10 g sodium bitartrate which has been dissolved in 50 ml hot H_2O. Dilute with ca 100 ml ethyl alcohol. Titrate with $TiCl_3$ to a yellowish end point. (Back titrate with Methylene Blue, if desired.)

Calculations:

$$1 \text{ ml of } 0.1\ N\ TiCl_3 = .0036 \text{ g Beta Naphthol.}$$

Dye coupling and analyses of known solutions of pure beta naphthol gave excellent recoveries, by the method given. Procedure was then applied to samples of the purified D&C Red No. 35, to which known amounts of beta-naphthol had been added, the latter in dilute alkaline solution.

COLLABORATIVE RESULTS

Samples of a commercial D&C Red No. 35 and a synthetically prepared sample containing 0.30% beta naphthol were submitted for collaborative analysis to P. T. Beeton and L. Krawer of Ansbacher-Siegle Corporation, and to C. Graichen of the Cosmetic Division of the Food and Drug Administration. Analytical results by the junior author are included.

It is of interest that in spite of the three divergent results, each collaborator duplicated his own results. On the basis of the foregoing, further work is required on the extraction procedure.

TABLE 1.—*Recovery of Beta Naphthol in sample of D&C Red No. 35*

BETA NAPHTHOL ADDED	DETERMINATION	TOTAL	BLANK	RECOVERY
per cent		*per cent*	*per cent*	*per cent*
0.05	(1)	0.045	—	0.045
	(2)	0.049	—	0.049
0.10	(1)	0.098	—	0.098
	(2)	0.100	—	0.100
0.15	(1)	0.15	—	0.15
	(2)	0.13	—	0.13
0.25	(1)	0.24	—	0.24
	(2)	0.23	—	0.23
	(3)	0.23	—	0.23
0.30	(1)	0.41	0.12	0.29
	(2)	0.41	0.12	0.29
	(3)	0.35	0.09	0.26
	(4)	0.37	0.09	0.28
0.50	(1)	0.49	—	0.49
0.60	(1)	0.68	0.09	0.59
0.80	(1)	0.93	0.12	0.81
	(2)	0.91	0.12	0.79
	(3)	0.87	0.12	0.75

TABLE 2.—*Collaborative results*

COMMERCIAL D&C RED NO. 35			SYNTHETIC SAMPLE CONTAINING 0.3% (9)			
COLLABORATOR	DETERMINATION	BETA NAPHTHOL	COLLABORATOR	DETERMINATION	RESULT	(MINUS) (BLANK)
		per cent				
1	(1)	0.38	1	(1)	0.34	(0.28)
	(2)	0.36		(2)	0.30	(0.24)
2	(1)	0.35	2	(1)	0.34	(0.28)
	(2)	0.33		(2)	0.34	(0.29)
3*	(1)	0.67				
	(2)	0.68				
4	(1)	0.45				
	(2)	0.46				

* Collaborator No. 3 commented (a) that some fine particles of dye may have been carried through into the filtrate, as the methylene blue back titration end point faded on standing; (b) a greater volume of the dilute acid should be employed for each extraction, to improve efficiency of the extractions.

It is recommended* that this work be continued, to be followed by further collaborative study.

REFERENCES

(1) *Allen's Commercial Org. Anal.*, Vol. II, p. 408.
(2) *J. Soc. Chem. Ind.*, **16**, p. 294.
(3) *A.O.A.C.* Bulletin #107 (1907).
(4) Allen's Commercial Org. Anal., Vol. III, p. 209.
(5) *Merck Index.*
(6) *Allen's Commercial Org. Anal.*, Vol. III, p. 234.
(7) *J. Soc. Chem. Ind.*, **16**, p. 294.
(8) Some of the batches of D&C Red No. 35 washed in this manner, gave a blank, which might be either residual beta naphthol or possibly dye or some decomposition product. This blank varied in amount in various batches, and is being investigated further, using D&C Red No. 35, recrystallized from chloroform.
(9) The sample of D&C Red No. 35 gave an analytical blank of 0.06%, which was subtracted from the total result.

REPORT ON LEAD IN LAKES (ALUMINUM) OF COAL-TAR COLORS

By LEE S. HARROW (Cosmetic Division, Food and Drug Administration, Federal Security Agency, Washington, D. C.), *Associate Referee*

At the 1947 meeting of the A.O.A.C. the Associate Referee presented methods for the determination of lead in aluminum coal-tar color lakes and in calcium, barium, and strontium coal-tar color lakes.[1]

Composite samples containing known amounts of lead were distributed to several laboratories for collaborative analysis by these methods. During the past year, reports were submitted by:

Ansbacher-Siegle Corporation—H. Holtzman reporting.

TABLE 1.—*Lead in D&C Blue No. 1, Aluminum Lake*

COLLABORATOR	LEAD FOUND	LEAD PRESENT
	p.p.m.	*p.p.m.*
1	23.5	23.6
	25.0	
	25.0	
2	73.0	76.6
	76.0	
3	23.0	23.6
	28.0	

* For report of Subcommittee D and action of the Association, see *This Journal*, 32, 51 (1949).
[1] *This Journal*, 31, 677 (1948).

H. Kohnstamm and Company, Inc.—I. Hanig reporting.
Cosmetic Division, Food and Drug Administration—C. Graichen reporting.
National Aniline Division, Allied Chemical & Dye Corp.—A. T. Schramm reporting.

The results submitted are shown in Tables 1 and 2.

TABLE 2.—*Lead in D&C Red No. 9, Barium Lake*

COLLABORATOR	LEAD FOUND	LEAD PRESENT
	p.p.m.	*p.p.m.*
1	75.0	76.0
2	35.0	36.0
	37.0	
	34.0	
	31.0	
3	56.7	56.0
	55.4	
	40.6	
4	51.0	56.0
	55.0	

Since so few collaborative reports were submitted, no recommendation of adoption of the method is made. It is recommended* that study on this topic be continued.

REPORT ON LEAD IN COAL-TAR COLORS

By NATHAN ETTELSTEIN (Cosmetic Division, Food and Drug Administration, Federal Security Agency, Washington, D. C.),
Associate Referee

A study of a dithizone method for the determination of lead in coal-tar colors was presented at the 1946 meeting of the Association.[1] The method there described has been submitted to collaborative study. The results obtained are presented in Table 1.

The dithizone-lead determination under study consists of a preliminary digestion of the dye with nitric, sulfuric, and perchloric acids. The resulting solution is neutralized and the *p*H adjusted to 8.5–9. The lead is then extracted with a chloroform solution of dithizone. The extracted lead dithizonate is decomposed with 1 per cent nitric acid which transfers the lead to the aqueous phase. The lead is finally determined electrolytically as described in Chapter 21, *Methods of Analyses*.

Samples were sent to three manufacturers who had expressed willing-

* For report of Subcommittee B and action of the Association, see *This Journal*, 32, 51 (1949).
[1] *This Journal*, 30, 552 (1947).

ness to collaborate and to three chemists in the Food and Drug Administration. Two of the three firms have not yet reported results, because of lack of the necessary electrolytic equipment. However, it is hoped that they will be able to report in the near future.

TABLE 1.—*Collaborative results*

COLLABORATOR	RECOVERY MICROGRAMS	DEVIATION FROM MEAN
Associate Referee	118	+1
	119	+2
2	116	−1
	117	0
3	118	+1
	119	+2
4	119	+2
	121	+4
5	110	−7
	109	−8
Mean	117	
Average Deviation		2.6
Standard Deviation		3.7

The results of the several collaborators (Table 1) give an average deviation from the mean of 2.6 micrograms and a standard deviation of 3.7 micrograms. The highest result is 121 micrograms; the lowest is 109 micrograms.

One collaborator commented that he "found this method better than the double extraction colorimetric method for several reasons, namely, (1) shorter time required; (2) less chloroform required; and (3) fewer pitfalls due to the final electrolytic method of determination."

The author wishes to thank P. A. Clifford, L. S. Harrow, N. Gordon, and H. Holtzman for collaborative results reported.

RECOMMENDATION*

It is recommended that the proposed method be adopted as official, first action, for the determination of lead in all straight coal-tar colors listed as certifiable under the regulations promulgated in accordance with the Federal Food, Drug, and Cosmetic Act, except those containing calcium, barium, or strontium.†

* For report of Subcommittee B and action of the Association, see *This Journal*, 32, 51 (1949).
† Details of the method are given in *This Journal*, 32, 88 (1949).

REPORT ON NON-VOLATILE, UNSULFONATED, AMINE INTERMEDIATES IN COAL-TAR COLORS

By LEE S. HARROW (Cosmetic Division, Food and Drug Administration, Federal Security Agency, Washington, D. C.), *Associate Referee*

3-NITRO-P-TOLUIDINE

D&C Red No. 35 (also known as Toluidine Red, C. I. 69) and D&C Red No. 38 (also known as Toluidine Maroon) are colors certifiable by the Food and Drug Administration for coloring drugs and cosmetics (1). The maximum amount of free intermediate, 3-nitro-p-toluidine, permitted in a certified batch of these colors is 0.2 per cent.

In a paper presented at the annual meeting of the A.O.A.C. (October 1947) a method was proposed for the separation and quantitative determination of p-nitroaniline in D&C Black No. 1 (2). The method described here is a modification of that method which makes it applicable to the separation and quantitative determination of 3-nitro-p-toluidine in D&C Red No. 35 or D&C Red No. 38. The 3-nitro-p-toluidine is extracted from the colors with petroleum benzin and titrated with titanium trichloride (3).

METHOD

Place a 10 g sample of D&C Red No. 35 or D&C Red No. 38 in a Soxhlet extraction thimble of suitable size and extract with petroleum benzin for 6–8 hours. Transfer extract to 500 ml wide-mouth Erlenmeyer flask. Rinse the Soxhlet flask with two 10 ml portions of petroleum benzin and add these to main extract. Add 50 ml of water to combined extracts and heat on steam bath until all petroleum benzin is removed, using a gentle air current to hasten the process. Remove flask from steam bath and add ca 15 g of sodium tartrate, heat soln to boiling, and titrate with standard 0.1 *N* $TiCl_3$ soln under a stream of CO_2 to disappearance of yellow color. The end point can be more readily detected when 1 ml of a standard soln of FD&C Green No. 2 (Light Green SF Yellowish) is added near end of the titration to serve as an indicator.

RESULTS

Samples of D&C Red No. 35 and D&C Red No. 38 were extracted with petroleum ether until intermediate free; known quantities of 3-nitro-p-toluidine were added to 10 gram portions of these intermediate free colors. Satisfactory recoveries of the added intermediates were obtained when these samples were analyzed by the proposed method. Results of these analyses are shown in Tables 1 and 2.

Further investigations are being conducted to determine the applicability of the method to 2-4-dinitroaniline and 2-nitro-p-anisidine in colors in which they may be encountered.

P-NITROANILINE

A collaborative study of the method proposed for the determination of p-nitroaniline in D&C Black No. 1 (2) has been made.

TABLE 1.—*Recovery of 3-nitro-p-toluidine from D&C Red No. 35*

3-NITRO-p-TOLUIDINE ADDED	3-NITRO-p-TOLUIDINE RECOVERED	RECOVERY
gram	*gram*	*per cent*
0.00	0.00	0.0
0.0205	0.0195	94.8
0.0224	0.0214	95.6
0.0195	0.0190	97.2
0.0400	0.0391	97.7
0.0395	0.0383	96.8
0.0417	0.0409	98.0
0.0592	0.0584	98.8
0.0629	0.0631	100.3
0.0603	0.0598	99.3
	Average Recovery.....	97.6

TABLE 2.—*Recovery of 3-nitro-p-toluidine from D&C Red No. 38*

3-NITRO-p-TOLUIDINE ADDED	3-NITRO-p-TOLUIDINE RECOVERED	RECOVERY
grams	*grams*	*per cent*
0.00	0.00	0.0
0.0224	0.0224	100.0
0.0196	0.0193	98.5
0.0230	0.0224	97.3
0.0408	0.0398	97.6
0.0399	0.0391	98.0
0.0404	0.0399	98.7
0.0590	0.0591	100.2
0.0603	0.0598	99.2
0.0613	0.0603	98.4
	Average Recovery.....	98.7

A composite sample of D&C Black No. 1 was prepared and sent to the following collaborators for study:

Ansbacher-Siegle Corporation—H. Holtzman reporting.

National Aniline Division, Allied Chemical & Dye Corp.—A. T. Schramm, reporting.

Calco Chemical Division, American Cyanamid Company—William Seaman, E. Z. Montgomery, and W. H. McComas, Jr. reporting.

Thomasset Colors, Inc.—A. Cohen reporting.
Wm. J. Stange Company—W. H. Kretlow reporting.
H. Kohnstamm and Company, Inc.—I. Hanig reporting.

The results submitted are given in Table 3.

TABLE 3.—*p-nitroaniline in D&C Black No. 1*

COLLABORATOR	p-NITROANILINE	COLLABORATOR	p-NITROANILINE
	per cent		*per cent*
1	0.15	5	0.12
	0.15		0.12
			0.13
2	0.19		
	0.19	6	0.12
			0.09
3	0.13		
	0.13	Average.....	0.14
4	0.17		

RECOMMENDATIONS*

It is recommended—

(1) That collaborative work be done on 3-nitro-p-toluidine in D&C Red No. 35 and D&C Red No. 38.

(2) That additional collaborative work be done on p-nitroaniline in D&C Black No. 1.

(3) That the topic Non-Volatile, Unsulfonated, Amine Intermediates in Coal-Tar Colors be continued.

REFERENCES

(1) S.R.A., F.D.C. 3, U. S. Food and Drug Administration.
(2) HARROW, L. S., *This Journal*, **31**, 594 (1948).
(3) KNECHT and HIBBERT, "New Reduction Methods in Volumetric Analysis," Longmans (1918).

REPORT ON SPECTROPHOTOMETRIC TESTING OF COAL-TAR COLORS

IDENTIFICATION OF FLUORESCEIN COLORS

By RACHEL N. SCLAR (Cosmetic Division, Food and Drug Administration, Federal Security Agency, Washington, D. C.), *Associate Referee*

There are fifteen individual colors of the fluorescein type (excluding the salts of the listed color acids) on the list of certifiable colors (1). In the qualitative analysis of food, drug, and cosmetic products for coal-tar dyes, it is usually an easy task to separate the fluorescein-type dyes from

* For report of Subcommittee B and action of the Association, see *This Journal*, 32, 51 (1949).

the other dyes present and establish their presence. The positive identification of the particular fluorescein dye, or dyes, present, however, is a more difficult task.

This report covers a preliminary study of the spectrophotometric char-

TABLE 1.—*Fluorescein colors, in order of increasing wave length of absorption maximum*

COLOR	OTHER NAME	WAVE LENGTH OF ABSORPTION MAXIMUM	REMARKS
D&C Yellow No. 7	Fluorescein	490	Strong fluorescence
D&C Orange No. 5	Dibromofluorescein	504	
*D&C Orange No. 14	Tribromodicarboxy-fluorescein	504	
*Ext. D&C Orange No. 2	Dinitrofluorescein	504	
*D&C Orange No. 8	Dichlorofluorescein	504	Strong fluorescence
*D&C Orange No. 10	Diiodofluorescein	508	
*D&C Red No. 24	Tetrachlorofluorescein	509	Strong fluorescence marked shoulder at 475 mμ.
D&C Red No. 21	Tetrabromofluorescein	517	
*D&C Red No. 29	Pentabromodicarboxy-fluorescein	517	
*D&C Orange No. 16	Diiododibromofluorescein	521	Very slight (almost no) shoulder.
*FD&C Red No. 3	Erythrosine Na salt of tetraiodo-fluorescein	527	
*Ext. D&C Red No. 3	Violamine R	530	No inflection, much broader curve.
*D&C Red No. 27	Tetrachlorotetrabromo-fluorescein	539	Marked shoulder at 505 mμ.
*Ext. D&C Red No. 4	Dichlorotetraiodo-fluorescein	546	
D&C Red No. 19	Rhodamine B	553	

* Commercial samples.
Color acids run in dilute NH_4OH.
Na salts run in plain H_2O.

acteristics of the certifiable fluorescein dyes. The work reported here gives a method for the spectrophotometric identification of any of the fifteen certifiable fluorescein colors, provided that only one color of this type is present.

The absorption spectra of the solutions of various fluorescein colors are, as would be expected, very similar in shape. The chief difference in the respective curves of colors of this type is the position of the absorption maximum. The wave length of the maximum extinction for dilute am-

moniacal solutions of the fifteen certifiable fluorescein colors is shown in Table 1.

Table 1 shows that in many curves the wave length of maximum absorption alone is sufficiently distinctive to identify the color. In several other curves where two dyes have very nearly the same wave length of maximum absorption, qualitative tests for the halogens and nitrogen will provide more definite identification.

It will be noted, however, that there are two pairs of colors, D&C Oranges No. 5 and 14, and D&C Reds No. 21 and 29, the members of which cannot be distinguished in this manner. Attempts to differentiate these dyes on the basis of differences in the location of the absorption peak in various solvents were unsuccessful. In an attempt to determine the isosbestic points of these colors, however, it was noted that there are observable differences in the spectrophotometric curves of these colors at certain *p*H values. Further work showed that these differences could be used to identify the colors.

EXPERIMENTAL

All optical measurements were made with a General Electric recording spectrophotometer equipped with slit adjustments for an 8-millimicron wave length band. To minimize the effect of the fluorescence of the dye, the cells containing the solutions used in this work were placed at the forward end of the transmission compartment, approximately five inches from the integrating sphere. Calculations indicate that under these conditions less than one per cent of the fluorescent light emitted by the sample should reach the integrating sphere.

The solutions used to obtain the data shown in Table 1 contained 0.5 ml of concd. ammonium hydroxide per 100 ml. For most of these colors the same curve is obtained at any *p*H above 6.0.

Solutions for the spectrophotometric determinations in the study of the effect of *p*H on the curves were prepared by pipeting 25 ml of the alcoholic dye solution into a 50 ml volumetric flask, and adding the required amount of buffer. The resulting solutions were made to volume with water. The buffer mixtures were prepared as directed by Clark and Lubs (2), except that the concentrations of the stock solutions were two to five times that specified to allow for subsequent dilution. (The *p*H values quoted are those the buffer mixtures would give in water alone. It is realized that these values may not represent the actual *p*H of the alcoholic solutions.)

DISCUSSION

Table 2 gives the data obtained on solutions of D&C Yellow No. 7, D&C Oranges No. 5 and 14, and D&C Reds No. 21 and 29, at various *p*H values. Typical sets of curves for each of these dyes are shown in Figures 1–5.

The data for fluorescein shows that the wave length of maximum ab-

TABLE 2.—*Wave length of absorption maximum and extinction at absorption maximum*

pH	FIG. 1 D&C YELLOW #7		FIG. 2 D&C ORANGE #5		FIG. 3 D&C ORANGE #14		FIG. 4 D&C RED #21		FIG. 5 D&C RED #29	
	WAVE LENGTH	EXTINCTION	WAVE LENGTH	EXTINCTION	WAVE LENGTH	EXTINCTION	WAVE LENGTH	EXTINCTION	WAVE LENGTH	EXTINCTION
	mμ	*% max.*	*mμ*	*% max.*	*mμ*	*% max.*	*mμ*	*% max.*	*mμ*	*% max.*
1.4							524	5.3	528	7.4
2.0							529	13.8	533	17.5
2.2							529	29.7	533	35.4
2.4							530	47.1	533	50.1
2.6							530	61.8	533	61.7
3.0			529	6.4	472	3.9	530	82.8	533	76.0
3.4			528	7.8	470–500	4.9				
3.8	454	1.6	525	10.0	516	7.8	529	98.8	531	88.8
4.0	454	2.9	517	19.0	514	21.3	527.5	97.1	528	91.3
4.2	454	5.8								
4.4	454 & 478	9.5	511	59.8	513	60.2				
4.8	481	20.3	510	83.6	512	77.5	523	98.3	525	96.5
5.2	489	35.9	510	93.4	511	87.3				
5.6	494	59.0								
6.0	495	78.8					522	100.0	524	100.0
8.0	496	100.0					522	100.0	524	100.0
8.4	496	100.0	509	100.0	510	100.0				

sorption shifts to shorter wave lengths as the *p*H is lowered. The extinction per milligram in the visible region decreases rapidly as the *p*H is lowered and is very low at any *p*H below 4. At all *p*H values less than 5, the curve shows a double peak.

