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CONTENTS

TUESDAY—MORNING SESSION

SIXTY-SECOND ANNUAL MEETING

The 62nd Annual Convention of the Association of Official Agricultural Chemists will be held October 11 to 13, inclusive, at the Shoreham Hotel, 2500 Calvert Street, N.W., Washington 8, D.C.

Hotel accommodations may not be readily obtainable even by next fall; and reservations should be arranged for directly with the hotel and well in advance of the meeting.

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ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS—OFFICERS 1948

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Subcommittee B: HARRY J. FISHER (1948), Conn. Agricultural Experiment Station, New Haven, Conn.; G. R. CLARK (1950), and F. H. WILEY (1952).

Subcommittee C: JOSEPH CALLAWAY (1950), P.A. CLIFFORD (1952), and A.H. ROBERTSON (1954).

Subcommittee D: C. S. FERGUSON (1948), State Department of Public Health, Boston, Mass.; KENNETH L. MILSTEAD (1950), and J. WALTER SALE (1952).

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METHODS OF ANALYSIS, 6th edition, 1945

The list price of the new edition will be $6.25, domestic postpaid.

EDWARD MONROE BAILEY, 1879–1948

EDWARD MONROE BAILEY

Edward Monroe Bailey died at his home in New Haven, Connecticut, on April 13, 1948, after an illness of several months. He was born at New London, Connecticut, on August 27, 1879, the son of Edward Monroe and Louise Maria (Hagan) Bailey. His father moved to a farm at Higganum, Connecticut, when he was a few months old, and it was here that Bailey grew up, attending local schools and graduating from the Middletown High School in 1898. It was never characteristic of Monroe Bailey to talk about himself and he revealed none of the details of his earlier life to his associates. Mrs. Bailey is responsible for one incident of interest: a friend offered to pay his expenses at Wesleyan University if he would agree to become a minister, but he declined.

After a short interlude as a country school teacher he entered the Sheffield Scientific School of Yale University and graduated in 1902. Immediately after his graduation (July 1902) he came to work at the Connecticut Agricultural Experiment Station as an assistant chemist and remained continuously at the Station thereafter until his retirement on October 1, 1945, a total of 43 years. In 1918 he was appointed acting head of the Department of Analytical Chemistry in the absence of J. P. Street, then in military service; the following year Mr. Street resigned and Bailey received the permanent appointment as chemist in charge that he held for the ensuing 26 years.

While working at the Station Bailey continued his advanced studies at Yale, receiving the master's degree in 1905 and the doctorate in 1910. His work under the late Lafayette Mendel on the composition of the banana was published in a series of articles in the Journal of the American Chemical Society and the Journal of Biological Chemistry.

He was married to Myrtle Mix Studley of New Haven on June 11, 1906. One son, Irving Monroe, was born on August 13, 1914, and is now living in Hartford. There is also a young granddaughter, Beverley.

Bailey's published papers covered a variety of investigations into the chemistry and analysis of foods and drugs: the studies on the banana previously mentioned, the analysis of noodles, the determination of vanillin, coumarin, and acetanilid in vanilla extract, the detection of sulphites in meat, the determination of starch in meat, tests for the anthraquinone drugs and a study of the carbohydrates and enzymes of the soy bean. He acquired a very extensive knowledge of the composition of common and uncommon foods, and it was under his direction that the Connecticut Agricultural Experiment Station undertook the analyses of nearly all the foods on the market that were frequently reproduced in diabetic manuals before the advent of insulin. He was responsible for the compilations of analyses of foods that appeared in several editions of Joslin's "Treatment of Diabetes Mellitus."

It was probably a combination of this familiarity with the composition of foods with his reputation for good judgment that was responsible for his appointment to two positions that he held for many years. In 1924 he was appointed by the A.O.A.C. as a member of the "Committee to Coöperate with Other Committees on Food Definitions," and from that date he served on the old Food Standards Committee until 1940, when the new U.S. Food Drug, and Cosmetic Act authorized the promulgation of standards that were no longer purely advisory. He probably would have been appointed to the new Standards Committee also had his health permitted. In 1930 he was appointed a member of the Council on Pharmacy and Chemistry of the American Medical Association and continued to serve until 1938. When this Council set up a Committee on Foods in 1931 he was an original member and

remained on the committee when it became a separate Council in 1936, resigning in 1938.

Almost from the first day he went to work for the Experiment Station Bailey took an active part in the work of the A.O.A.C. At the first meeting he attended—in 1904—he was appointed Associate Referee on Fruit Products, and the following year he presented a report on the determination of water in dried fruits. At various times he held refereeships on dairy products (1913), tea (1918–1919), cacao products (1924 and 1926) and coffee (1932–1933) and associate refereeships on cocoa and cocoa products (1906), the cryoscopic examination of milk (1921), alcohol in drugs (1928) and colorimetric methods for vitamins (1929). He was a member of Subcommittees B and C, and served as Chairman of the Committee on Recommendations of Referees continuously from 1926 to 1934. He was a member of the Executive Committee in 1923–1924, 1926–1928 and 1931, and was vice president of the Association in 1929 and President in 1930.

The fourth and fifth editions of the "Book of Methods" were prepared under his editorial direction; he was delighted when the Association showed its appreciation of the quality of these two compilations by presenting him with a silver service.

As chairman of the Committee to Confer with the American Public Health Association on Standard Methods of Milk Analysis from 1932 and 1943 he was responsible for the chemical section of several editions of that manual of the A.P.H.A.

His Connecticut duties included responsibility for administration of the feed and fertilizer laws. The laboratory under his direction not only made the usual chemical analyses of feeds but determined the ingredients microscopically and assayed vitamin D supplements by animal feeding. When the Association of American Feed Control Officials was organized he took an active part in this association as well as in the A.O.A.C., and in 1934 he served as its president.

This recital of Bailey's scientific accomplishments and the positions he held leaves out the most important qualities of the man himself. What will most be remembered about him by those who worked under him can be expressed in one simple word—his "goodness." I have never met a person who had one ill word to say about him. He did not tolerate slipshod chemical work or mental dishonesty in his subordinates, but he had a never failing friendliness and helpfulness. When the present writer was in the hospital for a serious operation some years ago there was never a day that Bailey did not drop by to see him. This kindliness and his excellent judgment were his outstanding characteristics. His knowledge of the chemistry and analysis of foods was encyclopedic, and this was united with a critical faculty in interpreting analytic evidence that was almost intuitive. In his field he will have few equals and no superiors; his personal character will always be an inspiration to those of us who knew him.

HARRY J. FISHER

TUESDAY—MORNING SESSION

REPORT ON VITAMINS

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

Vitamin A.—The Associate Referee made no report.

Vitamin B_1.—The recommendation of the Associate Referee, which reads as follows, is approved. That the thiochrome method for thiamine be made official, first action.

Vitamin B_2.—The recommendations of the Associate Referee, which read as follows, are approved:

(1) That further work be conducted on the fluorometric method that was the subject of this year's study.

(2) That further consideration be given to improvements that may be made in the present microbiological method.

Vitamin C.—The Associate Referee made no report.

Vitamin D. (Poultry).—The Associate Referee made no report.

Nicotinic Acids.—The Associate Referee made no report.

Pantothenic Acid.—The Associate Referee made no report.

Folic Acid.—The recommendation of the Associate Referee, which reads as follows, is approved: That the findings in this investigation of folic acid indicate the need for further study.

Carotene.—The recommendations of the Associate Referee, which read as follows, are approved:

(1) That the collaborative procedure for determination of carotene be further studied, with different samples of alfalfa meal, as well as other types of food and feed materials.

(2) That the collaborative procedure as outlined in his report be adopted as an alternative method for the determination of carotene in hays and dried plants.

No reports were given for vitamin A, vitamin C, or vitamin D (milk, poultry). For recommendations of the Associate Referee on vitamin B^2, see Referee report.

REPORT ON THIAMINE

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

In 1943 (1) it was recommended to this Association that the U. S. Pharmacopoeial method for the determination of thiamine by the thio-

chrome procedure be adopted as a tentative chemical method to be applied to cereal products and vegetable products. This recommendation was based upon the results of an extensive collaborative study that had been sponsored by the United States Pharmacopoeial Vitamin Advisory Board. The thiochrome method adopted at that time has served for the past four years as a tentative A.O.A.C. procedure and now appears in the Sixth Edition of the *Book of Methods*. During this time the results of wide experience with the method have demonstrated that it is a satisfactory one in all essential details. There have been a number of studies designed to test the reliability of various steps of the procedure but in the main the method as now used is that described in the 1943 report.

The American Association of Cereal Chemists, in connection with the program of control of the enrichment of bread and flour, has been vitally interested in chemical methods for the determination of thiamine. From a collaborative study reported in 1944 by Andrews (2) in which samples of flour and bread were examined in a large number of laboratories it was concluded that the oxidation of thiamine to thiochrome in the procedure was a critical step that required further study. Last year Watson, (3) of the Food and Drug laboratory at Winnipeg, and independently, Hall of the Vitamin Division laboratory, learned that the order and speed of addition of the reagents and sample solution used in the oxidation step, as well as the degree of shaking of the reaction mixture, are important factors in obtaining a maximum amount of thiochrome. Since less than 100 per cent of thiamine is converted to thiochrome a procedure carefully standardized at this point is extremely important. Watson points out that thiamine may be converted in weak alkaline solution to a carbinol form from which thiochrome is formed slowly. This was described by Zima and Williams (4) in their studies of crystalline thiamine. A strong alkaline solution depresses the conversion to the carbinol form and therefore favors optimum conversion to an activated form of thiamine which is rapidly oxidized by ferricyanide to the fluorescing thiochrome. Hall described before this Association last year studies that indicate that excessive shaking, following addition of the oxidizing reagents to the sample solution, reduces the amount of thiochrome formed because, he postulates, an excessive amount of oxygen is introduced into the solution. He also pointed out that too little shaking led to less than maximum thiochrome formation because of insufficient mixing, and incomplete oxidation. His experiments have further indicated, that in the presence of the amount of acid recommended, the use of 15 per cent sodium hydroxide is sufficient to give a concentration of alkali necessary for maximum conversion to thiochrome. The thiochrome method as it appears in the Sixth Edition of *Methods of Analysis* takes into account the results of these studies.

The American Association of Cereal Chemists has had for the past several years a program of check sample analyses which has included the

use of the thiochrome method. It is interesting to note in the report of Meredith (5) which describes the results obtained in the 1944–1945 study, that there was great improvement in the results obtained over those for the previous year.

In the Food and Drug Administration there are four laboratories in which flour and bread are routinely examined for thiamine content by the thiochrome procedure. In these four laboratories the A.O.A.C. method

TABLE 1.—*Thiamine values for flour check-samples*
(Assayed in Food and Drug Administration Laboratories)

SAMPLE	LABORATORY			
	I	II	III	IV
	Milligrams per pound			
1	1.99	1.92	2.00	2.28
2	1.83	1.98	1.81	1.89
3	2.23	2.24	2.16	2.21
4	1.87	1.78	1.81	1.98
5	3.10	2.85	3.04	2.95
6	1.70	1.65	1.68	1.53
7	2.41	2.38	2.34	2.51
8	1.64	1.66	1.61	1.65
9	1.94	1.93	1.96	1.98
10	2.40	2.38	2.42	2.25
11	1.18	1.21	1.23	1.15
12	1.80	1.77	1.77	1.77
13	2.20	2.28	2.26	2.31
14	1.88	1.94	1.94	2.01
15	2.30	2.29	2.13	2.15

Average Standard Deviation between laboratories = .075
Coefficient of Variation = ±3.7%

has been closely followed during the past year. In order that each of the analysts involved interpret properly the written procedure, and understand the more critical steps of the method, W. L. Hall of the Vitamin Division visited each of the laboratories and carried through the method with the analyst. To correlate the results obtained in these four laboratories a check sample analysis program has been in effect for some time. Mr. Alfend of the St. Louis Station has been in charge of preparation of these samples and of reporting the results of analysis. For more than a year samples of flour have been used as check samples with the examination of one each month. The results of analysis of 15 check samples are presented in Table 1.

Many types of products may be assayed by the thiochrome procedure with elimination of the base-exchange step. Flour is an example of such

a product and it is common practice to omit the use of the adsorption column in its examination. The results in Table 1 were obtained by the shortened procedure. The results given are in milligrams of thiamine per pound, on an air-dry basis. Thiamine content of the samples examined varies from 1.2 to 3.1 mg. per pound. Variation between laboratories has been calculated, by analysis of variance.

As indicated in the table, the coefficient of variation between laboratories is ±3.7%. This means that in 1 of 20 determinations the checks will exceed ±8% of the mean. The agreement between laboratories is exceed-

TABLE 2.—*Thiamine values for collaborative bread samples*
(Assayed in Food and Drug Administration laboratories)

SAMPLE	LABORATORY			
	I	II	III	IV
	Milligrams per pound—air-dry weight			
1	1.89	1.92	2.06	1.90
2	1.65	1.69	1.72	1.58
3	1.70	1.72	1.74	1.60
4	1.63	1.72	1.77	1.64
5	1.63	1.69	1.69	1.54
6	0.41	0.43	0.40	0.39
7	0.43	0.43	0.43	0.39
8	0.28	0.29	0.30	0.30
9	0.34	0.36	0.35	0.31
10	0.33	0.34	0.34	0.30

	ALL SAMPLES	SAMPLES 1 TO 5	SAMPLES 6 TO 10
Av. Stand. Dev. between laboratories—	.050	.068	.018
Coefficients of Variation—	±4.8%	±4%	±5%

ingly good for this type of determination, and illustrates the value of personal contact among analysts.

Bread is a type of product that requires the base-exchange step in its analysis by the thiochrome method. To test the effect of the adsorption technic on uniformity of results from different laboratories, a series of bread samples was prepared and distributed to the four laboratories. Samples of fresh bread were purchased in Washington, and dried to an air-dry weight in the Vitamin Division laboratory. In order to have a range of thiamine values, samples of both enriched and unenriched bread were obtained. These samples were dried at room temperature overnight, then at 50°C for 8 hours in a forced-draft oven. They were then allowed to come to moisture equilibrium in the air-conditioned laboratory, bottled, and distributed. The analytical procedure included the use of the base-

exchange column. The thiamine values reported are milligrams of thiamine per pound of bread on an air-dry basis and are presented in Table 2. Samples 1 to 5, labelled "enriched," ranged from 1.6 to 2.0 mg. per pound dry weight. Samples 6 to 10 were unenriched white breads.

Again for this type of determination, remarkably good agreement was obtained between laboratories. For all samples examined the average standard deviation between laboratories was .050 with a resulting coefficient of variation of $\pm 4.8\%$. This means that 1 in 20 comparisons will exceed ± 10 of the mean. Thiamin content of the sample had little effect upon the error of the method, as illustrated by the similarities of coefficients of variation for samples 1 to 5 and 6 to 10.

The results presented here serve as a basis for the following recommendation:

It is recommended* that the A.O.A.C. tentative thiochrome method for thiamine applied to cereals and vegetables be made official, first action.

Acknowledgments: Miss Lila Knudsen and her staff prepared the statistical evaluations of the data prescribed here. Analyses were conducted at the Philadelphia, St. Louis, and San Francisco Stations, and in the Vitamin Division Laboratory. Analyses reported were carried out by W. L. Hall, E. L. Combs, R. M. Hawkins, S. Alfend, V. H. Blomquist, L. H. McRoberts, N. E. Foster, A. M. Fleiss, F. C. Minsker. Bread samples were prepared by H. W. Loy. The efforts of all these are greatly appreciated.

REFERENCES CITED

(1) KLINE, O. L., *This Journal*, **27**, 534 (1944).
(2) ANDREWS, J. S., *Cereal Chem.*, **21**, 388 (1944).
(3) WATSON, H. A., *Ibid.*, **23**, 166 (1946).
(4) ZIMA, O., and WILLIAMS, R. R., *Ber.*, **73B**, 941 (1940).
(5) MEREDITH, W. O. S., *Cereal Chem.*, **23**, 585 (1946).

No reports were given on nicotinic acid or pantothenic acid.

REPORT ON CAROTENE

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

To find out what was wanted in a carotene procedure and what equipment was available for measuring carotene in the various laboratories of the country, a questionnaire was sent to some 120 control, research and industrial laboratories over the United States and Canada. Most of them

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* For report of Subcommittee A and action of the Association, see *This Journal*, 31, 44 (1948).

replied and their response brought forth a number of significant points, some of which are shown in Table 1.

State control laboratories in considerably less than half of the States are attempting to determine carotene in any feed or food products. Most of the research and industrial laboratories contacted were doing analyses.

The replies indicated that present and former A.O.A.C. methods for carotene are not widely used. About one-third of the replies indicated some use was being made of A.O.A.C. procedures and some of these considered them unsatisfactory. Nearly two-thirds favored discarding present A.O.A.C. methods in favor of some other method.

Among the many suggested substitutes, the methods of Wall and Kelley (1) and Silker, Schrenk, and King (2) were most frequently mentioned. Replies agreed almost unanimously that chromatography is an essential step in a satisfactory method. A few indicated they were making use of dichromate standards.

TABLE 1.—*Summary of results from questionnaire*

	STATE CONTROL LABORATORIES		STATE & FEDERAL RESEARCH	INDUSTRIAL	TOTAL
	FEED CONTROL	FOOD & DRUG			
Replies	35	13	21	19	88
Analyzing for carotene	17	1	20	13	51
Wish to collaborate	19	3	17	12	51
Have spectrophotometer	17	3	18	12	50
Have photoel. colorimeter	4	1	2	3	10
Have visual colorimeter	2	3	—	—	5

Spectrophotometers were available in the vast majority of the laboratories which expressed an interest in doing carotene analyses. Most of the remainder had photoelectric colorimeters.

Fifty-one replies indicated a willingness to collaborate on analyses. Some of these suggested names of others who would probably wish to participate. The test materials most frequently suggested were alfalfa—45, carrots—23, commercial feeds—8, and tomatoes—5.

COLLABORATIVE ANALYSES OF ALFALFA MEAL SAMPLES

Guided by the suggestions from the questionnaire, an attempt was made to provide a simple and rapid procedure based on what seemed to be the best features of the methods of Wall and Kelley (1), Silker, Schrenk, and King (2), and Zscheile and Whitmore (3). After several tests by different analysts in our laboratories, a procedure was sent out to 65 laboratories followed by two samples of dehydrated alfalfa meal—one fresh, the other nearly a year old. Our analysts had obtained average carotene values of

296 p.p.m. and 40 p.p.m., respectively, on the two samples. A sample of magnesium oxide adsorbent was included. Instructions were to hold samples at room temperature and to send in results within a week.

COLLABORATORS

State control laboratories: Maine, New Hampshire, Connecticut, Massachusetts, Maryland, Ohio, Kentucky, Indiana, Minnesota, North Dakota, South Dakota, Texas, California, Oregon, and Montreal, Canada.

State and federal research laboratories: Agricultural Experiment Stations of Delaware, Michigan, Kansas, Oklahoma, Utah, Arizona, California; Department of Botany, University of Chicago; Eastern, Southern, and Western Regional Laboratories; U. S. Food and Drug Laboratories at Washington, D. C., and St. Louis; Bureau of Dairy Industry, Beltsville; Fish and Wild Life Service, Bowie, Maryland; Q. M. Food and Container Institute, Chicago.

Industrial laboratories: Best Foods, Inc., Bayonne, N. J.; Food Research Laboratories, N. Y.; G. L. F. Mills, Inc., Buffalo; Eastern States Farmers' Exchange, Buffalo; General Biochemicals, Inc., Chagrin Falls, Ohio; Central Mills, Dunbridge, Ohio; General Mills (Larrowe Division), Rossford, Ohio; Cargill, Inc., Minneapolis, Minn.; Cerophyll Laboratories, Kansas City, Mo.; W. J. Small Co., Kansas City; Ralston-Purina Co., St. Louis; Merchants Exchange Laboratory, St. Louis; Elk Valley Alfalfa Mills, Independence, Kansas; National Alfalfa Dehydrating and Milling Co., LaMar, Colo.; Albers Milling Co., Seattle, Wash.; Laucks Laboratories, Inc., Seattle.

SUMMARY OF COMMENTS OF COLLABORATORS

Many collaborators called attention to two typographical errors (in the instructions), *i.e.* (1) the figure 100 which should have been 1000, in the formula for calculating results and (2) the number of the magnesium oxide adsorbent, given as 2462, which should have been 2642. Several wished the alfalfa samples had been larger. Some reported that a 100 ml. volumetric flask was not large enough to contain the eluate. It was anticipated that all collaborators would attach the chromatographic column to a suction flask and use a good water pump under which conditions in our laboratory, the final volume of eluate was found to be less than 100 ml. in a number of trials. The directions should have been more specific.

COMMENTS OF COLLABORATORS

Some of the more pertinent comments from individual collaborators are listed below:

No. 3.—First 50 ml of eluate, which contained no color, was not collected in the 100 ml flask used for photometry.

No. 6.—The chromatographic separation using the adsorbent provided gave excellent separation of the various bands.

No. 11.—The solutions seemed to pass through the column very slowly. Otherwise the method appears to be easy and quick.

No. 14.—The procedure used met with favorable comment from others in the laboratory. It is not time-consuming and is simple to carry out.

No. 15.—I would prefer to make a greater dilution (200 ml) on samples of high carotene content like Sample No. 1. The 50 ml of wash solution did not remove quite all of the carotene.

No. 17.—We were able to check our results better with the present A.O.A.C. procedure. Otherwise the collaborative procedure is satisfactory. There was very little difference in time required to make the determinations.

No. 18.—Collection of 100 ml of extract and 50 ml of acetone-hexane wash in 100 ml volumetric flask is confusing. With the use of a 1 mm capillary on adsorption column the use of suction would facilitate the passing of the extract through the column.

No. 22.—Petroleum ether (Skellysolve F) used. Carotenes did not pass through column rapidly. The first 25 ml or more of filtrate was colorless and more than 50 ml of wash solvent was needed before the carotene was washed completely through the column. This necessitated a final volume of eluate that exceeded 100 ml.

No. 26.—No difficulties were encountered in using the procedure as given. It would be of interest to know how well it would work with plant tissues other than alfalfa.

No. 27.—2 gm. sample for Sample #1 gave too concentrated an extract. 50 ml washing did not produce a colorless filtrate at end of washing. 100 ml is too much to put through column. Believe an aliquot would be just as effective.

No. 30.—The exact amount of the aliquot used for chromatography should be specified. If the whole extract is used, part of the carotene will come through the column.

No. 31.—The analytical procedure is essentially the same as the one I have been using for some time. It might be helpful if the reasons for adopting 436 millimicrons as the reference point, and for the use of the specific absorption coefficient of 196 were given in detail. The absorption curve shows no inflection at 436.

No. 32.—Preliminary study of the method using our magnesium oxide (Micron Brand #2642) seemed to show that 50 ml. was insufficient eluant to remove the carotene. We therefore ran some tests using larger volumes of eluant. Our adsorbent has different adsorptive power than that furnished with the alfalfa samples.

No. 35.—We find two points in the procedure ambiguous: (a) Under "Chromatography" what is meant by "15 cm of the dry adsorbent?" The amount used is fixed by the description of the column size in the first line, so this statement seems at best only unnecessarily confusing. (b) Also under "Chromatography" we assume that only an aliquot of the extract was to be chromatographed and we have used 50 ml aliquots throughout. This point could be clarified in the procedure.

No. 36.—Our adsorbent develops more quickly with the eluting mixture.

No. 37.—The collaborative procedure gives results in excellent agreement with the procedure used in our laboratory. The extraction procedure in the collaborative method is somewhat inconvenient since it requires constant attention due to bumping and spattering of the meal on the walls of the extraction flask. With certain samples such as silage which give many carotenoid bands, the failure of the collaborative procedure to give a sharp carotene band on adsorption might be a disadvantage. On the other hand, the fact that extracts need not be concentrated is a definite advantage. On the whole, the technique is quite similar to that used in our laboratory for some time and we think that it has many advantages over the other methods previously used in A.O.A.C. studies.

No. 37a.—The logic of using alpha = 196 at 436 mμ should be given careful con-

sideration. Personally I do not like chromatography where the band of interest does not segregate. Although the results may seem to be satisfactory, the excesssive eluant (acetone) present may also elute extraneous material in an unknown fashion. The method we prefer makes of alcoholic KOH saponification petroleum extraction and elution on a 3 MgO to 1 of Celite or sometimes a 1:1 mixture. Segregation and separation are then under visible control. Our procedure uses small columns and takes about the same total time as the one you have submitted.

No. 40.—30 ml acetone-hexane for original extraction can go dry easily if condenser doesn't fit snug. Directions have inverted numbers designating MgO brand number 2642. The 10 cm adsorption column is fully twice as long as necessary. This may be good precaution in a test set of this type. Final volume of eluate is 100 ml. This appears larger than necessary and makes the density value unnecessarily low.

No. 41.—With the sample high in carotene, we encountered some difficulty in the elution of pigments other than carotene through the column.