The absorption curves for solutions of D&C Oranges No. 5 and 14 show that below *p*H 4, there are distinct differences in the location of the absorption maxima and the shape of the curves. For D&C Orange No. 5, the absorption maximum moves toward the longer wave lengths as the *p*H is lowered, but the shape of the curve remains the same. For D&C Orange No. 14, however, the maximum present at *p*H 4 flattens out as the *p*H is decreased, and a new absorption maximum appears at 472 mμ. The wave length of maximum extinction at *p*H 3.8 for D&C Orange No. 5 is 525 mμ and for D&C Orange No. 14, 516 mμ. There is also a greater proportional increase in the extinction at *p*H 6 over that at *p*H 3.4 for D&C Orange No. 14 than for D&C Orange No. 5. The ratio of the extinction at *p*H 6 to that of *p*H 3.4 is 12.9 for D&C Orange No. 5 and 23.4 for D&C Orange No. 14.

Since solutions of D&C Yellow No. 7 below a *p*H of 4 show very little absorption at 510 mμ, moderate contamination of D&C Orange No. 5 with D&C Yellow No. 7 has little effect on the location of the absorption peak or extinction values at the peak for solutions of D&C Orange No. 5 at the low *p*H values. The ratio E*p*H 6.0/E*p*H 3.4 is, however, increased and may be used to estimate the amount of D&C Yellow No. 7 present in samples of D&C Orange No. 5.

The differences between D&C Reds No. 21 and 29 are not as great as

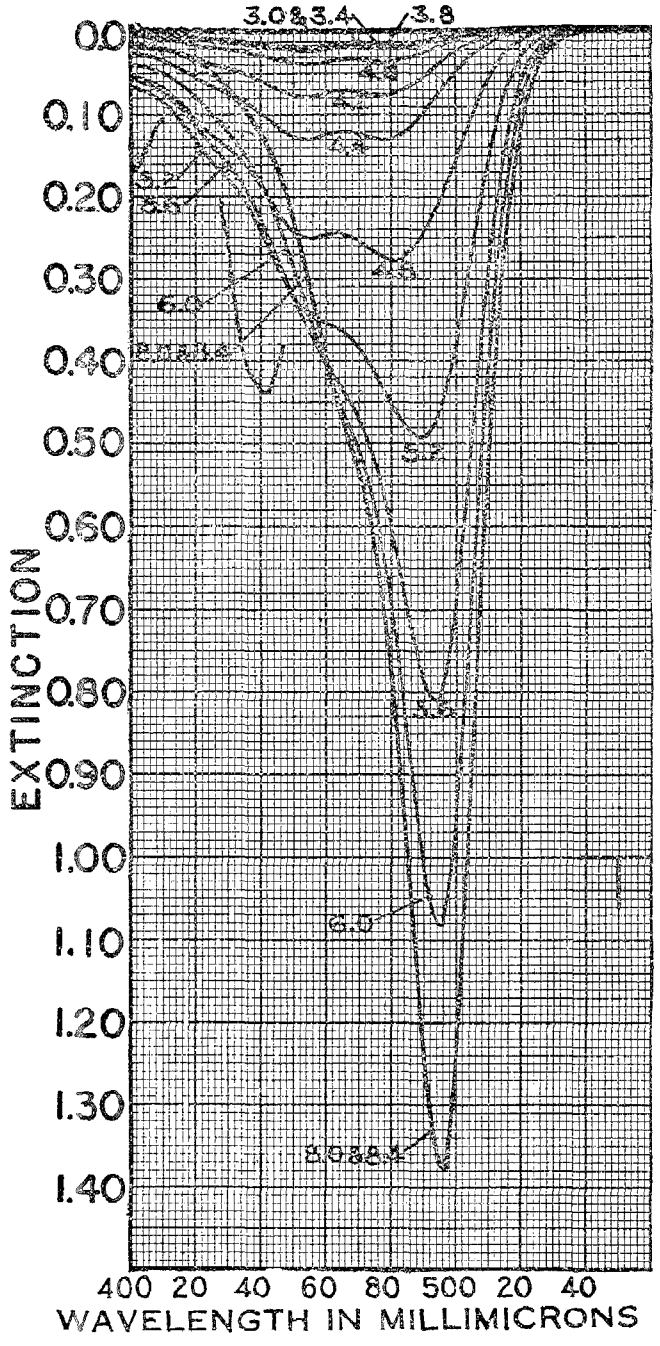

FIG. 1.—Extinction of solutions of D&C Yellow No. 7, 5.4 mg./liter, at various *p*H values. Solvent—50% alcohol. Cells—1 cm.

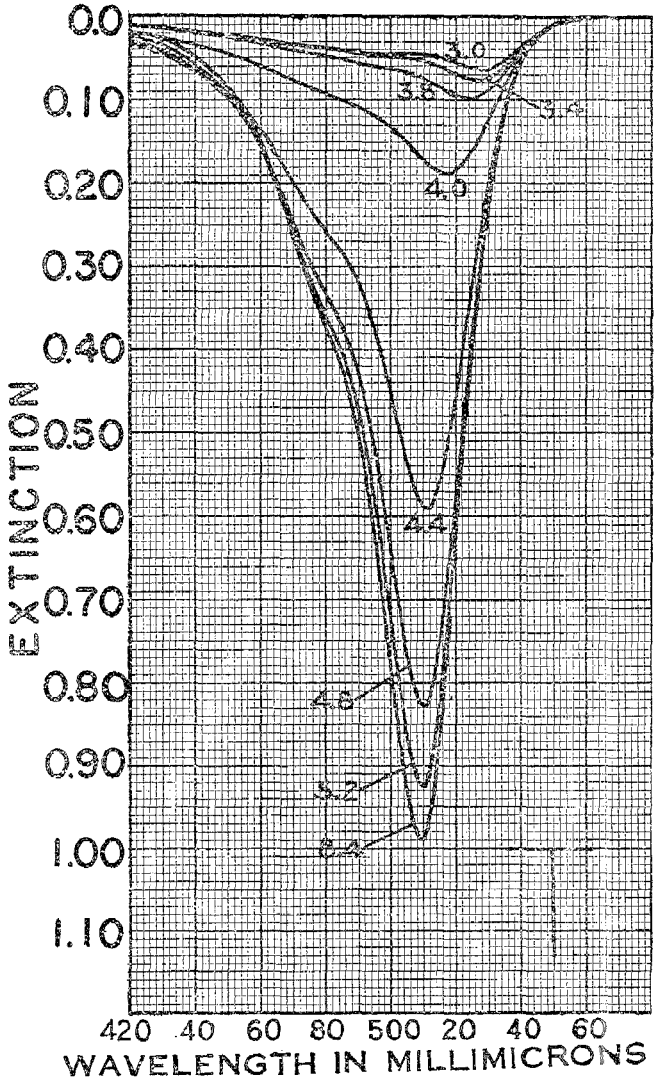

FIG. 2.—Extinction of solutions of D&C Orange No. 5, 6.0 mg./liter, at various *p*H values. Solvent—50% alcohol. Cells—1 cm.

those between D&C Oranges Nos. 5 and 14. Examination of the data, however, does show distinctive differences in the behavior of the two colors. For both colors, the absorption peak shifts steadily toward shorter wave lengths as the *p*H is raised. For D&C Red No. 29, the maximum extinction value increases continuously as the *p*H is raised until it reaches a limiting value at about *p*H 6. For D&C Red No. 21, however, the maximum

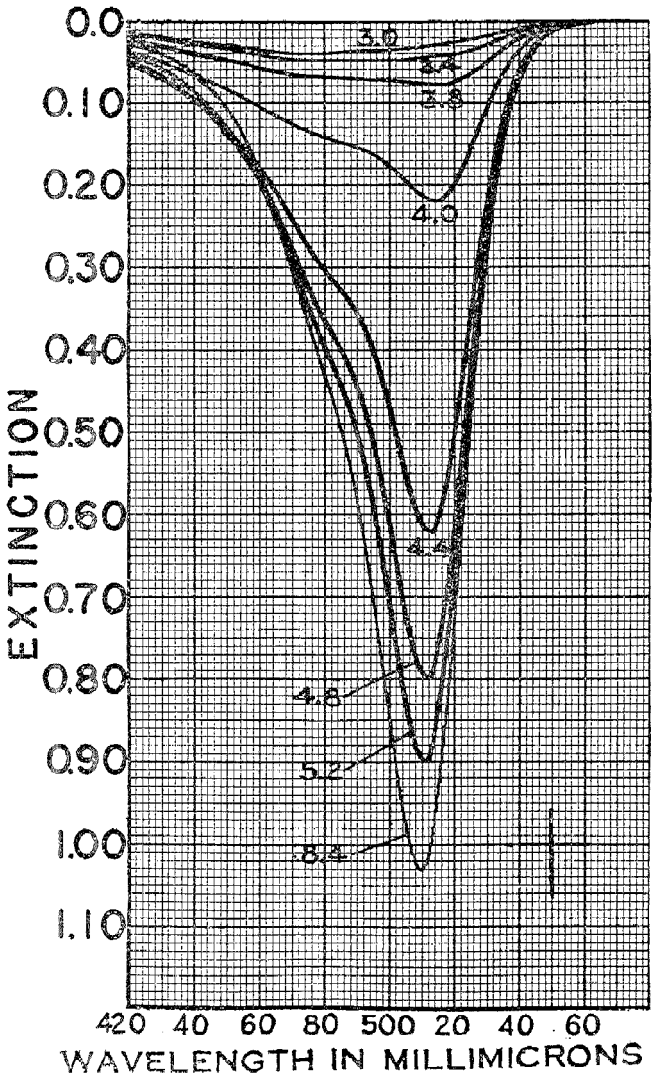

FIG. 3.—Extinction of solutions of D&C Orange No. 14, 7.0 mg./liter, at various *p*H values. Solvent—50% alcohol. Cells—1 cm.

extinction value increases until the *p*H is 3.8, is less at *p*H 4.0 and 4.4 than at 3.8, and finally increases to a limiting value at about *p*H 6.0.

The ratio E*p*H 3.8/E*p*H 2.0 is also useful as a supplementary means of identification. This ratio is 7.2 for D&C Red No. 21, and 5.2 for D&C Red No. 29.

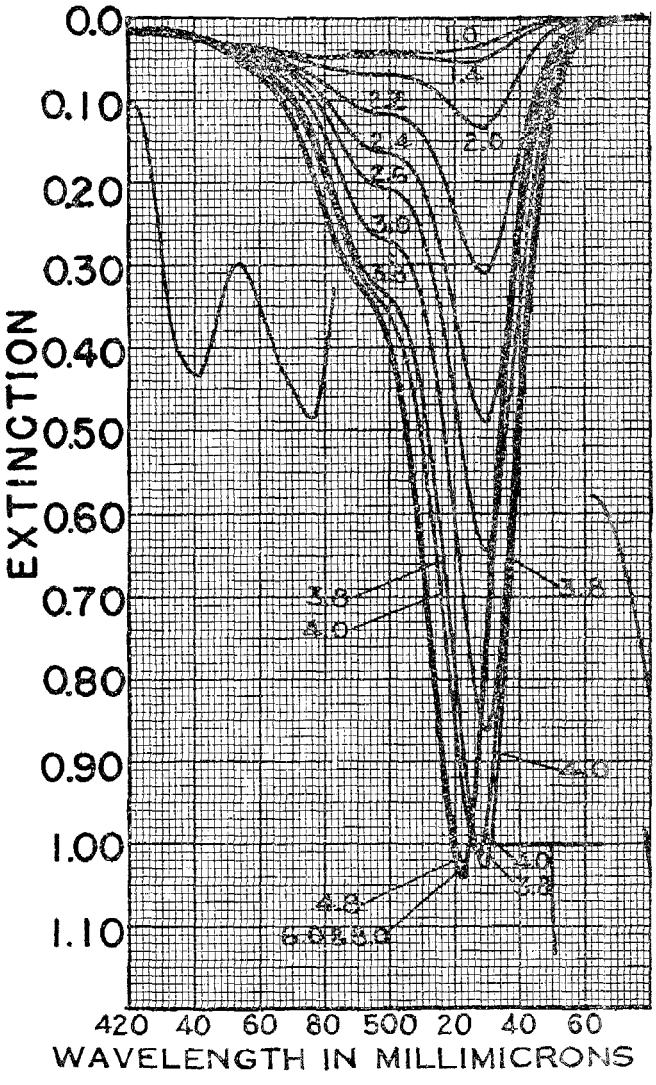

FIG. 4.—Extinction of solutions of D&C Red No. 21, 6.4 mg./liter, at various *p*H values. Solvent—50% alcohol. Cells—1 cm.

Curves for solutions of D&C Red No. 21 containing 2.0–22.0 per cent of added D&C Orange No. 5, at *p*H values of 2.0, 3.8, 4.0, and 4.4, were drawn. The distinctive decrease in the extinction for D&C Red No. 21 at *p*H 4.0 was not eliminated, although it became less apparent at the highest percentage of D&C Orange No. 5. The extinction value for the absorption peak at *p*H 4.4 increased very rapidly as the percentage of

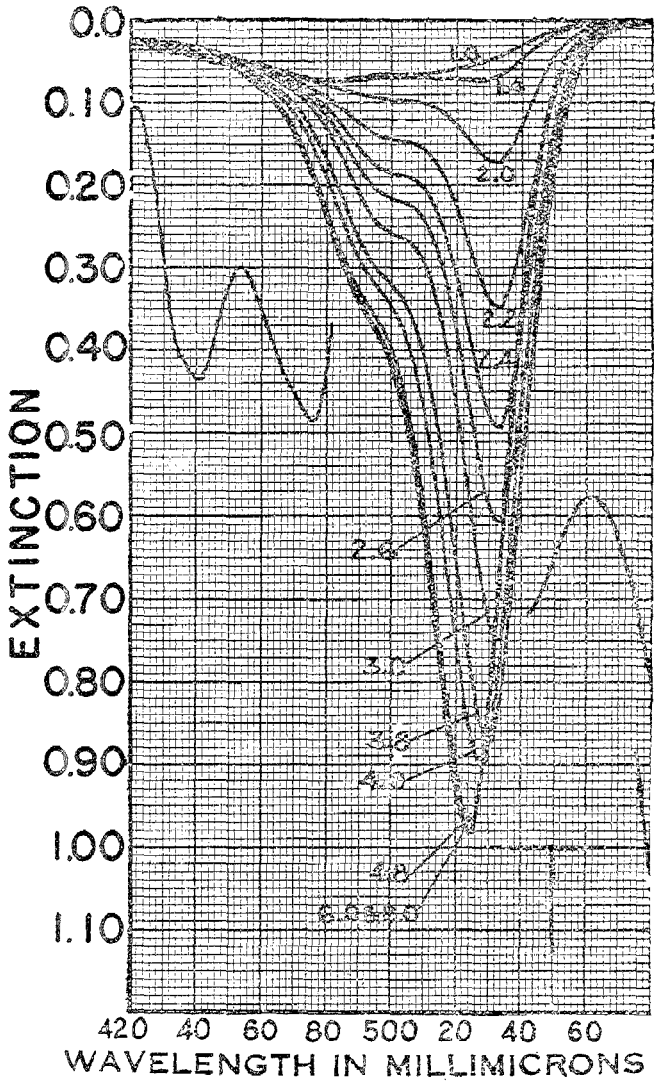

FIG. 5.—Extinction of solutions of D&C Red No. 29, 7.5 mg./liter, at various *p*H values. Solvent—50% alcohol. Cells—1 cm.

orange dye was raised and should provide an estimate of the amount of D&C Orange No. 5 present in the D&C Red No. 21.

APPLICATIONS

It should be noted that it is not necessary to obtain the curves at all the *p*II values listed in Table 2 to identify the dyes. Curves for solutions at

*p*H 3.0, 3.4, 3.8, and 6.0 are sufficient to identify D&C Oranges No. 5 and 14, while curves at *p*H 2.0, 3.8, 4.0, and 4.4 are sufficient to identify D&C Reds No. 21 and 29.

Samples of lipsticks were prepared containing the following percentages of commercial dyes:

Fluorescein dye	2.0
D&C Red No. 17	0.5
D&C Red No. 8	10.0

The samples were examined as unknowns and the fluorescein dye present identified without difficulty.

The data given in this report have been used to show that a sample of D&C Orange No. 5, which had a low bromine content, contained a considerable amount of unbrominated fluorescein.

It is planned to extend the study to determine the effect of *p*H on the absorption curves of solutions of the other certifiable fluorescein dyes and the non-certifiable fluorescein colors.

SUMMARY

Data on the wave length of maximum absorption for dilute ammoniacal solutions of the fifteen certifiable colors are given. These data will permit identification of most of these colors, provided only one color of this type is present. In other cases, qualitative analysis for halogens or nitrogen will be needed to complete the identification.

D&C Oranges No. 5 and 14 (and D&C Reds No. 21 and 29) cannot be differentiated on this basis. It is shown, however, that these colors can be identified from the spectrophotometric curves of solutions of the colors in fifty per cent alcohol solution at several *p*H levels.

Data on the location and magnitude of the absorption peak of solutions of D&C Yellow No. 7, D&C Oranges No. 5 and 14, and D&C Reds No. 21 and 29 at various *p*H values are presented and discussed.

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REPORT ON SPECTROPHOTOMETRIC ANALYSIS OF COAL-TAR COLORS

D&C GREEN NO. 6

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This report on the spectrophotometric determination of D&C Green No. 6 is a continuation of the program for the spectrophotometric analysis

of oil-soluble dyes certifiable under the Coal-Tar Color Regulations (1). Previous reports gave spectophotometric data for Ext. D&C Yellow No. 5 (2), D&C Red No. 35 (3), D&C Red No. 36 (3), Ext. D&C Orange No. 1 (4), and for D&C Violet No. 2 (5).

As before, conformity to Beer's law, location of the absorption peaks, and the extinction ratio at suitable wave lengths were determined for solutions of the dye.

EXPERIMENTAL

All the spectrophotometric data were determined with a General Electric recording spectrophotometer equipped with an automatic slit adjustment for an 8-millimicron wave length band.

Preparation of D&C Green No. 6 (Quinizarin Green)

One part of leucoquinizarin (obtained by reducing quinizarin—m.p. 195°C., with stannous chloride) was condensed with about eight parts of p-toluidine (m.p. 44°C.) in the presence of anhydrous boric acid and chalk. The resulting leuco dye was allowed to reoxidize in air to form quinizarin green. These processes were combined in one operation (6). The dye was boiled with dilute hydrochloric acid, washed with water until neutral, then boiled with dilute sodium hydroxide, washed, and dried. The dye was recrystallized three times from glacial acetic acid. Melting point (on Fisher block), 219–219.5°C. Recrystallization of this material from chloroform did not change the melting point. Literature, m.p. 218°C. (7).

The dye appears to pass through a transition stage at about 213–214°C. At that temperature it liquefies, but resolidifies upon further heating and finally melts sharply at 219–219.5°C. The pure dye, when heated in a crucible on a hot plate until completely liquid, cooled, and allowed to crystallize, melts at 219°C without preliminary liquefaction. Spectrophotometric examination of a solution of the standard dyestuff prior and subsequent to the heating treatment showed no change in spectrophotometric characteristics.

The purified material adsorbed on a column of activated alumina from petroleum ether and developed with benzene appeared homogeneous. A commercial preparation of D&C Green No. 6 when chromatographed in the same manner gave two bands (mauve and green). The mauve fraction was not definitely identified, but appeared to be unreacted quinizarin.

A portion of the commercial sample of D&C Green No. 6 was washed with hot dilute sodium hydroxide, hot water, hot dilute hydrochloric acid, dried and recrystallized once from glacial acetic acid. This material melted at 214°C. Two recrystallizations from chloroform raised the melting point to 218°C. Recrystallization from benzene brought the melting point to 218.5°C. The purified material was spectrophotometrically identical with the laboratory preparation and gave a homogeneous chromatogram. The sample prepared in this laboratory was, therefore, considered sufficiently pure to serve as a standard for D&C Green No. 6.

Spectrophotometric Data

The dye, weighed on a semimicro balance sensitive to 0.02 mg, was dissolved in about 50 ml of chloroform in a 100 ml volumetric flask. The solution was made to volume with chloroform, aliquot portions were diluted to a definite concentration, and the spectrophotometric curve determined. (All solutions were made to volume at the temperature of the room in which the optical measurements were made. U.S.P. chloroform was used throughout the experiment.)

In subsequent experiments identical spectrophotometric curves were obtained when gentle warming on a water bath was used to facilitate solution of the color in the solvent.

A typical set of data is shown in Table 1.