No. 42.—The procedure appears to work quite well. A shorter column seems to speed up the operation. The procedure given or some variation of it appears to be a great improvement on past collaborative procedures. Would suggest a larger sample for future work.

No. 46.—Method very satisfactory.

No. 50.— . . . the outlined method of chromatography is too time-consuming to be practical.

No. 53.—The eluate from #1 sample was made to a volume of 250 ml in order to keep the density readings in a normal position on the Beckman transmission scale.

No. 55.—Final solution from sample 1 was made up to 250 ml volume as original solution was too concentrated for accurate reading. 50 ml wash solution appears as perhaps inadequate for some samples. However, a greater volume of wash solutions with the acetone of specified concentration would probably cause inaccuracy due to xanthophylls which might be eluted.

No. 56.—Collaborative procedure more rapid than present method.

No. 57.—Too little solvent for refluxing gave erratic results. Not necessary to pass all of sample through adsorption column.

No. 60.—Your method of assay for dried vegetable material is rapid and easy to carry out.

RESULTS AND DISCUSSIONS

Analyses were made in 49 laboratories, 41 of which used the collaborative procedure. The individual results are shown in Table 2. The average results are grouped by type of laboratory and type of instrument and compared with results from other procedures in Table 3.

The individual results showed fairly good agreement. It is suspected that some of the low values resulted from placing the sample in a thimble during extraction. The directions were apparently not emphatic enough on this point. Some of the high results can be attributed to erroneous standards.

Results from the different types of laboratories showed similar deviations but remarkably good agreement in average values. No significant trend of values was shown for any of the different instruments used.

The results compared favorably with those obtained by two other methods but were generally lower than those obtained by past or present tentative A.O.A.C. methods.

TABLE 2.—*Individual results of collaborative study on alfalfa meal samples*

A. State control laboratories:

COLL. NO.	INSTRUMENT	COLL. METHOD		OTHER METHODS OR MODIFICATIONS		
		SPLE. I	SPLE. II	SPLE. I	SPLE. II	METHOD
		p.p.m.	*p.p.m.*	*p.p.m.*	*p.p.m.*	
1	Visual colorimeter			325	58	*A.O.A.C.*
3	Beckman	254	38			
4	Visual colorimeter			254	58	*A.O.A.C.*, p. 600
5	Coleman 11	320	53	318	—	*Analyst* **66**, 334 (1941)
6	Beckman	277	28	294	13	*Science*, **90**, 623 (1939)
7	Nessler tubes			292	49	Thimble used
9	Coleman 11			276	36	Wall-Kelley[1]
11	Cenco-Sheard	271	39			
12	Beckman	284	42			
14	Beckman	311	38			
15	Klett-Summerson	274	35	289	25	Wall-Kelley[1]
17	Beckman	272	38	264	45	*A.O.A.C.*
18	Beckman	248	38			
22	Evelyn	314	41	304	59	*A.O.A.C.*, 1940 Ed.
24a	Beckman	296	40			
	Av.	284	39			

B. State and federal research laboratories:

25	Beckman	282	37			
26	Beckman	307	45			
	Cenco-Sheard			275	43	*A.O.A.C.*, p. 145
27	Beckman	295	46	297	—	"Methods of Vitamin Assay"
29	Beckman	312	33			
30	Evelyn	308	44	286	21	Cold extraction overnight
31	Beckman	317	24			
32	Beckman	287	36	299	—	Column washed 100 ml. of solvent
32	Evelyn	296	36	—	45	Sample rehydrated
32				281	49	*Ind. Eng. Ch.* **35**, 1173 (1943)
33	Beckman	288	39	278	35	Silker, *et al.*[2]
34	Evelyn	299	34			
35	Beckman	264	38			
36	Klett-Summerson			282	32	Thimble used
37	Beckman	304	45	304	48	Wall-Kelley[1]
37a	Beckman	309	47	230	32	Different calculation
38	Hilger			282	30	Thimble used
38	Hilger			300	58	*Ind. Eng. Chem.* **13**, 600 (1941)
39	Evelyn	330	36			
40	Klett-Summerson	272	35			
	Av.	298	38			

C. *Industrial laboratories:*

COLL. NO.	INSTRUMENT	COLL. METHOD		OTHER METHODS OR MODIFICATIONS		
		SPLE. I	SPLE. II	SPLE. I	SPLE. II	METHOD
		p.p.m.	*p.p.m.*	*p.p.m.*	*p.p.m.*	
41	Cenco photel.	301	51	270	40	Silker, *et al.*[2]
42	Beckman	298	29			
43	Coleman 11	256	35			
44	Klett-Summerson			352	48	*A.O.A.C.*, p. 600
46	Lumetron 402 EF	263	30			
47	Beckman	273	33			
48	Beckman	270	30			
49	Klett-Summerson	288	42			
50	Klett-Summerson	309	44	307	29	Soda ash chromatog. after 72 hr. extn. with p.e.
51	Evelyn	324	56	360	68	*A.O.A.C.*, 1945
52	Beckman	286	25	282	25	Extn. 1 hr. on mech. shaker
53	Beckman	353	49	286	35	Wall-Kelley[1]
55	Klett-Summerson	290	35	291	35	Silker, *et al.*[2]
56	Coleman 11	287	39	270	53	Wall-Kelley[1]
57	Evelyn	266	33	283	36	25 ml. chromotographed
60	Beckman	307	38	340	48	By chart from carotene std.
	Av.	292	38			

TABLE 3.—*Comparison of results by groups*

A. *Collaborative procedure:*

	NUMBER OF CASES	AV. VALUES ON SPLE. 1	AV. VALUES ON SPLE. 2
1. Type of laboratory—			
State control	11	285	39
State & Federal research	15	298	38
Industrial	15	292	38
	41	Av. 292	38
2. Type of instrument—			
Beckman	23	291	37
Evelyn	7	305	40
Klett-Summerson	5	287	38
Coleman 11	3	287	42
Other	3	278	40

B. *Other procedures:*

A.O.A.C.	6	310	56
Wall-Kelley	5	285	39
Silker, *et al.*	3	280	37

RECOMMENDATIONS*

It is recommended—

(1) That the collaborative procedure be studied further with different samples of alfalfa meal as well as other types of food and feed materials.

(2) That the collaborative procedure as outlined for the determination of carotene in hays and dried plants be adopted as an alternative tentative method. Complete details of the methods are given in *This Journal*, **31**, 111–112 (1948), under "Changes in Methods of Analysis."

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REPORT ON FOLIC ACID

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

Folic acid in natural products has been assayed both biologically and microbiologically. The chick is commonly used for biological assays and usable procedures have been described by O'Dell and Hogan (1), Campbell, Brown, and Emmett (2), Hutchings, Oleson, and Stokstad (3), Scott and coworkers (4), and Lillie and Briggs (5). Day and Totter (6) have recently published an excellent review of published methods for the bioassay of the "Vitamin M group" (the factor variously called vitamin M, factor U, vitamin B_c, norite eluate factor, folic acid, and *L. casei* factor).

Not long ago, Stokstad and Hutchings (7) prepared an excellent summary of published procedures for the microbiological assay for this vitamin, and more recently Roberts and Snell (8) described a modified medium that should improve materially the assay procedure for pteroylglutamic acid. When *L. casei* is used as the test organism, the excess of strepogenin furnished in this new medium in the form of trypsinized casein should insure rapid early growth of the organism, thus overcoming one of the main objections to the use of *L. casei*. The identification of this new vitamin and the elucidation of the chemical structure of pteroylglutamic acid were greatly speeded by microbiological assays with lactic acid bacteria as test organisms. If, however, one reads the rapidly expanding literature in this field, it is apparent that "no rigorously tested or widely accepted method for quantitative extraction or determination of folic acid is yet available" (8). Kidder and Dewey (9) suggest that the protozoan *Tetrahymena* may be used in testing for folic acid, and they

* For report of Committee A and action by the Association, see *This Journal*, 31, 44 (1948).

mentioned that Dr. Bird, of Parke, Davis, and Company, is working on an assay method with this organism.

The collaborative study of the assay of folic acid made this year (1947) is the first undertaken under the auspices of the Association of Official Agricultural Chemists. For this study the microbiological procedure of Teply and Elvehjem (10), using *L. casei* as the test organism, was selected from the methods available in the literature. The constituents of this medium are found in most laboratories and the method has given good results in many hands. 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 which they were using routinely. The materials chosen for assay were (1) dehydrated, powdered spinach, and (2) ground dehydrated and defatted beef (from the round). Each laboratory co-operating in the project supplied its own folic acid standard, bacterial culture, and enzyme preparation, as well as all ingredients for the basal media. We realized that this policy increased the probability of variations in results even though specified and detailed directions were followed explicitly. Two laboratories, at the University of Missouri[1] and the University of Maryland,[2] agreed to assay the spinach by the chick method.

COLLABORATORS

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

O. D. Bird, Parke, Davis, and Company, Detroit, Mich.

H. W. Cromwell, Abbott Laboratories, North Chicago, Ill.

M. C. Caswell and E. Konopka, Merck & Company, Inc., Rahway, N. J.

Laura M. Flynn, Department of Agricultural Chemistry, University of Missouri, Columbia, Mo.

C. M. Hayes, Ayerst, McKenna, and Harrison, Ltd., Station "O," St. Laurent, Montreal, Province of Quebec, Canada.

Lloyd Hein, Wisconsin Alumni Research Foundation, Madison 6, Wis.

T. H. Jukes, Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

Dorothy J. Longacre, National Dairy Research Laboratories, 1403 Eutah Place, Baltimore 17, Md.

Henry W. Loy, Jr., Vitamin Division, U. S. Food and Drug Administration, Washington, D. C.

Bernard L. Oser, Food Research Laboratories, Inc. 48-14 Thirty-third Street, Long Island 1, N. Y.

L. R. Richardson, Department of Biochemistry and Nutrition, Agricultural & Mechanical College of Texas, College Station, Tex.

H. S. Schaefer, Ralston Purina Laboratories, St. Louis 2, Mo.

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[1] Boyd L. O'Dell, Department of Agricultural Chemistry, University of Missouri, Columbia, Mo.
[2] George M. Briggs, Department of Poultry Husbandry, University of Maryland, College Park Maryland.

Ruth Steinkamp, John R. Totter and Paul L. Day, Department of Biochemistry, School of Medicine, University of Arkansas, Little Rock, Ark.

F. M. Strong, Department of Biochemistry, University of Wisconsin, Madison, Wis.

Virginia R. Williams, Louisiana State University, Baton Rouge, La.

L. D. Wright, Sharp and Dohme, Inc., Glenolden, Pa.

RESULTS

The data submitted by the co-operating laboratories are summarized in Tables 1 and 2. To facilitate comparisons, numerical averages and median values are listed in Tables 1 and 2. In the calculation of these averages

TABLE 1.—*Results of 1947 A.O.A.C. collaborative study on folic acid* (Assays by Teply and Elvehjem Method using *Lactobacillus casei*)

LAB. NO.	SAMPLE I		SAMPLE II	
	SPINACH*		BEEF	
	FREE	TOTAL (CORRECTED FOR ENZYME)	FREE	TOTAL (CORRECTED FOR ENZYME)
	mmg/g	*mmg/g*	*mmg/g*	*mmg/g*
1	7.48	14.9	0.24	0.42
2	4.5	11.5	0.21	0.18
3	4.88	12.9	0.221	0.269
4	*Std. #1*		*Std. #1*	
	4.42	9.47	0.19	0.20
	4.26	9.64	0.22	0.21
	Std. #2			
	7.02	15.03	0.30	0.32
	6.76	15.30	0.35	0.33
5	5.25	12.0	0.25	1.01
6	12.4	29.8	0.57	0.59
7	10.5	*A.O.A.C.* Enz. Treatment 7.8; Routine Enz. Treatment 10.4	.13	*A.O.A.C.* Enz. Treatment 0.20; Routine Enz. Treatment 0.27
8	4.6	12.6	0.28	0.23
9		11.64		
	7.90	13.9	0.140	0.247
10			0.3	0.5
11	5.91	13.8	0.3	1.41
14	6.00	14.4	0.74	1.00
15	7.66	13.7	0.264	0.32
16	9.5	17.0	0.36	0.33
Mean	6.59	12.89	0.282	0.47
Median	(6.00–6.89)	(12.9–13.7)	0.264	0.325

* Result of assay by chick method:
Laboratory A—11 mmg/g
Laboratory B—16 mmg/g

and medians only one figure was used from each laboratory, an average of the several figures from each laboratory as shown in the tables.

Data from Laboratory 6 were not included in computing averages. The Associate Referee inadvertently sent to this laboratory, for use as a standard, a preparation of folic acid which had been partially inactivated

TABLE 2.—*Results of 1947 A.O.A.C. collaborative study on folic acid*
(Assays by methods other than that of Teply and Elvehjem)

LAB. NO.	SPINACH*		BEEF	
	FREE	TOTAL	FREE	TOTAL
	mmg/g	*mmg/g*	*mmg/g*	*mmg/g*
2	4.6	10.8	0.19	0.17
3	—	11.5	—	0.275
5	3.3	6.7	0.13	0.78
7	12.1	9.4	.13	.28
		8.4		.18
8	4.2	12.3	0.30	0.25
	5.2	11.4	0.33	0.30
9	—	16.8	—	0.383
10	3.6	3.2	0.4	0.5
	3.9	5.6	0.5	0.6
11	10.38	14.48	0.24	0.97
12	4.96	14.61	0.31	0.41
15	7.55	12.99	0.233	0.333
	—	12.35	—	0.26
16	10.35	17.25	0.32	0.32
17	3.48	12.48	0.243	0.37
Mean	6.52	11.86	0.26	0.42
Median	4.6	11.5	0.243	0.32
	4.96	12.3	0.30	0.333

* Result of assay by chick method:
Laboratory A—11 mmg/g
Laboratory B—At least 16 mmg/g

by over-heating. Laboratory 4 assayed each sample twice for free folic acid, and twice for total folic acid with each of two folic acid standards. Standard No. 1 and Standard No. 2 were from two different preparations of crystalline folic acid, and it was assumed that Standard No. 1 was better. The results from these two laboratories focus our attention upon the importance of securing a reference standard for folic acid at the earliest possible date.

Each value listed for Laboratory 7 is an average of twelve tests. This laboratory subjected one series of twelve samples to the enzyme treatment specified in the directions sent to collaborators, and subjected a second series of twelve samples to the enzyme treatment routinely used

in the laboratory. Weighings were made upon three different days. All extracts from both enzyme treatments were assayed according to the Teply and Elvehjem procedure as prescribed in the directions and also by the assay procedure routinely used.

A comparison of Tables 1 and 2 shows that as a rule when a laboratory carried out an assay by more than one method, all methods gave approximately the same results. Occasionally this was not true. Several laboratories reported values showing marked variations resulting from several methods as used in each laboratory.

One of the laboratories making a chick assay on the spinach reported 11 mmg. per gram; the other reported 16 mmg. per gram.

INFORMATION FROM QUESTIONNAIRES

Much helpful information was obtained from the questionnaires returned by the collaborators with their reports. A brief summary of this information follows.

Sensitivity of L. casein cultures.—Eight collaborators stated that they have encountered strains of bacteria identified as *L. casei* which are insensitive to folic acid or have lost their sensitivity with subculturing.

Routines for stock cultures.—Since it seemed possible that subculturing may cause changes in the requirements of *L. casei* for folic acid or unidentified growth factors, or both, an inquiry was made concerning precautions routinely taken to prevent changes in the culture of this test organism. Four of the laboratories commented that they use no special precautions. Six laboratories gave more or less detailed accounts of routines used in the maintenance of stock cultures of *L. casei.* These routines varied appreciably from laboratory to laboratory and their differences may well be reflected in varying responses of the stock cultures to folic acid or to the unidentified growth stimulants found in feeds and foodstuffs. Descriptions were given for four modifications of the agar medium for storage. The frequency of making transplants to agar varied from once a week to once a month. The laboratories choose differing formulas for preparation of liquid media for subcultures from agar.

Size of inoculum.—Methods routinely used for standardizing the inoculum in the several laboratories also show wide variations. About half of the laboratories reporting use 24-hour cultures; others prefer 15- to 18-hour cultures. Arbitrarily assuming that the inoculum cultures contained 10^8 bacteria per milliliter the Associate Referee calculated that various reporting chemists used for seeding a 10-milliliter culture inocula of the following different bacterial concentrations: 4×10^4, 2×10^5, 4×10^5, 1×10^6, 8×10^6, 1.5×10^7, and 3×10^7 bacteria.

L. casei vs. S. faecalis.—Most of the group routinely using *L. casei* prefer to use titration measurements in determining growth response. The group routinely using *S. faecalis* prefer turbidity measurements in determining the response to the vitamin. Half of the chemists reporting prefer *L. casei* as a test organism, the other half prefer *S. faecalis.*

Most laboratories carry stock cultures of *L. casei* for use in riboflavin tests. It appears that *L. casei* is more sensitive to folic acid than is *S. faecalis. L. casei* grows in proportion to the folic acid content of the medium over a range of from approximately 0.75 times 10^{-5} mmg to at least 10 times 10^{-5} mmg of the vitamin per milliliter of culture. Turbidity reading can frequently be made over a much longer range. With media in which a low blank can be secured one can obtain excellent acid production with this organism and the dose-response curve is steep. *L. casei,* being more

sensitive to the vitamin, can be used for assays of extracts which are very low in folic acid. The size of inoculum is more critical with *L. casei* than with *S. faecalis*.

With *S. faecalis* the amount of vitamin needed for a quantitative response is about ten times as much as with *L. casei*. Because of its lower sensitivity to the vitamin *S. faecalis* gives low blanks. There may be some difficulty with caramelization of the glucose in a citrate buffered medium for *S. faecalis*, unless the sterilization time is cut considerably. *S. faecalis* does not respond to folic acid conjugates.

Response in "Standard" cultures.—Collaborators were asked whether they found, with the Teply and Elvehjem medium, that the cultures from unknowns grow out more rapidly than cultures containing only standard folic acid solutions. They were also asked what corrections or adjustments they make if the unknowns show more rapid growth. Two of the group replying have had no difficulty with slowly growing standards; seven have made no observations on this point. Eight chemists indicated that under the conditions in their laboratories the cultures containing standard folic acid show a lower rate of early growth than is shown by cultures containing test extracts, with their almost inevitable unknown growth stimulants. Suggested remedies included assaying in reduced concentration and increasing the length of the incubation period. Several laboratories reported excellent results when enzyme digested casein is used. They found it possible with this material to obtain low blanks, more linear standard curves, and maximum growth at high levels of the vitamin.

Another inquiry on the questionnaire was concerned with the amount of acid produced during 72 hours' incubation as compared with the amount produced during an incubation period of 96 to 108 hours. Five replies indicated that the data on this point were insufficient to justify an opinion. One laboratory found no increase in acid production on lengthened incubation. Four replies indicated that acid production at the end of 96 to 108 hours' incubation was appreciably greater than at the end of 72 hours. Two of these four found that the increases in the standards and in the unknowns were so similar that either incubation period could be used with confidence in an assay. On the other hand, two laboratories reported that acid production in cultures containing standard solutions showed more change with increased incubation than was shown by cultures containing extracts of unknowns.

It was the consensus of opinion that norite-treated peptone is not satisfactory as a source of unidentified growth factors for *L. casei* in folic acid assays. The blanks were high, and the dose-response curve was not steep.

Dose-response curves.—The laboratories participating in this study were asked to submit specific data showing dose-response in cultures containing known amounts of pteroylglutamic acid. Data from the different laboratories showed appreciable variation in dose-response curves. With an ideal method and perfect techniques this would not be the case. Reports from several laboratories indicated that they were using the procedure of logarithmic plotting of dose-response curves as suggested by Wood (13) for evaluating their data and making calculations. It is the experience of the Associate Referee that the use of data in logarithmic form, either as numerical logarithms or by the use of graph paper with a log-log grid, is of great aid in the criticism and in the interpretation of microbiological assays.

DISCUSSION OF RESULTS

Each laboratory that participated in this study (1947) procured its own supplies of the ingredients necessary for these assays, including folic acid for a standard, the test organism, peptone as a source of the unknown growth stimulants, and the enzyme preparation for freeing bound folic

acid. Under the circumstances the circumstances the assay results as reported in Tables 1 and 2 show, as a rule, fairly good agreement.

Furthermore, we find agreement among the laboratories upon the following points:

(a) *Turbidity tests.*—Those who use *S. faecalis* for folic acid assays find that the amount of the vitamin can be estimated satisfactorily within 16 or 18 hours from the turbidity readings. Those who use *L. casei* find that the turbidity measurement lacks precision when the organism is grown in the Teply and Elvehjem medium. Titration of *L. casei* cultures at the end of 72 hours gives acceptable results.

(b) *Growth in "Blank" cultures.*—When the directions were followed there was too much growth in cultures to which no folic acid was added. In other words, the blanks were too high.

(c) *Need for improvement in method.*—When *L. casei* is the test organism improvements in the medium are desirable. Results were not reliable and reproducible when the specified procedures were followed.

We find disagreement among the laboratories upon the following points:

(a) *Purification of folic acid for standard.*—Several suggestions were made which indicated that the synthetic pteroylglutamic acid used as a standard had been subjected to recrystallization, or other purification procedures before use in a standard solution. Further study is needed to determine what procedures, if any, should be followed to prepare commercial pteroylglutamic acid for a reference standard. Steps looking toward the development of a reliable reference standard have been taken by the proper authorities. In the meantime it would seem wise, whenever possible, to characterize solutions to be used as standards by data on their absorption spectra at several wave lengths. Physical constants for folic acid, including extinction data, have been published by Parke, Davis, and Company (11) and by the Lederle Laboratories (12).

(b) *Choice of test organism.*—Seven of the collaborators expressed a marked preference for *S. faecalis*. Seven prefer *L. casei*. Three commented either that they have not tried *S. faecalis* or that their experience was too limited to justify an opinion. Only a few laboratories indicated that they have used both. Possibly the preferences expressed are the results in some cases of greater experience in the use of one or the other organism in microbiological assays.

Turbidity versus titration measurements.—One would expect preference for the method of evaluating growth response to vary from laboratory to laboratory. Such preference would depend upon the facilities of the laboratory, the extent of the technician's experience with each method, and the standardization and skill attained in the use of the method in any individual laboratory.

Routines for maintenance of stock culture, and preparation of inoculum.—The seemingly minor variations in these routines, as followed in the several

laboratories, may or may not be of significance in their effect upon the response of the test organism to the vitamin, or upon the rapidity of early growth in cultures containing standard folic acid.

Basal media for test.—Several laboratories have developed basal media of their own or made adaptations of published media. More extensive comparative tests are needed in order to select or develop the best possible basal medium for tests for this vitamin.

SUMMARY

The microbiological method for determining folic acid was subjected to collaborative assay in 1947, and results showed fair agreement between the laboratories. However, the findings in this first collaborative investigation of folic acid indicate the need for further study of its determination.

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PRESERVATIVES AND ARTIFICIAL SWEETENERS

By Margarethe Oakley (State Department of Health, Baltimore 18, Md.), *Referee*

This chapter is alive and now progressing in full vigor. During the past year nine subjects were assigned for study and from the progress made it is estimated that in another two years the chapter will be as up to date as the other subjects in the *Methods of Analysis*.

There is one more topic that should be added for study—namely, the new sweetener, 1-propoxy 2-amino 4-nitrobenzene. Investigations are al-

ready being conducted on its toxicity and recently a method for its determination was published (*Chem. Weekblad,* **43,** 283 (1947), through *C. A.* **41,** 5058 (1957).

Benzoate of Soda and Esters of Benzoic Acid

The Associate Referee reported plans to work on a method for the determination of ethyl vanillate; a sample of which he has recently secured.

Saccharin

The Associate Referee conducted tests on several methods for the extraction of baked goods but none proved sufficiently successful to submit to collaborative study.

Quaternary Ammonium Compounds

The Associate Referee completed collaborative studies on quaternary ammonium compounds in commercial preservatives, table sirup, bottled beverages containing fruit juice, and beer. He plans further studies on fruit juices, bottled sodas, milk, salad dressings, and pickled relishes.

Monochloracetic Acid

The Associate Referee completed collaborative studies on monochloracetic acid in carbonated beverages and fruit juices. He plans collaborative work on identification tests and further extension of the application of this method.

Dichloracetic Acid

The Associate Referee reported that the lateness of his appointment combined with the volume of his normal duties made it impossible for him to submit a report.

Mold Inhibitors-propionates

The Associate Referee completed collaborative studies on the detection of added propionate in bakery products and plans further work on propionate losses in baked goods and an investigation using chromatographic technique for the identification and estimation of acetic and propionic acid in these products. If time permits he also plans to investigate other mold inhibitors.

Thiourea

The Associate Referee submitted and recommended methods for thiourea, which are given in detail in *This Journal,* **31,** 100–104 (1948), under "Changes in Methods of Analysis."

Dulcin

The Associate Referee had been working on an alkaline-ether extraction

method on carbonated beverages and a qualitative test for the extracted dulcin but the Referee was unable to contact him recently and has received no report of his investigation.

Formaldehyde

The Associate Referee spent all his available time on another A.O.A.C. problem this year but hopes to begin work shortly on the development of a more modern method for the detection and determination of formaldehyde in food products and the deletion of many of the present A.O.A.C. methods.

RECOMMENDATIONS*

The Referee recommends—

(1) That work be continued on benzoate of soda and esters of benzoic acid.

(2) That the sublimation method for saccharin determination, *This Journal*, **30,** 494 (1947), be adopted as tentative. The first line to read "Transfer 200 ml of filtrate, **32.14,** to a separator and add 15 ml HCl."

(3) That studies on the determination of saccharin in baked goods be continued.

(4) That the ferricyanide method for quaternary ammonium compounds in commercial preservatives, *This Journal*, **29,** 312 (1946), be made official, first action.

(5) That the method for quaternary ammonium compounds in table sirup, *This Journal*, **29,** 325 (1946), be made official, first action.

(6) That the method for quaternary ammonium compounds in bottled beverages containing fruit juices, *This Journal* **29,** 322 (1946), be made official, first action.

(7) That the method for quaternary ammonium compounds in beer, *This Journal*, **29,** 325 (1946), be made official, first action.

(8) That further studies on quaternary ammonium compounds be conducted as outlined in the report of the Associate Referee.

(9) That the method for the determination of monochloracetic acid, *This Journal*, **27,** 199 (1944), be adopted as official, first action.

(10) That collaborative work be carried out on monochloracetic acid as outlined in the report of the Associate Referee.

(11) That a collaborative study of the method for identifying dichloracetic acid, *This Journal*, be made and/or any other studies the Associate Referee wishes to inaugurate.

(12) That the distillation procedure, described in the report of the Associate Referee on mold inhibitors-propionates, be adopted as tentative for the detection of added propionates in bakery products.

(13) That further investigations on propionates in bakery products be undertaken as described in the report of the Associate Referee.

* For report of Subcommittee C and action of the Association, see *This Journal*, 31, 53 (1948)

(14) That work on a method for the detection and/or estimation of thiourea in foods be continued.

(15) That studies on methods for detection and/or determination of dulcin in food be continued.

(16) That studies on present methods for the detection of formaldehyde be made in order to modernize that section of the chapter on preservatives and artificial sweeteners.

(17) That committee C take under advisement the deletion of the sections on B Napththol and Abrastol from *Methods of Analysis* if they consider the use of these compounds to be obsolete. (This recommendation is made in view of the increase in space needed by the introduction of the above recommended methods into the chapter on preservatives and artificial sweeteners.)

(18) That a Referee be appointed to develop a method for the detection and/or determination of the newly publicized sweetener, 1-propoxy 2-amino 4-nitrobenzene.

REPORT ON THIOUREA IN FOODS

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

Although thiourea has been used as a drug for a considerable time, its proposed use in foods, as far as the writer is aware, is of fairly recent date. Its proposed use in foods has been to serve two distinct purposes: (1) to prevent bacterial decay such as stem end rot in citrus fruits, and (2) to prevent browning (darkening) in fruits. It is possible that other uses may have escaped discovery.

REAGENTS

A number of methods exist for determining thiourea in the free state or in the presence of inorganic materials, but most of these methods are not sufficiently specific or adaptable to use in food products, nor are they sufficiently sensitive for this purpose. Probably the most sensitive and widely used types of reagent for the determination of thiourea and its derivatives are the pentacyanoferrates derived from sodium nitroprusside. The form most commonly used appears to be that developed by Grote (1) with some slight modifications (2). This is a hydrated form of pentacyanoferrate. There exists also the amino form in which the ammonia group has replaced the nitrogen group in the sodium nitrosopentacyanoferrate (sodium nitroprusside) (3).

In most of the work done by the Associate Referee, a slightly modified form of Grote's reagent has been used but the amino form is also being investigated. Although this reagent, as well as the amino form, gives re-

actions with a great many different compounds, the conditions of reaction, and the colored products differ greatly. In dilute acid solution, the number of compounds giving a blue chromogen, similar to thiourea and its derivatives, is decidedly limited. Under these conditions (*p*H 3.5 to 6.8) the reaction can be made quite specific for these compounds by measuring the chromogen at the proper wave length and insuring a low concentration of other materials which produce a similar color. Thiourea produces a much more intense blue color in minute quantities than is given by any other substances known to the writer. This makes possible the diluting out of other substances, if present. The reaction appears to be a gentle oxidation followed by coupling. It is rather sensitive to the environment and is affected by a considerable number of materials. The reagent (essentially that of Grote) (1) is also sensitive and its effectiveness is quite markedly affected by the manner of its preparation. Identical conditions should always be followed, therefore, in its preparation.

The amino form of the reagent has not received as much attention, but it would appear that its preparation can be more easily controlled than that of Grote (hydrated form). It is being used at present as a qualitative test for the chemical in orange juice.

METHODS FOR FOODS

A study has been made of the application of the reaction to two foods, oranges (or orange juice) and frozen dessert peaches. Some difficulty was encountered in applying the reaction to oranges. They contain an inhibitory substance which prevents the formation of the chromogen. The interfering substance is, in the Associate Referee's opinion, doubtless a reducing substance. In order to make a quantitative determination, the Associate Referee has resorted to extraction of the thiourea with an immiscible solvent to separate the chemical from the interference. Although determinations have been made using clarifying reagents, the removal of the interference depends upon its occlusion with precipitates of other material. No clarifying agent that gives a precipitate with the interference and is safe to use with thiourea has been found. Removal of the interference by occlusion with a precipitate often carries with it some of the thiourea and such methods are therefore not deemed satisfactory.

Another possibility is the oxidation of the interference at the same time oxidizing the thiourea only to the disulfide stage which also gives a reaction with the pentacyanoferrates. Qualitative tests for presence of thiourea, based on this principle, are given in detail in *This Journal*, **31**, 104 (1948).

A study is being made to see if these tests can be made quantitative and thereby eliminate the necessity of extraction, but as yet this has not been accomplished.

THIOUREA IN ORANGE JUICE

A quantitative method for thiourea has also been proposed, and details

of the procedure are published in *This Journal*, **31**, 100 (1948) under "Changes in Methods of Analysis."

FROZEN DESSERT PEACHES

The other food to which thiourea has been added at times is frozen dessert peaches. As is well known, many fruits darken on being cut and exposed to the air, due to the enzyme systems in the fruit. To prevent this darkening, various substances have been added to fruits at times. The enzyme systems also attack the thiourea and precautions must be taken to prevent this during the course of the analysis. The nature of this reaction is the subject of another paper and will not be discussed here.

The method as proposed for thiourea determination in frozen dessert peaches is given in detail in *This Journal*, **31**, 102 (1948).

RECOMMENDATIONS*

It is recommended:

(1) That the qualitative test for thiourea in orange juice, using the pentacyanoammonioferrate, be adopted as tentative.

(2) That the method for quantitative determination of thiourea in orange or orange juice be adopted as tentative.

(3) That the method for determination of thiourea in frozen dessert peaches be adopted as tentative.

(4) That the work on methods of thiourea determination in foods be continued.

REFERENCES

(1) *J. Biol. Chem.*, **93**, 25 (1931).
(2) *Ibid.*, **152**, 202 (1944).
(3) *Analyst*, **71**, 562 (1946).

REPORT ON BENZOIC AND VANILLIC ACID ESTERS

By W. J. McCarthy (Food and Drug Administration, Federal Security Agency, 501 Post Office Building, Cincinnati 2, Ohio), *Associate Referee*

During the year experimental work on the recovery of ethyl vanillate from various food products was planned. However, when attempt was made to procure ethyl vanillate through the usual channels, chemical supply houses, it was learned that the production of ethyl vanillate is still in the development stage and the product is not available commercially at present.

Inquiry was made of the authors of the research work done at the Institute of Paper Chemistry, Appleton, Wisconsin, as to methods they might

* For report of Subcommittee C and action of the Association, see *This Journal*, **31**, 54 (1948).

have used in recovery experiments. Mr. Irwin A. Pearl, research associate, in his reply said they had not studied the point directly but that recoveries of vanillic acid esters had been made from various organic materials and he described the general method used in most cases, as follows:

The material is extracted with ether (or benzene) and the ether extract is evaporated. The residue is boiled with dilute alkali to hydrolyze the ester. The mixture is cooled and extracted with ether to remove the alcohol formed upon hydrolysis. The alkaline solution is acidified with dilute sulphuric acid and again extracted with ether. The ether is evaporated and the residue dried to give vanillic acid. The vanillic acid is estimated by methoxyl analysis. In most cases the vanillic acid may be determined by actual weight along with melting point.

Since ethyl vanillate is not available commercially, Mr. Pearl very kindly sent me a laboratory sample of the product for experimental use.

It is recommended* that further study be given this problem during the coming year.

REPORT ON SACCHARIN

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

A package of unsweetened muffin mix was used to prepare cookies containing known amounts of saccharin. To 350 grams of the mix 70 mg. of saccharin were added, in order to have a finished product containing approximately 200 p.p.m. Sufficient water was added along with the saccharin solution to produce a dough of such a consistency that it could be dropped by spoonfuls onto cooky sheets. The cookies were baked at ca 230°C., allowed to air dry overnight, and weighed. Calculated on this weight, the baked material contained 226 p.p.m. of saccharin. After comminuting the cookies by passing through a meat grinder the crumbled material was stored in glass jars.

Fifty gram samples theoretically containing 11.3 mg. of insoluble saccharin were used for all determinations.

1. A Soxhlet extractor was first tried, and after a two hour extraction with petroleum benzine to remove fats, the benzine was discarded and a six hour alcohol extraction to remove the saccharin was carried out in the same apparatus. The alcohol extract was dark brown with extraneous material some of which carried over when the extract was sublimed in an endeavor to purify the saccharin. The yield was so small that this method was abandoned.

2. A mixture of the sample in water was made alkaline to bring the

* For report of Subcommittee C and action of the Association, see *This Journal*, 31, 53 (1948).

saccharin into solution, centrifuged, and the supernatent liquid acidified before extraction with ether. Acidification threw down such a mass of extraneous solid material that a Palkin extractor was tried; however, the ether brought the solid to the surface in the form of a gummy mass which was not a suitable medium for this type of extractor. This method was finally abandoned in favor of a modification of the official general method for the preparation of solid preparations, sec. **32.14** (c).

3. The sample was treated with hot water, acetic acid, and lead acetate. Instead of trying to filter the huge precipitate, it was centrifuged and the supernatent liquid strained through glass wool before acidification with hydrochloric acid and extraction with ether. It was necessary to break the emulsions formed by centrifuging. The ether extract was transferred to a sublimator with warm alcohol and sublimed at 140–160° for 1½ hours at 2 mm. pressure. The results from this method seemed promising and the sublimates appeared quite free from extraneous material. However, it was decided to check the purity of the residues by sulfate determinations. These showed the sublimates to be only a little over half saccharin, and the problem was right back where it was in 1924, when M. G. Wolf [(*This Journal*, **7**, 43 (1924)] abandoned the determination of saccharin baked foods because he could not get any better than 50 per cent recovery.

The Associate Referee is willing to make one more try to determine saccharin in baked goods before giving up the problem and therefore recommends that the study be continued another year.

REPORT ON QUATERNARY AMMONIUM COMPOUNDS IN FOODS

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

Procedures for the determination of quaternary ammonium compounds in ten different kinds of foods were published in *This Journal* about one year ago.[1] Upon his appointment the Associate Referee decided to submit to collaborative study the ferricyanide method for examination of commercial preservatives, the two procedures for fruit juice, and several of the procedures for other foods. Accordingly, eight samples were prepared and sent out to collaborators. The samples were made up as described below.

A quantity of lauryldimethylbenzylammonium chloride which had been analyzed by the ferricyanide method and found to contain about 92 per cent of the anhydrous compound was used to make up the samples.

1. For the sample similar to commercial preservative, 25 grams of the

[1] *This Journal*, **29**, 311 (1946).

salt was dissolved in water and made up to one liter, giving a concentration of 2.3 grams per 100 ml.

2. A quantity of table sirup containing about 15% maple sirup and 85% sugar sirup was purchased. 80 mg of preservative, corresponding to 73.6 mg of anhydrous quaternary ammonium salt was dissolved in some of the sirup and thoroughly mixed until there was a total of 1600 grams, corresponding to 4.6 mg. per 100 grams.

3. A sample of orange juice was prepared from canned orange juice by dissolving 0.16 gram, corresponding to 0.1472 gram of the anhydrous quaternary and made to four liters, which corresponds to 3.68 mg per 100 ml. of juice. After mixing as well as possible, 400 ml of juice was measured into each pint bottle, loosely stoppered with a rubber stopper, and heated in the steam sterilizer for 30 minutes and when cool the stoppers were driven in. Sterilized glassware was used throughout, but in spite of these precautions mold grew in some of the samples.

4. For the orange beverage containing about 15 per cent of fruit juice, a case of commercial still beverage was purchased. The bottles were opened and the contents transferred to a sterilized two-gallon bottle. 300 mg of the quaternary compound, corresponding to 276 mg of lauryldimethylbenzylammonium chloride, was dissolved in a few ml of H_2O added to the beverage mixed in a total volume of 5 liters. The beverage now containing 5.52 mg of the quaternary compound per 100 ml, was filled into the original bottles which were loosely stoppered with rubber stoppers and heated in a steam sterilizer for 30 minutes; after gradual cooling, the stoppers were driven in.

5. A case of imitation grape soda was purchased, the several bottles opened and transferred to a sterilized two-gallon bottle. 100 mg preservative, corresponding to 92 mg of lauryldimethylbenzylammonium chloride was dissolved in a portion of the beverage, then mixed with the main body of liquid which was made up to 5 liters, filled into the original bottles, and recapped. Since this beverage was supposed to contain no fruit juice, and was carbonated, it was unnecessary to sterilize it, although sterilized glassware was used in making up the sample to prevent a heavy contamination which might have proved troublesome. None of these samples spoiled in any way. They contained 1.84 mg lauryldimethylbenzylammonium chloride per 100 ml.

6. Sample 6 was designed to show the efficacy of the method in detecting quaternary ammonium compounds in milk accidentally contaminated by improper rinsing of equipment which has been cleansed with these compounds. A water solution containing 0.23 mg of lauryldimethylbenzylammonium chloride per 1 ml was to be mixed with milk by the collaborator, at the rate of 5 ml per 500 ml of mixture.

7. Sample 7 was designed to show the efficacy of the method for milk when the quaternary compound was added as a preservative. The water

solution containing 2.07 g of lauryldimethylbenzylammonium chloride in 500 ml was to be diluted as under (6) yielding milk containing 4.14 mg of quaternary compound per 100 ml.

8. A case of beer was purchased and the bottles cooled in ice water for several hours. A solution of 300 mg of the preservative was dissolved in water and made to 50 ml in a volumetric flask. When cold, each bottle was opened separately, 2 ml of preservative solution was pipetted into it, the bottle recapped immediately, and mixed thoroughly. In all cases only a slight head formed when the original cap was removed. In no case did a bottle foam over. This procedure yielded a beer containing 11.04 mg lauryldimethylbenzylammonium chloride per bottle or 3.11 mg per 100 ml provided the bottle contained 12 fluid ounces (355 ml).

Samples were sent to the collaborators listed below, for whose assistance we wish to express grateful appreciation.

V. D.—Halver C. Van Dame, Food & Drug Administration, Cincinnati, Ohio.
J.—Curtis R. Joiner, Food and Drug Administration, St. Louis, Mo.
We—Louis C. Weiss, Food & Drug Administration, Los Angeles, Cal.
K—Gardner Kirsten, Food & Drug Administration, New York, N. Y.
M—Dorothy Montgomery, Food & Drug Administration, Washington D.C.
W—John B. Wilson, Food & Drug Administration, Washington D. C.

The results obtained by collaborators are given in Table 1. Unfortunately, the sample of orange juice became moldy and several of the collaborators did not analyze it.

The results reported in Table 1 indicate that the "Ferricyanide Method for Quaternary Ammonium Compounds in Commercial Preservatives" should be recommended for adoption, without further work, as official, first action.[2]

The results obtained in analyzing sirup (average found, 4.48 mg/100 ml, recovery 97%); orangeade (average found, 4.97 mg/100 ml, recovery 90%), and beer (average found, 2.73 mg/100 ml, recovery 88%) warrant the adoption of these methods as official, first action.

More collaborative work should be done on the following food products: Orange juice, grape soda, and milk (preservative quantities).

Evidently an error was made in the preparation of the sample of orange juice, since collaborators found more than was to be added. In previous work in no case was a recovery of 100 per cent obtained. The results are therefore being rejected with a view to repeating the work next year.

Only two collaborators obtained good results on milk sample (No. 7). It is hoped that further experience will enable collaborators to obtain better results on next year's samples.

Since all collaborators found some quaternary ammonium compound in the "accidentally contaminated milk" containing about 2 p.p.m. of

[2] Details of the Method are published in *This Journal*, 31, 105 (1948).

TABLE 1.—*Quaternary ammonium compounds in foods*

NO.	KIND OF FOOD	ADDED G/100 ML	FOUND					
			W	M	J	V.D.	K	W$_E$
			g/100 ml					
1	Preservative	2.300	2.235	2.205	2.264	2.228	2.218	2.266
			2.245	2.201	2.266	2.231	2.215	2.266
		mg/100 ml	mg/100 ml					
2	Sirup	4.6	4.3	4.4	4.6	5.0	4.7	3.8
			4.3	4.4	4.6	5.0	4.6	
			4.2					
			4.4					
3	Orange Juice	3.7[a]	4.5	3.8	3.8	[d]		4.8
			4.2	3.8	3.9			5.2
		[b]	4.0	2.2	4.3			4.1
			3.6	2.2	4.4			4.2
4	Orangeade	5.5	5.0	4.6	4.9	5.2	5.4	4.7
			5.3	4.8		5.1	5.3	4.5
			5.2[c]					
			5.4[c]					
5	Grape Soda	1.8	1.8	1.6	1.6	1.7	1.2	1.3
			1.7	1.6	1.6	1.7	1.2	1.3
			1.9					
6	Milk	0.23	0.04	0.10	0.06	0.09	0.05	0.08
			0.05	0.10	0.05	0.12	0.05	0.08
			0.04		0.08			
7	Milk	4.1	3.9	3.2	0.8	3.9	1.5	2.9
			3.9	3.3	1.0	3.9	1.4	2.7
					2.7			
					1.8			
8	Beer	3.1	2.7	2.5	3.2	2.3	2.9	3.1
			2.6	2.5		2.4	3.0	3.1
			2.5					

[a] By method for fruit juices (29, 318, 1946).
[b] By shorter method for fruit juices (29, 319, 1946.)
[c] Liquid phase was also steam distilled.
[d] Sample had spoiled.

lauryldimethylbenzylammonium chloride, further work on amounts in the category will not be done at this time.

In the method for sodas the volume of bromophenol blue reagent used (3 ml = 1.2 mg) probably does not furnish a sufficient excess of reagent to insure complete extraction of the quaternary compound. In next year's

study 5 to 10 ml of the reagent will be commended, with the hope that the low results obtained by some collaborators will be avoided.

RECOMMENDATIONS*

It is recommended:

(1) That the ferricyanide method for quaternary ammonium compounds in commercial preservatives. *This Journal.* **29,** 312 (1946), be made official, first action.

(2) That the method for table sirup, *This Journal* **29,** 325 (1946), be made official, first action.

(3) That the method for bottled beverages containing fruit juice, *This Journal,* **29,** 322 (1946), be made official, first action.

(4) That the method for beer, *This Journal,* **29,** 325 (1946), be made official, first action.

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

(a) Method for fruit juices (*This Journal,* **29,** 318).

(b) Shorter method for fruit juices (*This Journal,* **29,** 319).

(c) Method for bottled sodas (*This Journal,* **29,** 323), subject to increasing the volume of bromophenol blue reagent to 5–10 ml.

(d) Method for milk (*This Journal,* **29,** 324), on samples containing preservative quantities of quaternary ammonium compounds.

(6) That collaborative study be made of the methods for mayonnaise, salad dressings, sandwich spreads, pickles, and relishes.

REPORT ON MONOCHLOROACETIC ACID

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

For the collaborative work on monochloroacetic acid, four samples were submitted to the nine collaborators listed, with the instructions given below. Samples 1, 2, and 3 were water solutions, whereas sample 4 consisted of orange juice which was heated to 100°C in an "Arnold Sterilizer" for 30 minutes after bottling.

COLLABORATORS

G—E. H. Grant, Food and Drug Administration, Boston Mass.
Ma—Alex P. Mathers, Alcohol Tax Unit, Washington, D. C.
Me—H. J. Meuron, Food and Drug Administration, San Francisco, Calif.
Mo—Dorothy Montgomery, Food and Drug Administration, Washington, D. C.
N—E. K. Nealon, Alcohol Tax Unit, Detroit, Mich.
O—Harold F. O'Keefe, Food and Drug Administration, Chicago, Ill.
S—Angus J. Shingler, Food and Drug Administration, Atlanta, Ga.

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* For report of Subcommittee C and action of the Association, see *This Journal,* **31, 54** (1948).

T—John A. Thomas, Food and Drug Administration, New Orleans, La.
W—John B. Wilson, Food and Drug Administration, Washington, D. C.

DIRECTIONS FOR COLLABORATORS

(For analysis of samples containing monochloroacetic acid)

Samples 1–4 inclusive:

A. Determine monochloroacetic acid in 100 ml by the quantitative method (*This Journal*, **27**, 199, 1944), in duplicate.

Note: In the titration of the excess $AgNO_3$ with NH_4CNS it has been found that the end point is more easily observed if the precipitate is removed just before the final pink color appears. To accomplish this, add the NH_4CNS soln carefully until the pink color formed by such addition fades slowly on mixing the soln. When this point is reached, shake the soln for ½ min. and filter through a folded filter into another flask. When the first flask is empty, wash down the sides with about 50 ml of water and add this to the filter after the soln has all gone through. When the wash water has passed through, complete the titration.

B. Make qualitative tests as follows:

Extract 4 separate 175 ml portions of each sample in automatic extractors, adding 6 ml of H_2SO_4 to each and extracting for about 2 hours. Test the extracts as follows:

1. *Barium test.* Treat extract (1) as directed in *This Journal*, **27**, 446, 1944, beginning "Transfer the ether extract to a separatory funnel . . . Examine the crystals under the polarizing microscope."

Note: The microscopic examination (*This Journal*, **27**, 447, 1944) should be made by a district analyst familiar with the procedure, and the remaining crystals should be forwarded to the Microchemical Section for check analysis.

2. *Indigo test on barium salt.* Treat extract (2) as above except apply the indigo test to the crystals following the procedure "Indigo Test" on the mimeographed sheet (same as *This Journal*, **28**, 303, 1945).

Note: Use the entire crop of crystals if less than 0.17 g is obtained and if 50 mg or less is obtained make up in a 5 ml graduated cylinder and add only 0.3 ml. of 1 *N* H_2SO_4.

3. *Indigo test.* To extract (3) add 3 ml of anthranilic acid reagent and evaporate the ether at a low temperature. 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 to a 50 ml beaker. Now apply the test as under (2) beginning "Test with litmus paper, etc."

4. *Pyridine test.* Treat extract (4) by the method (*This Journal*, **29**, 104, 1946) under the heading "Qualitative Method Applicable to Beverages and Fruit Juices," parts (1) and (2).

The results obtained by the quantitative method are given in Table 1.

In the case of sample 1 the analyses by "V" and "Mo" were made within a few days after the preparation of the sample. As several collaborators mentioned that a growth of mold was found in the samples, "W" repeated the determination about 6 months later and found about 1 mg monochloroacetic acid per 100 ml. It appears, therefore that the mold had been growing upon the monochloroacetic acid, which accounts for the low results obtained by the remaining collaborators. From a quantitative view-

point these determinations, except those made by "W" and "Mo" soon after preparation, should be disregarded. They have been reported in the table because of their bearing upon the qualitative tests.

"G" and "S" found their continuous extractors did not take out all of the monochloroacetic acid in 2–3 hours, as recommended in the method. They were advised to test the efficiency of these extractions using a solution of monochloroacetic acid containing 1 g per liter. Both found that 5

TABLE 1.—*Monochloroacetic acid (quantitative method)*

SAMPLE	ADDED MG/100 ML	FOUND—MG/100 ML								
		W	Mo	S	G	O	T	Me	Ma	N
1	8.0	7.2 7.7 7.8 7.7	7.2 7.0	2.2 2.2	3.2 3.4	2.4 2.8	5.0 5.0	3.2 3.0	3.4	3.5
2	22.0	21.2 21.2 21.3	20.1 20.5	18.8 21.3	19.8 20.3	22.5 21.7	21.8 21.9	20.5 20.8	20.8	20.0
3	46.0	44.9 45.4 45.9	43.0 43.7	42.7 44.4 44.9	44.4 45.8	45.9 47.1	44.8 44.3	43.5 43.2	45.4	43.0
4	17.7	15.4 15.2 16.0	13.3 13.3	11.5 10.8 13.8 11.2 10.9	14.0 14.7	13.7 13.5	14.9 13.3	14.0 14.0	14.3	12.5

hours were required. "G" obtained the following recoveries with 5 hours extraction:

Present	*Recovered*
Mg	*Mg*
50.0	49.8
15.0	14.7
3.5	3.4

These collaborators would prefer to make the extractions in separatory funnels.