TABLE 1.—*Extinction values of solutions of D&C Green No. 6 in U.S.P. chloroform*

Typical data

CURVE NO. (CHART 1)	CONCENTRATION	EXTINCTION			$E_{645\,m\mu}$	$\frac{E_{630\,m\mu}}{E_{660\,m\mu}}$
		630 mμ	645 mμ	660 mμ	CONCENTRATION	
	mg./liter					
1	7.70	.288	.304	.272	.0395	1.059
2	15.41	.572	.608	.544	.0395	1.051
3	30.81	1.156	1.220	1.092	.0396	1.059
				Average....	.0395	1.06

Typical extinction curves for chloroform solutions of D&C Green No. 6 are shown in Figure 1. The curves show a minor absorption peak at approximately 412, and a characteristic double peak in the red area. The major absorption peak is at 645 ± 2 mμ. (All wave lengths were corrected to ± 2 mμ with the aid of didymium glasses calibrated by the National Bureau of Standards; see footnote to Figures 1 and 2.)

The average extinction per milligram per liter for D&C Green No. 6, calculated from the results of 30 determinations (at various concentrations) made from ten portions of the dye is 0.0395. The average deviation from the mean for these determinations was 0.2 per cent, and the maximum deviation 0.5 per cent.

Point readings of extinction values were taken at arbitrarily chosen wave lengths, 630 mμ and 660 mμ on opposite sides of the major absorption peak. The ratio of extinction values ($E_{630\,m\mu}/E_{660\,m\mu}$) at these wave lengths was $1.06 \pm .01$ (see Table 1).

A chloroform solution of the dye, stored for three days in the dark, gave spectrophotometric data identical with that of the freshly prepared solutions.

Application to Commercial Samples

Three samples of certified D&C Green No. 6 (straight colors) were analyzed spectrophotometrically. Weighed samples were dissolved in

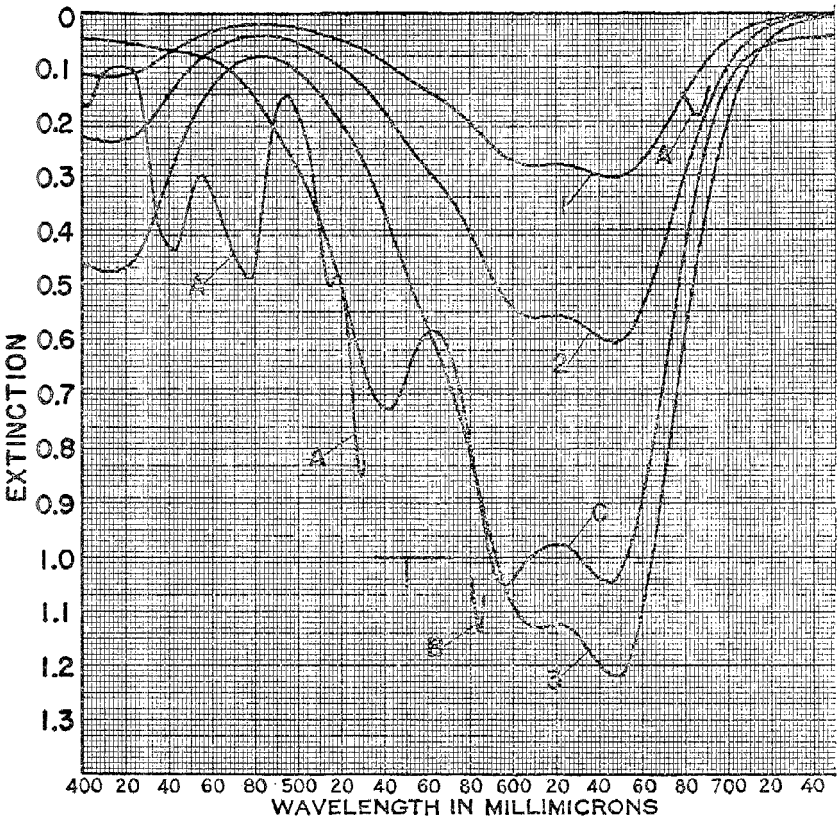

FIG. 1.—Solutions of D&C Green No. 6 in U.S.P. chloroform.
Curve 1— 7.70 mg./liter
Curve 2—15.41 mg./liter
Curve 3—30.81 mg./liter
Cells—1 cm.

A = Corning Didymium Glass 512, 6.0 mm. (Absorption peaks at 400.4, 441.4, 477.1, 529.0, and 684.8 mμ).
B = Corning Didymium Glass 592, 4.02 mm. (Absorption peak at 583.7 mμ).
C = Signal Lunar White Glass-H-6946236.

U.S.P. chloroform by warming on the steam bath. The solutions were transferred to 100 ml flasks, cooled, and made to volume at room temperature. Extinction measurements were made on appropriately diluted aliquots. The curves are shown in Figure 2, and the data in Table 2.

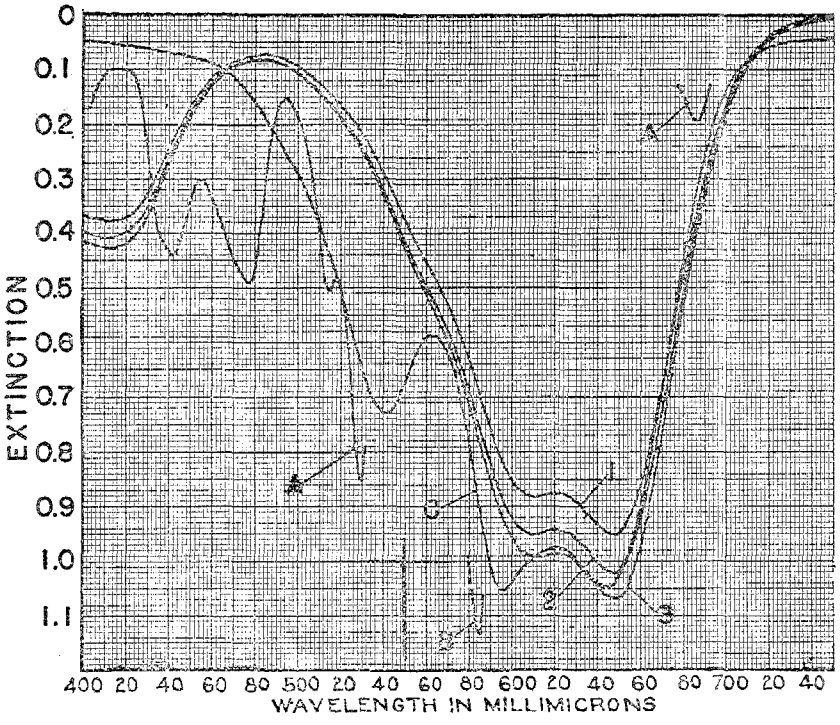

FIG. 2.—Certified samples of D&C Green No. 6.
Curve 1—24.69 mg./liter
Curve 2—26.74 mg./liter
Curve 3—27.60 mg./liter
Cells—1 cm.

A = Corning Didymium Glass 512, 6.0 mm. (Absorption peaks at 400.4, 441.4, 477.1, 529.0, and 684.8 mμ).
B = Corning Didymium Glass 592, 4.02 mm. (Absorption peak at 583.7 mμ).
C = Signal Lunar White Glass-II-6946236.

TABLE 2.—*Analysis of certified samples of D&C Green No. 6*
Straight color

SAMPLE NO.	M.P.	CONCENTRATION OF SAMPLE	$E_{633\,m\mu}$	DYE* SPECTROPHOTOMETRICALLY	DYE FROM NITROGEN CONTENT
		mg./liter		*per cent*	
1	213–214	24.69	0.952	97.6	97.2
2	216.5–217.5	26.74	1.020	96.6	98.0
3	218	27.60	1.067	97.9	98.3

* The dye content was calculated by using 0.0395 (Table 1) as the extinction value for 1 mg./liter of pure D&C Green No. 6.

DISCUSSION

The ratios of extinction to concentration in Table 1 show that at 645 mμ chloroform solutions of D&C Green No. 6 containing 7 to 31 mg of color per liter obey Beer's law. The "pure dye" content of a sample of this color can therefore be determined from the ratio of the extinction of a chloroform solution of the sample to that of a standard solution of the pure dye at 645 mμ.

SUMMARY

Spectrophotometric data for chloroform solutions of pure D&C Green No. 6 are presented. The dye is shown to follow Beer's law. The major absorption peak is at 645 ± 2 mμ. The extinction per milligram per liter at 645 is $.0395 \pm .0002$. The extinction ratio $E_{630\ m\mu}/E_{660\ m\mu} = 1.06 \pm .01$. Chloroform solutions of the dye are stable for at least three days if stored in the dark.

Application is made of these data to the determination of the pure dye content of commercial samples of the color. Typical results are given.

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REPORT ON SUBSIDIARY DYES IN COAL-TAR COLORS

SPECTROPHOTOMETRIC DETERMINATION OF D&C ORANGE NO. 4 IN SAMPLES OF FD&C ORANGE NO. 1

By MEYER DOLINSKY (Cosmetic Division, Food and Drug Administration, Federal Security Agency, Washington, D. C.), *Associate Referee*

The Coal-Tar Color Regulations permit a maximum of 5.0 per cent D&C Orange No. 4 in certifiable batches of FD&C Orange No. 1 (1). The tentative A.O.A.C. method (2) for the determination of D&C Orange No. 4 in samples of FD&C Orange No. 1 is not very satisfactory for routine analytical work, since it requires numerous extractions and takes $1\frac{1}{2}$ to 2 hours for each analysis.

It was found that the number of extractions and the time required could

be cut in half by taking a 50 to 100 mg sample and determining the extracted D&C Orange No. 4 spectrophotometrically. However, even this modified procedure was felt to be too slow. Furthermore, results obtained by the extraction procedure are usually low because of mechanical losses and incomplete separation of D&C Orange No. 4 from the FD&C Orange No. 1. Recoveries of D&C Orange No. 4 obtained when extracted alone and in the presence of FD&C Orange No. 1 are shown in Table 1.

TABLE 1.—*Recoveries of D&C Orange No. 4 by extraction procedure*

FD&C ORANGE NO. 1	D&C ORANGE NO. 4	D&C ORANGE NO. 4 RECOVERED
mg	*mg*	*per cent*
98	12 (modified procedure)	93
0	2 " "	91
0	50 " "	83
977	63 (A.O.A.C. procedure)	65

Because of the disadvantages of the extraction procedure it was felt that a more rapid and precise method would be desirable. In this connection it was decided to investigate a spectrophotometric method for the determination.

The absorption curves of neutral solutions of FD&C Orange No. 1 and D&C Orange No. 4 are quite similar, but there is a marked difference

TABLE 2.—*Spectrophotometric analysis of solutions containing purified D&C Orange No. 4 and FD&C Orange No. 1*

FD&C ORANGE NO. 1		D&C ORANGE NO. 4		RECOVERY OF D&C ORANGE NO. 4
ADDED	FOUND	ADDED	FOUND	
per cent	*per cent*	*per cent*	*per cent*	*per cent*
90.9	91.4	9.1	8.6	95
90.9	90.8	9.1	9.2	101
90.9	91.6	9.1	8.4	92
95.2	94.7	4.8	5.3	110
95.2	95.4	4.8	4.6	96
95.2	96.0	4.8	4.0	83
97.5	97.6	2.5	2.4	96
97.5	97.3	2.5	2.7	108
98.0	98.5	2.0	1.5	75
99.0	98.8	1.0	1.2	120
99.0	98.8	1.0	1.2	120
		Average Recovery.....		100
		Average Error.....		11
		Maximum Error.....		25

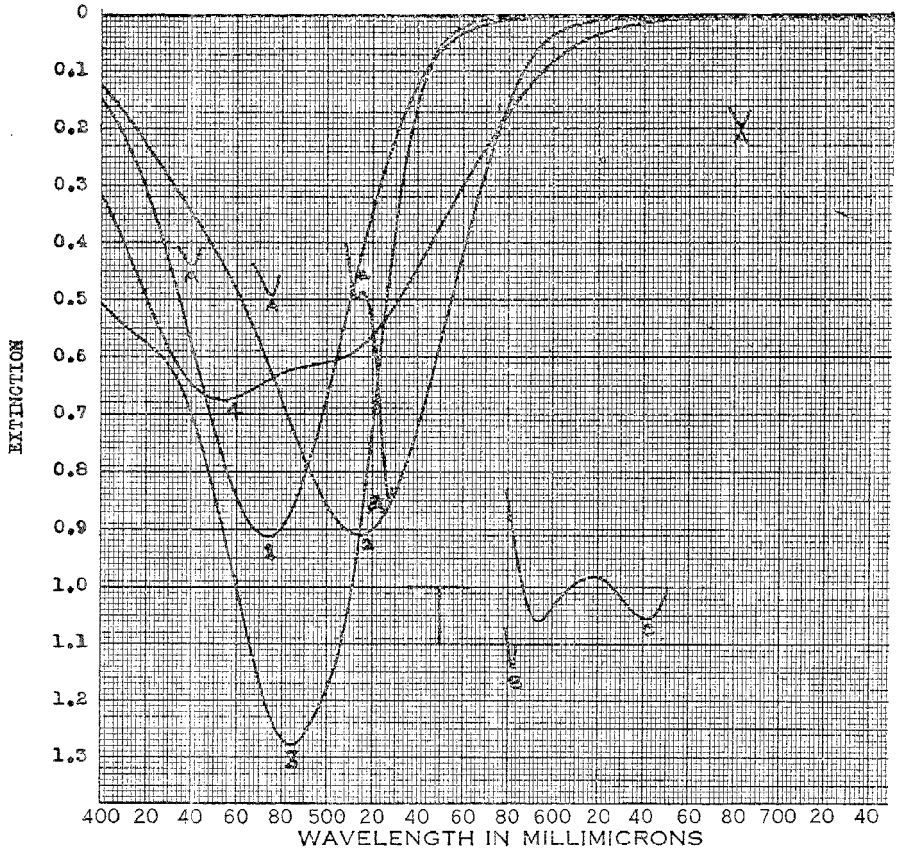

FIG. 1.—Absorption curves of FD&C Orange No. 1 and D&C Orange No. 4 in neutral and in 0.1 *N* NaOH solution.

Curve 1—FD&C Orange No. 1—Neutral —10 mg./liter.
Curve 2—FD&C Orange No. 1—In 0.1 *N* NaOH—10 mg./liter.
Curve 3—D&C Orange No. 4 —Neutral —20 mg./liter.
Curve 4—D&C Orange No. 4 —In 0.1 *N* NaOH—20 mg./liter.
Cells —1 cm.

A = Corning Didymium Glass 512, 6.0 mm. (Absorption peaks at 400.4, 441.4, 477.1, 529.0, and 684.8 mμ).
B = Corning Didymium Glass 592, 4.02 mm. (Absorption peak at 583.7 mμ).
C = Signal Lunar White Glass-H-6946236.

when the colors are dissolved in 0.1 *N* sodium hydroxide solution (Fig. 1). Each of the dyes follows Beer's law in 0.1 *N* sodium hydroxide solution. It is possible, therefore, to calculate the amount of each dye present in a mixture of the two from the absorption spectra of solutions of the mixture and the individual components by the use of simultaneous equations.

EXPERIMENTAL

Optical measurements were made with a General Electric Recording spectrophotometer and with a Beckman Quartz spectrophotometer.

The purified D&C Orange No. 4 used as a standard in these studies was obtained by twice recrystallizing a certified sample from alcohol. The purified FD&C Orange No. 1 was obtained by twice recrystallizing a certified sample from alcohol-water. Both colors were dried at 135°C. prior to use.

A suitable aliquot of a 0.02% solution of purified D&C Orange No. 4 was added to a definite amount of a 0.02% solution of FD&C Orange No. 1 and the mixture diluted with water and sufficient 0.4 *N* sodium hydroxide to give the solutions for spectrophotometric analysis. The densities of the mixture and the standards at 455 mμ and 515 mμ were determined and the percentage of each dye in the mixture calculated by the method of simultaneous equations. Results are shown in Table 2.

Two certified samples of FD&C Orange No. 1 were analyzed for D&C Orange No. 4 by both the extraction and spectrophotometric procedures. Results are shown in Table 3.

TABLE 3.—*Spectrophotometric determination of D&C Orange No. 4 in commercial samples of FD&C Orange No. 1*

SAMPLE	D&C ORANGE NO. 4 SPECTROPHOTOMETRICALLY	D&C ORANGE NO. 4 BY EXTRACTION
	per cent	*per cent*
No. 1	3.1 (Beckman) 3.0 (G.E.) 2.9 (G.E.) 2.4 (G.E.)	2.1 (Average of four determinations) (A.O.A.C. and Modified Procedure)
No. 2	4.0 (Beckman) 3.6 (G.E.)	2.9 (Modified Procedure)

SUMMARY

A rapid spectrophotometric method for the determination of D&C Orange No. 4 in samples of FD&C Orange No. 1 is presented.

The method is shown to be applicable to mixtures containing 1.0 to 9.1 per cent of pure D&C Orange No. 4 and 99.0 to 90.9 per cent of pure FD&C Orange No. 1, with an average error of 11.1 per cent. Values obtained for D&C Orange No. 4 in two commercial samples of FD&C Orange No. 1 by the spectrophotometric method were higher than those obtained by the A.O.A.C. extraction procedure.

It is recommended* that the method for D&C Orange No. 4 in FD&C Orange No. 1 be submitted to collaborative study, and that the topic be continued.

* For report of Subcommittee B and action of the Association, see *This Journal*, 32, 51 (1949).

REFERENCES

(1) SRA. F.D.C. 3—Food and Drug Administration.
(2) *Methods of Analysis, A.O.A.C.*, 6th Ed. (1945).

REPORT ON PURE DYE IN LAKES AND PIGMENTS

By KENNETH A. FREEMAN (Cosmetic Division, Food and Drug Administration, Federal Security Agency, Washington, D. C.), *Associate Referee*

The tentative procedure (1) for the determination of pure dye in lakes of D&C Red No. 8 and D&C Red No. 31 has been shown to be applicable to D&C Red No. 7 (2) and to D&C Red No. 10 (3). Since the previous report was presented at the meeting of the Association of Official Agricultural Chemists in October 1947, samples of D&C Red No. 14, D&C Red No. 34, and Ext. D&C Red No. 2 have been prepared, purified, and analyzed for one or more elements. The average purities calculated from these results were 99.9, 99.3, and 99.4 per cent, respectively. These calculated values for purity and the titanium trichloride titration figures differed by less than 1 per cent in each case.

Samples of D&C Red No. 14, Sodium Lake, D&C Red No. 34, Calcium Lake, and Ext. D&C Red No. 2, Barium Lake, were prepared and submitted to various laboratories for collaborative analysis. These lakes, prepared from the purified colors, contained about 35 per cent pure color. A portion of each of these lakes was submitted to the collaborators for analysis. The second samples submitted to the collaborators were prepared by diluting the above lakes with known amounts of talc.

Samples with directions for the analysis were sent to the following, listed alphabetically:

Ansbacher-Siegle Corporation—H. Holtzman reporting.
Calco Chemical Division American Cyanamid Company—Wm. Seaman reporting.
Harmon Color Works, Inc.—Vincent C. Vesce reporting.
Hilton-Davis Chemical Company—Anna Bartruff reporting.
H. Kohnstamm and Company, Inc.—Louis Koch reporting.
National Aniline Division, Allied Chemical and Dye Corp.—A. T. Schramm reporting.
Cosmetic Division, Food and Drug Administration—Charles Graichen and S. S. Forrest reporting.

In order to reduce the collaborators' work load to a minimum, it was requested that the results be reported in terms of ml of 0.1 *N* titanium trichloride required to titrate 0.5 gram of the lake.

The collaborative results reported last year showed considerable variation (2). It was suspected that this variation might be due to a lack of uniformity in standardization of the titanium trichloride solution. To

minimize this source of error, each collaborator was requested to standardize his titanium trichloride solution by a method supplied with the directions for the pure dye determination.[1]

The results are listed in Table 1 in the order in which they were received.

Most of the collaborative results are in good agreement with one another and the calculated value. The only real difficulty was encountered with sample No. 3. Two collaborators were unable to obtain consistent values on this sample while another reported a value nearly 25 per cent below the average (collaborator No. 1). The Associate Referee encountered considerable difficulty in mixing sample No. 3. All samples were milled for several hours in a laboratory ball mill. Sample No. 3 had a marked tendency to stick to the sides of the bottle and to the balls. To insure thorough mixing, the color was scraped off frequently, but it may be that, even with this precaution, uniform mixing was not obtained. The results of six of the eight collaborators, however, are close to the calculated value.