"G's" experiments show that when 180 ml of solution containing monochloroacetic acid is shaken with 100 ml of ether, 58 per cent of chloroacetic acid goes into the ether layer. Four such extractions would remove 96.9 per cent of the monochloroacetic acid; six would remove 99.5 per cent, and eight, 99.9 per cent.

"W" has found that if an equal volume of ether is used, about 80 per

cent of the monochloroacetic acid goes into the ether layer. With this ratio, 3 extractions should yield 99.2 per cent recovery. In most cases, three extractions should suffice.

"G" made a check on sample 4 shaking out in separatory funnels and found 15.1 mg, as against 14.0 and 14.7 mg using the continuous extractor.

"S" also made a comparison between the two means of extraction as follows:

Sample	1	1	4	4
Continuous extractor 5 hours	2.2	2.2	11.2	10.9
Separatory funnels	2.4	2.2	8.5	12.6

There was some difficulty with emulsions when the orange juice was shaken in separatory funnels.

The recoveries of monochloroacetic acid are summarized in Table 2.

TABLE 2.—*Recoveries of monochloroacetic acid*

SAMPLE	2		3		4	
	mg	*per cent*	*mg*	*per cent*	*mg*	*per cent*
Maximum	22.5	102.3	47.1	102.3	16.0	94.1
Minimum	18.8	85.4	42.7	92.8	10.8	63.5
Average	20.9	95.0	44.6	97.0	13.5	79.4

The results obtained by collaborators on samples 2 and 3, together with those reported in previous publications[1] warrant the adoption of the method as "Official—First Action." From a regulatory standpoint low results such as were obtained on sample 4 work no hardship upon food manufacturers, but from a scientific standpoint they show the need for a more efficient method of extraction. Considerable objection has been registered by analysts in the Food and Drug Administration to the time consumed in the extractions, and several have expressed a desire to make extractions by hand using separatory funnels. Before recommending final action on the method, a further comparison should be made between automatic extraction and hand extraction in separatory funnels. Two suggestions have been made to improve the extraction procedure. The first is the salting out of the monochloroacetic acid, and the second the use of a more efficient extractor. Yakowitz, of our San Francisco Station, has designed a new extractor, which is described in a paper soon to be published in this *Journal*. Mathers, of the Alcohol Tax Unit, demonstrated that 113 mg out of 115.3 mg of monochloroacetic acid can be extracted in 30 minutes using a continuous extractor designed in that laboratory.

QUALITATIVE TESTS

The results of the qualitative tests are given in Table 3.

[1] *This* Journal, **25**: 145 (1942); **27**: 195 (1944).

"G" is the only collaborator who obtained crystals of barium monochloroacetate from sample 1. To accomplish this he used carbonic acid to remove excess barium hydroxide, acidified the filtrate with a drop of 0.01 *N* acetic acid to discharge the pink phenolphthalein color formed on heating, and made the final crystallization from a small quantity of alcohol.

"O", who has had considerable experience with the indigo test, prefers to use a mixture of equal parts of sodium hydroxide and potassium hydrox-

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

TEST	SAMPLE	W	Mo	S	G	O	T	Me	Ma
Barium	1	−	−	−	+	−	−	−	−
	2	+	+	+	+	+	−	−	+
	3	+	+	+	+	+	+	−	+
	4	−	−	−	−	−	−	−	−
Indigo Barium	1	+	−	−	[a]	−	+	−	
	2	+	+	−		+	+	−	
	3	+	+	+		+	+	−	
	4	+	+	+		+	+	−	
Indigo	1	+	+	−	[a]	+	+[b]	−	
	2	+	+	+		+	+	+	+
	3	+	+	+		+	+	+	+
	4	+	+	+		+	+	−	
Pyridine	1	+	+	−	+[c]	+	−	−	−
	2	+	+	+	+	+	−	+[d]	+[f]
	3	+	+	+	+	+	−	+[e]	+[g]
	4	+	+	+	+[c]	+	−	−	−

[a] Tests were not made but collaborator said "I can detect reasonably small amounts by the other tests."
[b] Indigo precipitate was not noticeable in the solution but was apparent on the filter paper.
[c] Tests tests made by C. S. Purcell, who was more experienced with the method.
[d] m.p. 157°C.
[e] m.p. 158°C.
[f] Wt. of crystals corresponds to 21.5 mg $CH_2ClCOOH$.
[g] Wt. of crystals corresponds to 46.6 mg $CH_2ClCOOH$.

ide, instead of all sodium hydroxide, in the 50% alkali solution, in which case the fusion is carried out at 225°–240°C. with less danger of losing indigo through overheating. However, in the present instance he followed the prescribed procedure.

The barium test is adapted for use on comparatively pure solutions when 20 mg or more per 100 ml are present. None of the collaborators obtained a positive barium test on the orange juice.

The indigo test on the ether extract yielded more positive results than when performed on the barium crystals; it seems advisable to confine the qualitative tests to the simple indigo test and the pyridine test which seem to be running neck and neck. The work on these two tests should be extended to other types of foods.

RECOMMENDATIONS*

It is recommended—

(1) That the method for the determination of monochloroacetic acid (applicable to carbonated beverages and fruit juices containing 5 to 150 mg CH_2Cl COOH in 150 ml), *This Journal*, **27**, 199 (1944) with the addition under "Apparatus," of the statement, "2. Use any equally efficient means of extraction." be adopted as official, first action.

(2) That collaborative work be done to establish the advisability of permitting extractions to be made in separatory funnels as an alternate procedure.

(3) That collaborative work be done on other fruit juices and beverages.

(4) That collaborative work be done on the application of the method to other types of food.

(5) That collaborative study of the indigo test and the pyridine test be continued.

No reports were given on dichloroacetic acid or on formaldehyde.

REPORT ON MOLD INHIBITORS, PROPIONATES

By LEWIS H. MCROBERTS (Food and Drug Administration, Federal Security Agency, San Francisco, Calif.),
Associate Referee

DETERMINATION OF PROPIONATES IN BAKERY PRODUCTS

Under provisions of section 701 of the Federal Food, Drug and Cosmetic Act, a hearing has been held and proposals issued for establishment of standards of identity for various types of bread (1). At this hearing evidence was presented indicating that the use of mold and "rope" inhibitors was a not uncommon bakery practice. The proposed standards of identity limit the quantities of preservatives (with appropriate label declaration) to the following:

Calcium or Sodium propionate	
In white bread	=0.32 part/100 parts flour
In whole wheat bread	=0.38 part/100 parts flour
Vinegar—100 grain or equivalent	=1 pint/100 lb. flour
Sodium diacetate	=0.4 part/100 parts flour.

Whether or not the proposed standards are adopted it is desirable that methods for detection and estimation of these preservatives be available. The determination of propionic acid should serve well in the estimation of added propionates for there is no evidence that this acid is normally present in bakery products in other than calculated trace amounts. Acetic acid is normally present in amounts that would have to

* For report of Subcommittee C, and action of the Association, see *This Journal*, 31, 52 (1948).

be taken into account in estimating the proportion of vinegar or diacetate added. The determination of both of these acids through adaptation of the volatile acid technique (2, 3, 4, 5) appeared promising. For supplementary confirmation the use of the chromatographic column (6) also offered possibilities.

EXPERIMENTAL

In preliminary studies the writer applied the Official Method (**23.36 (b)**) for volatile fatty acids in dried eggs to the analysis of samples of commercial bakery products, some of which were believed to contain added propionate; and to samples of bread prepared in the laboratory, without propionate, and with varying amounts of propionic acid added.

Some of the same samples were also analyzed by a modification of this procedure, which provided for ether extraction of the aqueous sample solution obtained after treatment with phosphotungstic acid and filtration (first step in preparation of the sample by the official method above cited). The extraction was carried out on an aliquot of the filtrate, acidified with sulfuric acid in a large Palkin extractor. Trial with pure formic, acetic, and propionic acids had indicated that such extraction would be 90–100 per cent complete in five hours. Following extraction, water was added to the extract, rendered alkaline to phenolphthalein with 1 *N* sodium hydroxide, and the ether evaporated. The aqueous phase was made to a convenient volume and an aliquot subjected to the usual distillation procedure (sec. **24.11**).

The purpose of the modification was to remove carbohydrates which, it was suspected, might break down during distillation to contribute volatile acids to the distillate. Incidental to the application of the modification certain observations gave rise to a suspicion that volatile acids, particularly acetic, might be contributed by the ether used for extraction. For that reason the ether was thoroughly washed with water just prior to use.

Results of these preliminary trials are given in Table 1. There is no indication, in these results, of any contribution of formic or propionic acids from a breakdown of carbohydrates during distillation. The implications are not entirely clear-cut in respect to acetic acid. The results may imply some derivation of acetic acid from carbohydrates and also from the use of unwashed U.S.P. ether in the extraction. As far as propionic acid is concerned the unmodified method gives results more closely in agreement with the theoretical.

In the course of routine operations a considerable number of samples of bakery products, suspected of containing added propionate, have been submitted to this laboratory for analysis. Table 2 presents the results obtained on a representative group. In some instances the batch formula customarily employed was known, although there is no assurance that

such formula was actually adhered to in preparing the particular batches analyzed. The bread analyses have been converted to the terms of the batch formula on the convention that 150 lbs. of fresh product is equivalent to 100 lbs. of flour used in preparing it.

While lack of authenticity of the samples prevents any quantitative conclusions as to the percentage recovery, it is noteworthy that the anal-

TABLE 1.—*Determination of volatile acids and recovery of added propionic acid by the official method for volatile acids in dried eggs, and by an ether extraction modification*

SAMPLE	OFFICIAL METHOD			"C" VALUE	ETHER EXTRACTION MODIFICATION			"C" VALUE
	FORMIC	ACETIC	PROPIONIC		FORMIC	ACETIC	PROPIONIC	
Laboratory prepared bread no added propionate	12	56	None	2.6	8	20	15	2.0
	13	34	4	2.4	9	34	6	2.3
	13	30	7	2.2				
Same—with 149 mg/100 gm added propionic acid	12	51	151	1.8	6	35	165	1.8
	14	54	151	1.8	9	37	161	1.7
Same—with 75 mg/100 gm added propionic acid	11	32	83	1.8	11	9	98	1.7
	13	39	78	1.8	10	13	97	1.7
Commercial Bread A	15	32	13	2.2	11	19	23	1.9
	16	30	13	2.2	8	21	20	2.0
Commercial Bread B[1]	14	28	83	1.8	13	57	62	2.0
Commercial Bread C[1,2]	27	67	117	1.9	25	88	87	2.0

[1] Ether used for extraction modification on these samples was not washed.
[2] Analyzed fresh and results computed on the fresh basis.

yses in most instances indicate a sodium propionate content materially lower than the batch formulas would have provided. Inasmuch as the method employed had yielded virtually complete recovery of propionic acid added to air-dry, propionate-free bread (Table 1), the results may reflect baking losses and/or losses of propionic acid during air-drying of the sample.

To provide material for further study as well as for collaborative samples, authentic batches of bread containing known added amounts of propionate were baked in the laboratory. Details of preparation were as follows:

Ten pounds of "enriched bleached flour" was thoroughly mixed by rolling in a large container and then separated into three portions that were used in the preparation of three 3-loaf batches of bread. A commercial propionate obtained from a local

TABLE 2.—*Volatile fatty acids in commercial bakery products determined by official method for dried eggs*

PRODUCT	FORMIC MG/100 G	ACETIC MG/100 G	PROPIONIC MG/100 G	"C" VALUE	SODIUM PROPIONATE COMPUTED TO PARTS PER HUNDRED PARTS OF FLOUR USED	
					AS DETERMINED	FROM FORMULA
Samples air-dried and analysis for individual acids reported on air-dry basis.						
White bread —D	12	29	41	2.0	0.06	0.18
E	14	28	83	1.8	0.12	0.19
F	12	50	100	1.8	0.15	0.25
G[3]	4	43	35	2.0	0.04	0.08
H	14	56	55	2.0	0.08[2]	N. R.
Wheat bread—A	11	51	46	1.9	0.08	0.19
B	20	48	55	2.0	0.08	0.18
C	15	67	31	2.1	0.04	0.09
D	23	76	10	2.3	0.01	0.06
E[3]	10	38	57	1.8	0.09	0.06
F[3]	6	16	58	1.7	0.09	0.06
G[3]	6	40	38	2.0	0.06	0.08
Rye bread —A	11	173	44	2.2	0.06[1]	N. R.
Doughnuts —A[3]	15	None	263	1.6		N. R.
B[3]	11	None	298	1.5		N. R.
Samples analyzed fresh and analysis for individual acids reported on fresh basis.						
Cup cakes	8	19	None	2.6		N. R.
Fruit cake	72	124	54	2.2		N. R.
Light cake	36	None	138	1.6[2]		N. R.
Sweet rolls	16	31	None	2.5		N. R.
Jelly roll	23	None	74	1.6		N. R.
Chocolate cake—A	63	100	12	2.3		N. R.
B	22	55	96	1.9		N. R.

[1] Presence of propionic acid in distillate confirmed chromatographically, *This Journal*, 28, 644.
[2] Presence of propionic acid in distillate confirmed chromatographically and also by redistillation, *Ibid.*, 21, 688. [3] Analyses by J. V. Beck. N. R.—Not reported.

bakery was added to two of the portions of flour in the amounts of 0.32% and 0.16%. The following described recipe was used:

1.5 cakes yeast
1.5 tbs. sugar
1.5 pts. milk
2.5 lbs. prepared flour (±2 gms)
0.7 tbs. salt
3 tbs. shortening.

Baked at 450°F. for 15 min. and then lowered to 375°F. for 45 min.

Precautions were taken to insure that all of the 2.5 lbs. of prepared flour were incorporated in the bread. The three batches of bread designated as "A," "B," and "C" were allowed to cool for one hour prior to weighing. After 24 hours the loaves were then sliced and air dried for about 48 hours, re-weighed, crushed and ground to pass 20 mesh. The following data and calculations were recorded:

Bread Batch	"A"	"B"	"C"
Propionate—% in flour	0.32	0.16	None
Wt. (1 hr. after baking) oz.	56	56	58
Wt. (24 hrs. after baking) oz.	55.32	56.06	54.21
Ratio: Bread/Flour	1.38	1.40	1.36
Wt. Air dried, oz.	42.6	43.0	42.7
Ratio: Air Dry/Fresh	.7701	.7607	.7868

Assuming no baking or air drying loss, batches "A" and "B" (air dry) were then calculated to contain 0.30 per cent and 0.15 per cent of propionate, or 0.230 per cent and 0.115 per cent propionic acid, respectively.

The propionate, the flour and the mixtures of propionate and flour were analyzed for propionic acid.

Five grams of the propionate was made to 500 ml, and a 50 ml aliquot, acidified with sulfuric acid and diluted to 150 ml, was subjected to the regular distillation procedure (sec. **24.11**). The average of two determinations was 76.2 per cent of propionic acid, equivalent to 99.1 per cent of sodium propionate.

The flour and the flour-propionate mixtures were analyzed by the procedure for dried eggs, with results as follows:

Flour

SAMPLE	VOLATILE ACIDS (MG/100 G)			EQUIVALENT SODIUM PROPIONATE	
	FORMIC	ACETIC	PROPIONIC	*mg/100 g*	*per cent*
Flour Mix A	4.4	None	265	344	0.34
Flour Mix B	3.7	None	136	177	0.18
Flour Mix C	4.3	None	3	4	0.00

The bread samples were analyzed by four different procedures.

(1) The official method for dried eggs.
(2) The ether extraction modification described above.
(3) A preliminary dispersion of the sample in 100 ml of water, acidified with 25 ml 1 *N* sulfuric acid by means of a Waring Blendor. Modification (4) was then applied. The purpose of this preliminary treatment was to test whether low results might be due to occlusion of propionate in the bread particles, rendering the initial aqueous extraction incomplete.
(4) The detailed procedure given below and issued to collaborators for trial. This modification retains the basic principles of the official procedure for dried eggs, varying the technique of sample preparation solely for reasons of convenience in handling samples of bakery products.

TABLE 3.—*Volatile acids recovered from propionate-containing bread by four modifications of the official method for dried eggs*

(Results expressed as mg/100g air-dry sample.)

MODIFICATION	BREAD A			BREAD B		
	FORMIC	ACETIC	PROPIONIC	FORMIC	ACETIC	PROPIONIC
1	23	36	172	13	5	99
2	15	32	166	7	28	87
3	21	33	165	14	8	97
4	21 22	29 20	172 180	13 13	23 24	83 84
Propionic acid added air-dry basis			230			115

Results obtained by the four modifications are given in Table 3.

The results are in harmony with previous conclusions in regard to the absence of any advantage in the ether extraction (modification 2) and they fail to demonstrate any advantage in the more vigorous disintegra-

TABLE 4.—*Collaborative assay of sodium propionate*

COLLABORATOR*	ASSAY		AVE. "C" VALUE DETERMINED	STD. "C" VALUE PROPIONIC ACID
	PER CENT	AVE.		
Lewis H. McRoberts—Assoc. Ref., San Francisco	99.7, 98.5	99.1	1.61	1.58
William Horwitz—Minneapolis	96.7, 99.2, 98.3, 100.4, 99.4, 98.6	98.8	1.59	1.58
V. E. Munsey—Food Div., Washington, D.C.	99.2, 99.2	99.2	1.61	1.69
H. M. Bollinger—Los Angeles	98.5, 98.6	98.6	1.61	1.63
Mrs. D. W. Montgomery—Food Div., Washington, D.C.	99.4, 99.4	99.4	1.63	1.63
H. O. Fallscheer—Seattle	98.7, 98.9	98.8	1.69	1.64
H. I. Maycomber—Baltimore	99.6, 99.7	99.7	1.60	1.61
Meyer Matluck—Boston	98.5, 98.9	98.7	1.59	1.57
Average		99.0	1.62	1.62

* All collaborators are with the U. S. Food and Drug Administration, stationed at the laboratories indicated.

TABLE 5.—*Collaborative results—volatile acids in air-dried bread*
(Results expressed as mg/100 g air-dry sample)

COLLABORATOR	BREAD A—230 MG PROPIONIC ACID ADDED PER 100 G AIR DRY SAMPLE					BREAD B—115 MG PROPIONIC ACID ADDED PER 100 G AIR DRY SAMPLE					BREAD C—NO ADDED PROPIONIC ACID					STANDARD C VALUES		
				AVE. C VALUE					AVE. C VALUE					AVE. C VALUE				
	FORMIC	ACETIC	PROPIONIC	DET'D.	CORREC. FOR FORMIC	FORMIC	ACETIC	PROPIONIC	DET'D.	CORREC. FOR FORMIC	FORMIC	ACETIC	PROPIONIC	DET'D.	CORREC. FOR FORMIC	FORMIC	ACETIC	PROPIONIC
L. H. McR.	21	29	172	1.75	1.67	13	23	83	1.79	1.71	13	26	4	2.43	2.20	2.97	2.44	1.58
	22	20	180			13	24	84										
W. H.	19	63	141			10	37	74			8	34	None					
	15	31	171			9	41	72	1.87	1.85	7	28	None	2.54	2.45	3.10	2.48	1.58
	16	45	159	1.80	1.72	8	38	76			6	30	None					
	16	42	151															
	15	49	159															
V. E. M.	24	79	150	1.87	1.90	14	None	133	1.64	1.55	12	41	None	2.60	2.50	3.03	2.40	1.69
	23	61	166			15	None	133			11	46	None					
H. M. B.	17	29	177	1.79	1.73	9	20	93	1.76	1.73	7	25	1	2.53	2.42	3.07	2.48	1.63
	17	36	177			9	20	93			7	25	2					
D. W. M.	25	29	189	1.78	1.69	18	27	93	1.86	1.76	13	24	11	2.32	2.13	3.11	2.45	1.63
	25	32	185			18	30	88			14	27	10					
H. O. F.	24	30	176	1.78	1.69	14	12	95	1.80	1.70	16	10	10	2.42	2.09	3.10	2.49	1.64
	25	36	176			17	7	98			16	15	6					
H. I. M.	N.R.	25	187	N.R.	1.68	N.R.	21	91	N.R.	1.75	N.R.	5	None	N.R.	2.65	2.97	2.39	1.61
		27	187				32	83				6	None					
M. M.	26	11	192	1.74	1.64	18	26	84	1.80	1.68	16	16	9	2.36	2.09	2.99	2.41	1.57
	29	32	162			19	9	92			15	18	6					
Average propionic det'd.			175					93					4					
Apparent baking and air-drying loss (per cent)			24					19										

N. R. Not reported.

tion treatment constituting modification 3. The fourth modification gives entirely comparable results and was selected for collaborative study because of its greater convenience. Its details are given in the preceding number of *This Journal*, **31**, 99 (1948).

COLLABORATIVE STUDY

The Associate Referee has been fortunate in receiving the cooperation of seven collaborators, representing six different laboratories. Each collaborator was provided with subdivisions of air dried breads, A, B, and C,

TABLE 6.—*Volatile acids in air-dry bread determined by chromatographic procedure*

(Results expressed as mg/100 g air-dry sample)

ACID	BREAD A		BREAD B		BREAD C	
	V.E.M.	D.W.M.	V.E.M.	D.W.M.	V.E.M.	D.W.M.
Acetic	23	37	18	26		27
	24	38	15	27	16	29
Propionic	147	168	70	87		
	136	168	66	87	2	None

described above, and a portion of propionate used in their preparation. They were advised as to the results obtained by the Associate Referee in assay of the propionate sample, and were requested to determine and report its calculated sodium propionate content as a check on the standardization of their volatile acid apparatus.

Results are given in Table 4.

The collaborators were not advised of the amounts of propionic acid added to the collaborative bread samples. They were requested to determine the volatile acids by the procedure described above, a copy of which was provided, and to report the data presented in Table 5.

It was also suggested that the collaborators apply the chromatographic technique (6) on distillates of the bread samples if time and facilities were available. Munsey and Montgomery report the results presented in Table 6.

DISCUSSION

On the whole, the results reported for propionic acid are reasonably consistent and imply that the suggested distillation procedure can be relied upon to detect added propionate in amounts greater than the equivalent of 20 mg propionic acid per 100 grams of sample analyzed. The results for acetic acid and formic acid are not as consistent but neverthe-

less demonstrate that these acids are naturally present in bakery products in significant amounts.

The apparent total baking and/or air-drying loss (if the loss is due to these circumstances) is quite substantial. If such loss is incurred in air-drying, it can, of course, be eliminated by analyzing the fresh sample. It was essential that the method of analysis be proven reliable before investigation of this loss could be undertaken intelligently. The extent and character of the collaborative results is believed to be such as normally to support a recommendation for adoption of the procedure as a quantitative method for determination of propionic acid. Pending clarification of the apparent baking and/or drying loss, however, the method must be viewed as qualitative only, notwithstanding the consistency of the collaborative results.

ACKNOWLEDGMENT

The Associate Referee acknowledges with appreciation the assistance of the collaborators whose names are given in Table 4 and whose results are identified by their initials elsewhere.

RECOMMENDATIONS*

It is recommended:

(1) That the distillation procedure described herein be adopted as tentative for the detection of added propionate in bakery products.

(2) That investigation of the nature and cause of the loss of propionic acid, referred to herein as "apparent baking and/or air-drying loss," be undertaken.

(3) That further investigation of the chromatographic technique, for the identification and estimation of acetic and propionic acids in bakery products, be undertaken.

REFERENCES

(1) *Federal Register*, Vol. 8, 10782–3, 8/3/1943.
(2) HILLIG, *et al.*, *This Journal*, **22**, 116–118 (1939).
(3) HILLIG, FRED, *Ibid.*, **25**, 176–195 (1942).
(4) ——, *Ibid.*, **26**, 198 (1943).
(5) ——, *Ibid.*, **27**, 204–223; 237–240 (1944).
(6) RAMSEY and PATTERSON, *Ibid.*, **28**, 644–656 (1945).

No report was given on diacetates.

No report was given by the General Referee on eggs and egg products.

* For report of Subcommittee C and action of the Association, see *This Journal*, **31**, 53 (1948).

REPORT ON ADDED GLYCEROL IN EGGS

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

The present quantitative method for added glycerol in eggs was adopted tentatively in 1934 with the recommendation that further studies be made. In 1939 a brief progress report described some preliminary work on separating glycerol by distillation with superheated steam. No further reports have been submitted.

A survey of the literature indicates increasing use of the Malaprade periodate oxidation method for determining glycerol in mixtures. Amerine and Dietrich (*This Journal*, **28**, p. 408, 1943) use this method for glycerol in wine, and Shupe (*Ibid.*, **28**, 249, 1943) describes periodate methods for glycerol and glycols in cosmetics. The determination is based on the oxidation of glycerol to formaldehyde and formic acid by means of periodic acid or by periodate salt. Glycerol may be calculated from the amount of acid formed, as determined by direct titration, also by measurement of the excess periodate. The oxidation proceeds at room temperature and is more selective than a dichromate oxidation. However, certain substances such as dextrose, levulose, and certain organic acids among others, react with periodate and hence must be removed.

The above method was applied to samples of whole egg clarified by the present method for glycerol in egg. Blanks were high, ranging from 0.2–0.3 per cent of apparent glycerol. In view of the results, work was diverted to the problem of isolating glycerol in products entirely free from interfering egg constituents. A number of procedures based on published methods for glycerol in various food products have been examined. Blanks as low as 0.08 per cent have been obtained, but further work will need to be done before a method can be submitted.

It is recommended* that studies on methods for determination of added glycerol be continued.

REPORT ON ACIDITY OF FAT IN EGGS

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

In accordance with the recommendation of Subcommittee C (*This Journal*, **30**, 50) methods for the determination of acidity of fat in eggs were studied.

The present official method I(1) for dried eggs is based upon the process of drying the eggs at 55°C. in a vacuum oven to constant weight, extracting the fat in a continuous extractor with anhydrous ether, and then

* For report of Subcommittee C and action of the Association, see *This Journal*, 31, 51 (1948).

titrating the ether extract dissolved in neutral benzene with sodium ethylate. The results are reported as ml of 0.05 *N* sodium ethylate per g of ether extract. Method II, (1) the present rapid tentative method for dried eggs is essentially the same as Method I except that the eggs are extracted with ether without preliminary drying, and instead of using a continuous extractor, repeated extractions are made of the egg material in a small flask, the clear ether layer being decanted through a filter into a weighed flask. The ether is then evaporated, the residue dried, weighed, and titrated with sodium ethylate as in Method I.

Kline and Johnson (2, 3), in a recent study of lipolytic activity in stored dried eggs, have shown that a specific method for measuring glyceride hydrolysis is necessary. In this work they have shown that as much as 70 per cent of the total acidity of ether extracts of egg powders as obtained by the A.O.A.C. method is due to cephalin. The cephalin fraction of animal phospholipids acts as monobasic acid and is completely titrated in solvents of low dielectric value, with phenophthalein as indicator. They have also shown that the acidity of the ether extract increases with the increase in moisture content of the eggs since larger amounts of phospholipids are extracted by the ether at higher moisture levels.

This paper further describes a method whereby the true fatty acid content of dried eggs may be determined by extracting the fat and fatty acids with acetone. The phospholipids are only slightly soluble in acetone and may be completely removed by treatment with alcoholic magnesium chloride.

Kline and Johnson have shown that by their proposed method preliminary drying is unnecessary. Further work indicated that a possible source of error in the determination of free fatty acids in dried eggs, particularly in eggs where the acids have developed in the liquid state before drying may be due to the formation of a fatty acid-protein complex. Complete recovery of the fatty acids cannot be made unless the *p*H of the egg mixture is adjusted to 4 prior to drying. However, fatty acids which have developed during storage in the dried eggs may be completely extracted at the normal *p*H of the powder, since the medium is nonaqueous and the fatty acids have no opportunity to combine with the bases present in the egg.

The method proposed by Kline and Johnson is as follows:

Transfer 2 g egg powder to a 40 ml conical, graduated centrifuge tube and add 15 ml of acetone. Let stand for 0.5 hour with frequent stirring. Centrifuge and decant into centrifuge bottle. Repeat extraction 4 times using 10 ml portions of acetone, allowing 5–10 min. extraction time. To the combined acetone extracts, which should be kept to a volume of 60 ml, add 1 ml of a satd soln of magnesium chloride in absolute alcohol. Mix well by swirling and then centrifuge. Decant supernatant liquid into a 500 ml separatory funnel. Wash the residue twice with 5 ml portions of acetone, centrifuging after each washing; and combine washings with the original supernatant liquid in the separatory funnel.

Add 30 ml of petroleum ether to the acetone soln and thoroly mix. Add 200 ml of

H_2O and agitate contents of funnel gently to prevent emulsion formation. Allow phases to separate and discard the aqueous acetone. Wash the petroleum ether with two 25 ml portions of water and transfer the petroleum ether quantitatively to a 125 ml Erlenmeyer flask. Add 50 ml of isopropanol and 10 drops of 10% ethanolic phenolphthalein. Titrate the soln with 0.025 *N* sodium ethylate.

Samples of powdered egg yolk and whole egg were obtained by the Associate Referee to compare results for the acidity of fat as determined by the present A.O.A.C. methods and the proposed method of Kline and Johnson.

Results for acidity of fat

	ML Na ETHYLATE/2 G SAMPLE	
	EGG YOLK	WHOLE EGG
A.O.A.C. Method I Vacuum oven 55°C.	4.35	1.90
A.O.A.C. Method I Vacuum oven 100°C.	3.95	1.85
A.O.A.C. Method I Without drying	4.10	2.15
A.O.A.C. Method II	4.50	2.25
Proposed acetone method without drying	1.67	1.13
Proposed acetone method after drying, vacuum oven 100°C.	1.70	0.90

DISCUSSION

The above results confirm the work of Kline and Johnson that the acetone extraction method gives results for the acidity of fat which are approximately 60 per cent less than the A.O.A.C. methods, the difference being due to the presence of titratable phospholipids which are extracted by ether but are insoluble in acetone.

RECOMMENDATIONS*

It is recommended that the proposed acetone method for determining acidity of fat in eggs be further studied.

REFERENCES

(1) *Methods of Analysis, A.O.A.C.* (1945, 6th ed.), **23.30, 23.31.**
(2) Leo Kline and C. M. Johnson, *Ind. Eng. Chem., Anal. Ed.*, **18,** 35 (1946).
(3) *Ibid.*, **18,** 617 (1946).

* For report of Subcommittee C and action of the Association, see *This Journal*, **31,** 51 (1948).

REPORT ON DECOMPOSITION IN FOODS

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

The report on "Decomposition in Shellfish" includes collaborative data on the indole content of shrimp, oysters, and crabmeat of varying stages of decomposition. Earlier data of individual analysts on a considerable number of samples of each of the above shellfish corroborate the usefulness of indole as a criterion of decomposition in these products. The results support the recommendation that the method for indole be adopted as official, first action. The Referee also concurs in the other recommendations of the Associate Referee.

The Associate Referee's progress report on "Decomposition in Fruits" describes some of the attempts to correlate rot in apples with a chemical test. At the present stage of development, no recommendation is made toward adoption of a definite procedure, but only that further study be made on the problem in an effort to select that chemical test with the best promise of becoming useful for regulatory purposes.

It is recommended* that studies be continued on fish and dairy products.

No reports were given on decomposition in fish products or in dairy products.

REPORT ON DECOMPOSITION IN FRUIT PRODUCTS

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

The use of considerable amounts of rotten fruit in the manufacture of fruit juices, jellies, and butters is easily concealed because their presence cannot be detected by odor or taste. Methods in use at present for detecting rot in fruit products are (1) The Mold Count Method and (2) The Rot Fragment Count. These methods have been used with much success on fruit products that have been processed in such a way as not to remove the mold or rot fragments, but they are of little value when applied to clear jellies and juices. Other means are desirable for establishing the presence of rotten apples, when used, where present methods are inapplicable, and in other cases, to supplement the findings based on mold or rot fragment count. The purpose of the work summarized in this report was to study the composition of juice from rotten apples with the view of developing such a chemical method.

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* For report of Subcommittee C and action of the Association, see *This Journal*, **31**, 51 (1948).

† Present address: Chemical Section, Insecticide Division, Livestock Branch, Production and Marketing Administration, U. S. Dept. of Agriculture, Beltsville, Md.

PREPARATION OF MATERIAL FOR STUDY

Preliminary experiments on good and rotten apple juice to ascertain qualitative chemical differences made it clear that large quantities of juice would be required in order to isolate a sufficient quantity of the components for chemical characterization.

Approximately 20 bushels of rotten apples were macerated, a filter aid was added ("supercel" was found to be suitable), and the juice was pressed out. The apples had been discarded as unfit for use. Those individual apples containing appreciable quantities of sound tissue were either not used or the sound tissue was removed. The juice from these apples will be referred to in this work as 100% rotten juice. Approximately 50 gallons of juice were obtained and immediately concentrated in vacuo to give a dark colored thick sirup with a solids content of 60 per cent. This concentrated juice was poured into 1-quart ice cream containers and stored at 0°F. for further study.

Twelve bushels of good apples (grade 2) were processed in exactly the same manner as above. The concentrated good apple juice soon solidified to a gel, but the rotten juice did not, thus indicating a partial breakdown of the pectin present.

PECTIN DEGRADATION PRODUCTS

One of the first problems undertaken in this study was the search for the presence of some product or products of pectin degradation which would be indicative of rot. The micro-organisms that invade the damaged fruit and cause it to rot have long been known to constitute a rich source of pectic enzymes, and it appeared reasonable that certain degradation products of pectin would be present in rotten apple juice, and would either be absent or present in much smaller quantities in good juice. In the case of apples, no naturally occurring pectinase activity has been reported, and in the present investigation, none could be demonstrated.

Three such products of pectin degradation were found to be present in rotten apple juice, and two of these will be mentioned only briefly, since neither appeared very promising as the basis of a method for detecting rot

Alcohol-Soluble Non-Dialyzable Substance:

This substance was obtained by precipitating a concentrated solution of rotten apple juice from 80% ethyl alcohol to remove the pectin and dialyzing the filtrate in a cellophane tube overnight against cold running tap-water. A small quantity of precipitate separated from solution during dialysis, which gave a strong positive test for pentose. A quantity of good apple juice treated similarly gave no such substance, but the quantity of the substance obtained from the rotten apple juice was too small to be of any consequence.

Alcohol-soluble Furfural-yielding Substances:

McKinnis (1) investigated the presence of free pentoses in an 80–90%

alcohol filtrate of "Delicious" apples and concluded that, if any were present, they were there only in traces. He failed to get an estimable precipitate of furfural phloroglucide but he did not indicate the weight of starting material.

Ehrlich (2) found a substance which he called araban, and which could easily be separated from pectin by virtue of its solubility in 70% alcohol. It seemed likely that as a result of enzyme action increasing quantities of free pentoses would be released in the juice, and to answer this question the following experiments were carried out:

Two hundred g of good apples were macerated and extracted with water and the extract concentrated to a weight of 65 g. To this was added with stirring 346 ml of 95% ethyl alcohol, and the precipitated pectin was removed by filtration with added filter-aid. After removal of the alcohol in the filtrate by distillation in vacuo, the solution was diluted to 250 ml in a volumetric flask. A furfural determination on a 10 ml aliquot of the solution according to the method of Duncan (3) showed the presence of 81.3 mg of pentose (calculated as arabinose) in 100 g of apples.

The same quantity of rotten apple tissue treated similarly was found to contain 219 mg of pentose per 100 g of tissue.

One of the aims in this work was to find, if possible, some substance in rotten apple juice that was absent in good juice, and for this reason no further study of the pentose content was made.

D-Galacturonic Acid:

Ehrlich (4) described a specific test for galacturonic acid in which a brick-red precipitate was obtained on heating a basic lead acetate solution of this substance. When the test was applied directly to 1 ml of 100% rotten apple juice, a faint positive test was given. A more distinct and characteristic test was obtained on a solution of the acids obtained from the juice by precipitation with lead acetate from 80% alcohol solution, after removal of the pectin. This finding looked most promising, particularly since very little if any free galacturonic acid could be detected in good apple juice. Galacturonic acid was subsequently isolated from rotten apple juice as the *p*-bromphenylhydrazone *p*-bromphenylhydrazine derivative.

A method was devised for the quantitative determination of galacturonic acid in apple juice after removal of the sugars and other interfering substances. The galacturonic acid was determined in the final solution by a modification of the method of Deichmann and Dierker, and this modified method is given in detail below.

REMOVAL OF SUGAR, PECTIN, AND OTHER INTERFERING SUBSTANCES

Concentrate 100 ml of apple juice at reduced pressure, in a 500 ml Claisen distilling flask with a water aspirator, to ca 25 g, and transfer to a weighed 400 ml beaker. (If the soln is concd much below 25 g, there is danger of the concd juice gelling, a result which makes transfer to the beaker difficult.) The concd juice, plus the water used in the transfer, must not be permitted to exceed 30 g in weight. Add 2 g of filter-aid (Celite was found to be suitable) to the 30 g of concentrate, and add

160 ml of 95% ethyl alcohol, with stirring to precipitate the pectin, and filter the mixture with suction. Wash the residue, after pressing with a glass stopper, with ca 10 ml of 80% ethyl alcohol, and discard it. Then transfer the filtrate and washings to a 400 ml beaker and precipitate the acids by adding 2 g of neutral lead acetate ($Pb(CH_3COO)_2 \cdot 3H_2O$), dissolved in ca 10 ml of warm 80% alcohol. Add 2 g of filter aid to the suspension of lead salts, and filter the mixture on a 7 cm Büchner funnel. Press the mixture of Celite and lead salts on the funnel with a flat-top glass stopper, and transfer to a 50 ml beaker. Wash the mixture twice, by stirring with a minimum of 80% alcohol, filtering with suction each time, then transfer to a 250 ml Erlenmeyer flask. Add 100 ml of distilled water, disintegrate the mixture with a stirring rod, and decompose the salts with a current of H_2S gas. Remove the filter aid and PbS by filtration with suction, and concentrate the filtrate and washings to remove the excess H_2S, and then dilute to 100 ml with distilled water in a volumetric flask.

DETERMINATION OF GALACTURONIC ACID

Weigh 50 mg of naphthoresorcinol on an analytical balance, transfer to a 25 ml glass-stoppered cylinder, and add 1 ml of the soln of acids from apple juice, 2 ml of 50% alcohol, and 2 ml of concd HCl. Shake the mixture to dissolve the reagent, and place the stoppered cylinder in a water bath at 50°C. for 90 min. Cool the mixture to room temp., add ethyl ether to the 25 ml mark, and extract the pigment by shaking vigorously for about 10 min. After standing for ca 10 min., decant the ether extract into a colorimeter tube containing a small quantity of Na_2SO_4. Stopper the tube with a cork, whirl a few times to clear up the solution; and determine the optical density at 580 mμ (a Lumetron colorimeter, Model 400 A, was used).

The galacturonic acid content of three samples of apple juice, determined according to the above procedure, is shown in Table 1.

TABLE 1.—*Galacturonic acid content of apple juice*

SAMPLE	GALACTURONIC ACID
	micrograms/ml juice
Grade 1 juice ("Red Delicious")	35
Grade 2 juice (variety composite)	81
100% rotten juice (variety composite)	1580

It was of interest to determine the influence of variety on the content of galacturonic acid in apple juice and the data are given in Table 2.

TABLE 2.—*Relationship between apple variety and galacturonic acid present*

VARIETY	GALACTURONIC ACID
	micrograms/ml juice
Red Delicious	24
York Imperial	25
Black Twig	54
Lowry-1	32
Lowry-2	38
Western Winesap	13
Grimes Golden	40
Ben Davis	20
Jonathan	19

ACCURACY OF THE METHOD

The accuracy of the above-described method will be influenced by the completeness of precipitation of the galacturonic acid with lead acetate, and by the presence of substances other than galacturonic acid in the lead acetate precipitate which may react like galacturonic acid in the final color reaction. To determine the completeness of precipitation of the galacturonic acid by lead acetate, the following experiment was carried out:

To 100 ml of 80% ethyl alcohol, containing the quantities of sugar, malic acid, and galacturonic acid shown in Table 3, was added 2 g of filter aid and 2 g of $Pb(CH_3COO)_2 \cdot 3H_2O$ dissolved in 5 ml of hot 80% alcohol. The suspension of lead salts was filtered with suction using a flat-top glass stopper for pressing the filter cake as previously described. The lead salts were washed twice by suspending in a minimum of 80% alcohol and filtering, after which they were decomposed with H_2S, filtered, concentrated to remove the excess H_2S, and diluted to 100 ml with H_2O. One ml of this solution was analyzed for galacturonic acid as previously described.

TABLE 3.—*Recovery of galacturonic acid in lead acetate precipitate*

VOL. SOLN.	SUCROSE ADDED	MALIC ACID ADDED	GALACTURONIC ACID	
			ADDED	FOUND
	g	*g*	*micrograms/ml*	
100	0	0.5	10	12
100	0	0.5	25	26
100	0	0.5	50	45
100	0	0.5	100	92
100	5	0.5	50	48
100	10	0.5	50	48

As seen in Table 3, there is practically quantitative recovery of the galacturonic acid in the precipitate. Occlusion of the lead galacturonate with the relatively large quantity of lead malate is probably an important factor in the quantitative precipitation of galacturonic acid in such small quantities.

The naphthoresorcinol reaction has frequently been criticized for lack of specificity, but the modification of Deickmann and Dierker (5) represents a great advance in improving the specificity of this reaction. A number of other acids which could conceivably be present in fruit juices were tested and the results are shown in Table 4.

GALACTURONIC ACID AS AN INDEX OF ROT IN APPLES

The enzyme galacturonase, which is a member of the pectinase group, and which liberates galacturonic acid from polygalacturonides, was found by Kertesz (6) to be present in tomatoes but absent in apples. The in-

crease in galacturonic acid in apples on rotting is evidently due to the action of galacturonase of microbial origin.

Galacturonic acid liberated from pectin by bacterial enzymes would appear to be a satisfactory compound upon which to base a method for detecting rot in certain apple products. It is reasonably heat-stable, non-volatile, and can be determined, after removal of interfering substances, in microgram quantities. Its presence in normal fruit will place some limitations on its use as an index of rot, but does not render such a method useless.

In some cases commercial apple juice is clarified, prior to pasteurization and filtration, by certain enzyme preparations which are known to liberate

TABLE 4.—*Color given by various acids in the naphthoresorcinol reaction*

ACID	CONCENTRATION	OPTICAL DENSITY
	micrograms/ml	*580 mu*
Galacturonic	50	0.20
Pyruvic	1,000	.04
l-Ascorbic	250	.01
Oxidized ascorbic[1]	250	.05
Levulinic[2]	250	.05
2-Ketogluconic	250	.01
5-Ketogluconic	250	.01
Gluconic	250	.01
Citric	250	.01
Malic	10,000	.01
Tartaric	250	.01
Reductinic	250	.01

[1] Oxidized by aeration.
[2] Impure product.

galacturonic acid from the pectin present. Obviously a method based upon galacturonic acid would be of no use on such a product, but could be applied to such products as apple butter, jelly, and juice not clarified by enzymes.

It is felt that the present method is a step forward in the solution of the difficult problem of rot in fruit products, and particularly as applied to apples. Some work remains to be done to thoroughly test the usefulness of the method.

RECOMMENDATIONS*

It is recommended:

(1) That the proposed method for galacturonic acid in apple products be studied further in order to more definitely define its potential usefulness for detecting rot in apple products;

* For report of Subcommittee C and action of the Association, see *This Journal*, 31, 51 (1948).

(2) That substances other than galacturonic acid be investigated as indices of the use of rotten apples in the manufacture of apple products.

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(6) Kertesz, Z. I., *Food Research*, **3**, 481 (1938).

REPORT ON DECOMPOSITION IN SHELLFISH

INDOLE IN SHRIMP, OYSTERS, AND CRABMEAT

By R. E. Duggan (Food and Drug Administration, Federal Security Agency, New Orleans, La.), *Associate Referee*

W. I. Patterson[1] has recently reviewed the general subject of the chemical detection of decomposition in foods; therefore there is no need for a review of those fundamental principles in their application to the specific problem of decomposition in shellfish.

Shrimp, oysters, and crabs form the major commercial catch of shellfish. The commercial catch of clams, lobster, and crayfish is much smaller. The search for methods, other than organoleptic, for the detection of decomposition in these products has been in progress for a number of years.

King, Flynn, and Gowanloch[2] proposed the use of the indole content of canned oysters to detect the use of decomposed raw stock. They found that fresh oysters contain little or no indole, and that the indole content of oysters allowed to spoil before canning is much greater. A small amount of some substance reacting as indole to p-dimethylamino-benzaldehyde was found in fresh oysters and evidence was presented indicating that this substance was not indole. Beacham[3] substantiated the work of King, Flynn, and Gowanloch on oysters in an investigation of the decomposition of oysters and clams. Beacham concluded that indole could be used as an index of decomposition in oysters and clams. He also reported that small amounts of volatile acids were found in fresh clams and that substantially larger amounts of volatile acids were present in decomposed clams. In addition to confirming the conclusion of King, Flynn, and Gowanloch concerning the presence of indole-reacting substance (not indole) in fresh oysters, Beacham reported that larger quantities of a similar substance were found in fresh clams.

Duggan and Strasburger[4] investigated the relationship of indole and

[1] *This Journal*, **28**, 233 (1945).
[2] *Ibid.*, **28**, 385 (1945).
[3] *Ibid.*, **29**, 89 (1946).
[4] *Ibid.*, **29**, 177 (1946).

organoleptic decomposition in shrimp. No indole was found in fresh shrimp. Large amounts of indole were found in decomposed shrimp and correlated with the organoleptic degree of decomposition. They reported that different types of decomposition produced varying amounts of indole. The decomposition characterized as ammoniacal produced smaller amounts of indole than was found in the putrefactive type of decomposition. It was also found that some lots of decomposed shrimp contained insignificant quantities of indole.

An investigation of the significance of indole in crabmeat is being carried out at the present time by the U. S. Food and Drug Administration. The results of this investigation are not available at this time, and the value of indole as an index of decomposition in crabmeat has yet to be evaluated. However, since collaborative determinations of indole in oysters and shrimp were being undertaken, it was decided to extend the study to include crabmeat. Very little additional expense was incurred in the collection of this information on crabmeat. The same determinations, made later on as a separate project, would have involved a much larger outlay of time and money.

The basic method for the determination of indole in the above reports was the distillation procedure of Clarke, *et al.*[5] They reported that the method had been successfully used by five chemists in two different laboratories. No collaborative work was reported in connection with the investigations of indole in shellfish. Since indole has been shown to be significant in the detection of decomposition in shellfish[2,3,4] it was felt that the collaborative study of the method was indicated. The methods studied were those used by King, Flynn, and Gowanloch, Beacham, and Duggan and Strasburger, in their respective investigations. The procedure used in the collaborative work incorporated the worthy contributions of each of the above investigators to the basic procedure of Clarke, *et al.*

Several unsuccessful attempts were made by the Associate Referee to eliminate the substance in fresh oysters which reacts like indole. Although the error involved is small, it is advisable to eliminate such interferences where possible, and additional attempts will be made to eliminate this substance without the loss of significant quantities of indole.

COLLABORATIVE STUDY

Samples of shrimp, oysters, and crabmeat were selected in order to approximate the range of indole contents which might be encountered in commercial samples. The collaborative work on shrimp was performed approximately a year in advance of the determinations on oysters and crabmeat. Since satisfactory results were obtained by the collaborators on the recovery of indole added to shrimp, it was not considered necessary to repeat recovery experiments on oysters and crabmeat.

[5] *Ibid.*, 20, 475 (1937).

The collaborative sample of each product consisted of a portion of a finely ground, well mixed sample. The different portions were frozen and shipped under dry ice refrigeration to the participating laboratories. Drained meats of canned oysters and canned crabmeat were used as samples of these products. The samples of shrimp were prepared from raw stock. Details of the method are given, under "Changes in Methods of Analysis," *This Journal*, **31**, 96 (1948). Results are shown in the table on the next page.

DISCUSSION OF RESULTS

The tabulation presents the results obtained by nine chemists in seven laboratories in different cities. An inspection of the results shows that the greatest variations were encountered in the shrimp samples. Undoubtedly some of the differences were due to sampling error, and others were probably due to the inexperience of the chemists in the use of the method. The differences were not considered of sufficient magnitude to warrant repeating the experiment. In connection with the determination of indole in shrimp, two collaborators[6] reported that traces of chlorine in the distilled water were responsible for the partial or complete destruction of indole in the determination. They suspected that traces of this element in ordinary distilled water was very common. The Associate Referee also found that indole was destroyed by traces of bromine.

The results obtained by the collaborators on the samples of oysters and crabmeat are excellent.

It is apparent that duplicable results can be obtained by different chemists having a minimum of experience with the method.

RECOMMENDATIONS*

It is recommended—

(1) That the method presented for the determination of indole in shellfish be adopted as tentative.

(2) That the investigation of methods to eliminate the indole reacting substance found in fresh oysters be continued.

(3) That substances other than indole be investigated as indices of decomposition in those types of decomposition which form little or no indole.

ACKNOWLEDGMENTS

The Associate Referee wishes to acknowledge his indebtedness to D. Banes, Chicago Station; H. W. Conroy and H. P. Bennett, Kansas City Station; Dona Clarke, Minneapolis Station; M. L. Dow, St. Louis Station; L. La Grange, New Orleans Station; H. Reynolds, Food Division; and H. C. Van Dame, Cincinnati Station, for their splendid cooperation and interest in this collaborative study.