Most collaborators reported that it was necessary to employ an indicator in the titration of samples Nos. 5 and 6. Since the use of an indicator is stated in the method as optional with the analyst, such procedure is not precluded.

Collaborative analyses have now been completed and reported on the following certifiable coal-tar color lakes:

D&C Red No. 7, Calcium Lake
D&C Red No. 8, Sodium Lake
D&C Red No. 10, Sodium Lake
D&C Red No. 14, Sodium Lake
D&C Red No. 31, Calcium Lake
D&C Red No. 34, Calcium Lake
Ext. D&C Red No. 2, Barium Lake

The collaborative analyses show that the method is applicable to all of the colors studied. While strontium lakes have not been included in the studies thus, far, it has been the experience of the color certification laboratory that they behave no differently than sodium, calcium, and barium lakes. It is, therefore, felt that they may, without collaborative study, be included in the lakes to be analyzed by the method.

The Associate Referee believes that the work done has shown the method to be reliable and convenient for the determination of pure dye in lakes of D&C Red Nos. 6–16, inclusive, 31, 34, and Ext. D&C Red No. 2.

It should be noted that not all permitted lakes of these colors have been studied collaboratively. To do so would impose an undue burden upon the collaborators. Rather, it has been the purpose of this work to study representative lakes of the more commonly certified azo colors. This phrase of the topic has now been completed.

[1] This method is described in the report of the Associate Referee on standardizations of titanium trichloride solutions (*This Journal*, p. 589) as Method II.

TABLE 1.—*Collaborative results*

COLLABORATOR	D&C RED NO. 34, CALCIUM LAKE				D&C RED NO. 14, SODIUM LAKE				EXT. D&C RED NO. 2, BARIUM LAKE			
	SAMPLE NO. 1		SAMPLE NO. 2		SAMPLE NO. 3		SAMPLE NO. 4		SAMPLE NO. 5		SAMPLE NO. 6	
	0.1 *N* $TiCl_3$/0.5 g	PURE DYE	0.1 *N* $TiCl_3$/0.5 g	PURE DYE	0.1 *N* $TiCl_3$/0.5 g	PURE DYE	0.1 *N* $TiCl_3$/0.5 g	PURE DYE	0.1 *N* $TiCl_3$/0.5 g	PURE DYE	0.1 *N* $TiCl_3$/0.5 g	PURE DYE
	ml	*per cent*	*ml*	*per cent*	*ml*	*per cent*	*ml*	*per cent*	*ml*	*per cent*	*ml*	*per cent*
1	15.7		16.7		14.6*		19.5		11.5		10.7	
2	15.5		16.5		18.0		19.6		11.5		11.1	
3	15.3		16.2		—		19.1		11.1		10.7	
4	15.6		16.7		18.4		19.6		11.4		11.3	
5	15.8		16.4		18.5		19.5		12.0		11.3	
6	15.2		16.1		18.0		19.7		11.4		10.9	
7	15.4		16.4		18.3		19.4		11.3		10.8	
8	15.3		16.1		18.0		20.0		11.8		10.9	
Average Value	15.5	35.7	16.4	37.7	18.2	28.6	19.6	30.8	11.5	37.6	11.0	35.9
Calculated Value	15.7	36.1	16.5	38.0	18.4	28.9	19.6	30.8	11.4	37.3	11.0	35.9
Difference	−0.2	−0.4	−0.1	−0.3	−0.2	−0.3	0	0	+0.1	+0.3	0	0

* This figure disregarded in computing the average value.

RECOMMENDATIONS*

It is recommended—

(1) That the method for the determination of pure dye in lakes of D&C Reds Nos. 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 31, and 34, and Ext. D&C Red No. 2 be adopted as official, first action.

(2) That the topic be continued.

REFERENCES

(1) *Methods of Analysis, A.O.A.C.*, 6th Ed. **21.41**(d).
(2) FREEMAN, KENNETH A., *This Journal*, **31**, 595 (1948).
(3) CLARK, G. R., *Ibid.*, **28**, 761 (1945).

No report was given on buffers and solvents in titanium trichloride titrations, or on ether extract in coal-tar colors; identification of certified coal-tar colors; sulfonated amine intermediates; sulfonated phenolic intermediates; intermediates derived from phthalic acid; mixtures of coal-tar colors for drug and cosmetic use; subsidiary dyes in D&C colors; or hygroscopic properties of soal-tar colors.

The paper by K. A. Freeman and L. S. Harrow, entitled "A New Method for Determining the Boiling Range of Pseudocumidine in FD&C Red No. 1," is published in *This Journal*, **32**, 127 (1949).

The paper by M. Dolinsky, entitled "Spectrophotometric Analysis of D&C Red No. 19 (Rhodamine B)" is published in *This Journal*, **32**, 130 (1949).

* For report of Subcommittee B and action of the Association, see *This Journal*, **32**, 51 (1949).

WEDNESDAY—MORNING SESSION

REPORT ON FEEDING STUFFS

By L. S. WALKER (Vermont Agricultural Experiment Station, Burlington, Vt.), *Referee*

RECOMMENDATIONS*

It is recommended—

(1) That further study be made on the following:

(a) Mineral mixed feeds (calcium and iodine)
(b) Lactose in mixed feeds
(c) Fat in fish meal
(d) Adulteration of condensed milk products
(e) Crude fat of ether extract
(f) Microscopic examinations
(g) Fluorine
(h) Crude fiber
(i) Protein evaluation in fish and animal products
(j) Hydrocyanic acid glucosides
(k) Sampling and analysis of condensed buttermilk
(l) Tankage (hide, hoof, horn, and hair content)

(2) It is recommended that the tentative methods for calcium and phosphorus, *This Journal*, **31**, 98 (1947), and the tentative acetone method for fat in fish meal, *Ibid.*, be made official, first action.

REPORT ON MINERAL MIXED FEEDS—IODINE†

By ALFRED T. PERKINS, *Associate Referee*, and J. F. MERRILL, Kansas Agricultural Experimental Station, Manhattan, Kans.

The Elmslie-Caldwell method as published in the 1945 A.O.A.C. *Book of Methods* is a tentative method for the determination of iodine in mineral mixed feeds. Correspondence has indicated there are questions regarding the accuracy of this method for various types of feeds, especially feeds high in content of organic matter. The Associate Referee has understood that the method is intended to be applicable for mineral mixed feeds low in organic matter. The method has been tested as a method for high mineral feeds, and no attempts have been made to adapt the method for organic feeds. A not very recent publication[1] reports extensive tests on the method, and the work of the current year has been largely devoted to re-checking the method and ascertaining the effect of variations in the published procedure.

* For report of Subcommittee A and action of the Association, see *This Journal*, **32**, 42 (1949).
† Contribution No. 374, Department of Chemistry, Kansas Agricultural Experiment Station.
[1] *This Journal*, **21**, 597 (1938).

The ashing time and temperature has been found satisfactory for all mineral mixtures tested, but is not adequate for feeds high in content of organic matter. The recommended time and temperature is insufficient to ash organic feeds so a rapid filtration can be made.

The method has been tested to learn the effect of additions of excess bromine, and results show that such excesses do not interfere with analytical results. Sufficient bromine must be added to oxidize the iodine to iodate, and the excess bromine demands only a longer boiling time to removed this excess.

The boiling time has been tested and no loss of iodine has been found to occur with prolonged boiling.

Tests have been made on the effect of varying the amount of phosphoric acid required, and the work of 1938 has been checked. One hundred per cent of the iodine has been recovered with phosphoric acid additions of 1 ml in addition to the amount required to reach the methyl orange endpoint. Larger additions of phosphoric acid do not interfere with the titration, but smaller additions will not return all of the iodine and will result in an unstable end point.

There is an indication that cooling the solution to 10° gives a minor increase in the iodine titration. However, studies of the effect of the temperature of the solution during titration fail to show a significant difference in results due to cooling.

It is recommended that new collaborative tests be made looking to the adoption of the Elmslie-Caldwell method as official.

REPORT ON THE ACTIVITY OF YEAST

By H. C. SCHAEFER (Manager, Nutrition Research Laboratories, Ralston Purina Company, St. Louis, Mo.), *Associate Referee*

Two years ago a rather detailed report of our work on the activity of yeast was made.[1] Since that time, in trying to get further information regarding current interest in yeast added to feed for fermentation, it was found that several of the large producers have discontinued production of yeast for this purpose, and that yeast of this type is being sold by a relatively small number of producers.

The addition of live, dry yeast to feeds, and allowing it to ferment, is not a practice to be encouraged or recommended. From our present knowledge of nutrition, it appears that this practice destroys carbohydrates, and apparently does not create any other nutrients to compensate for that loss; hence it does not appear to be an economical operation. In our previous work it was found that yeast, with aging, loses its viability.

[1] *This Journal*, **30**, 599 (1947).

In view of the fact that there is little interest, and nothing definite to measure, it has been recommended that this work be discontinued, and that no more work be done until it is demonstrated that yeast activity creates some measurable nutrient; and that in the meantime yeast be considered in feeds merely on the basis of its value as a source of protein and water-soluble vitamins.

REPORT ON MINERAL CONSTITUENTS OF MIXED FEEDS

SAMPLE PREPARATION FOR CALCIUM AND PHOSPHORUS IN FEEDS USING NITRIC-PERCHLORIC ACID

By J. L. St. John (*Associate Referee*) and Edith Eng Huey (Division of Chemistry, Agricultural Experiment Stations and State Chemist's Laboratory, Pullman, Washington)

This is a continuation of the work reported in 1947 and 1948. The methods are those described in *This Journal*, **30**, 606 (1947).

Two different commercial mixed feeds were secured from a commercial mill.* Sample No. 4 is a mixed ration labeled Calf Meal and sample No. 5 is a Dog Meal. One basis of selection was the fact that the Dog Meal was higher in calcium and phosphorus than the Calf Meal. The ingredients were such as are found in commercial feeds of this type, including some fortification with vitamins and minerals.

The samples were somewhat finely ground when received. They were thoroughly mixed and sampled, but purposely sent out without further

Table 1.—*Collaborative results*

Collaborators	Calcium in feeds				Phosphorus in feeds			
	Sample 4		Sample 5		Sample 4		Sample 5	
	A.	B.	A.	B.	A.	B.	A.	B.
	per cent		*per cent*		*per cent*		*per cent*	
W. R. Flach		1.18		2.36		0.75		1.49
C. Tyson Smith		1.12		2.34		0.71		1.45
W. C. Geagley	1.17	1.13	2.18	2.15	0.59	0.59	1.39	1.29
George E. Grattan		1.40		2.33		0.70		1.50
Fred E. Randall	1.12	1.15	2.27	2.28	0.81	0.81	1.57	1.58
O. R. Alexander		1.07		2.52				
P. B. Curtis		1.60		3.00		0.57		1.49
M. P. Etheredge		1.47		2.39		0.74		1.49
E. E. Huey	1.29	1.34	2.58	2.58	0.72	0.72	1.49	1.49
L. V. Burns		1.56		2.99		0.81		1.65
Averages		1.30		2.49		0.71		1.49

A.—A.O.A.C. Method.
B.—Nitric-perchloric Method.

* Centennial Flouring Mills, Spokane, Washington.

grinding. The collaborators were requested to further grind and mix before analyzing. The results are reported in Table 1. Only three collaborators determined calcium and phosphorus by the A.O.A.C. method.

The results of the different collaborators are in fairly good agreement, although it might be anticipated that the agreement would not be quite as close as would have been obtained had the samples been more finely ground by the Associate Referee. Perhaps more important are the cases where a comparison is possible. There is excellent agreement between the results obtained by the A.O.A.C. method and by the nitric-perchloric acid method of sample preparation.

Based on three years' results it is recommended† that the nitric-perchloric acid method of sample preparation be made official, first action.

REPORT ON CRUDE FIBER

By VAN P. ENTWISTLE and WM. L. HUNTER (*Associate Referee*) (Feed Control Laboratory, California Department of Agriculture, Sacramento, California)

Crude fiber is the result of an arbitrary method of treatment of a material and does not represent a definite measure of a specific substance or group of substances. Crude fiber consists largely (97%) of cellulose and lignin (1). It does not represent all of the cellulose and lignin initially present. Cellulose recovery in crude fiber is approximately 60–80%, and in lignin recovery, 4–67%. Considerable variation in the lignin content of the crude fiber fraction is found. Highly lignified materials do not necessarily yield crude fiber fractions high in lignin. Thus the crude fiber fraction obtained does not bear any definite relationship to the structural constituents of a material.

Since the crude fiber method is definitive and does not measure a fixed portion of the materials tested, it can be changed by changing the definition, with the consent of those involved. In order for a new method or modification to gain sufficient support to be adopted, it must present improvements over the present method in that it will be quicker, easier to carry out, more reproducible, or represent the structural constituents of the material more truly. It is, of course, desirable that the results obtained be comparable to those of the present official A.O.A.C. method in order to retain the value of our present data.

The present official method is based on a method developed nearly a century ago by Henneberg, Stohmann, and Rautenberg (2) in the agricultural experiment station at Weende bei Göttigen in Germany. Only slight modifications have been made in the Weende method since its development. There have been numerous attempts to develop other methods which were easier or more exact.

† For report of Subcommittee A and action of the Association, see *This Journal*, **32**, 43 (1949).

A number of investigators have been of the opinion that we should attempt to approach pure cellulose and they have developed methods and evaluated them to this end.

Schulz (3) in 1856 developed a method for freeing cell membranes from encrusted lignin with nitric acid and potassium chlorate.

König (4) used glycerin and sulfuric acid to determine crude cellulose which some investigators look upon as crude fiber. This method was modified by Bellucci (5) to widen its application.

Hladík (6) used CCl_4 extracted samples and digested first with nitric and acetic acids. After pouring into cold water and washing by decantation the sample is heated with dilute sulfuric acid.

Scharrer and Kürscher (7) developed a method especially for feeding stuffs for which they claimed a higher purity for the end product (freer from lignin and pentosans). It is a single step method refluxing the sample with glacial acetic acid, nitric acid, and tri-chloracetic acid. Since lignin is removed more extensively than in the Weende (official) method, the crude fiber values found are lower. It is claimed to be more reproducible than the Weende method due to lignin removal.

Tang, Yen, and Hsü (8) developed a method using 1.5% chlorindioxide solution, then treatment with 30% pyridine solution, and allowing the sample to stand overnight in a sodium hydroxide (7%)–sodium chloride (3%) mixture.

Other investigators have taken the view that crude fiber should be directly relative to the indigestible matter in a material. Therefore, enzymatic digestive methods were developed which purport to give a much closer correlation to the digestibility of the material under question.

Remy (9) used dilute pepsin-hydrochloric acid solution, then malt diastase solution, and finally digestion with pancreatin-sodium carbonate solution, all operations carried out at 37°C.

Horwitt, Cowgill, and Mendel (10) modified the procedure using a special takadiastase (clarase) for the malt diastase and trypsin for pancreatin. This method took 6 days to complete.

Woodson and MacKenzie (11) simplified the procedure for application to cereals. This method called for boiling the sample with water to gelatinize the starch, cooling to 38°C., digestion with pangestin for 48 hours; adjustment of the *p*H to 7.5–8.0, and an additional 48-hour digestion with pangestine.

All of the enzymatic methods give considerably higher results than are obtained with the Weende method. Also these methods are very lengthy and do not meet the need for a speedy analysis which most laboratories require.

The term fiber in plant materials is commonly applied to cellulose; yet it appears that crude fiber values would be more valuable as an indicator of the material's feeding value if the crude fiber contains as much

as possible of both the cellulose and the lignin. This would eliminate those methods which are attempting to approach pure cellulose. The enzymatic methods, while claiming a close correlation to the feeding value, are too slow to be of value in control work. Until a method is developed to meet the above requirements and be practical for control work, we should continue attempts to improve our present method.

The A.O.A.C. method has been reexamined at intervals with numerous helpful criticisms, but with no basic changes. Bidwell and Walton (12), Bidwell and Bopst (13), Francis (14), Hanson (15), and others have studied thoroughly the various steps in our present official method. The results of their findings are:

(1) Size and shape of flask are not critical so long as the material does not adhere to sides of flask out of digestion solution.

(2) Any efficient condenser is satisfactory.

(3) 350-mesh wire filters gave results comparable to cloth filters.

(4) Non-fat extracted samples gave slightly higher results.

(5) Volume of digestion solution not critical. 400 ml. of solution did not lower results appreciably.

(6) The fineness of grind affects results. The finer the sample is ground the lower the crude fiber result.

(7) Neutralization of the acid, rather than filtering off, may give high results because of precipitation of substances previously made soluble in the acid digestion.

(8) Intensity of heat applied affects results. Vigorous boiling gives lower results than a less violent boiling.

(9) Filtering aids showed little effect. Asbestos caused slightly lower results on some materials, slightly higher on others.

(10) Delay in filtering. A delay of 7 minutes in the acid filtration will cause a lower result due to the continued action of the acid. A similar delay in the alkali filtration has the same effect for the same reason at first, but if the delay continues, substances made soluble in the hot alkali may precipitate as the solution cools. This is especially true of samples high in protein. Neubert, Van Amburgh, and St. John (16) found that samples which filtered difficultly in the final filtration could be made to filter rapidly by the addition of potassium sulfate. Results of this method agree very closely to the official method on easily filtered samples, but give somewhat lower values on those which filter slowly. However, it is felt that this modification yields results more valid than the official method, because the long time involved for the final filtration introduces errors in the official method.

The official method (17) allows latitude in the size and type of digestion flask, type of condenser, filtering cloth and in the choice of Gooch or alundum crucibles for the final filtration. It specifies the use of asbestos as a filter aid. A survey conducted by Hunter (18), of 69 participants in the American Association of Feed Control Officials collaborative work, shows that the various laboratories vary considerably on these optional points and also on others where no option is allowed. This is done in spite of the necessity of close adherence to an empirical method, as stated by Bidwell and Walton (12), Hunter (18), and others. A study of the results obtained in this collaborative series does not show however, that there is any

correlation between results obtained and equipment and technique used. Of the 69 participants surveyed, only seven were found who were using the same method throughout the determination. The survey showed the following types of equipment and technique used:

TABLE 1.—*Equipment and technique of 69 collaborators*

	Digestion Vessel Size (ml)						
	300	400	500	600	750	800	1000
Number using	1	1	23	20	10	1	13

	Vessel Shape		
	Beaker, Tall form	*Beaker*, Regular	*Flask*, Erlenmeyer
Number using	37	9	23

	First Filtration			
	Cloth Filter	*Wire Filter*	*Neutralization*	*Other Types Filter*
Number using	55	6	3	4

	Source of Heat	
	Gas	*Electric*
Number using	27	41

	Type of Condenser		
	Air	*Running Water*	*Flasks Filled with Water*
Number using	9	58	2

	Filter Aid		
	Asbestos	*Glass Wool*	*None*
Number using	34	1	33

	Final Filtration		
	Gooch	*Alundum*	*Other*
Number using	38	27	3

This shows clearly that 500 or 600 ml tall form beakers are the choice of the majority. Cloth filters for the acid filtration, electric heat, and running water condensers are wide favorites. Asbestos, while specified in the official method, is used by approximately only half of those surveyed. Gooch crucibles are a slight favorite for the final filtration. It is interesting to note that of the 38 using Gooch crucibles, 22 (58%) used asbestos,

whereas of the 27 using alundum crucibles, only 6 (22%) used asbestos. We have found that asbestos speeds the final filtration if the proper type of asbestos is used. All asbestos is not satisfactory, even so-called Gooch grades. Bakers "Powminco" grade acid washed and fired is the only type which we have found that is satisfactory. Unreported variations are doubtless being made which also have their effect on the crude fiber value found.

One point that the A.A.F.C.O. collaborative work demonstrates is the fallacy of reporting crude fiber results to the second decimal place. A study of the crude fiber results of this collaborative work indicates that for the present, crude fiber results should not be reported closer than to the nearest quarter per cent. Using the crude fiber average and allowing a tolerance of one quarter per cent, about one third, at best, of the collaborators will still be outside of the range. To continue to report to the second decimal place is to continue to place an absolutely false value on the reliability of the result found. Reporting to the closest quarter per cent will still leave many laboratories who will need to improve their technique and follow the official method more closely in order to fall within the suggested tolerance.

Popov (19) and Lepper (20) have suggested radical changes in the Weende (A.O.A.C.) method. Popov reports that by increasing the concentration of the acid and alkali digestion solutions to 2.1% the digestion

TABLE 2.—*Comparative data on time of digestion*

TYPE OF MATERIAL	LEPPER METHOD 10 MIN.	A.O.A.C. METHOD 30 MIN.
	per cent	*per cent*
Broiler feed	5.9	5.7
Rabbit pellets	6.9	6.3
Meat and bone scraps	2.9	2.5
Dairy feed	8.1	8.2
Turkey starter mash	6.0	5.8
Turkey feed	6.6	6.3
Turkey feed	7.0	6.7
Turkey finish mash	6.0	5.8
Dairy feed	8.0	7.0
Ground barley	6.9	6.7
All purpose mash	5.8	5.6
Calf meal	7.7	7.8
Meat and bone scraps	1.3	1.6
Cottonseed meal	9.7	10.0
Ground barley	7.6	7.7
Corn gluten meal	4.2	4.1
Wheat bran	9.6	10.0
Copra	9.5	10.3
Alfalfa meal	26.8	27.8

time can be shortened to 15 minutes, and still yield results comparable with the usual 30-minute digestion with 1.25% solutions. Lepper advocates using 3.125% acid and alkali solutions and shortening digestion time further to 10 minutes. He also claims results comparable to the Weende (A.O.A.C.) method. Lepper tried to shorten the digestion time to 5 minutes by further increasing the concentration of the digestion solutions, but it was not satisfactory. We have made a short investigation of Lepper's method. The results obtained are shown in Table 2.

These data definitely indicate a possibility of shortening the time required for the determination without disturbing present concepts of crude fiber.

It appears that Lepper's method or that of Popov may at least have a place in industrial control work where results are needed in a hurry. The data also suggests that laboratories located at high altitudes might vary the digestion solution concentration or digestion time to compensate for reduced boiling temperatures.

It is strongly urged that one or both of these shorter methods be given collaborative study to determine their merit as a possible replacement for the present official method.

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No reports were given on lactose in mixed feeds, fat in fish meal, adulteration of condensed milk products, crude fat or ether extract, microscopic examination, fluorine, protein evaluation in fish and animal products, hydrocyanic acid glucosides, sampling and analysis of condensed buttermilk, and tankage (hide, hoof, horn, and hair content).

No report was given on waters, brine, and salt, including boron in water and fluorine in salt.

CORRECTIONS IN FEBRUARY JOURNAL

Report on Changes in Methods, Vol. 32, No. 1

Page 74. Pyrogallol in Hairdyes, Quantitative determination: Under "reagents," line 2, change "$FeDo_4$" to read "$FeSO_4$."

Page 75. Line 18. Delete "s" on "*Solutions.*" Line 3. Change "*Aluminum*" to "*Alumina.*" Under "Standardization," change formula to read "$k = \frac{D}{c}$" (Insert = sign). Under Liquid Dyes "Continuous Extraction," line 11, change "in" to "into."

Page 76. Henna Powder Mixture, line 1, after "Weigh 0.9 to 1.1" insert "g."

ANNOUNCEMENT

L. M. Beacham, Food and Drug Administration, Washington, D. C., has been appointed Referee on Processed Vegetable Products, in place of V. B. Bonney.

CONTRIBUTED PAPERS

THE ANALYSIS OF CASTOR OIL IN LIPSTICK*

By S. H. NEWBURGER (Division of Cosmetics, Food and Drug Administration, Federal Security Agency, Washington 25, D. C.)

A principal ingredient of nearly all lipsticks, castor oil consists essentially of the glyceryl ester of ricinoleic acid, an unsaturated hydroxy fatty acid. The most widely used analytical methods for the determination of castor oil employ the acetylation of the free hydroxyl group as the basis of the analysis. Modern lipsticks, however, contain many substances in addition to castor oil which can be acetylated. Beeswax has an acetyl value of 15.2, carnauba wax 55.2, and lanolin 23. Most of the organic lakes, fluorescein dyes, and oil soluble colors have active hydroxyl or amino groups. Still other easily acetylated materials which may be encountered are glyceryl monostearate and cetyl alcohol. It is obvious, therefore, that the application of the acetylation technique to the determination of castor oil in lipstick requires the preliminary removal of interfering substances.

The following is a brief summary of the method developed: The lipstick was boiled with benzene, centrifuged, the liquid decanted, and the residue of insoluble lakes discarded.[1] The benzene was evaporated, the residue treated with hot glacial acetic acid and with hot light mineral oil. Most of the organic material was extracted into the mineral oil layer; while the castor oil, together with some of the dyes and other materials, was drawn off in the glacial acetic acid. The glacial acetic acid was diluted with water and extracted with ether. The ether solution in turn was extracted with dilute aqueous alkaline alcohol to remove fluorescein dyes. Evaporation of the ether left a residue which consisted of the castor oil, oil soluble color, and small amounts of other substances. The residue was saponified and the unsaponifiable matter discarded. This eliminated the oil soluble dye, any higher alcohols, and entrained mineral oil. Finally, the fatty acids including those of the castor oil were extracted. By acetylation of these acids it was possible to determine the castor oil content of the lipstick. A more detailed account of the procedure is as follows:

MATERIALS USED IN THE PREPARATION OF THE EXPERIMENTAL LIPSTICKS

(1) *Lipstick base.*—A castor oil-free lipstick base was prepared. It is doubtful whether such a base is of practical value; however, it was desired to incorporate a number of ingredients which would interfere with the acetylation procedure. The following ingredients were melted together at 100°C and. then cooled to room temperature to form the experimental lipstick base:

* Presented at the Annual Meeting of The Association of Official Agricultural Chemists, held at Washington, D. C., October 11, 12, and 13, 1948.

[1] The method used for the elimination of the lakes was suggested in a private communication from K. A. Freeman of this Division.

	Gms.
U.S.P. White Beeswax	30
U.S.P. Anhydrous Lanolin	10
U.S.P. Heavy Mineral Oil	5
U.S.P. Petrolatum	5
Ceresin	5
Glyceryl Monostearate	5
Carnauba Wax	5
Cetyl Alcohol	3

(2) *Lake mixture.*—A combination of certified lakes was prepared:

Lake	*% Pure Dye*	*Substratum*	*Gms.*
D&C Red No. 9 Ba Lake	36	Gloss White	2
D&C Red No. 10 Na Lake	83	Gloss White	2
D&C Red No. 31 Ca Lake	44	Gloss White	2
D&C Orange No. 15 Ca Lake	35	Gloss White	2

(3) *D&C Red No. 21.*—A certified straight color containing 98% dye.
(4) *D&C Red No. 17.*—A certified straight color containing 94% dye.
(5) *Castor Oil.*—A U.S.P. product.

The various experimental lipsticks were prepared by heating together at 100°C., lipstick base, lake mixture, D&C Red No. 21, D&C Red No. 17, and castor oil.

METHODS OF ANALYSIS

PROCEDURE

Isolation of castor oil fatty acids:

Weigh ca 4 gm of lipstick into a 250 ml beaker, add 50 ml of benzene, cover beaker with watch-glass, and heat to boiling for several minutes. Cool the mixture to room temperature and transfer to centrifuge tube. Rinse the original beaker with 10 ml of hot benzene and add to the contents of the centrifuge tube. Centrifuge mixture and decant liquid thru cotton plug into a 150 ml beaker. Add 25 ml of hot benzene to residue in centrifuge tube, mix thoroly, again centrifuge and also decant into the 150 ml beaker. Evaporate the benzene on the steam bath, add 10 ml of glacial acetic acid to the residue, cover beaker with watch-glass, boil for several minutes, and transfer the hot mixture to a 250 ml separatory funnel. Rinse beaker with 10 ml of hot glacial acetic acid and 30 ml of hot (100°C.) U.S.P. light mineral oil and add rinsings to the contents of the separatory funnel. Shake mixture well.[2] Fasten stopcock of separatory funnel with a rubber band, partially immerse the separatory funnel in a steam bath for several minutes and then draw off the acetic acid layer into a 100 ml beaker. Continue the extraction with 2 additional 20 ml portions of hot glacial acetic acid taking care to keep the mixture hot by immersing the separatory funnel in the steam bath for a few minutes each time. Combine the acetic acid extracts, cool to room temperature with stirring, and allow to stand for ½ hour. Slowly add 3 ml of water with stirring and allow to stand for another five minutes. Filter the mixture slowly by gravity or gentle suction thru a Gooch crucible containing a ¼" to ⅜" layer of sand over an asbestos mat. Wash the filter with a solution of 15 ml of acetic acid and 1 ml of water. Transfer the filtrate and washings to a 500 ml separatory funnel with the aid of 100 ml of ether, add 250 ml of water and shake well. Draw off the aqueous layer and re-extract with 2 additional 50 ml aliquots of ether. Wash the combined ether extracts with two 50 ml portions of water. Discard washings and extract the ether with two 100 ml portions of a 20% alcohol solution containing 3% KOH. Continue the extraction with 50 ml portions

[2] In extracting hot mixtures a cotton glove was worn to protect the hand.

of the alkaline alcohol solution until any extracted color shows little or no fluorescence. Discard the alkaline extracts, acidify the ether solution with HCl, wash with two 50 ml portions of water, and evaporate the ether on the steam bath. Saponify the residue by refluxing for 2 hours with a mixture of 50 ml of benzene, 25 ml absolute alcohol, and 1 gm KOH. Transfer the hot saponified mixture to a separatory funnel, add 50 ml of hot water and shake well.[3] Draw off the aqueous layer and extract with 2 additional 20 ml portions of hot benzene. Wash the combined benzene extracts with two 25 ml portions of 30% alcohol containing 1% KOH. Discard the benzene solution. Combine the two alkaline alcohol wash solutions, extract with 20 ml of ether to remove dye, discard the ether, and add the alkaline wash solution to the original extracted alkaline aqueous solution. Acidify the alkaline solution and extract with 30, 20, and 20 ml portions of benzene. Combine the benzene extracts, wash with water, and filter thru a cotton plug into a tared beaker. Evaporate the benzene on the steam bath, dry residue at 100°C. for 10 minutes in oven, cool, and weigh as fatty acids.

Acetylation of the hydroxy fatty acids:

Dissolve the isolated fatty acids in benzene, transfer to a 50 ml volumetric flask, dilute to mark with benzene, and mix. Pipet a 25 ml aliquot into a 125 ml acetylation flask and reserve the remaining solution for a blank. Evaporate the benzene on the steam bath with the aid of a glass tube, connected to the vacuum, and projecting into the acetylation flask. Add 5 ml of acetic anhydride and reflux for 2 hours. Add 25 ml of hot water, allow to stand on steam bath for 30 minutes with occasional swirling, add 15 ml of toluene, and transfer the hot mixture to a 250 ml separatory funnel. Rinse the acetylation flask with 25 ml of toluene and add rinsings to the contents of the separatory funnel. Fasten stopcock of separatory funnel with a rubber band, partially immerse the separatory funnel in the steam bath for a few minutes, and then shake well for about five minutes. Again partially immerse the separatory funnel in steam bath for a few minutes and then draw off the aqueous layer. Continue the extraction with 15 ml portions of hot water, heating each time in the steam bath, until the aqueous layer requires less than 0.1 ml of 0.1 *N* NaOH to turn phenolphthalein pink. Filter the toluene solution thru a cotton plug into a tared 150 ml beaker, and wash the separatory funnel and cotton plug with benzene. Evaporate the toluene-benzene solution on the steam bath, dry the residue at 100°C. for 15 minutes in an oven, cool, and weigh the acetylated material.

Transesterification of the acetylated material and subsequent saponification of the ethyl acetate:

The procedure is based on the Sclar and Clark[4] modification of Freudenberg's method. This is an indirect method as the acetyl group of the acetylated hydroxy acids is converted into ethyl acetate which is in turn saponified.

The apparatus is described in *This Journal*, **27**, 473 (1944).

Surround the receiver with an ice bath.

Dissolve and transfer the acetylated material to the distilling flask with 20 ml of hot absolute alcohol. Dissolve and transfer 2 gm. of *p*-toluenesulfonic acid to the same flask with another 10 ml of absolute alcohol.

Distill from a vigorously boiling water bath; 20 minutes after the first distillate drops into the receiver, add 25 ml of absolute alcohol and distill for 20 minutes; then add another 25 ml of absolute alcohol and distill for another 20 minutes.

Remove the boiling water bath, disconnect the receiver, stopper and label as

[3] Troublesome emulsions can be broken by fastening the stopcock of the separatory funnel with a rubber band and partially immersing the separatory funnel in a steam bath until the contents begin to boil.

[4] R. N. Sclar and G. R. Clark, *This Journal*, **27**, 472 (1944).

distillate No. 1. Connect another receiver, replace boiling water bath, add 25 ml absolute alcohol to the distilling flask and distill for 20 minutes. Disconnect the receiver and label the distillate No. 2.

Add 25 ml of 0.1 *N* NaOH to distillate No. 1 and 10 ml of 0.1 *N* NaOH to distillate No. 2, connect the receivers to reflux condensers, and immerse the flasks in a boiling water bath for 15 minutes. Wash down the condenser of receiver No. 1 with 50 ml of water and that of No. 2 with 15 ml of water. Cool the two solutions to room temperature, add 5 drops phenol red indicator (0.1% alcohol solution) to distillate No. 1 and 3 drops to distillate No. 2. Titrate with 0.1 *N* HCl to a yellow end point.

Standardize the 0.1 *N* HCl against 0.1 *N* NaOH by pipeting 25 ml of the standard alkali into a solution of 75 ml of absolute alcohol and 50 ml of water. Add 5 drops of phenol red indicator solution and titrate with the HCl to a yellow end point. Calculate the normality of the HCl.

Run a blank on the reserved unacetylated fatty acids as follows: Transfer the approximately 25 ml of reserved benzene solution to the distillation flask, evaporate the benzene, and add 2 gm *p*-toluenesulfonic acid and 30 ml of absolute alcohol. Proceed as described for the acetylated material beginning with: "Distill from a vigorously boiling water bath . . . ."

Calculation of per cent castor oil.—Subtract the HCl titers of the acetylated material from the corresponding titers of the blank and add the two differences. Use this value in the following formula to calculate the percentage of castor oil.

$$\frac{\text{Ml of 0.1 } N \text{ HCl} \times 0.004001 \times 2 \times 100}{0.1177 \times 0.951 \times \text{w't of sample (gm)}} = \% \text{ Castor Oil.}$$

Ml of 0.1 *N* HCl × 0.004001 = gm of NaOH required to saponify the acetylated material.

0.1177 = gm of NaOH required to saponify compounds obtained from the acetylation of 1 gm of castor oil fatty acids; *see* Table 2.

0.951 = fatty acid fraction of castor oil; *see* Table 1.

The factor of 2 is used because only one-half of the sample is acetylated.

EXPERIMENTAL RESULTS

Castor oil was saponified and the unsaponifiable matter and fatty acids determined. The procedure was similar to that already described with the exception that chloroform instead of benzene was used to extract the fatty acids. The results are given in Table 1.

TABLE 1.—*Saponification of castor oil*

SAMPLE U.S.P. CASTOR OIL	UNSAPONIFIABLE MATTER	FATTY ACIDS
gm	*gm*	*gm*
3.462	0.019 (0.5%)	3.291 (95.1%)

The castor oil fatty acids were acetylated, transesterified, and the ethyl acetate saponified according to the described procedure. The data are given in Table 2.

It should be noted that the saponification values in Table 2 are based on

TABLE 2.—*NaOH required to saponify acetylated castor oil fatty acids*

SAMPLE CASTOR OIL FATTY ACIDS	WEIGHT OF ACETYLATED MATERIAL	NaOH REQUIRED TO SAPONIFY ACETYLATED ACIDS
gm	*gm*	*gm NaOH/gm fatty acids*
0.414	0.453	0.1167
0.211	0.232	0.1187
		Av. 0.1177

TABLE 3.—*Analysis of castor oil in lipstick*

SAMPLE		WEIGHT OF FATTY ACIDS	WEIGHT OF ACETYLATED MATERIAL	CASTOR OIL	
				ADDED	FOUND
	gm	*gm*	*gm*	*per cent*	*per cent*
Lipstick base	3.500	0.138	0.069	None	0.8
Lake mixture	0.400				
D&C Red No. 21	0.080				
D&C Red No. 17	0.020				
U.S.P. castor oil	0.000				
Total	4.000				
Lipstick base	3.300	0.350	0.183	5.5	6.2
Lake mixture	0.400				
D&C Red No. 21	0.080				
D&C Red No. 17	0.020				
U.S.P. castor oil	0.221				
Total	4.021				
Lipstick base	3.100	0.611	0.329	12.5	13.3
Lake mixture	0.400				
D&C Red No. 21	0.080				
D&C Red No. 17	0.020				
U.S.P. castor oil	0.515				
Total	4.115				
Lipstick base	2.700	0.871	0.472	20.2	20.1
Lake mixture	0.400				
D&C Red No. 21	0.080				
D&C Red No. 17	0.020				
U.S.P. castor oil	0.811				
Total	4.011				

the original weight of the fatty acids rather than the weight of the acetylated product.

Lipstick samples varying in castor oil content were prepared and analyzed by the outlined method of analysis. The results are given in Table 3.

DISCUSSION

The data shown in Table 3 indicate that the castor oil content of a lipstick can be determined within 1%. As there is a blank of 0.8% on the castor oil free ingredients, the values should be high. This was observed with two of the experimental lipstick preparations. With the third preparation, highest in castor oil content (20.2%), approximately the theoretical result was obtained. A loss of some castor oil was probably counterbalanced by the blank.

If one assumes that all the castor oil fatty acids have been recovered, then, from the data in Table 3, the weight of non-castor oil fatty acids varies from 0.100 gm to 0.140 gm. It is conjectured that most of this material is contributed by the glyceryl monostearate.

It is unfortunate that the final titers are rather small. The compounds obtained from the acetylation of one gram of castor oil fatty acids require only 29.4 ml of 0.1 *N* NaOH for saponification. The blank on the unacetylated material never exceeded 0.3 ml of 0.1 *N* NaOH. This blank is, therefore, of doubtful necessity when only a rough estimate is required of the castor oil present.

As it is not possible to easily obtain two 25 ml aliquots from 50 ml of solution, the blank contains a little less material than the aliquot taken for acetylation. However, this difference is not significant since the blank is small.

CONCLUSION

An analytical method has been developed for the determination of castor oil in lipstick. Typical results indicate that the castor oil can be determined to within 1%.

THE ASSAY OF PROCAINE HYDROCHLORIDE SOLUTION, N.F.

By SAM D. FINE, Food and Drug Administration, Federal Security Agency, Cincinnati, Ohio)

The September, 1948, issue of the *Journal of the American Pharmaceutical Association* contains a paper (1) on the spectrophotometric determination of procaine in procaine penicillin G. The method is quite simple, involving merely the weighing of a sample, making two dilutions, and measuring optical density at 290 mμ against water as a blank. The

concentration of the solution is obtained by calculation from an extinction coefficient determined on a sample of pure procaine hydrochloride.

If there is no interference from the other ingredients, it should be possible to assay procaine hydrochloride solution, N.F., by diluting to a suitable concentration with water, measuring the optical density against a water blank and calculating the concentration from an extinction coefficient as above. The National Formulary product contains 20 mg of procaine hydrochloride per milliliter in isotonic salt solution; various preservatives may be used, including chlorbutanol, cresol, and sodium bisulfite.

EXPERIMENTAL

Apparatus.—Transmittancy measurements were made with a Beckman quartz spectrophotometer equipped with one centimeter silica cells and ultraviolet radiation source operating with a spectral band width of approximately 1–5 mμ.

Effect of Interferences.—The effect of the interference by sodium chloride, chlorbutanol, cresol, and sodium bisulfite was investigated. A solution of each was prepared of the same concentration that is present upon dilution of the N.F. product as directed by the recommended spectrophotometric procedure. Transmittancy measurements were made on these solutions from 220 to 320 mμ. The results are shown in Table 1. Transmission of all is greater than 95% at 290 mμ.

TABLE 1.—*Transmission (per cent) of sodium chloride, sodium bisulfite, chlorbutanol, and cresol*

WAVE LENGTH	SODIUM CHLORIDE	SODIUM BISULFITE	CHLORBUTANOL	CRESOL
mμ	*0.8 mg/1000 ml*	*0.3 mg/1000 ml*	*1.0 mg/1000 ml*	*0.3 mg/1000 ml*
220	91.5	94.7	89.2	87.0
230	93.6	96.5	92.5	93.5
240	94.9	97.7	94.3	96.9
250	95.5	98.5	95.0	97.5
260	96.0	98.5	95.2	97.2
270	96.2	98.5	95.5	97.3
280	96.4	98.8	95.8	97.3
290	96.8	98.8	96.1	97.4
300	97.0	98.4	96.2	97.7
310	97.2	98.3	96.5	98.2
320	97.3	98.6	96.7	98.4

Comparison of Spectrophotometric and National Formulary (2) Methods.—A solution was prepared containing 20 mg/ml of procaine hydrochloride and 5 mg/ml of chlorbutanol in isotonic salt solution. Transmittancy measurements from 220 to 320 mμ made on a dilution of this solution disclosed that maximum absorption was at 290 mμ. Assay by the National Formulary method gave 19.8 mg/ml; assay by the spectrophotometric

method gave 20.2 mg/ml. A series of samples from different manufacturers were assayed by the spectrophotometric procedure and the National Formulary method. The results are shown in Table 2.