[6] Messrs. Bennett and Conroy, Kansas City Station, U. S. Food and Drug Administration.

* For report of Subcommittee C and action of the Association, see *This Journal*, **31**, 51 (1948).

LABORATORY	CHEMIST	RECOVERY INDOLE ADDED TO SHRIMP	INDOLE—MICROGRAMS/100 g										
			SHRIMP, SAMPLE NO.					OYSTERS, SAMPLE NO.			CRABMEAT, SAMPLE NO.		
			1	2	3	4	5	1	2	3	1	2	3
		per cent											
A	A	88.7	0	17.8	787	1.1	91	5.0	16.0	23.0	13.0	68	180
		93.3	0	17.3	774	1.0	92	5.0	16.0	22.0	15.0	66	160
		95.3											
		90.7											
B	B	97.5	1.2	20.0	1000	0	106	2.2	15.0	24.8	11.3	64	189
		100	1.5	20.2	1000	0	106	2.2	15.0	27.2	10.6	64	190
		101											
		101											
C	C	—	0	21.0	920	3.0	101	2.5	15.0	21.5	11.2	58	181
	D	—	0	19.4	916	3.0	100	2.4	15.0	22.7	11.2	60	184
D	E	97	0	17.4	1249	4.8	98	0.4	16.0	22.8	8.8	57	208
		105	0	17.6	1250	4.0	99		14.2	22.6	9.6	57	212
		97											
E	F	101	1.2	10.6	830	6.0	110						
		86	1.6	9.0	870	4.2	111						
		93											
	G	—	0	18.6	960	3.2	99	2.2	13.6	20.0	11.6	65	208
			0	17.6	904	2.0	96	2.2	13.6	22.8	11.0	68	207
F	H	97	0	5.6	820	2.4	101						
		98	0	6.2	767	1.2	101						
G	I	—						2.4	16.2	24.8	11.4	67	198
								2.5	16.2	25.4		67	202
Average			0.4	15.6	932	2.6	101	2.6	15.2	23.3	11.3	63	193

REPORT ON GELATIN, DESSERT PREPARATIONS, AND MIXES

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

The Associate Referee has submitted a report recommending that changes be made in paragraph **9.6** (p. 104, *Methods of Analysis*, 6th Ed.), for determining the jelly strength of plain gelatine.

Your General Referee recommends* that the changes in the method as outlined in the report of the Associate Referee be made and that the revised method be adopted by the Association as tentative. It is further recommended that this new revised method under paragraph **9.6,** for the determination of jelly strength of plain gelatin, be studied collaboratively with a view to making the method official.

Another method which needs further study is the method for jelly strength of gelatin dessert powders under paragraph **9.12.** This method deals solely with a determination of the jelly strength of the finished dessert powder and is not a method for the determination of the amount of gelatin it contains. It is recommended that the principles of the method for determining the jelly strength of plain gelatin be given consideration in a study of the method for the jelly strength of gelatin dessert powders as set forth under paragraph **9.12,** and that the method be studied collaboratively with a view to making it official.

REPORT ON JELLY STRENGTH

By Paul A. Kind (Kind and Knox Gelatin Company, Camden, N. J.), *Associate Referee*

Under the heading **9.6,** Jelly Strength (gelatine—tentative), there is given a method for the determination of jelly strength which is essentially the same as the method adopted and used for over twenty years by the Edible Gelatin Research Society of America, Inc. However, this method has one extremely significant variation, namely that the concentration of gelatine under test is set at 7.5 gm of sample in 100 ml of water, instead of the 7.5 gm of sample in 105 gm of water as employed by the Gelatin Association. This is a very serious variance. Its magnitude is illustrated by the following determinations with gelatines of various types.

7.5 gm of gelatine *105 gm of water*	*7.5 gm of gelatine* *100 gm of water*
135	148
172	182
175	182

* For report of Subcommittee C and action of the Association, see *This Journal*, 31, 51 (1948).

7.5 gm of gelatine 105 gm of water	*7.5 gm of gelatine 100 gm of water*
176	188
200	213
225	241
250	268
288	307

In 1924 the Edible Gelatin Research Society of America, Inc., an association of approximately 75 per cent of the American manufacturers of food gelatines, adopted the Bloom Gelometer and the Viscosity Pipette, and general procedures for the determinaton of jelly strength and viscosity as had been previously adopted by the National Association of Glue Manufacturers at their meeting at Atlantic City, October 10, 1923. The apparatus and methods of procedure as adopted were reported in *J. Ind. Eng. Chem.*, **16**, 310–315 (1924). Later publications on these methods have appeared in the Analytical Edition *Ind. Eng. Chem.*, **2**, pages 348–351 (1930). The Edible Gelatin Research Society of America, Inc. followed the identical procedure as outlined for glue, but instead of using 15 gm of glue for each 105 gm of water the Gelatin Association employs 7.5 gm of gelatin for each 105 gm of water.

This procedure and this concentration is now used by all American gelatine manufacturers and values recorded by these methods are accepted as general practice by nearly all American consumers. The methods may be considered as international because the British Association of Gelatin Manufacturers evaluate their products with the Bloom Gelometer at 7.5 gm for each 105 gm of water. Hence, in order that the A.O.A.C. methods may be in accord with national and international procedures for the determination of jelly strength, it is recommended* that sec. **9.6**, page 104, 6th Ed. of the A.O.A.C. *Methods of Analysis*, be deleted and substitute adopted to read as follows:

"To 7.5 g of sample add from pipet, with stirring, 105 ml of water at 10° to 15°C. Let stand for one hour and then bring to 62° in 15 min. by placing in a water bath regulated at 65°; the sample may be swirled several times to aid solution. Finally mix by inversion, let stand 15 min., and place in a water bath controlled at 10° ±0.1°. Chill for 17 hours. Determine jelly strength on Bloom Gelometer (2)† adjusted for a 4 mm depression and to deliver 200 g shot/5 seconds (±5 g), using the ½" plummet."

REPORT ON MEAT AND MEAT PRODUCTS

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

For the reasons given by the Associate Referee on soybean flour and

* For report of Subcommittee C and action of the Association, see *This Journal*, 31, 51, 74 (1948).
† Reference number refers to the selected references, *Methods of Analysis*, 6th Ed., p. 104.

dried skim milk in meat products (ff.), it is apparent that it will prove very difficult to work out an accurate method for the determination of soybean flour. It is thought, therefore, that another Associate Referee should be appointed to take up the work on dried skim milk in meat products. The General Referee is already in possession of a satisfactory method for the determination of this substance, and there remains only the necessity of some minor experimental work before the method is ready for collaborative testing. It is hoped that an Associate Referee can be secured to conduct this work.

During the war, a method for the direct determination of creatin was developed which is reported to yield more accurate results than the official method, by which the creatin is determined by difference. It is believed this method should be studied during the coming year. The following recommendations* are made for work on Meat and Meat Products during 1948:

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

(2) That an Associate Referee be appointed to continue work on a method for the determination of dried skim milk in sausage and similar products.

(3) That study be undertaken on a method for the direct determination of creatin.

No report on dried skim milk was given; see Referee report.

REPORT ON SOYBEAN FLOUR IN SAUSAGE AND SIMILAR PRODUCTS

By ORAN L. BENNETT (Meat Inspection Division, Bureau of Animal Industry, U. S. Department of Agriculture, Washington 25, D. C.), *Associate Referee*

The problem of developing a practicable method for the determination of soybean flour in meat food products is a complex one. This is in part due to the various added substances such as cereal, dried skim milk, spice, cures, etc., one or all of which may be added in the preparation of these products. Another difficulty in the way of development of an accurate method is the variation in the composition of commercial soybean flours, due to different methods of processing. This variation will obviously cause a difference in the results obtained by any method. This fact was

* For report of Subcommittee C and action of the Association, see *This Journal*, 31, 52 (1948).

demonstrated by Munsey[1] in his report on soybean flour in cereal products and confirmed by the author in his own work.

Several methods for the approximate determination of soybean flour in certain meat products have been published in the past several years but none of them satisfy the requirements for a practical analytical procedure for control work. This need has been accentuated by the recent decision of M. I. D. permitting sausage manufacturers to add soybean flour to their product.

The author, with the assistance of other interested chemists, has attempted to devise an approximate but rapid method which would be suitable for control work. In devising such a method, endeavors have been made to use the hemicellulose constituent of soybean flour as a means of its measurement, since knowledge gained through previous personal experience and consideration of published methods indicates this to be the best avenue of approach to the problem. Several methods, all of which utilized the hemicellulose component as means of measurement, were developed, but the two appearing to have the most merit were chosen for further study. The methods are not applicable to those products containing other materials of high cellulose content such as dried vegetables. Such ingredients are not permitted in Federally inspected sausage.

Each of the two methods employs a potassium hydroxide solution in alcohol to dissolve the meat from the soybean and cereal flour. Both methods are empirical and each employs a separate appropriate factor for conversion of percentage of hemicellulose found to percentage of soybean flour present.

Method I is an adaptation of Hendry's method[2] and was developed principally by chemists in the St. Louis Meat Inspection laboratory. This method is based on the fact that a certain proportion of the hemicellulose of soybean flour may be separated and converted by acid hydrolysis to nonfermentable reducing sugars, the amount of which, multiplied by a predetermined factor, indicates the amount of soybean flour present. Since lactose may be present through the inclusion of dried skim milk in the product, its removal is accomplished by extraction with hot water before digestion with the potassium hydroxide-alcohol solution. The hemicellulose contained in any cereal flour which may be present in sausage is also hydrolyzed to non-fermentable reducing sugars, but a correction is made for this, depending upon the amount of cereal present. While this is not a short method, it has been demonstrated that it yields fairly consistent results in the hands of different analysts.

Method II, developed by the author, also utilizes the hemicellulose constituent as a basis for measurement of soybean flour content. It takes advantage of the fact that the hemicellulose of soybean flour and the

[1] *This Journal*, 30, 187 (1947).
[2] *Ind. Eng. Chem., Anal. Ed.*, 11, 611–613 (1939).

starch of the cereal flour may be separated by means of their different solubilities in different concentrations of hydrochloric acid. After separation the hemicellulose may be re-precipitated by the addition of 95% alcohol, centrifuged at a given speed for a period of time, and measured volumetrically. Volume in ml × a predetermined factor gives approximate % of soybean flour. The same factor is not applicable to product containing over 4 per cent of soybean flour. In such cases a smaller sample must be taken and a corresponding change made in the calculation. This method is comparatively short but sometimes gives results which are somewhat inconsistent.

METHOD I

Weigh 10 gm of the finely chopped meat into a 100 ml centrifuge tube and add gradually, with constant stirring, 60 ml of hot water. Suspend the tube in a water bath, kept just below the boiling point, for 15–20 min. with occasional stirring. Wash off the rod with 2–3 ml of hot water. Centrifuge the tube 5 min., return to bath and add a slurry of 1 gm filtercel in 5 ml hot water. Stir thoroly without disturbing the meat and again centrifuge 10–15 min. Pour off and discard the supernatant liquid, which will probably be cloudy, especially in the presence of dried skim milk. Add 50 ml of 8% KOH in 95% alcohol to the tube, return to the water bath stirring until the alcohol boils. Reduce the temperature of the bath to ca 80 degrees C. and continue heating with occasional stirring for 10 min. after the particles of meat have disappeared (about 20 min. in the water bath is necessary). Filter the alkaline soln thru a Gooch crucible having a thin pad of asbestos covered with filtercel, using gentle suction and keeping the mixture well stirred during filtration. Wash the tube and crucible with four 25 ml portions of 95% alcohol. Remove the excess alcohol with strong suction. Invert the Gooch over a large stem funnel in a 200 ml Erlenmeyer flask and tap gently until the cake falls out. (The cake usually comes out in one piece although sometimes it will be necessary to remove the asbestos with a glass rod.) After moistening with a few ml of 2.5% HCl, break up the cake and wash it into the flask with a stream of 2.5% HCl from a wash bottle. Wash the centrifuge tube, Gooch, and rod, with the HCl using a rubber-tipped rod, and add the wash soln to the Erlenmeyer flask. The total volume of the acid soln used should approximate 60 ml. Thru a rubber stopper attach an air-reflex condenser to the flask. Suspend in a boiling satd salt soln for three hours, boiling temperature about 105°C. Remove from the salt bath, cool, and add 1 ml of 20% phosphotungstic acid. Neutralize to *p*H 6.5–7.0, first adding 6 ml of 25% NaOH, then complete neutralization with 10% NaOH from a dropper. Make to volume in a 100 ml flask and filter about 75 ml. Centrifuge, in a 100 ml tube, 10 ml of a 25% yeast suspension (Fleischmann or similar preparation). Discard the liquid and dry the sides of the tube. Add about 75 ml of the neutralized filtrate, shake, and allow to stand 1 hour with occasional mixing. Centrifuge until clear. Remove a 50 ml aliquot and determine reducing sugars according to the Munson-Walker method[3], using sodium thiosulfate for the titration. Subtract a blank of 2.3 mg of Cu_2O before computing dextrose. If no cereal is present in the sausage the per cent non-fermentable sugars as dextrose X 14.4 = per cent soybean flour in the sample. If cereal is present, 0.33% for each per cent of cereal must be subtracted from this total.

METHOD II

Weigh directly into a 100-ml oil tube (A.S.T.M. conical form, stem graduated from 0 to 3 in 0.1 ml), 10 g of finely divided sample. Add 50 ml of 8% soln of KOH

[3] *Official and Tentative Methods of Analysis*, 6th Ed., (1945), p. 571, par. 34.38.

TABLE 1.—*Soybean flour in sausage*

ANALYST	METHOD NO. 1				METHOD NO. 2		
	SAMPLE NO.	SOYA FLOUR ADDED	CEREAL ADDED	SOYA FLOUR FOUND	SOYA FLOUR ADDED	CEREAL ADDED	SOYA FLOUR FOUND
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	1	1.0	0	.8	3.0	1.0	3.0
	2	2.0	0	2.1	1.0	1.0	1.2
	3	3.0	0	3.1	2.0	2.0	2.1
	4	4.0	0	4.3	3.0	3.0	3.2
	5	5.0	0	5.3	3.0	3.0	4.0
	6	3.0	1	3.3	4.0	4.0	5.1
	7	1.0	1	1.0	4.0	4.0	4.6
	8	2.0	2	1.8	4.0	4.0	5.1
	9	3.0	3	2.6	4.0	4.0	5.2
	10	4.0	4	3.8	5.0	5.0	4.8
2	1	3.7	2.7	3.6	3.7	2.7	3.8
	2	2.0	2.0		2.0	2.0	2.2
	3	2.9	2.4	2.7	2.9	2.4	
	4	3.6	3.2	3.1	3.6	3.2	4.0
	5	5.0	3.2	4.6	5.0	3.2	6.3
3	1	2.0	2.0	2.1	1.0	1.0	1.0
	2	2.0	2.0	2.3	3.0	2.0	3.3
	3	2.0	2.0	2.4	2.0	3.0	2.1
	4	2.0	2.0	2.0	1.0	1.0	.7
	5	2.0	0	2.0	2.0	2.0	1.6
	6	4.0	0	4.0	2.0	3.0	2.3
	7	3.0	0	2.5	1.0	1.0	.9
	8	4.0	0	4.1	0	2.0	0
	9	2.0	0	2.1	2.0	3.0	1.6
	10	2.0	0	1.9	3.0	2.0	2.7
4	1	2.5	1.0	2.8	1.0	1.0	.9
	2	2.5	1.0	2.6	1.0	2.0	.8
	3	4.0	2.0	3.8	2.0	3.5	1.8
	4	4.0	2.0	3.8	2.0	1.5	1.9
	5	4.0	1.0	4.2	3.0	2.5	3.0
	6	2.0	.0	2.1	3.0	0	3.1
	7	3.0	.0	2.9	3.0	0	3.3
	8	4.0	.0	4.0	3.5	2.0	3.4
	9	5.0	.0	5.0	3.5	0	3.6
	10	6.0	.0	6.1	4.0	1.0	4.3
5	1	3.0	0.0	2.6	0	0	0
	2	3.0	0.0	2.4	3.0	0	3.3
	3	3.0	3.0	2.1	3.0	0	3.3
	4	3.0	3.0	1.9	0	3.0	.6*
					3.0	3.0	3.0
					3.0	3.0	2.7
6	1	3.0	0	Dry Yeast 3.2	3.0	0	4.3
	2	3.0	0	Dry Yeast 2.2	3.0	0	3.9
	3	3.0	0	Fresh Yeast 2.7	3.0	0	3.9
	4	3.0	0	Fresh Yeast 2.9	3.0	0	3.6

* This precipitate apparently was due to a small amount of hemicellulose from cereal present in sample.

in alcohol and digest on steam bath for 20 min., with occasional stirring to facilitate digestion. Remove from bath and make up to 100-ml mark with 95% alcohol. Shake well and centrifuge for 4 min. Decant and discard KOH-alcohol soln, and wash residue with 25 ml of 95% alcohol, using a glass rod to stir up the sediment. Centrifuge, decant, and discard alcoholic soln.

To the residue in tube add 50 ml of 1 + 3 HCl, and stir residue in stem with rod to reduce any lumpy material. Shake for one min., after which centrifuge for 4 min. at a speed of ca 2000 r.p.m. Add 25 ml of this clear soln to 75 ml of 95% alcohol in another oil tube (conical form, stem graduated from 0 to 1 in .05 ml), shake well, and let stand for 1 hour. Centrifuge for exactly 2 min. at a speed of 1500 r.p.m., accelerating to 1500 r.p.m. in 1 min. and using a head 6¾ inches in diameter. (International Equipment Co., Catalog No. 235.) Read volume of residue in tube and multiply by the factor 6, to obtain the percentage of soybean flour present.

Results of analysis of samples of sausage containing known amounts of soybean flour by several analysts, using the two methods, are given in Table 1.

Samples of sausage containing various amounts of soybean flour were prepared by the Associate Referee and submitted to three collaborators for analysis. Results of these determinations are given in Table 2.

TABLE 2.—*Collaborative results*

	METHOD NO. 1—COLLABORATOR			METHOD NO. 2—COLLABORATOR		
	NO. 1*	NO. 2	NO. 3	NO. 1	NO. 2	NO. 3
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
Sausage containing 1% soybean flour		.6	1.3	.6	.7	1.1
Sausage containing 3% soybean flour		3.1	3.1	2.8	2.8	2.3
Sausage containing 1.5% soybean flour plus 1.5% cereal		1.1	1.9	.9	.9	1.6
Sausage containing 2% soybean flour plus 1.5% dried skim milk		1.9	2.1	1.7	1.6	2.0

* No analysis made.

While the two methods under study appear to yield only approximate results in their present form, it is believed that they are the best methods available for control work at this time. Further, your Associate Referee believes that these methods can be definitely improved by the adoption of some changes suggested by the collaborators and by further study.

It is recommended,* therefore, that work be continued along the same lines during the coming year.

ACKNOWLEDGMENT

Acknowledgment is made for valuable assistance in this work to collaborators H. R. Kraybill, American Meat Institute; M. L. Laing, Armour and Company; C. C. Zigler, Swift and Company; and to the several BAI meat inspection chemists who participated in this study.

* For report of Subcommittee C and action of the Association, see *This Journal*, 31, 52 (1948).

REPORT ON MICROBIOLOGICAL METHODS

By GLENN G. SLOCUM (Food and Drug Administration, Federal Security Agency, Washington, D. C.), *Referee*

This is the first report of your Referee on Microbiological Methods since his appointment in May 1946. The past year and a half has been largely a period of orientation, and there is little to report in the way of progress. Much of the work on microbiological methods was necessarily curtailed or interrupted during the War and subsequently by the change in General Referee. During the next year work on these methods will be activated again and it is hoped that considerable progress can be reported at the next meeting.

The Sixth Edition of *"Methods of Analysis"* contains the following methods which have been adopted as official, first action:

1. Examination of Eggs and Egg Products.
2. Detecting and Estimating Numbers of Thermophilic Bacteria in Sugar.
3. Examination of Canned Vegetables.

The method for eggs and egg products is generally satisfactory but will require some slight modification and additional collaborative work. The method for sugar also appears to be satisfactory and probably can be made official in the near future. The same is probably true of the method for canned vegetables. However, methods for other non-acid canned foods, specifically canned meats and canned fish, have not been presented in form for proposal as tentative methods. It is highly desirable that methods for these products be developed along with the methods for canned vegetables and that they be so coordinated that the control laboratory will not be confronted with widely divergent procedures requiring a considerable variety of culture mediums and incubation times and temperatures. The latter is, of course, true for all microbiological methods.

The Sixth Edition of *Methods of Analysis* contains tentative methods for the examination of canned fruits and other acid canned food, and for nuts and nut products. The former method will need additional checking and possibly some modification and the appointment of an Associate Referee to fill immediately the vacancy created by the death of Mr. Linden is recommended. The methods for nuts and nut products have been under study by the Associate Referee and these studies will be continued during the year.

The report of the Associate Referee on frozen fruits and vegetables is being presented at this meeting. The General Referee concurs in this report.

In his reports as General Referee on Microbiological Methods, Dr. Hunter more than once referred to relationship of the American Public Health Association and this Association in the development of such

methods. It is apparent that these organizations are following parallel courses; and it is imperative that a close coordination of effort must be obtained not only to avoid any conflict in procedures but also to prevent unnecessary duplication of effort by a proper division of labor. Dr. Hunter carefully laid the ground work for such coordination and your General Referee will continue to work to that end.

RECOMMENDATIONS*

It is recommended—

(1) That the methods in the report of the Associate Referee on frozen fruits and vegetables be studied further.

(2) That work be continued on eggs and egg products, sugar, canned vegetables, canned meat, canned fish, acid canned foods, nuts and nut products, and frozen fruits and vegetables.

No reports were given on microbiological methods in canned fishery products, canned meats, canned tomatoes and other acid vegetables and fruit products, or eggs and egg products.

REPORT ON MICROBIOLOGICAL METHODS FOR FROZEN FRUITS AND VEGETABLES

By H. E. GORESLINE (Production and Marketing Administration, Poultry Branch, Washington, D. C.), *Associate Referee*

The following methods for the examination of frozen fruits and vegetables are presented in order to form the basis for the development of complete methods. It is suggested that the few methods forming this report be printed, and that reprints be forwarded to at least 50 laboratories, colleges, experiment stations, and State and Government agencies, for the purpose of obtaining criticism and collaboration. At the end of a suitable trial period these groups will be contacted and the results of the findings reported to the Association in the form of recommended methods.

SUGGESTED METHODS FOR THE MICROBIOLOGICAL EXAMINATION OF FROZEN FRUITS AND VEGETABLES

EXAMINATION OF FROZEN VEGETABLES

From the lot to be examined select a suitable number of packages and transport in dry ice to the laboratory for analysis. Place the samples in a refrigerated (0°F.) storage chest until they are to be analyzed.

PREPARATION OF SAMPLE

A. Peas, lima beans, cut corn, whole or regular cut green beans, etc.

1. *Macroscopic examination.*—Open the package and note condition of the prod-

* For report of Subcommittee C and action of the Association, see *This Journal*, 31, 53 (1948).

uct. If ice crystals are present on the inner walls of the package and the vegetables appear somewhat shriveled, it is indicative of thawing and subsequent refreezing, Record observations and note any abnormalities, such as unnatural color or odor. or pink colonies of torulae which are indicative of improper handling practices.

2. *Sampling for plate counts.*—Break the sample, if not loose-frozen, into small units by tapping the unopened package sharply against a table edge or by sharply striking the packing with a dull instrument, being careful not to break open the package. Open the package and remove sample with sterile spoon, taking the sample from various parts of the broken-out package, *e.g.*, from the center and corners.

Weigh a 50-g sample aseptically into a sterile borosilicate glass mechanical blender jar. Add 450 ml of sterile water to the jar and blend the contents of the jar for 2 mins. If a Variac transformer is available it is advisable to increase the speed of the motor gradually and then run the blender for the required time at the 100 V. setting of the transformer.

Allow the sample to stand for 2–3 min. to permit foam to subside.

Pipet 1 ml of the mixture into a 99 ml sterile water blank. Replace the cap on the diln bottle and shake the bottle briskly 25 times thru a 1-foot arc.

Pipet 1 ml aliquots of mixture into each of 2 petri dishes (1:1000 diln) and also 0.1 ml aliquots into each of 2 petri dishes (1:10,000 diln). (A 1:100 diln may be obtained by pipeting 0.1 ml aliquots of the original mixture into each of two petri dishes. Dilns. of 1:100, 1:1,000, and 1:10,000 will usually suffice for commercially packed frozen vegetables, although further dilns should be made if the history or the appearance of the samples warrants.)

Pour melted tryptone glucose extract agar (*p*H 7.0) cooled to 45°C. into the petri dishes immediately, and thoroly mix the diln water with the agar by gently rotating the plates in a figure 8 motion with slight tilting; harden, invert, and incubate at 32°C. for 4 days.