TABLE 2.—*Comparison of spectrophotometric method and National Formulary method*

SAMPLE	PRESERVATIVE		PROCAINE HYDROCHLORIDE, MG/ML	
			SPECTROPHOTOMETRIC METHOD	N.F. METHOD
		per cent		
1	Chlorbutanol	0.5	21.4	21.3
	Sodium bisulfite	0.1		
2	Sodium bisulfite	0.1	19.8	20.0
3	Chlorbutanol	0.5	19.5	19.8
4	Sodium bisulfite	0.1	19.9	19.7
5	Chlorbutanol	0.25		
	Sodium bisulfite	0.1	20.7	20.4
6	Sodium bisulfite	0.1	20.2	19.9
7	None		20.8	20.4
8	Chlorbutanol	0.25	20.3	20.4
9	Cresol	0.1	20.1	19.8
10	Chlorbutanol	0.25	20.0	19.9

Effect of Decomposition of Procaine Hydrochloride: Spectrophotometric Assay.—One sample assayed by the recommended spectrophotometric procedure was found to contain less than the declared amount of procaine hydrochloride. Assay by the National Formulary method gave erratic results. Assay was made by A.O.A.C. Methods, **39.102** (3) (bromination) and **39.104** (3) (distillation). Both methods determine total procaine originally present, and the results were 19.3 and 19.7 mg/ml, or approximately the declared amount. Procaine hydrochloride decomposes by hydrolysis into *p*-amino benzoic acid and *β*-diethylamino ethyl alcohol. A study of the ultra-violet absorption of procaine hydrochloride and of its decomposition products was made. Transmittancy measurements were made for each from 220 to 320 mμ; the results are shown in Figure 1. The maximum absorption for procaine hydrochloride is at 290 mμ; the maximum for *p*-amino benzoic acid is at 268 mμ; *β*-diethylamino ethyl alcohol does not absorb ultraviolet light, the transmission being greater than 95% in the region where the other two compounds exhibit maxima.

Transmittancy measurements were made on the partially decomposed sample from 220 to 320 mμ; the results are also shown on Figure 1. The maximum is at 286 mμ, in contrast to the maximum of 290 mμ found for the prepared sample. These results indicated that both procaine hydrochloride and *p*-amino benzoic acid were present.

After extraction from ammoniacal solution with chloroform as in the National Formulary method, the solution of this sample was acidified and

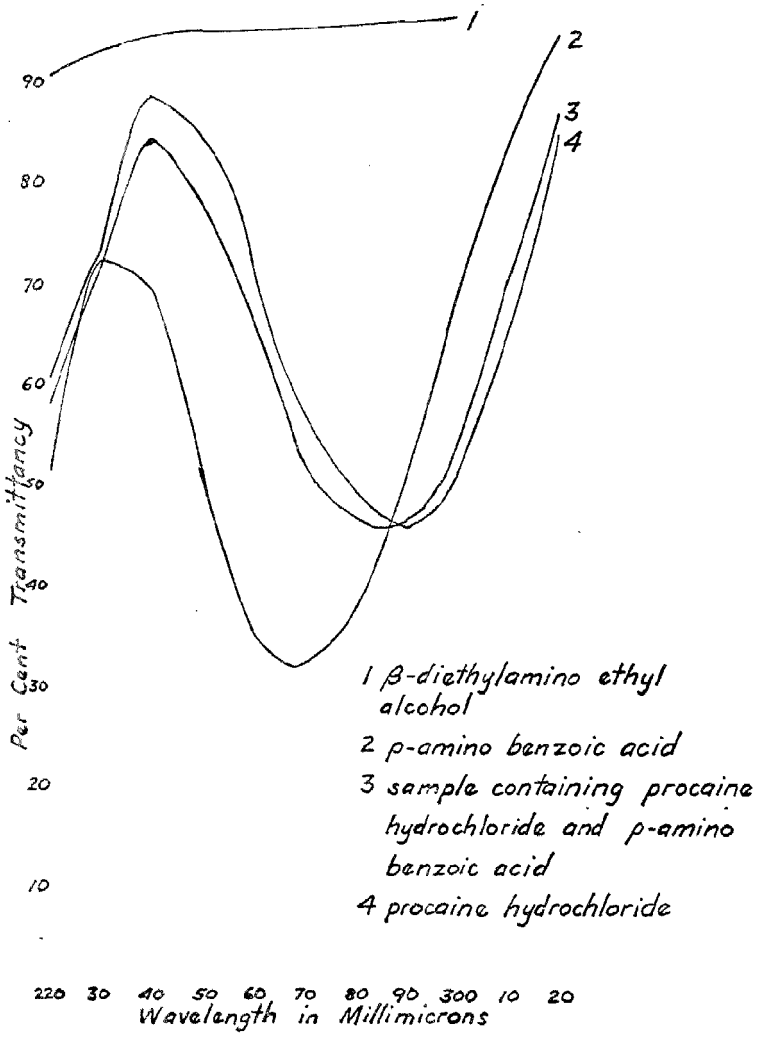

FIG. 1

extracted with ether. The extract, after evaporation of the ether, was dried and the melting point was found to correspond with that of *p*-amino benzoic acid. The *p*-amino benzoic acid was determined quantitatively by bromination after extraction of the procaine. Results were 5.3, 5.5 mg/ml of *p*-amino benzoic acid.

A spectrophotometric method of assay for both the procaine hydrochloride and the *p*-amino benzoic acid was devised. The optical densities

of standards and of the sample were measured at 268 and 290 mμ, and a set of two equations in two unknowns established. Let

E_1 = extinction coefficient for procaine hydrochloride at 290 mμ.
E_2 = extinction coefficient for *p*-amino benzoic acid at 290 mμ.
E_3 = extinction coefficient for procaine hydrochloride at 268 mμ.
E_4 = extinction coefficient for *p*-amino benzoic acid at 268 mμ.
Cp = concentration of procaine hydrochloride as mg./100 ml.
Cb = concentration of *p*-amino benzoic acid as mg./100 ml.
D290 = optical density of the unknown at 290 mμ.
D268 = optical density of the unknown at 268 mμ.

Then

$$D290 \times 1000 = E_1Cp + E_2Cb$$
$$D268 \times 1000 = E_3Cp + E_4Cb$$

Solving for Cp and Cb

$$Cp = \frac{1000\ (E_4D290 - E_2D268)}{E_1E_4 - E_2E_3}$$

$$Cb = \frac{1000\ (E_1D268 - E_3D290)}{E_1E_4 - E_2E_3}$$

Using this procedure, 11.0 mg/ml of procaine hydrochloride and 4.5 mg/ml of *p*-amino benzoic acid were found. The 4.5 mg of p-amino benzoic acid is equivalent to 9.0 mg of procaine hydrochloride. This corresponds to a total of 20.0 mg/ml of procaine hydrochloride originally present. (The figures found for total procaine hydrochloride by the two A.O.A.C. (3) methods were 19.3 and 19.7 mg/ml.)

In order to check this method, a solution containing procaine hydrochloride and *p*-amino benzoic acid was prepared and assayed. Recovery of the added procaine hydrochloride was 97.1%, and of the added *p*-amino benzoic acid 102.0%.

Effect of Decomposition of Procaine Hydrochloride: The National Formulary Assay.—The effect of the second decomposition product, β-diethylamino ethyl alcohol was considered. It is soluble in both water and organic solvents. It is the product that is distilled over in A.O.A.C. (3) method **39.104,** and if present in the residue after evaporation of the chloroform, would consume standard acid. The results obtained on the partially decomposed sample by the National Formulary method were 16.8, 13.9, 15.9, 14.1, 15.6, and 12.6 mg/ml procaine hydrochloride. Further determinations by the National Formulary method were made, with the chloroform being evaporated under varying conditions. Dependent upon these conditions, more or less standard acid was consumed. A laboratory sample of 2% procaine hydrochloride solution was assayed by the National Formulary method, and the residue after evaporation of the chloroform was allowed to remain on the steam bath for over a minute. There was no appreciable loss of procaine base, as determined by consumption of standard acid.

When applied to a partially decomposed solution of procaine hydrochloride, the procedure specified in the National Formulary monograph results in β-diethylamino ethyl alcohol being present along with procaine in the chloroform extract. The variation in the results is due to the variation in the evaporation of this volatile constituent. The National Formulary method is the same as A.O.A.C. (3) method **39.103.** This latter method bears the parenthetical statement that it determines only undecomposed procaine. However, a review of the collaborative work leading to its adoption as official, failed to disclose that it was ever tried on a partially decomposed procaine hydrochloride solution.

Recommended Spectrophotometric Procedure—Pipet 10 ml of the sample into a 1000 ml volumetric flask and make to volume with distilled water. Transfer 20 ml of this dilution to a 1000 ml volumetric flask, dilute to volume with distilled water, and measure the optical density of the resulting soln at 290 mμ against water in the reference cell. Obtain the concentration of the soln by calculation from an extinction coefficient determined on a sample of pure procaine hydrochloride. Determine if any decomposition of the procaine has occurred by checking the wave length of maximum absorption. Maxima at less than 290 mμ indicate decomposition. By use of "two color" analysis, proportion of procaine hydrochloride and *p*-amino benzoic acid can be determined. (Clean absorption cells thoroly before use. Take the average of the readings for several adjustments of the instrument.)

SUMMARY

A spectrophotometric method for the assay of procaine hydrochloride solution, N.F., has been described. Accuracy, compared with the National Formulary method, is good where no decomposition of the procaine hydrochloride has occurred. The National Formulary method and the A.O.A.C. (3) method **39.103** are not applicable to decomposed procaine hydrochloride solutions. Decomposition of such solutions can be detected by determination of the wave length of maximum absorption. By the use of "two color" analysis, the extent of decomposition can be determined.

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THE ANALYSIS OF MIXTURES OF FATTY OILS WITH HYDROCARBONS

By K. A. WILLIAMS (Consulting Chemist, 161–165 Rosebery Avenue, London, England)

S. H. Newburger (1) has worked out a method, based on a statement of the author (2), for the separation of hydrocarbons from their mixtures with wool grease by chromatographing a solution of the mixture in

petroleum benzin (B.P. 30–75°C.) through an activated aluminum oxide column. Under the conditions he describes, both hydrous and anhydrous lanolin are adsorbed to an extent greater than 99 per cent; hydrocarbons are not adsorbed at all, but may be recovered quantitatively from the percolate from the column.

For more general application, especially for the determination of small percentages of mineral oil in admixture with fatty oils, it is not possible to apply the chromatographic technique directly to the oil-hydrocarbon mixture. In such cases, the unsaponifiable matter should first be prepared and, after solution in light petroleum or petroleum benzin, should be passed through the column of aluminium oxide. If suitable conditions are employed for the chromatographing, hydrocarbons percolate through the column and may be recovered from the percolate quantitatively, while the rest of the unsaponifiable matter remains adsorbed. There is abundant evidence (1, 2, 3, 4) that the percolate consists of pure hydrocarbon. The technique of S. H. Newburger (3) may be employed for the separation. The following alternative technique has been in use in the author's laboratory for a number of years.

METHOD

PREPARATION OF AL_2O_3 COLUMN

Apparatus.—Constrict a glass tube, 30 cm. to 40 cm. long, and 1.5 cm. in internal diameter, at one end and join the constricted end to a short piece of glass tubing of 0.5 cm. internal diameter. Fix the tube vertically by passing the narrower end thru a cork into a filter flask. Tamp a 1.5 cm. cotton plug into the bottom of the wide part of the tube.

Column.—Mix aluminium oxide of suitable grade (Alorco grade F-20 mesh 80–200 or B.D.H. aluminium oxide for chromatographic analysis) with sufficient light petroleum (B.P. 40–60°C.), or petroleum benzin (B.P 30–75°C.) to form a thin slurry from which air trapped in the Al_2O_3 separates easily. Pour the slurry carefully into the chromatograph tube thru a funnel so that it distributes itself evenly. Apply very light suction to the filter flask and, while maintaining constantly a head of petroleum over the Al_2O_3, build up the depth of this in the tube to a total depth of approximately 15 cm. by further additions of the slurry. Maintain a layer of at least 2 cm. of petroleum over the top surface of the column from this point until the chromatography is finished.

DETERMINATION

Prepare the unsaponifiable matter from a suitable weight of the oil, quantitatively by a recognised standard method. In the case of most fatty oils, the weight of oil taken should be from 2.0 to 2.2 grams; if the percentage of unsaponifiable matter exceeds 5 per cent, the weight taken should be reduced to approximately 1 gram. The method of most general application for the quantitative separation of the unsaponifiable matter is the S.P.A. method (5); if the oil concerned in the test is not of marine origin, use either the method of the A.O.C.S. (6) or that of Bolton and Williams (7).

Dissolve the unsaponifiable matter so prepared in from 50 to 70 ml of light petroleum (B.P. 40–60°C.), or of petroleum benzin (B.P. 30–75°C.), and pour the solution gradually thru the column. Take care not to disturb the upper surface of the Al_2O_3 in adding the solution; assist filtration by gentle suction applied to the

filter flask, adjusting the pressure in the flask so that the rate of filtration does not exceed about 2 drops per second of liquid leaving the tube. Keep the Al_2O_3 continuously covered with a layer of solvent as previously described. When the solution has nearly all passed into the oxide layer, wash the column with light petroleum or petroleum benzin, passing in all about 150 ml of this thru the column. The tube should be nearly filled at each addition of the washing solvent, and the layer above the Al_2O_3 should be allowed to reduce to about 2 cm. in depth before the next addition of solvent is made.

Transfer the contents of the filter flask thru a filter to a weighed flask, rinsing and washing the filter and the flask into the weighed flask. Evaporate the solvent in the usual manner by distillation from a hot water bath, dry the residue in an oven at 100°C. for 10 minutes, cool in a desiccator, and weigh. Repeat the drying until the weight is constant to 1 mg.

Divide the weight of hydrocarbon obtained by the weight of oil originally taken and multiply by 100 to give the percentage.

DISCUSSION

This method has been used extensively in this laboratory for the separation of squalene from olive oil (4), and for the detection and determination of mineral oil in fatty oils that do not normally yield appreciable amounts of hydrocarbon when pure. Such oils include linseed oil, teaseed oil, and rapeseed oil; the method is also suitable for examining lanolin and sperm oil and certain sulphonated oils used in the leather industry. As is shown by the data recorded below, mineral oils have been found in the past few years to occur to varying small extents in the above oils.

TABLE 1.—*Recovery of hydrocarbons from pure fatty oils*

MATERIAL EXAMINED	UNSAPONIFIABLE MATTER (S.P.A. METHOD)	HYDROCARBON SEPARATED
	per cent	*per cent*
(a) Oil prepared in laboratory:		
Linseed oil no. 1	1.18	0.05
Linseed oil no. 2	1.25	0.07
Linseed oil no. 3	1.12	0.04
(b) Oils believed to be free from mineral oil:		
Sperm oil no. 1	42.9	0.10
Sperm oil no. 2	43.4	0.07
Linseed oil no. 4	1.16	0.05
Linseed oil no. 5	1.14	0.03
Teaseed oil no. 1	0.89	0.02
Teaseed oil no. 2	0.81	0.03
Rapeseed oil no. 1	1.07	0.06
Rapeseed oil no. 2	1.16	0.04

The hydrocarbon extracts from the oils known and believed to be uncontaminated with mineral oil were usually yellow in fluorescence when examined in ultra-violet radiation. In daylight they appeared colourless.

All the extracts from the oils of Table 2, and those from the last two oils of Table 3 were also colourless in daylight but showed a pale-blue fluorescence in ultra-violet radiation.

It is interesting to note that if the figures for the hydrocarbon content of the linseed oils are plotted against the respective percentages of unsaponifiable matter in a graph, the points lie close to a straight line of unit slope. From this and the other facts adduced, it is inferred that the percentage of hydrocarbon extracted is less than 0.1 per cent higher than the percentage of adventitious mineral oil present in a sample.

TABLE 2.—*Recovery of hydrocarbons from commercial fatty oils*

MATERIAL EXAMINED	UNSAPONIFIABLE MATTER (S.P.A. METHOD)	HYDROCARBON SEPARATED
	per cent	*per cent*
Oils imported to Great Britain 1947/8		
Linseed oil no. 6	1.59	0.40
Linseed oil no. 7	1.29	0.19
Linseed oil no. 8	1.39	0.26
Linseed oil no. 9	1.81	0.58
Linseed oil no. 10	2.39	1.23
Linseed oil no. 11	1.95	0.83
Linseed oil no. 12	2.23	0.98
Linseed oil no. 12	3.17	2.01
Teaseed oil no. 3	2.28	1.34
Teaseed oil no. 4	1.37	0.52
Teaseed oil no. 5	2.77	1.79
Teaseed oil no. 6	2.46	1.70
Rapeseed oil no. 3	1.48	0.33

TABLE 3.—*Recovery of hydrocarbons from known mixtures*

MATERIAL EXAMINED	UNSAPONIFIABLE MATTER (S.P.A. METHOD)	HYDROCARBON SEPARATED
	per cent	*per cent*
Linseed oil no. 1	1.18	0.05
Same oil+0.2 per cent of mineral oil	1.40	0.23
Same oil+0.5 per cent of mineral oil	1.67	0.51

SUMMARY

A chromatographic method is described for the recovery of hydrocarbons from the unsaponifiable matter of fatty oils. Results are given for the application of the method to pure and contaminated oils; it is shown to yield figures within 0.1 per cent of the amount of mineral oil present in samples of linseed, teaseed, and rapeseed oil. The method is of

general application to oils which do not yield hydrocarbons in a pure state.

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STUDIES ON COAL-TAR COLORS

FD&C YELLOW NO. 6 AND C.I. NO. 26

By CHARLES STEIN (Division of Cosmetics, Food and Drug Administration, Federal Security Agency, Washington, D. C.)

FD&C Yellow No. 6 (Sunset Yellow), the disodium salt of 1-(4 sulfophenol)-2-naphthol-6-sulfonic acid, is listed as certifiable for use in foods, drugs, and cosmetics under the authority of the Federal Food, Drug, and Cosmetic Act of 1938 (1). This study was undertaken in order to make available a sample of pure FD&C Yellow No. 6, which is to be used to check the validity and accuracy of the procedures ordinarily employed in the examination of this color.

The sodium salt of 1-phenyl-azo-2-naphthol-6-sulfonic acid (C.I. No. 26) (2) is a possible contaminant in commercial samples of FD&C Yellow No. 6. Since the regulations specify the amount of subsidiary dye allowable in certifiable samples of FD&C Yellow No. 6, a sample of C.I. No. 26 was prepared to aid in the development of a method for subsidiary dyes in FD&C Yellow No. 6.

The purified materials were also employed in our investigation of the spectrophotometric characteristics of coal-tar colors. Solutions of the colors were tested spectrophotometrically to determine the location of the absorption peaks, the conformity of the colors to Beer's law, and the effect of *p*H on the absorption spectra as an aid in identifying the color.

EXPERIMENTAL

Schaffer's acid (2-naphthol-6-sulfonic acid) was prepared according to the procedure of Engel and Hutchison (3). Commercial β-naphthol (100 gms.) was sulfonated with 100 ml. of 100% sulfuric acid at 85° for 75 minutes. The reaction mixture was poured into 500 gm. of ice and water.

The resulting solution was filtered and the Schaffer's acid precipitated by saturating the cold solution with dry hydrogen chloride gas. The precipitated material was filtered on a fritted glass Büchner, washed with cold concd. hydrochloric acid, then dissolved in water and again precipitated with hydrogen chloride gas. This procedure was repeated until the precipitated material gave a negative test for sulfuric acid. The product was then placed in a desiccator over solid potassium hydroxide until free of hydrogen chloride. A small amount of the chloride-free material was dried in a desiccator over sulfuric acid to form the monohydrate. This melted at 129°, as stated by Engel and Hutchison (3).

The bulk of the Schaffer acid was not dried. Its moisture content was determined before use by drying a sample at 135° overnight. Allowance for the moisture content was made in weighing portions of the material for use.

Sulfanilic acid was purified by repeated recrystallization of commercial material from water.

Aniline was purified by distillation.

PREPARATION OF FD&C YELLOW NO. 6

Sulfanilic acid, 8.7 gms. (0.05 mole), was dissolved in 100 ml. of hot water containing 2.65 gm. of sodium carbonate. After cooling to 20°C, 3.7 gm. of sodium nitrite was added, and the solution poured into a beaker containing 50 gm. of ice and 10 ml. of concd. hydrochloric acid. The temperature of the solution was kept below 5°C for 15 minutes. An excess of urea was then added to destroy the excess nitrous acid.

The suspension of the diazonium salt was poured into a cold (5°C) solution of 11.2 gm. (0.05 mole) of Schaffer's acid and 6.0 gm. of sodium hydroxide dissolved in 250 ml. of water. After stirring for one hour, the solution was removed from the ice bath and allowed to come to room temperature.

The acidity of the reaction mixture was adjusted to a *p*H of 2, the solution was boiled for 4–5 minutes to destroy carbonates and bicarbonates, and then evaporated to dryness on the steam bath. The solid material was dissolved in 200 ml. of water, and the solution heated to boiling. The dye was precipitated by the addition of 1000 ml. of alcohol, filtered with suction, and washed with a 5:1 alcohol-water mixture. The color was reprecipitated twice using 100 ml. of water and 500 ml. of alcohol each time. After a preliminary drying on the steam bath, the purified color was dried at 135°C. Yield: 19.2 grams.

Analytical data on the purified FD&C Yellow No. 6 are shown in Table 1.

PREPARATION OF C.I. NO. 26

Aniline, 6.52 gm., (0.07 mole) was agitated with 25 ml of hot water, and 17.5 ml of concd. hydrochloric acid was added in a thin stream. The

TABLE 1.—*Analytical data*—Purified FD&C Yellow No. 6*

DETERMINATION	FOUND	CALCULATED
Nitrogen, per cent	6.1	6.19
Sulfur, per cent	14.1	14.2
Sodium (from sulfated ash) per cent	9.9	10.2
Titration (ml. 0.1 *N* $TiCl_3$ per gram)		
a. Sodium citrate as buffer	88.45	88.45
b. Sodium bitartrate as buffer	88.2	
Inorganic sulfates, chlorides, and carbonates	Nil	—

* All samples were dried in an Abderhalden drier at 135°C. and 6 mm pressure.

solution was allowed to cool to 40° and sufficient ice was added to lower the temperature to 0°, leaving a slight excess of ice. A solution of 4.9 gm. of sodium nitrite in 25 ml. of water was added rapidly with stirring. After several minutes, the excess nitrous acid was destroyed with urea.

The solution of benzene diazonium chloride was added slowly, with stirring, to a cold 5°C solution of 15.7 gm. (0.07 mole) of Schaffer's acid and 10.5 gm. of sodium hydroxide in 300 ml. of water. After 1 hour the solution was removed from the ice bath and permitted to warm to room temperature.

The dye was then isolated and purified in the same way as the FD&C Yellow No. 6. The yield of purified product was low, 8 gm., but spectrophotometric analysis of the combined filtrates revealed the presence of 13.3 gm. of the dye.

Analytical data on the purified material are shown in Table 2.

TABLE 2.—*Analytical data—Purified C.I. No. 26**

DETERMINATION	FOUND	CALCULATED
Nitrogen, per cent	7.8	8.0
Sulfur, per cent	9.1	9.15
Titration (ml 0.1*N* $TiCl_3$ per gram)	112.5	114.2
Inorganic sulfates, chlorides and carbonates	Nil	—

* Sodium salt of 1-(phenylazo)-2-naphthol-6-sulfonic acid.

DISCUSSION

The analytical data on the purified samples indicate a purity of at least 99 per cent for the FD&C Yellow No. 6 and over 98 per cent for the C. I. No. 26. Both samples appear to be sufficiently pure to serve as standards.

The data show that the titanium trichloride titration ordinarily used for the determination of the "pure dye" content of samples of these colors is accurate to within ± 1 per cent. Essentially the same result is obtained in the titration of FD&C Yellow No. 6 with either sodium citrate or sodium bitartrate as the buffer.

When the purified FD&C Yellow No. 6 was analyzed by the A.O.A.C. procedure for lower sulfonated (4) subsidiary dyes in FD&C Yellow No. 6, no subsidiary dye was found. When known amounts of C.I. No. 26 were added to the purified FD&C Yellow No. 6, 98 per cent of the added dye was recovered by this procedure. Three commercial samples of FD&C Yellow No. 6 were examined for subsidiary dyes by the A.O.A.C. procedure and found to contain 0.2–0.9 per cent subsidiary dye; however, the spectrophotometric curve of the extracted dye appeared to correspond more closely to that of D&C Orange No. 4 than to that of C.I. No. 26.

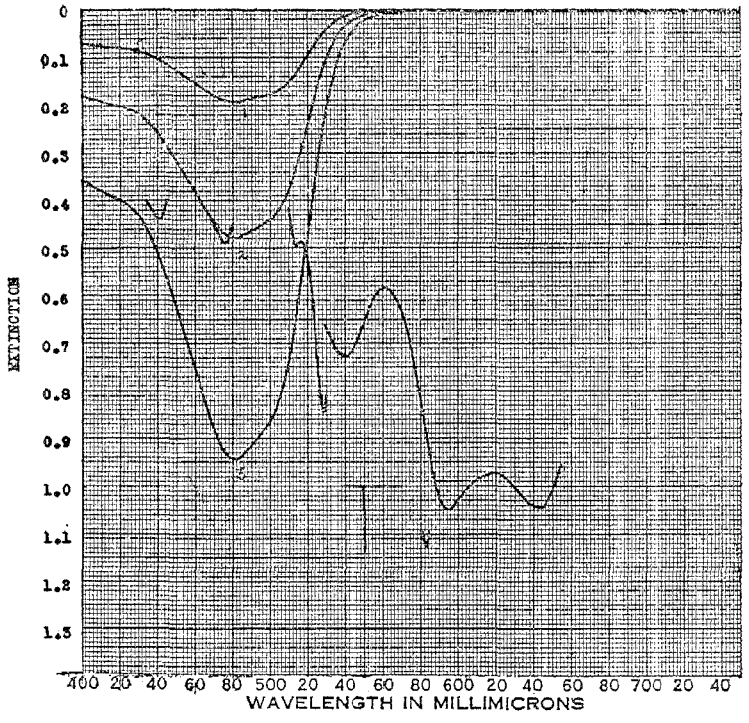

FIG. 1.—FD&C Yellow No. 6

Solvent: 0.02 *N* $NH_4C_2H_3O_2$
Concentration:

Curve 1— 3.48 mg. per liter
Curve 2— 8.70 mg. per liter
Curve 3—17.41 mg. per liter
Cells—1 cm.

A = Corning Didymium Glass 512, 6.0 mm.
(Absorption peaks at 400.4, 441.4, 477.1, 529.0, and 684.8 mμ)
B = Corning Didymium Glass 592, 4.02 mm.
(Absorption peak at 583.7 mμ)
C = Signal Lunar White Glass H-6946236.

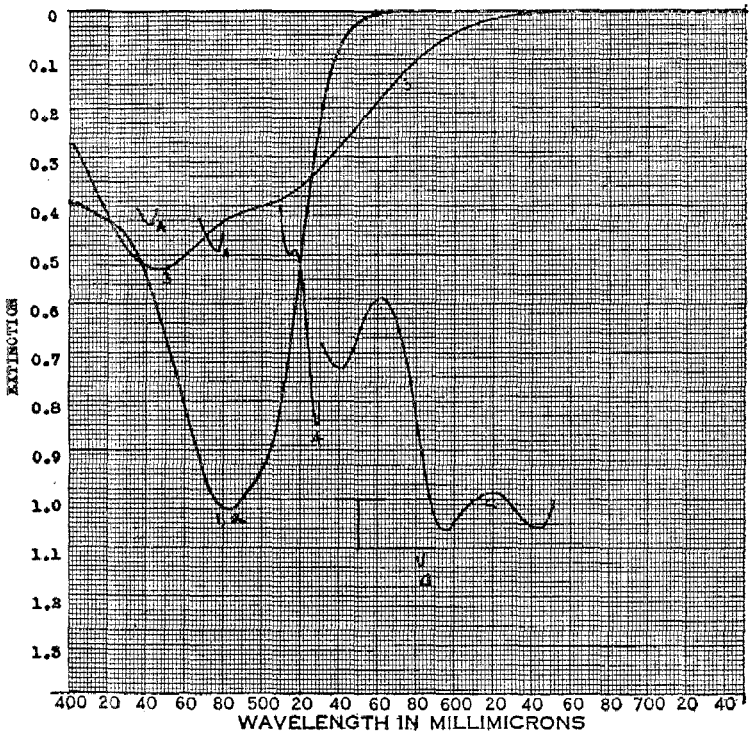

FIG. 2.—FD&C Yellow No. 6

Concentration: 1850 mg. per liter
Solvent:

Curve 1—0.02 *N* $NH_4C_2H_3O_2$
Curve 2—0.1 *N* HCl
Curve 3—0.1 *N* NaOH
Cells—1 cm.

A = Corning Didymium Glass 512, 6.0 mm.
(Absorption peaks at 400.4, 441.4, 477.1, 529.0, and 684.8 mμ)
B = Corning Didymium Glass 592, 4.02 mm.
(Absorption peak at 583.7 mμ)
C = Signal Lunar White Glass H-6946236.

SPECTROPHOTOMETRIC DATA

All spectrophotometric measurements were made with a General Electric Recording Spectrophotometer equipped with an automatic slit adjustment for an 8 mμ wave length band.

The solutions for spectrophotometric analysis were prepared by dissolving a weighed portion of dye in water and diluting to exactly 1000 ml. To aliquots of this solution the appropriate buffer was added and the solution diluted to a definite volume. All solutions were made to volume

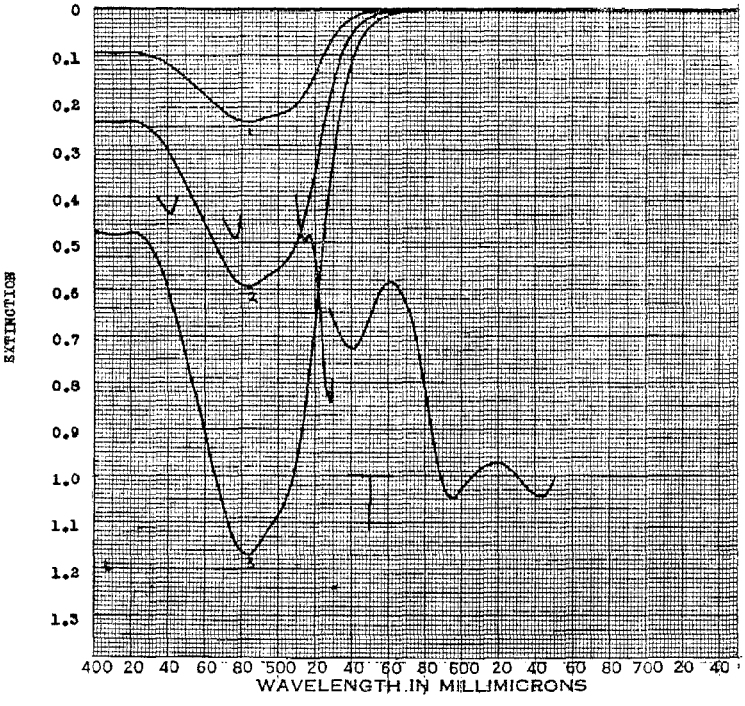

FIG. 3.—C.I. No. 26

Solvent: 0.02 *N* $NH_4C_2H_3O_2$
Concentration:

Curve 1— 3.97 mg. per liter
Curve 2— 9.94 mg. per liter
Curve 3—19.87 mg. per liter
Cells—1 cm.

A = Corning Didymium Glass 512, 6.0 mm.
(Absorption peaks at 400.4, 441.4, 477.1, 529.0, and 684.8 mμ)
B = Corning Didymium Glass 592, 4.02 mm.
(Absorption peak at 583.7 mμ)
C = Signal Lunar White Glass H-6946236.

at the temperature (25 ± 5°C) of the room in which the optical measurements were made.

Typical sets of curves for neutral solutions of FD&C Yellow No. 6 are shown in Figure 1 and for neutral, acid, and alkaline solutions of the color in Figure 2. Curves for solutions of C.I. No. 26 are shown in Figures 3 and 4.

The curve for neutral and acid solutions of FD&C Yellow No. 6 are identical with a peak at 482 ± 2 mμ and a shoulder at about 420 mμ. In strongly alkaline solution, the peak is shifted to 445 mμ with a broad shoulder at about 500 mμ.

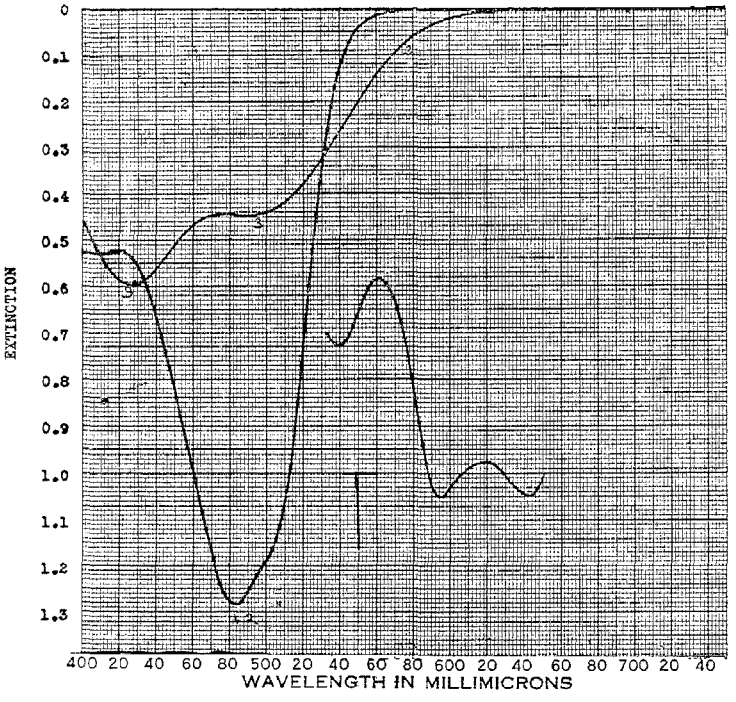

FIG. 4.—C.I. No. 26

Concentration: 21.77 mg. per liter
Solvent:

Curve 1—0.02 *N* $NH_4C_2H_3O_2$
Curve 2—0.1 *N* HCl
Curve 3—0.1 *N* NaOH
Cells—1 cm.

A = Corning Didymium Glass 512, 6.0 mm.
(Absorption peaks at 400.4, 441.4, 477.1, 529.0, and 684.8 mμ)
B = (Corning Didymium Glass 592, 4.02 mm.
(Absorption peak at 583.7 mμ)
C = Signal Lunar White Glass H-6946236.

Neutral solutions of FD&C Yellow No. 6 containing 4 to 25 mg. of dye per liter conform to Beer's law at the absorption peak. In six sets of three determinations each, the average extinction per milligram per liter at 482 mμ was 0.0547 with an average deviation of 0.34 per cent and a maximum deviation of 0.7 per cent.

The curves obtained on neutral, acid, and basic solutions of FD&C Yellow No. 6, which had aged for 24 hours, were identical with those of freshly prepared solutions.

The curves for neutral and acid solutions of C.I. No. 26 are identical

with a major peak at 484 ± 2 mμ and a secondary peak at 410 ± 2 mμ. In strongly basic solution, the dye gives peaks at 428 and 490 mμ.

Neutral solutions of C.I. No. 26, containing 4 to 20 mg. of color per liter, obey Beer's law to within ±1 per cent. The average extinction per milligram per liter at 484 mμ was found to be 0.0597. Neutral, acid and basic solutions of the color are stable for at least 24 hours.

C. I. No. 26 can be differentiated spectrophotometrically from FD&C Yellow No. 6 by its secondary peak at 410 mμ in neutral solution, or more readily by its curve in alkaline solution.

Since both of the colors covered in this report obey Beer's law, each dye can be determined conveniently by spectrophotometric examination if other colored substances are absent.

Spectrophotometric curves are now drawn routinely for all samples of FD&C Yellow No. 6 submitted for certification. For ten samples selected at random from the files the average deviation between the spectrophotometric and titration values for "pure dye" was 0.9 per cent and the maximum deviation, 2.2 per cent.

SUMMARY

Samples of FD&C Yellow No. 6 and C. I. No. 26 sufficiently pure to serve as analytical standards have been prepared.

Each of these dyes can be accurately titrated with titanium trichloride.

The A.O.A.C. procedure for subsidiary dyes in FD&C Yellow No. 6 will determine C.I. No. 26 practically quantitatively. The three commercial samples of FD&C Yellow No. 6 examined for subsidiary dyes did not contain appreciable amounts of C.I. No. 26.

Spectrophotometric data on solutions of the two colors is presented. The curves of the two dyes are quite similar, with a major absorption peak at 480–485 mμ, but the two colors can be differentiated readily spectrophotometrically.

Neutral solutions of both the dyes follow Beer's law; hence, they may be determined spectrophotometrically if interfering substances are absent.

The spectrophotometric and titration values for "pure dye" content of most commercial samples of FD&C Yellow No. 6 agree to within ±1 per cent.

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ACKNOWLEDGMENT

The author wishes to thank Mr. M. Dolinsky for making the spectrophotometric measurements.

VOLUMETRIC DETERMINATION OF CHLORINE AND BROMINE IN ORGANIC COMPOUNDS

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The work done in this laboratory requires the determination of halogens in a number of coal-tar colors, intermediates, and other organic compounds. In the compounds analyzed, chlorine, bromine, and iodine occur alone and in various combinations. Simple, accurate, volumetric methods for iodine and bromine in such compounds are available (1, 2). Several volumetric methods for chlorine in organic compounds are described in the literature, but none of these methods is very satisfactory when considerable accuracy is required. For that reason, chlorine is usually determined gravimetrically. When a large number of determinations are to be made, an accurate volumetric method for chlorine would be a decided advantage.

Sendroy (4) has described a titrimetric method for inorganic halides based on the reaction of the halide ion with solid silver iodate which is simple, convenient, and accurate. This paper describes a method developed in this laboratory for the determination of chlorine and bromine in organic compounds using Sendroy's titration for the final determination.

METHOD

REAGENTS

Silver iodate.—Suitable for the determination of chloride. (Obtainable from Merck and Company, Rahway, New Jersey. See reference (4) for method of preparation.)

Sodium nitrite, 10%.

Sulfuric acid, 6 N.

Sulfamic acid, 10%.

Standard sodium thiosulfate, 0.05–0.1 N.—Standardize against KIO_3.

PROCEDURE

Accurately weigh a sample which contains at least 15 mg of Cl or 30 mg of Br. Oxidize the sample and absorb the evolved halogen in a mixture of 15 ml of 1% hydrazine sulfate plus 5 ml of 10% NaOH as directed in the official A.O.A.C. procedure for bromine in halogenated fluorescein dyes (3).

Transfer the absorbing soln to a 200 ml beaker and wash the absorption flask with two 5–10 ml portions of H_2O. Complete the washing with 5 ml of the nitrite soln and 10 ml of 6 *N* H_2SO_4, and add these reagents to the beaker. Thoroly mix the resulting soln and allow to stand for at least 2 min. Wash down the sides of the beaker with 10 ml of the sulfamic acid soln and stir the mixture for 2 min. Add an excess (0.4–0.8 gram) of solid $AgIO_3$, and mix vigorously for at least 2 min. Transfer the mixture to a 100 ml volmetric flask, cool to room temp., and make to volume with

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water. Mix thoroly and filter thru a dry fluted filter. Discard the first few ml of filtrate.

Dilute an aliquot of the filtrate to ca 100 ml with water, add 2 g of KI and titrate the liberated I with $Na_2S_2O_3$, using starch soln as the indicator.

$$1 \text{ ml } 0.1\ N\ Na_2S_2O_3 = 0.591 \text{ mg Cl}$$
$$= 1.337 \text{ mg Br}$$

If the sample contains both Br and Cl, dilute the soln to exactly 100 ml before the addition of silver iodate. Neutralize an aliquot of this soln (use at least half the soln) with 30 per cent NaOH and determine Br by the official A.O.A.C. procedure (3). To the remaining soln, add solid $AgIO_3$, shake vigorously for at least 2 min., filter, and titrate an aliquot of the filtrate as directed above to obtain the total halide content (in mols).