After the incubation period is complete count the plates, using a suitable colony counter. Record results as "Plate count of microorganisms per gram."

3. *Direct microscopic count method.*—The direct microscopic method has an advantage over the plate count method in that it is quicker and requires less equipment and glassware. Also it detects dead microorganisms, and indicates sanitary history, irrespective of the viable count.

Weigh 50 g of the vegetable into a 250 ml iodine flask.

Add 100 ml water, stopper flask, and shake briskly 50 times thru a 1-foot arc. Using a Breed pipet, transfer 0.01 ml of the washings to a microscope slide and using a needle spread the drop evenly over a one square centimeter area of the slide. Dry and fix with heat or with methyl alcohol. Stain with Gray's double dye stain (1), or with North aniline oil methylene blue stain (2), rinse, dry and examine under the microscope, using oil immersion. Use an ocular micrometer, such as a Wipple disc or Howard disc, with the microscope tube so adjusted that the side of the graduations is equal to 0.1 mm (area of field 0.01 sq mm). Count the cells in 100 fields, and multiply the number by 20,000 to bring to the gram sample basis. Express results as "Direct microscopic estimate, microorganisms per gram."

In the direct microscopic method the following assumptions are made. (A) That all the cells are removed from the surface of the vegetable by the washing. (B) that the suspension of bacterial cells is uniform, and (C) that the drop of liquid is evenly spread over one square centimeter.

B. Frozen Spinach

For proper comminution of spinach in a "blender" it is necessary to allow the package of spinach to defrost partially by standing at room temp. for 1½–2 hours. Open the package and weigh 50 g of the contents into a sterile borosilicate mechani-

cal blender jar. Assemble the sample from various portions of the package taking care to select petiole and blade portions in about the same ratio as in the sample as a whole. Add 450 ml sterile water, blend for two min., and proceed as in analysis of A (peas, lima beans, etc.).

C. Frozen Broccoli and Cauliflower

Allow to defrost partially at room temp. Using a sterile scalpel cut portions from the curd and stem of several representative pieces of the vegetable. Aseptically transfer 50 g of these portions into the sterile borosilicate glass mechanical blender jars, add 450 ml sterile water, and proceed as directed for A (frozen peas, etc.).

D. Frozen Asparagus

Partially defrost at room temp. Cut spears into short lengths using sterilized scalpel. Transfer aseptically 50 g of the segments into a sterile borosilicate glass mechanical blender jar, add 450 ml sterile water and proceed as in analysis of A (frozen peas, etc.). When weighing out sample portions, a number of spears should be selected, and the proportion of butts and tips maintained.

(The "cuts and tips" type of asparagus pack can be weighed directly into the blender cup.)

EXAMINATION OF FROZEN FRUITS

PREPARATION OF SAMPLE

Hold the package of frozen fruit at room temp for 1–2 hours before opening. While the fruit is still partially frozen, cut portions from various parts of the contents of the package using a sterilized scalpel. The proportion of fruit to syrup should approximate that of the whole package. Weigh 50 g of fruit and syrup into a sterile borosilicate glass mechanical blender jar, add 450 ml sterile water and blend for two min. Make a 1:1000 diln by adding 1 ml of the blended mixture to 99 ml sterile water and further dilns in the usual manner. Plate 1 ml portions from the various dilns on tryptone glucose extract agar, and incubate for 3 days at 32°C. Count colonies under a suitable colony counter, and record results as "Plate count of microorganisms per gram."

Make direct microscopic counts for molds and yeasts according to the methods given in Official Methods, A.O.A.C. (3) for microscopic analysis of tomato products. Care must be taken to distinguish between mold hyphae and fruit setae.

REFERENCES

(1) GRAY, P. H. H., *J. Milk Tech.* **6** (2); 76 (1943) "Two-Stain Method for Direct Bacteria Count."

(2) NORTH, W. R., *This Journal*, **28**, 424 (1945), "Aniline Oil—Methylene Blue Stain for the Direct Microscopic Count of Bacteria in Dried Milk and Dried Eggs."

(3) *Methods of Analysis, A.O.A.C.*, 6th Ed., 1945, p. 788.

REPORT ON NUTS AND NUT PRODUCTS

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

The work on methods for nuts has been of an exploratory nature. There is no satisfactory method of preparing very oily nut meats for analysis. All methods in the chapter on nuts are tentative. As there appears to be

greater need of methods for moisture, or volatile matter, and fat these were selected for study.

INSTRUCTIONS TO COLLABORATORS

Preparation of Sample

Preserve sample in glass-top fruit jar or similar air-tight container at 35 to 50°F. or 5 to 10°C. Prepare sample for analysis as follows:

(*A-1*) *Nuts in Shell.*—Remove meats from shells, being careful to remove all particles of shells from the meats, and prepare meats as in A-2.

(*A-2*) *Nut Meats, Shredded Coconut, and Similar Sized Pieces.*—Grind not less than 250 grams twice thru Enterprise No. 5 food chopper, equipped with a revolving knife blade and plate with holes ca ⅛ inch in diameter. Any other make of food chopper that will give similar results may be used. The nut meats may also be comminuted by a mechanical grating device instead of food chopper that will comminute to similar condition and size of pieces. Mix ground sample well and store in air-tight glass container. (Comments on any other equipment that will satisfactorily prepare nut meats will be appreciated.)

(*B*) *Nut Butters and Pastes.*—Transfer sample to container of convenient size and mix carefully with stiff bladed spatula or knife. A semi-solid product may be warmed and then mixed with an electric malted milk mixer or similar equipment. Store sample in air-tight glass container.

Moisture Determinations

Method 1: Determine moisture by A.O.A.C. Method **27.3.** If necessary to secure a thin layer of the material, add a few ml of H_2O or ethanol and mix thoroughly.

Method 2: Same as Method 1 except dry at 70 to 75°C.

Method 3: Determine moisture by A.O.A.C. Method **27.4** *and* **27.5,** except substitute 500 ml Florence flask for 250 ml Erlenmeyer flask, as it is necessary to use 100 gm sample for most nut products.

Fat Determinations

Method 1: Determine fat by A.O.A.C. **27.24** and **27.25,** using Soxhlet extractor. Use the dried residue from moisture determination, Method 1 or Method 2.

Method 2: Determine fat as directed under A.O.A.C. **19.23,** using 250 ml extraction flask instead of 150 ml Erlenmeyer flask. On nut butters or pastes it may be desirable to use equal volume of filter-cel to facilitate filtration. No work is desired on fat constants or proteins at this time. In place of the Knorr extraction tube, a fritted glass Büchner funnel may be used, but in such case it is desirable to use a layer of the purified asbestos next to the fritted glass. A 30 ml Büchner type funnel with fritted disc of coarse or medium porosity, listed in Corning Catalogue as 38060, or an Allihn tube with fritted disc of medium porosity listed in Corning Catalogue as 37730, is suitable.

Method 3: Determine by A.O.A.C. Method **30.6.** Do not use any of this method beyond the sentence in line 6: "Dry dish and contents at 100° for 30 minutes, cool, and weigh."

The results are given in Tables 1 and 2.

COLLABORATIVE RESULTS

T. A. Pickett reported results on Methods 1 and 2 for drying five hours instead of drying to constant weight. He also reported on drying for five

hours at the temperature of boiling water, with results 7 to 10 per cent lower on peanuts and 33 per cent lower on peanut butter; and for drying at 135°C. for two hours, with results 2 to 3 per cent higher for peanuts and 10 per cent higher for peanut butter.

TABLE 1.—*Collaborative results on methods for moisture in nuts*

PRODUCT AND COLLABORATOR	METHOD 1		METHOD 2		METHOD 3	
	per cent		*per cent*		*per cent*	
Almonds:						
Munday	4.78	4.81	4.36	4.35	4.6	4.6
Coconut:						
Munday	6.39	6.29	5.96	5.85	6.5	6.4
Peanuts:						
Pickett, Sample #1	6.86	6.86	6.35	6.47	6.36	—
	6.93	6.88	6.35	6.45		
Sample #2	6.70	6.60	6.01	6.19	—	—
	6.66	6.72	6.01	6.11		
Sample #3	6.10	6.03	5.51	5.62	—	—
	6.06	6.06	5.60	5.53		
Pecans:						
Rokita	2.91	2.93	2.73	2.75	3.0	3.0
Walnuts:						
Banes	4.36	4.35	3.89	3.79	4.42	4.28
Peanut Butter:						
Banes	1.73	1.76	1.27	1.30	1.81	—
Munday	1.46	1.45	1.03	1.07	1.9	1.9
Pickett	1.47	1.60	1.12	1.10	1.67	1.62
	1.43	1.53	1.05	1.06		
Rokita	1.28	1.30	1.04	1.00	1.8	—

The "Rapid Method for Determining Oil Content of Tung Kernels" by Joseph Hamilton and Seymour G. Gilbert[1] suggests the use of a fat solvent in a Waring Blendor as an aid in determining fat or oil in nuts. Ella M. Baer and Sylvia F. Hoisington report results by the above method as well as by a modification of Method 1 wherein they use a Waring Blendor with petroleum ether for disintegrating the sample before extraction in the Soxhlet apparatus. They obtain higher results with the

[1] Analytical Chemistry, Vol. 19, p. 453.

Tung kernel method but report trouble with colloidal material that does not settle. Mrs. Rokita reports that method **30.6** is not suitable to nut butters, as it is hard to transfer the sample to the flask without loss of chloroform by volatilization, which tends to give high results. Method

TABLE 2.—*Collaborative results on methods for fat in nuts*

PRODUCT AND COLLABORATOR	METHOD 1		METHOD 2		METHOD 3	
	per cent		*per cent*		*per cent*	
Almonds:						
Munday	55.24	55.00	54.41	54.18	51.87	52.18
Coconut:						
Munday	45.42	45.76	33.02	33.97	33.72	33.89
Peanuts:						
Pickett, Sample #1	45.94	46.14	45.40	44.92	45.80	46.40
	46.01		45.16		46.90	
Sample #2	45.37	45.47	43.90	43.87	45.62	45.97
	45.38		43.38		46.05	
Sample #3	47.71	47.62	45.48	46.29	48.03	48.92
	47.62	47.70	44.29		48.77	
Pecans:						
Rokita	73.41	73.81	70.38	70.30	73.2	73.3
Walnuts:						
Banes	71.0		57.7		63.0	62.9
Peanut Butter:						
Banes	51.0		48.0		49.8	49.6
Munday	56.77	56.53	49.91	50.11	54.01	63.71
Pickett	47.31	47.52	47.28	47.59		
	48.25	47.92	47.94	47.12		
Rokita	50.47	50.09	48.34	48.49	52.2	53.7

19.28 gives low results, probably from incomplete extraction due to large particles. Mrs. Rokita and others report trouble with Method **27.25** on peanut butter, as starch contaminates the extract and it is necessary to treat the extract with petroleum ether.

Drying at 70°C. in vacuum gives lower results than at 100°C. in vacuum or distillation with toluene. Mr. Munday and Mrs. Rokita report that under some conditions the toluene distillation method gives high results which may be due to high humidity.

In the preparation of the sample, Baer and Hoisington recommend the Waring Blendor. The Griscer grater has been recommended for preparing cheese samples and is probably suitable for nuts. Hamilton and Gilbert recommend a flaking machine. Small-sized food choppers have also been used for preparing nut samples for analysis.

Grateful appreciation is expressed to the following collaborators who participated in this work:

Ella M. Baer, Florida Agricultural Experiment Station, Gainesville, Fla.
Daniel Banes, U. S. Food and Drug Administration, Chicago, Ill.
Sylvia T. Hoisington, Florida Agric. Experiment Station, Gainesville, Fla.
William H. Munday, U. S. Food and Drug Administration, Kansas City, Mo.
T. A. Pickett, Georgia Experiment Station, Experiment, Ga.
Phyllis B. Rokita, U. S. Food and Drug Administration, Atlanta, Ga.

RECOMMENDATIONS*

It is recommended—

(1) That methods for preparation of sample be studied.
(2) That methods for moisture or volatile matter be studied.
(3) That methods for fat determination be studied.
(4) That the other methods be studied if time is available.

REPORT ON VEGETABLE DRUGS AND THEIR DERIVATIVES

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

Only three Associate Referees sent reports, as the following will indicate.

RECOMMENDATIONS†

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

Physostigmine in Ointments.—A report was received describing an improved procedure for the determination of physostigmine in tablets with the recommendation that the improvement be incorporated into the present tentative method and that it then be adopted as official (first action). It was further recommended that work on the determination of physostigmine in ointments be continued. The Referee concurs in these recommendations.

Theobromine and Phenobarbital.—A progress report was received by the Referee. No final report was received. It is recommended that the subject be continued.

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* For report of Subcommittee C and action of the Association, see *This Journal*, 31, 53 (1948).
† For report of Subcommittee B and action of the Association, see *This Journal*, 31, 45 (1948).

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

Quinine.—No report was received, but the Associate Referee in correspondence outlined progress in the comparison of the method described by Herd[1] with the A.O.A.C. method (pages 669 and 670). This report also included a statement regarding a new quantitative procedure which may prove to be better than either of the two methods referred to above. The Referee recommends that the subject be continued.

Ephedrine.—A report was received. It is recommended that the subject be continued.

Spirit of Camphor.—A report was submitted. The Associate Referee recommends that the proposed modification of the N.F. VIII method be adopted as tentative and that the subject be closed. The Referee concurs.

Chemical Methods for Penicillin.—No report was received. It is recommended that the subject be continued.

REPORT ON PHYSOSTIGMINE SALICYLATE AND PHYSOSTIGMINE IN OINTMENTS

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

The present official method for the determination of physostigmine salicylate is dependent upon an accurate titration of the free base which has initially been completely extracted from sodium bicarbonate solution with chloroform. G. M. Johnson (1), the author of the method, and his collaborators were able to obtain consistent, but slightly low (97.5%) recoveries (2) of physostigmine from a lactose mix, taking the precaution only to avoid carbonate error in titration and to completely extract the alkaloid from sodium bicarbonate solution. The estimation of the methyl red end point was left to the judgment of the analyst.

Kolthoff (3) reports the stoichiometric point of physostigmine to occur at a *p*H of 5.0. Johnson checked this figure electrometrically using pure physostigmine and arrived at a value of 4.8, or just on the acid side of the methyl red end point. The shape of his titration curve near the equivalence point indicated that either another indicator should be used or that the color of the methyl red indicator should be checked against that of a buffered solution. The latter proved to give the best results and was therefore incorporated into the method.

A dry mixture of lactose and 2.43 per cent by weight of physostigmine salicylate was prepared and submitted for collaborative study. The purity of the alkaloid based on the nitrogen content was 99.5 per cent.

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[1] *J. A. Ph. A., Scien. Ed.*, XXXI, No. 1, Jan. 1942.

DETERMINATION

Follow *Methods of Analysis* 39.99 thru the sentence "Add methyl red indicator 39.8(b), and titrate the excess acid with 0.02 *N* NaOH." Then insert the following "until the color of the soln exactly matches that of 15 ml of standard buffer (*p*H =5.0; 50 ml M/5 KH Phthalate plus 23.65 ml. M/5 NaOH diluted to 200 ml) containing one drop of methyl red indicator or until a *p*H of 5.0 is reached as indicated by a *p*H meter." Finally continue with last sentence, "1 ml of 0.02 *N* H_2SO_4, etc."

TABLE 1.—*Collaborative results*

COLLABORATOR	PHYSOSTIGMINE SALICYLATE FOUND	RECOVERY
	per cent	*per cent*
J. H. McGee,	2.36	97.1
St. Louis, Mo.	2.36	97.1
	2.33	95.9
S. D. Fine,	2.39	98.3
Cincinnati, Ohio	2.32	95.5
H. R. Bond,	2.43	100.0
Kansas City, Mo.	2.42	99.6
	2.40	98.8
W. F. Kunke,	2.31	95.1
Chicago, Ill.	2.35	96.7
	2.36	97.1
G. S. Keppel,	2.42	99.6
Minneapolis, Minn.	2.45	100.8
M. L. Dow,	2.45	100.8
St. Louis, Mo.	2.39	98.3
	2.39	98.3
Ave.	2.37	97.9

The results indicate that slightly better recoveries are possible by using the revised method.

An experiment was conducted to determine whether physostigmine was hydrolyzed by the sodium bicarbonate to any extent. A portion of the lactose mix was allowed to remain in an excess of sodium bicarbonate solution for varying periods of time up to one hour without any untoward results.

In view of this and the consistency of the collaborators' results, it is recommended that the revised method used above be adopted as official (first action).

This proposed method was applied to the determination of physostig-

mine in ointments with very unsatisfactory low results. In addition the tentative prostigmine method (4) and a standard acid extraction method were tried upon the suggestion of the General Referee, but the results were too erratic to be of any use.

The ointment was prepared by mixing powdered physostigmine salicylate into a molten mixture of lanolin and petrolatum and stirring for several minutes after congealing had taken place. Three determinations using the above proposed method (the ointment was first dissolved in ether) were made and low results were obtained. The ointment was then spread out on a flat plate and mixed for ten minutes with a glass rod. The determinations were repeated with the same erratic low results. The ointment was stirred thoroughly for a third time and the determinations repeated with no better results.

It is apparent that some way must be found to obtain complete recovery of the alkaloid from the ointment. Ether, chloroform, and petroleum benzene were used to dissolve the ointment with about the same recoveries obtained in each instance. Ether has the advantage of producing less emulsions. An easily and accurately determined "tracer" compound mixed in with the ointment was contemplated but was not tried because of the lack of time.

It is recommended* that the work on the determination of physostigmine in ointments be continued, with particular attention being paid to obtaining complete recovery of the alkaloid from the ointment.

LITERATURE CITED

(1) GEORGE M. JOHNSON, *This Journal*, **14**, 816 (1941).
(2) ———, *Ibid.*, **14**, 817 (1941).
(3) J. M. KOLTHOFF, "Die Dissoziationskonstante, das Loslichkeits product und die Titnerbarkeit von Alkaloiden."
(4) *Methods of Analysis*, A.O.A.C., 6th Ed., p. 693.

REPORT ON EPHEDRINE

By LLEWELLYN H. WELSH (Food and Drug Administration, Federal Security Agency, Washington, D. C.), *Associate Referee*

At the 1946 meeting of the Association, the Associate Referee proposed a general method[1] for the determination of ephedrine in inhalants, nasal jellies, syrups, and solutions. Results obtained on preparations of known composition and on products produced by various pharmaceutical companies indicated that the method was of value, and it was recommended that collaborative study be undertaken. The present report includes the

* For report of Subcommittee B and action of the Association, see *This Journal*, **31**, 45 (1948).
[1] *This Journal*, **30**, 467 (1947).

results of collaborative study on three different samples, each corresponding to at least one dosage form of the drug.

Sample I consisted of pure, recrystallized 3,4-dimethyl-2,5-diphenyloxazolidine, m.p. 72–73° (corr.), prepared by reacting benzaldehyde with *l*-ephedrine base.[2] On acid hydrolysis it yields ephedrine theoretically equivalent to 65.22 per cent of its weight. This substance is used in some inhalants containing a light liquid petrolatum base. Since observations in this laboratory have indicated that the compound undergoes some decomposition when liquid petrolatum solutions of it are stored, with the result that recovery figures for ephedrine are low, collaborators were instructed to mix the sample with liquid petrolatum when beginning the analysis. Results of collaborative work on this sample correspond to those obtainable from oily solutions of ephedrine which has been chemically combined with carbonyl compounds, and are also representative of those obtainable from solution of the free base in aromatized oils.

Sample II had the following composition: ephedrine sulfate 0.4400 g. Syrup of Cherry N.F. 90 ml, ethanol (95%) 22 ml, water *q.s.* 200 ml. This mixture contained the equivalent of 0.1697 g ephedrine base per 100 ml and approximately 12% of alcohol by volume. It contained the lowest concentration of ephedrine and the highest concentration of ethanol which the Associate Referee has encountered in commercial ephedrine syrups, and it was chosen for collaborative study because it seemed reasonable that if the method proved satisfactory for this mixture it probably would be suitable also for similar preparations containing less alcohol and more ephedrine.

Sample III was a mixture of ephedrine hydrochloride (10%) and potato starch (90%) and contained the equivalent of 8.192% of ephedrine base. This composition was prepared to simulate those of tablets and the contents of capsules. At this point, the use of sodium carbonate in the assay instructions for sample III might be explained. In the report of last year,[1] the statement was made that although the method had not as yet been applied to tablets and capsules of ephedrine salts, no difficulty was anticipated with these dosage forms. It was found, however, that when aqueous suspensions of potato starch were made alkaline with sodium hydroxide there resulted a semi-solid translucent gel which could not possibly be extracted successfully by shaking with solvents. The use of sodium carbonate instead of the hydroxide provided a *p*H high enough to liberate ephedrine base but not sufficiently high to cause swelling of the starch grains and consequent jellification.

INSTRUCTIONS TO COLLABORATORS

The directions followed by the Associate Referee and sent to collaborators 1, 2, and 4 were as follows.

[2] *J. Chem. Soc.*, 1932, 1580.

Sample I. Accurately weigh a sample of about 0.15 gram and dissolve it in 5 ml of benzene contained in a 125 ml Erlenmeyer flask. After mixing the solution with 10 ml of light mineral oil, add 10 ml of 5% sulfuric acid and frequently agitate and swirl the mixture while it is boiled for ten minutes on a hot plate. Cool the flask, transfer the contents to a separatory funnel, and rinse the Erlenmeyer with portions of benzene totaling about 15 ml in order to remove all oily matter from it. It is desirable that the flask be provided with a lip in order to facilitate the quantitative transfer of the contents to the funnel. Shake the funnel containing the acid and benzene rinsings, drain off the acid layer into a second separatory funnel, and extract the benzene-oil phase with three 5 ml portions of water which have been previously used to rinse the flask. (In the transfer of ephedrine from organic solvent to aqueous phase, and *vice versa*, shakeouts should be conducted for a full minute.) Wash the aqueous soln of ephedrine sulfate with 3 ml of chloroform, add 2.5 ml of 20% sodium hydroxide, and extract the ephedrine with four 15 ml portions of chloroform. Filter the extracts thru a pledget of cotton into a tared 100 ml beaker, previously dried at 110° and cooled in a desiccator, rinse the cotton with chloroform and add five drops (0.2 ml) of conc. hydrochloric acid to the filtrate and washings. Heat the beaker on a steam bath in a current of air until the volume of liquid has been reduced to about 1 or 2 ml. Then cautiously heat, without air current, until the odor of hydrogen chloride has disappeared and the residue is apparently dry. Heat the beaker in an oven at 110° for one-half hour, cool in a desiccator and weigh: weight of residue $\times 0.8192$ = weight of ephedrine base. Report results as per cent of ephedrine base in sample.

Sample II. Measure out 10 ml of sample in a 10 ml volumetric flask and transfer to a separatory funnel by means of small portions of water totaling 10 ml. Make the mixture alkaline with about 3 ml of 20% sodium hydroxide and quantitatively extract the ephedrine with five 20–25 ml portions of ether. Shake the combined ether extracts with 5 ml of 10% sulfuric acid, then with four 5 ml portions of water, and continue the assay as described for Sample I, beginning with the chloroform wash of the acid soln, up to the point at which the chloroform extracts are evaporated in the presence of hydrochloric acid in a 100 ml beaker, which, in this case, need not be tared. Continue evaporation until the chloroform, but not the excess hydrochloric acid, has been removed. Direct a fine stream of redistilled reagent grade methanol around the inside of the beaker to dissolve the hydrochloride, and repeat the process immediately with a stream of chloroform. Transfer the methanol-chloroform soln to a tared 20 ml beaker, previously dried at 110° and cooled in a desiccator, and repeat the methanol and chloroform rinsings until the ephedrine salt has been quantitatively transferred. Evaporate the soln on a steam bath, in a current of air, until the salt begins to crystallize. Continue the removal of solvent by cautious heating, to avoid loss from crepitation, until the residue is apparently dry and there is no odor of hydrogen chloride. Dry the residue as previously described and weigh. Report results as grams ephedrine base/100 ml.

Sample III. Accurately weigh a sample of about 1.0 gram, suspend in 20 ml of water in a separatory funnel, add 0.5 gram of anhydrous sodium carbonate, and, beginning with the ether extractions, proceed with the assay as described for Sample II with the exceptions that the acid extract of ephedrine sulfate is basified with 1.0 gram of anhydrous sodium carbonate, and that the chloroform extract plus hydrochloric acid is evaporated in a tared 100 ml beaker as described for Sample I. Report results as per cent ephedrine base in sample.

Using an Anschütz or calibrated thermometer, determine the capillary melting point of one residue from each sample.

As a result of consideration of the results and comments of these col-

laborators, the original directions were modified. The modification included a test for complete extraction of the drug, and instructed: that care be exercised to avoid superheating and bumping in the acid hydrolysis of the oxazolidine; that all shakeouts be conducted as vigorously as practicable; that, instead of adding a specified quantity of sodium hydroxide or carbonate to acid extracts of ephedrine sulfate prior to extraction of the base, the acid extracts be rendered slightly alkaline before adding a specified excess of alkali. The modified instructions were sent to collaborators 5 and 6.