The bromine and chlorine content of the sample is calculated from the equations:

$$\text{Br (mg)} = T_1 \times N_1 \times 39.96 \times \frac{100}{A}$$

$$\text{Cl (mg)} = [T_2 \times N_2 \times \frac{100}{B} - (3 \times T_1 \times N_1 \times \frac{100}{A})] \times 5.91$$

where

T_1 = titration for Br determination
T_2 = titration for total halide determination
N_1 = normality of $Na_2S_2O_3$ used in T_1
N_2 = normality of $Na_2S_2O_3$ used in T_2
A = aliquot used for the Br determination
B = aliquot used for the total halide determination.

DISCUSSION

The chromic-sulfuric acid digestion employed in this study is a simple, convenient method for the decomposition of a large number of compounds. One advantage of this digestion is that iodine is oxidized to iodate and is not carried into the absorbing solution with the bromine and chlorine. Although other methods of destroying the organic material have not been investigated, it appears probable that the alkaline hydrazine absorbent could be used with several other decomposition procedures described in the literature.

Sendroy (4) has discussed in detail the principles of his method. The accuracy depends somewhat on the amount of halide present but, for concentrations above a minimum of about 5 millimoles per liter, the results are accurate to within a few parts per thousand. The final titration is simple and precise and the end point is sharp and permanent.

Sendroy recommends the use of dilute phosphoric acid (0.085 mole) in the chloride determination, but notes that the acidity is not critical unless iodide is present. The reaction between hydrazine and nitrite does

not appear to be complete unless the *p*H of the solution is low. In our experiments, essentially the same results were obtained whether the determination was carried out in dilute (0.06 *N*) sulfuric acid or in phosphoric acid. (See Table 1.)

TABLE 1.—*Recoveries of chloride and bromide*

HALOGEN	AMOUNT	RECOVERY IN PRESENCE OF:		
		H_3PO_4	H_2SO_4	ABSORPTION SOLUTION
	mg	*per cent*	*per cent*	*per cent*
Chloride	15.3	100.0	100.5	100.3
Chloride	30.6	100.0	100.3	—
Chloride	61.2	99.6	99.6	—
Bromide	16.8	102.1	101.4	—
Bromide	33.6	100.2	100.3	99.9
Bromide	67.2	99.8	99.6	—

The effectiveness of the procedure used to remove the excess hydrazine was checked by the addition of the specified reagents to 25 ml. of the mixed absorbing solution. The final solution neither reduced iodate nor oxidized iodide; an aliquot of standard potassium iodate solution liberated the same amount of iodine from iodide in the presence of this mixture as an equal aliquot did in the presence of dilute sulfuric acid. The results obtained, when known amounts of chloride and bromide were added to the alkaline hydrazine solution and the mixture analyzed by the method described, are shown in Table 1. These results show that the reagents used do not interfere in the determination.

The only difficulties encountered in numerous determinations of chloride and bromide by this method have been occasional low results obtained in early experiments. These were found to be due to incomplete reaction between the halide and the silver iodate. No difficulty from this source will arise if the mixing is carried out as specified in the directions.

Typical results obtained in the analysis of organic halogen compounds by the proposed method are shown in Table 2. The average deviation from the calculated value for these compounds is 0.5 per cent and the greatest deviation 1.0 per cent.

In calculating the results shown in Table 2 no correction has been made for the difference in recovery at different halide concentrations. The precision of the final titration might justify such a correction, but variations introduced in steps prior to the titration are probably greater than this correction. For the same reason no correction has been made for the volume (about 0.1 ml.) occupied by the solid material.

In the determination of both bromine and chlorine, bromine is deter-

TABLE 2.—*Chlorine or bromine in organic compounds*

COMPOUNDS	HALOGEN	
	FOUND	CALCULATED
	per cent	*per cent*
o-Chlorobenzoic acid (N.B.S. standard sample No. 144)	22.6 22.7	22.65
4-Bromoacetanilide	37.4 37.1	37.34
4-Chlororesorcinol	24.7 24.6 24.6	24.54
2,5-Dichloracetanilide	34.5 34.6 34.6	34.76
Tetrabomphthalic anhydride	68.4 68.2	68.9
Tetrachlorophthalic anhydride	49.4 49.2 49.6	49.7
Tetrabromfluorescein diacetate	43.6 43.8	43.68
Tetrachlorfluorescein diacetate	25.7 25.7	25.60

TABLE 3.—*Mixtures of bromine and chlorine*

MIXTURE NO.	BROMINE*		CHLORINE*	
	ADDED	FOUND	ADDED	FOUND
	mg	*mg*	*mg*	*mg*
1	33.6	33.5	15.2	15.0
2	83.9	83.3	15.2	15.3
3	83.9	83.2	15.2	15.4
4	67.1	66.8	30.3	30.4
5	67.1	66.7	30.3	30.5
6	33.6	33.3	151.6	151.8
7	33.6	33.2	151.8	150.8

* Added as KBr and NaCl, respectively.

mined directly and chlorine by difference. Both of these determinations must be made quite accurately if the results are to be satisfactory. Typical results obtained in the analysis of mixtures of chloride and bromide by the method proposed in this paper are shown in Table 3. The results are not quite as accurate as those usually obtained when the total halide content is determined gravimetrically, but are sufficiently accurate for most purposes.

SUMMARY

A simple, rapid, volumetric method for the determination of bromine and chlorine in organic compounds has been presented.

The compound is oxidized with chromic and sulfuric acids and the halogen evolved is absorbed in alkaline hydrazine. The excess hydrazine is destroyed with nitrite in acid solution and the excess nitrite removed with sulfamic acid. The halide is then determined by adding solid silver iodate and determining the iodate ion liberated as proposed by Sendroy.

If both bromine and chlorine are present, bromine is determined in a separate aliquot by the A.O.A.C. procedure and the chlorine content calculated by difference from the total halogen determined.

Typical results are given.

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THE DETERMINATION OF GAMMA-BENZENE HEXACHLORIDE IN INSECTICIDE PRODUCTS

By THOMAS H. HARRIS (Insecticide Division, Livestock Branch, Production and Marketing Administration, U. S. Department of Agriculture, Beltsville, Maryland)

Several independent methods for determining gamma-benzene hexachloride have recently been published (1–9). The conditions for separating the isomers of benzene hexachloride on a partition chromatographic column were worked out by Ramsey and Patterson (10), and this work furnished the basis for a quantitative method described by Aepli, Munter, and Gall (1). The method of Aepli and co-workers has been in use in this laboratory for some time and its accuracy and precision have been confirmed. The method is not entirely suitable, however, for routine work where the analysis of large numbers of samples are required. The purpose of the work reported here was to simplify this method and retain the accuracy and precision of the original method.

The procedure of Aepli and co-workers requires the use of 100 g of silicic acid and the collection of 30 40 ten ml fractions after 100 ml of solvent have pased through the column. The gamma isomer is located by evaporation of the fractions and identification by its characteristic crystalline appearance.

It was apparent after some study of this method that the number of fractions collected could be reduced if some means could be found of visually locating or marking the position of the gamma isomer band as it moved down the column. For this purpose a number of oil-soluble coal-tar colors appeared promising because of their solubility in, and distribution between, the two immiscible solvents, nitromethane and normal hexane. One of these dyes, D and C Violet No. 2 (1-hydroxy-4-p-toluinoanthraquinone) when added to a solution of gamma-benzene hexachloride to be chromatographed, was observed to move down the column just ahead of the gamma isomer, and largely with the alpha isomer which precedes the gamma isomer. With this dye present and serving as a marker for the position of the gamma isomer the collection of fractions could be delayed until all but the last trace of the dye had left the column.

A modified partition chromatographic procedure, using the above mentioned dye as a marker for the front of the gamma isomer band, is described and has been found useful in this laboratory for routine work.

APPARATUS

(1) *Partition column.*—The column and reduction valve are shown in Figure 1. The column is 72 cm long and 2.5 cm in diameter. A fritted glass disc is sealed in place and 5 cm below the disc is attached a ball and socket joint No. 18/7. The lower end of the female part of the joint is constricted to a diameter of about 5 mm. The column shown was constructed of standard wall Pyrex glass tubing by the Scientific Glass Apparatus Company.[1] The oxygen-type reduction valve was obtained from the Southern Oxygen Company.[1] Pressure is supplied from a laboratory air pressure line.

(2) *Solvent evaporator.*—The solvent evaporator is shown in Figure 2. The fractions are evaporated to dryness under reduced pressure at 60°C., with the aid of a water pump. The solvent is recovered in a trap consisting of a Kjeldahl flask immersed in a mixture of salt and ice.

(3) *Erlenmeyer flask.*—125 ml.

(4) *Graduated cylinders.*—Two 10 ml.

(5) *Pipets.*—Ten ml volumetric and 5 ml serological.

(6) *Volumetric flask.*—50 ml glass-stoppered.

REAGENTS

(1) *Normal hexane.*—Phillip's Petroleum Company[1] commercial grade.

(2) *Nitromethane.*—Commercial Solvents Company.[1] Redistilled before use.

(3) *Silicic acid.*—Mallinckrodt's[1] reagent grade.

(4) *Dye solution.*—Dissolve 25 mg of D and C violet No. 2[2] (1-hydroxy-4-p-

[1] The mention of these products does not imply that they are endorsed or recommended by the U. S. Department of Agriculture over others of a similar nature not mentioned.

[2] Available from Pylam Products, 799 Greenwich Street, New York, 14, N. Y.

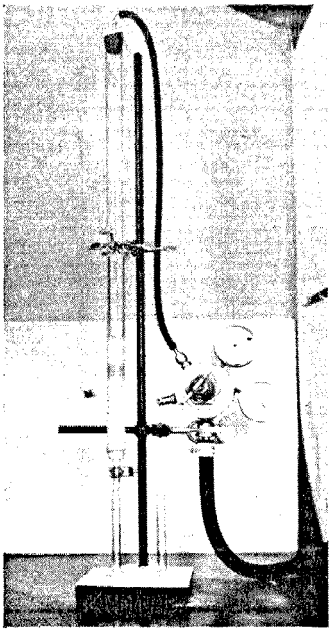

FIG. 1.—Partition column.

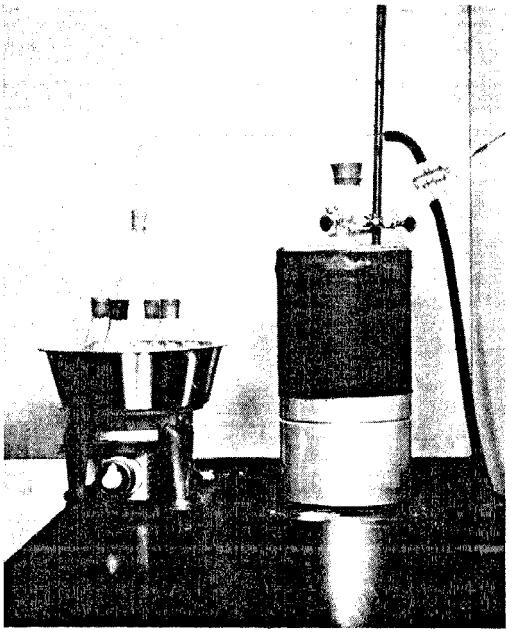

FIG. 2.—Solvent evaporator.

toluino-anthraquinone) in 50 ml of mobile solvent and store in a glass-stoppered bottle.

(5) *Mobile solvent.*—This is a saturated solution of nitromethane in normal hexane. Shake vigorously 2 liters of normal hexane with an excess of nitromethane in a glass-stoppered bottle. Decant the mobile solvent from the nitromethane as needed.

PREPARATION OF SAMPLE

(a) *Powders with more than 10% gamma-benzene hexachloride.*—Weigh a quantity of finely powdered sample sufficient to provide 150–200 mg of gamma-benzene hexachloride and transfer to a 125 ml glass-stoppered Erlenmeyer flask. Add 25 ml of mobile solvent, heat just to boiling, and allow to cool to room temperature with occasional shaking. Then decant the extract thru a small conical filter paper into a 50 ml glass-stoppered volumetric flask containing 1 ml of dye solution. Make a second hot extraction with 5 ml of mobile solvent. Rinse the residue 4–5 times using 5 ml portions of mobile solvent at room temperature. Finally dilute to volume with mobile solvent and mix.

(b) *Powders containing less than 10% gamma-benzene hexachloride.*—Transfer the weighed sample to a Soxhlet extractor and extract overnight with ethyl ether. Evaporate most of the ether on a steam bath and, finally, the remainder at room temperature under reduced pressure. Extract the gamma-benzene hexachloride from the residue with mobile solvent as directed above. (Samples containing less than 10% gamma-benzene hexachloride are difficult to extract directly with a minimum of mobile solvent owing to the bulk of siliceous filler generally present.)

(c) *Emulsion concentrates.*—Weigh a sample and transfer to 125 ml glass-

stoppered Erlenmeyer flask. Add 1–2 g of silicic acid to retain the water and extract with mobile solvent as directed above.

(d) *Solutions in petroleum distillate.*—Weigh a sample and dilute to 50 ml in a volumetric flask with mobile solvent.

PREPARATION OF COLUMN

Weigh 50 g (±.5 g) of silicic acid and transfer to a Waring blendor. Add 150 ml of mobile solvent and, with mixing, add 27 ml of nitromethane. After mixing for 15 seconds in the blendor, pour the mixture quickly into the column thru a glass funnel. Wash down the sides of the column with a few ml of mobile solvent and then apply 2–3 pounds pressure until all of the solvent is just forced into the column of silicic acid. Release the pressure by cautiously removing the rubber stopper at the top of the column so as not to disturb the silicic acid.

OPERATION OF THE COLUMN

Pipet out 10 ml of the solution and allow to flow slowly down the inside of the column without disturbing the surface of the silicic acid. Wash down the side of the column with 1–2 ml of mobile solvent and force the solution into the silicic acid by applying 2–3 pounds pressure. Release the pressure and slowly add 150 ml of mobile solvent, down the inside wall of the column. Apply 2–3 pounds pressure, which forces the solvent thru the column at a rate of about 3–4 ml per minute. Just before the last trace of dye leaves the column, begin to collect 5 ml fractions, alternately using two 10 ml graduated cylinders. Transfer each fraction to a 125 ml Erlenmeyer flask and evaporate to dryness, using the solvent evaporator. The fractions should be evaporated without boiling. If boiling occurs, raise the flasks momentarily from the water bath.

The appearance of the gamma isomer will be recognized upon evaporation by its tendency to cover the bottom of the flask as a white residual film. When the first residue of gamma isomer is recognized begin to collect 10 ml fractions until all of the gamma isomer is obtained, usually about 8 fractions. Dissolve the residue in each flask with normal hexane and transfer to a weighed 125 ml Erlenmeyer flask by means of a 5 ml serological pipet. Evaporate the solvent by using the solvent evaporator. Evacuate the flask[3] for about 5 minutes at room temperature with a vacuum pump. Release the vacuum, wipe the flask with a clean, moist towel and allow to stand for 5 minutes. Weigh and calculate the percentage of gamma-benzene hexachloride in the original sample.

Prepare the column for the next analysis as follows:

Extrude the silicic acid by applying gentle pressure. Clean the column with a long handled brush, wash with water and acetone, and dry by attaching to a vacuum line.

DISCUSSION

Following the procedure previously outlined, the data shown in Tables 1 and 2 were obtained. The accuracy and precision of the results shown may be expected in this method after one becomes experienced with the technique of preparing and operating the column.

The present method has been applied to a number of insecticidal powders and none of the other ingredients present have been found to cause

[3] There appears to be little or no danger in evacuating a 125-ml Erlenmeyer flask. A larger size Erlenmeyer flask, however, is likely to collapse under vacuum.

any interference. The wetting agents generally used in the wettable-powder-type products appear to be only slightly soluble in the solvents employed.

DDT causes no interference and is easily separated from the gamma isomer on the column. The quantitative determination of DDT by this same procedure is at present being investigated in this laboratory. Monochloronaphthalene, dichloronaphthalene, and trichloronaphthalene pres-

TABLE 1.—*Determination of gamma-benzene hexachloride in the presence of the other isomers*

SOLUTION	BHC ISOMERS ADDED				GAMMA ISOMER RECOVERED
	ALPHA	BETA	DELTA	GAMMA	
	mg				*mg*
1	—	—	—	100	100.8
2	25	—	—	200	198.4
2	25	—	—	200	199.0
3	100	10	20	100	101.0
3	100	10	20	100	99.9
4	100	—	—	12.5	13.2
4	100	—	—	12.5	12.4
4	100	—	—	12.5	12.3

TABLE 2.—*Analysis of commercial insecticide products. Precision of method*

SAMPLE	PER CENT GAMMA-BENZENE HEXACHLORIDE	
	(1)	(2)
a.	2.89	2.92
b.	3.44	3.42
c.	5.18	5.20
d.	11.3	11.3
e.	13.9	14.1
f.	15.3	15.5
g.	36.4	36.5

ent in one product analyzed caused no interference in the determination of gamma-benzene hexachloride. Some difficulty has been experienced, however, in the analysis of certain liquid concentrates containing methyl naphthalenes.

Numerous advantages result from the use of the dye to locate or mark the position of the gamma isomer on the column. In reducing the quantity of silicic acid, from 100 g as recommended in the method of Aepli and coworkers, to 50 g in the present method the same sharp separation of the gamma isomer is achieved if 5 ml fractions instead of the 10 ml fractions are collected. In using the smaller quantity of silicic acid

there is a sufficient space in the column above the silicic acid to hold the necessary volume of solvent required for analysis, thus eliminating the need for a solvent reservoir as required in the Aepli method. The time required to chromatograph a solution is reduced from about three hours to approximately 45 minutes.

Furthermore, the simplified apparatus required in the present method makes it feasible to operate a battery of several columns simultaneously.

ACKNOWLEDGMENT

The author wishes to thank Dr. G. Robert Clark of the Cosmetic Division, Food and Drug Administration, for supplying the coal-tar colors used in this study.

SUMMARY

A modified partition chromatographic method is presented for the determination of gamma-benzene hexachloride in insecticide products. A violet dye, D and C Violet No. 2 (1-hydroxy-4-p-toluino anthraquinone) is added to the solution aliquot to be chromatographed and serves as a visible marker for the front of the colorless gamma-benzene hexachloride band as it moves down the column. The use of this dye as a marker results in a number of advantages, chief among which are fewer fractions collected, simplified apparatus, and more rapid analysis. Furthermore, the simultaneous operation of several adsorption columns is made feasible. DDT, chloronaphthalenes, and other ingredients often present in these products, offer no interference. The accuracy and precision of the method are satisfactory for the analysis of most commercial insecticide products containing gamma-benzene hexachloride.

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BOOK REVIEW

Trace Elements in Food. By G. W. Monier-Williams. 511 pp. text, refs. and index. John Wiley and Sons, New York. Price $6.00.

The scope of the book is best presented by quotation and paraphrase of the author's preface: "The term 'trace element' is usually applied . . . to those elements which, in extremely small amounts, play some part in the nutrition of plants or animals. It may equally well be applied to the larger number which have . . . a toxicological rather than nutritional interest, and the presence of which in food must, on public health grounds, be subject to close scrutiny and control." It is pointed out that, within limits, some are innocuous or even essential and the necessity of defining the upper limits, or tolerances, often confounds the food chemist. "He must therefore have . . . knowledge . . . of all the questions involved; the biochemistry, nutritional significance and toxicology . . . ," also, of existing laws and regulations. The source of trace elements in foods, natural or adventitious, is considered and lastly, methods of analysis are outlined for each.

Trace elements are defined as those present in certain foods and in the human body in amounts up to .005 per cent (50 parts per million). Twenty-eight chapters are devoted, respectively, to copper, lead, zinc tin, arsenic, antimony, selenium, iron, nickel, cobalt, manganese, iodine, bromine, fluorine, boron, silicon, aluminum, silver, cadmium, chromium, bismuth, mercury, molybdenum, vanadium, titanium, indium, barium and strontium, and lithium and other metals. This listing of trace elements is stated to depend partly upon their relative importance as constituents of food and partly upon chemical relationships.

Each chapter comprises a discussion of the biological significance of the element; whether or not it is essential to plant or animal life; its toxicity, if any; the sources and amounts found in foods; legal limits if any; special topics; and, lastly, outlines of methods for its determination in foods. This discussion of methods comprises roughly about half of each chapter. Methods are usually given in outline, but some are presented in sufficient detail to serve as a "laboratory guide."

The book is profusely referenced (over 250 refs. in the copper chapter alone) and should be a source of much information for the toxicologist and food chemist.

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