COMMENTS OF COLLABORATORS

Charles F. Bruening.—"On Sample I . . . some determinations were lost because of bumping . . . . Possibly the method can be improved by using beads and constantly agitating to eliminate bumping."

Harry Rogavitz.—"Sample I. Boiling a two phase system for 10 minutes on a hot plate is difficult to control. Despite constant agitation, one determination was ruined because of superheating and loss of sample. Unless boiling is absolutely essential for success of the method, it is suggested that the directions be modified so that the sample would be warmed on steam bath for a period of time.

"Instead of the specific direction 'add 2.5 ml of 20% sodium hydroxide,' it is suggested that some general direction be used, as, 'make the soln alkaline to litmus with about 2.5 ml of 20% sodium hydroxide.' Because of the possible variation in strength of the reagents as ordinarily prepared in the laboratory, there is not enough margin to insure alkalinity when 2.5 ml of 20% sodium hydroxide are added to 10 ml of 5% sulfuric acid. In the actual determinations, it was found that after extraction with chloroform, one of the residual aqueous solns was faintly alkaline to litmus and the other was neutral. There was quite a discrepancy in the weights of the residues obtained. The residual aqueous solns were made distinctly alkaline to litmus by the subsequent addition of 0.5 ml of 20% sodium hydroxide and further chloroform extractions were made. When the weights of residues from supplemental extractions were added to that obtained from original extractions, concordant results were obtained. The data reported represent the sum of the original and supplemental extractions.

"If a general method should be adopted, it is suggested that some test for complete extraction be included, as 'Insure complete extraction by a fifth shaking with 15 ml of chloroform, addition of 0.2 ml conc. hydrochloric acid, and evaporation of the solvent in a separate container.' "

"Sample II. On heating residue in oven at 110°C., the residue turned brownish."

William F. Kunke.—"The results were obtained by following the respective method, particularly with reference to the volumes and the number of extractions, the length of time of each shaking was very appreciably more than one minute.

"In the case of Sample III it appears advisable to report the results found by making four more successive chloroform extractions of 15 ml each. The weights of the ephedrine hydrochloride were 0.0081 gram and 0.0028 gram, respectively. Including these results gives the total ephedrine base content as 7.94% and 7.85%.

"The incomplete extraction of the ephedrine base by four successive chloroform extractions of 15 ml each was confirmed by duplicate controls using 0.0958 gram ephedrine hydrochloride, 5 ml of 10% sulfuric acid, 20 ml of water, and 1.0 gram of anhydrous sodium carbonate."

Paul S. Jorgensen.—"Residues from each sample after drying at 110°C. for thirty minutes were slightly brown in color."

Sidney Gottlieb.—"It would seem advisable to routinely call for six extractions

instead of four in the final chloroform extraction. I found appreciable amounts of residue in the fifth and sixth extractions in all the samples, but negligible amounts in the seventh and eighth."

DISCUSSION

Five of the six collaborators, including one with no previous experience in proximate assays, obtained results on Sample I which ranged between 98.1 and 101.0% of the theoretical. These results are considered very good, and the average of 98.6% for all collaborators is considered satisfactory.

Results of six collaborators ranged between 96.1 and 108.4% for Sample II. Of the four collaborators who were sent the original instructions, the results of three were in the range of 100.8–103.1%, while those of the fourth ranged from 96.1–100.4%. The fact that the results of the remaining two collaborators, whose modified instructions included testing for complete extraction, obtained results of from 106.1 to 108.4% suggests that the apparently better results of the first group were due to the compensating factors of incomplete extraction of ephedrine and the presence of extraneous matter in the residue. As applied to syrups of ephedrine content as low as that of this collaborative sample, the method really represents an attempt to gravimetrically determine semimicro amounts of ephedrine by means of a proximate assay. The residues of ephedrine hydrochloride amounted to approximately 20 mg., and a difference of 1 mg. resulting from the presence of substances having solubilities similar to those of ephedrine, or arising for any other reason, would represent an error of 5 per cent. Increasing the size of the sample in order to obtain a much larger residue is an obvious recourse. Although this would render the method more cumbersome, it might represent an over-all advantage. From the collaborative results, it must be concluded that, when applied to syrups of composition similar to that of the collaborative sample, the method tends to give results some 5 per cent higher than those usually considered acceptable. However, in view of the fact that there are no official methods of analysis for ephedrine syrups of any type, the method might be adopted on a tentative basis.

Four of the six collaborators obtained what the Associate Referee considers to be good results (97.4–99.2%) on Sample III, and the average for the six collaborators was 97.5%. Two of the collaborators reported results representing 94.4–96.9% recoveries. The directions sent to Collaborator 4 did not include a test for complete extraction of the alkaloid and did not direct that a measured excess of carbonate be added before undertaking the chloroform extraction. Although his results include the ephedrine hydrochloride residue obtained from four additional extractions, it is not known whether complete extraction of ephedrine was effected, and it is possible that additional extraction would have raised the recovery figures. Modified instructions were sent to Collaborator 6,

TABLE 1.—*Results of collaborators*

COLLABORATOR	SAMPLE	EPHEDRINE BASE FOUND	PER CENT RECOVERY	M.P. OF RESIDUE °C. CORR.
		per cent		
1. Charles F. Bruening, U. S. Food & Drug Admin., Baltimore, Md.	I	64.8	99.4	217.2-218.0
	II*	0.173	101.9	215.4–216.8
		0.173	101.9	
	III	8.11	99.0	218.0–218.5
		8.13	99.2	
2. Harry Rogavitz, U. S. Food & Drug Admin., New York, N. Y.	I	65.9	101.0	216–217
		65.9	101.0	
	II*	0.174	102.5	213–216
		0.175	103.1	
	III	8.11	99.0	214–216
		7.98	97.4	
3. L. H. Welsh, U. S. Food & Drug Administration, Washington, D. C.	I	64.0	98.1	216–217.5
		64.2	98.4	
		64.5	98.9	
	II*	0.171	100.8	214–217
		0.172	101.4	
	III	8.03	98.0	216–217
		8.03	98.0	
4. Wm. F. Kunke, U. S. Food & Drug Administration, Chicago, Ill.	I	64.7	99.2	216–219
		64.6	99.0	
	II*	0.1704	100.4	
		0.1654	97.5	218–220
		0.1630	96.1	
	III	7.94	96.9	
		7.85	95.8	218–219
5. Sidney Gottlieb, U. S. Food & Drug Administration, Washington, D. C.	I	65.2	100.0	217.5–219
		65.7	100.8	
	II*	0.180	106.1	214.5–216.5
		0.181	106.7	
	III	8.07	98.5	217–218.5
		8.12	99.1	
6. Paul S. Jorgensen, U. S. Food & Drug Administration, San Francisco, Calif.	I	61.08	93.7	219.5†
		61.03	93.6	
	II*	0.184	108.4	216.5†
		0.182	107.2	
	III	7.73	94.4	219†
		7.75	94.6	

* For sample II, results shown as g/100 ml.
† Uncorrected, not included in m. p. average.

however, and it is assumed that the extraction test was used and that the transfer of ephedrine from water to chloroform was complete. Low recoveries would then most probably be due to failure to attain equilibrium in the ether extractions or be the result of incomplete separation of the aqueous and ethereal phases.

Average melting ranges of Sample I (216.5–218.5°) and Sample III (216.5–218.0°) fall within the 216–220° limits of the U.S.P. XII, although in one case the beginning of fusion of the residue from Sample III was reported as two degrees lower than the U.S.P. minimum. The lower purity of residues from Sample II is reflected in the average melting range (215.0–217.5°), in which the beginning of fusion is 1.5° lower than the averages for the other two samples. Also indicative of the lower purity of Sample II residues are the high recovery percentages reported and the fact that Collaborators 1, 2, 3, and 5 observed that residues from this sample were more highly colored than those from the other samples.

The instructions sent to the collaborators were reworded into a general method, in which modifications are described for adapting the method to dosage forms (water-soluble jellies, petroleum jellies, solutions of ephedrine salts, etc.) which were not subjected to collaborative study. Complete details of the method have been published in *This Journal*, **31**, 113 (1948). It has been adopted as official, first action. Samples representing these three types of preparations were not included in the study because it was felt that their inclusion would unduly burden the collaborators. The majority of the collaborative figures (Table 1) reasonably parallel those of the Associate Referee, who obtained satisfactory results in adapting the method to the three dosage forms in question. In the opinion of the Associate Referee, the adaptations are of a nature which would justify the conclusion that a collaborative study on these dosage forms would yield results paralleling those of the present study.

RECOMMENDATIONS*

It is recommended that the proposed method for the determination of ephedrine in its various dosage forms be adopted as tentative and that the subject be closed.

No reports were given on chemical methods for ergot alkaloids, on theobromine and phenobarbital, or on aminopyrine, ephedrine, and phenobarbital; for quinine, see report of Referee.

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* For report of Subcommittee B and action of the Association, see *This Journal*, 31, 45 (1948).

REPORT ON SPIRIT OF CAMPHOR

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

Spirit of Camphor was a U.S.P. XII product, but the preparation was omitted from the U.S.P. XIII. The same product and assay is now official in the National Formulary VIII.

Work this year was confined to a collaborative study of the determination of camphor by the official National Formulary VIII assay and by a modification of that assay. The latter method was sent to collaborators in a revised form from its original (*This Journal*, **28**, 719, 1945). Reagents specified were the same, except that following "(2) Aldehyde-free alcohol," the words "U.S.P. XII, p. 649," were deleted.

Details of the method are published in *This Journal*, **31**, 115 (1948).

COLLABORATIVE WORK

One sample of Spirit of Camphor, containing 9.60 g camphor in 100 ml at 20°C., was sent to collaborators. The camphor used in the prepara-

TABLE 1.—*Collaborative results*

COLLABORATOR*	CAMPHOR		RECOVERY	
	N.F. VIII METHOD		MODIFIED N.F. VIII METHOD	
	g/100 ml	*per cent*	*g/100 ml*	*per cent*
M. L. Dow	9.49	98.9	9.50	99.0
	9.43	98.2	9.53	99.3
			9.51	99.1
Sidney Williams	8.07	84.1	9.55	99.5
	8.42	87.7	9.56	99.6
	8.12	84.6	9.58	99.8
Rupert Hyatt	9.27	96.6	9.56	99.6
	9.08	94.6	9.60	100.0
Daniel Banes	8.34	86.9	9.52	99.2
	8.32	86.7	9.56	99.6
J. A. Thomas	9.13	95.1	9.49	98.9
	9.16	95.4	9.48	98.8
	8.99	93.6	9.49	98.9
Associate Referee	8.93	93.0	9.55	99.5
	9.05	94.3	9.58	99.8
Averages	8.84	92.1	9.54	99.4

* Addresses in order of listing: St. Louis, Mo., Minneapolis, Minn., Cincinnati, Ohio, Chicago, Ill., New Orleans, La., Kansas City, Mo. All of the U. S. Food and Drug Administration.

tion was purified according to C.A. **34**, 7302, 1940, and aldehyde free alcohol was prepared by A.O.A.C. Method **25.36(a)**.

COLLABORATORS COMMENTS

Rupert Hyatt.—N.F. Method, determination one, the precipitate was orange colored; No. 2 was lighter color and more of a yellow.

M. L. Dow.—The precipitate obtained in the modified N.F. VIII method was more granular and much more easily washed free of H_2SO_4 than the ppt. obtained following the N.F. VIII Method. The pressure bottles were kept in metal beakers as a precautionary measure.

J. A. Thomas.—As specified, the spirit of camphor was brought to 20°C. before taking aliquots. However, no attempt was made to bring the pipets to 20°C., as the high humidity in New Orleans would have caused condensation of moisture. With the pipet at 33°C., undoubtedly a volume change occurred when the camphor solution at 20°C. was transferred with the warm pipette.

DISCUSSION

The recovery of camphor by the modified N.F. VIII Method averaged 99.4 per cent and the maximum deviation from this value of any single determination was 0.6 per cent. As pointed out by Collaborator Thomas the measuring of a solution at 20°C. with a pipet at room temperature of 33°C. would cause a volume change which would be reflected in a slightly lower result. Collaborative work was done in the late spring and summer months.

Most of the camphor recoveries by the Official N.F. VIII Method were too low. Collaborators were requested to note the temperature of the refluxing condenser to determine how this factor affected recovery. While these varied from 18 to 30°C., the poorest yields were obtained at the two lower temperatures.

The Associate Referee believes that the following precautionary measure suggested by Collaborator Dow should be incorporated in the modified N.F. VIII Method under "Procedure," sentence 3, "close pressure bottle; immerse it in a covered metal beaker of water."

RECOMMENDATIONS*

It is recommended—

(1) That the proposed modification of the N.F. VIII Method be adopted as tentative.

(2) That the subject be closed.

No report was given on chemical methods for penicillin.

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* For report of Subcommittee B and action of the Association, see *This Journal*, 31, 46 (1948).

REPORT ON SYNTHETIC DRUGS

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

For the past year 11 topics were assigned to the Referee. One additional topic was studied with a view to determining whether the present A.O.A.C method for phenolpthalein in chocolate preparations should be modified in certain details. Each topic and recommendation will be discussed separately.

RECOMMENDATIONS*

Quinacrine Hydrochloride.—A method which is shorter than the U.S.P. procedure has been recommended for adoption as an official method. It was further recommended that the topic be closed. The results obtained were good and the Referee recommends that the method be adopted as official, first action, and that further study be discontinued. The Associate Referee has left his position with the U. S. Food & Drug Administration but has expressed a desire to continue to serve the Association as Associate Referee.

Dihydrocodeinone.—The Associate Referee has submitted a report which includes a collaborative study. The results were generally close to the theoretical. The Associate Referee recommends adoption of the method as official, first action, and that the subject be closed.

Thiouracil.—A report was submitted and the Associate Referee has recommended that the method, which was studied collaboratively with reasonably concordant results, be adopted as tentative and that the topic be closed.

The Referee concurs.

Trichlorethylene.—An attempt was made by the Associate Referee to use the A.O.A.C. method for tetrachlorethylene. The results were not satisfactory and a further study was recommended by the Associate Referee.

The Referee concurs.

Carbromal.—Because of insufficient time the Associate Referee was unable to develop a method suitable for collaborative study. He has recommended that the study be continued.

The Referee concurs.

Butacaine Sulfate.—Some exploratory work was done on this problem but no formal report was submitted. The Associate Referee recommended that the subject be continued.

The Referee concurs.

Propadrine Hydrochloride.—The Associate Referee had no formal report, but has done some preliminary work. He experienced some difficulty in securing the pure salt for experimental use and recommends a continuance of the study.

* For report of Subcommittee B and action of the Association, see *This Journal*, 31, 46 (1948).

The Referee concurs.

Methylene Blue.—The Associate Referee did some work on this topic but because of urgent regulatory work was unable to complete any conclusive determinations. Since methylene blue has been to a considerable extent replaced in medicine largely by the sulfa drugs and penicillin, the Referee recommends that this topic be dropped.

Sulfanilamide Derivatives.—No formal report was submitted, although the Associate Referee has undertaken a survey of the literature and has attempted the application of a number of methods. It was recommended that the subject be continued.

The Referee concurs.

Demerol.—The Associate Referee submitted collaborative results by the method previously published (*This Journal*, **28**, 711, 1945) and recommended that the method be advanced to official, first action, and the subject be closed.

Phenolpthalein in Chocolate Preparations.—Our attention was called to an article in *This Journal*, **26**, 194, 1943. A number of improvements over the present A.O.A.C. method were recommended. In order to investigate the matter an Associate Referee was appointed. He has conducted some experiments and finds merit in some of the proposals and recommends that the topic be continued with a view to collaborative study.

The Referee concurs.

Spectrophotometric Methods.—No report was received. It is recommended that the subject be continued.

No report was given on methylene blue, or on sulfanilimide derivatives.

REPORT ON ATABRINE (CHINACRIN, QUINACRINE)

By HAROLD C. HEIM (Food and Drug Administration, Federal Security Agency, San Francisco, Calif.), *Associate Referee**

In a previous report[1] the Associate Referee reported the results of collaborative study of an extraction method for the assay of quinacrine hydrochloride and quinacrine hydrochloride tablets. This method has been found to yield slightly high results. As a topic for further collaborative study the Referee has suggested a method which has been in use for several years and which closely resembles the U.S.P. XII official method.

EXPERIMENTAL

A sample of U.S.P. XII quinacrine hydrochloride was assayed for total chlorine by a sodium peroxide bomb fusion with the following results:

* Present address: Shool of Pharmacy, University of Colorado, Boulder, Colo.

[1] *This Journal*, **27**, 354 (1944).

TABLE 1.—*Results of chlorine analysis*

Cl FOUND	THEORY BASED ON ($C_{23}H_{30}OClN_3.2HCl.2H_2O$)	PURITY CALCULATED FROM Cl FOUND
per cent	*per cent*	*per cent*
20.62	20.90	98.66
20.71		99.09
Average		98.88

Samples of this quinacrine hydrochloride and a simulated tablet mixture consisting of quinacrine hydrochloride, talc, and starch were sent to collaborators with instructions, which are given with the method as published in *This Journal*, **31**, 115 (1948).

TABLE 2.—*Results with quinacrine hydrochloride*

ANALYST	FOUND	THEORY	RECOVERY (AVERAGE OF DUPLICATES)
	per cent	*per cent*	*per cent*
O. H. Miller	98.72; 98.89	98.88	99.9
M. L. Yakowitz	98.72; 98.69		99.8
A. G. Buell	98.69		99.8
R. D. Stanley	99.70; 99.95		100.9
Harry Isacoff	98.5 ; 98.6		99.7
H. C. Heim	98.68; 98.95		99.9
Average per cent recovery			100.0

TABLE 3.—*Results with simulated tablet mixture*

ANALYST	FOUND	THEORY	RECOVERY
	per cent	*per cent*	*per cent*
O. H. Miller	37.90; 37.93	37.95	99.9
M. L. Yakowitz	37.09; 37.45		98.2
A. G. Buell	37.67		99.3
R. D. Stanley	38.49; 38.43		101.3
Harry Isacoff	37.6; 37.7		99.2
H. C. Heim	37.43; 37.33		98.5
Average per cent recovery			99.4

The theoretical value for the simulated tablet mixture was calculated on the basis of a quinacrine hydrochloride of 98.88 per cent purity.

SUMMARY AND RECOMMENDATIONS*

(1) A method for the assay of quinacrine hydrochloride and quinacrine hydrochloride tablets has been subjected to collaborative study.

(2) Recoveries very closely approximating the theoretical values are included in this report.

(3) It is recommended that the method be adopted as official and that the topic be closed.

A REPORT ON THE COLLABORATIVE STUDY OF METHODS FOR THE DETERMINATION OF DEMEROL

By MERLIN MUNDELL† (Food and Drug Administration, Federal Security Agency), *Associate Referee*

Two methods for the determination of Demerol were reported[1] at the 1945 meeting of the A.O.A.C. At that time no collaborative work had been done on these methods. A sample was prepared for collaborative study which contained a mixture of Demerol hydrochloride, starch, lactose, and stearic acid; 102.0 grams of the mixture contained 17.0 grams of demerol hydrochloride. Samples were given to several collaborators who were asked to assay the samples by the two methods mentioned above. In printing the directions for the distillation method a typographical error was made. On page 712, line 12, of the paragraph headed "Determination" should have read "distill over a fourth 100 cc. portion of water." This correction was made in the copies of the methods sent to the collaborators.

Reports have been received from the following collaborators: Miss Muriel Drucker, New York Station, Food and Drug Administration, and Mr. Harold F. O'Keefe, Chicago Station, Food and Drug Administration. Their results are tabulated below.

TABLE 1.—*Per cent of demerol hydrochloride*

COLLABORATOR	BY DISTILLATION METHOD	BY EXTRACTION METHOD
#1	16.6	16.8
	16.7	16.7
	16.7	16.8
		16.7
#2	17.0	16.4
	17.0	16.5

* For report of Subcommittee B and action of the Association, see *This Journal*, **31**, 46 (1948).

† Present address, P.O. Box 793, Kilgore, Tex.

[1] *This Journal*, **28**, 711 (1945).

The values obtained by the collaborators agree closely with the 16.67% Demerol Hydrochloride contained in the sample.

The following comments were made by Mr. O'Keefe:

"I did not have the distillation set up described by you, so used instead the Hortvet type shown on page 186 of the *Book of Methods*.

"I wonder if it might not be practicable to use a greater excess of N/50 H_2SO_4 as in routine laboratory work one cannot be certain of having 0.1 gm or less of the drug."

"I don't see what advantage the standardization of the solution against sulfamic acid has over the official A.O.A.C. methods. I found that the factors obtained by the two methods checked very closely."

DISCUSSION

At the time the methods were presented the criticism was made that the accuracy of the method was not great enough to justify four significant figures in the factor for converting ml of 0.02 *N* sulfuric acid to g of demerol hydrochloride. It would therefore be better to use .00567 as the factor.

In regard to Mr. O'Keefe's comment that standardization of the solution against sulfamic acid has no advantage over the official A.O.A.C. methods, the Associate Referee feels that sulfamic acid has an advantage, in that it can be easily obtained in a pure form and can be maintained in the laboratory in dry form without change in strength. Standard solutions of the desired strength can be easily prepared from weighed samples. As Mr. O'Keefe pointed out, factors obtained by using sulfamic acid check very closely with the factors obtained by using the official A.O.A.C. methods. It would therefore be advisable to modify the wording of the methods to permit use of solutions standardized by the official A.O.A.C. methods.

Both the distillation and the extraction methods gave good results in the hands of the collaborators and no difficulties were reported. Details of the methods are published in *This Journal*, **31**, 115, 116 (1948).

RECOMMENDATIONS*

It is recommended that the methods, with the minor changes discussed above, be adopted as official, first action.

No report was given for propadrine hydrochloride; see report of Referee.

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* For report of Subcommittee B, and action of the Association, see *This Journal*, **31**, 46 (1948).

REPORT ON CARBROMAL

By RUPERT HYATT (Food and Drug Administration, Federal Security Agency, Cincinnati, Ohio), *Associate Referee*

Carbromal is official in the N.F. though no preparations containing it are listed and no assay is given. Some studies were made to find a suitable method for the assay of the crystalline material and commercial 5 grain tablets.

Three methods of assay were tried, *viz.*, extraction and weighing, nitrogen determination, and bromine determination. The extraction method gave low and variable results. The residue is appreciably volatile at 80°. This would make the drying of an extract difficult. The extraction method appears unsatisfactory.

The nitrogen determination appears promising. It would be necessary to use a separate portion for qualitative tests.

The bromine determinations were made by refluxing with alcoholic potassium hydroxide, the pressure bottle chloroform method, and charring methods similar to that for iodides (*Methods of Analysis*, 6th. Ed., 39.202). Slightly higher results were obtained by this method. The reason for this was not determined. No collaborative work was done.

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*

Attempt has been made to use the existing method for tetrachloroethylene, with slight variation (*Methods of Analysis*, 6th Ed., p. 711). It is unlikely that chlorine can be quantitatively removed from a compound of this kind by any less drastic treatment than is there described. A sample of trichloroethylene purified by three distillations and having a density of 1.4556 25°/4° was used for the determinations.

Results have not been quite satisfactory. Recoveries between 99 per cent and 99.4 per cent have been obtained occasionally, but not consistently. Apparently slight differences in conditions can cause rather large differences in percentage recovery. Recently, recoveries over 98 per cent have been obtained consistently. It seems likely that with further study of details the method can be made to give results consistently close to 100 per cent, which will justify collaborative work.

Factors which appear to increase percentage recovery are:

1. Sodium not too finely divided.

* For report of Subcommittee B and action of the Association, see *This Journal*, 31, 46 (1948).

2. Greater dilution with xylene (50 ml or over).
3. Fairly vigorous boiling.
4. Addition of amyl alcohol at the right intervals.

Further study is recommended.*

REPORT ON DIHYDROCODEINONE

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

In accordance with the recommendation of Subcommittee B of the Association (*This Journal* **30,** 46), a method for the determination of dihydrocodeinone was subjected to collaborative study.

Dihydrocodeinone ($C_{18}H_{21}O_3N$), commonly known as Dicodid, is an alkaloid with a physiological action midway between codeine and morphine. It is used particularly as preliminary to operations to relieve postoperative pain, and is usually administered in the form of the bitartrate or hydrochloride.

Dicodid hydrochloride was selected as the material to be used for collaborative work and a 20% mixture of this substance with milk sugar as an excipient was submitted to collaborators to be assayed by the proposed method, details of which are given in *This Journal,* **31,** 117 (1948).

The results reported by collaborators are as follows:

COLLABORATOR	DIHYDROCODEINONE HYDROCHLORIDE	COLLABORATOR	DIHYDROCODEINONE HYDROCHLORIDE
	per cent		*per cent*
1	19.98		
	19.91	5	19.26
			19.08
2	19.21		
	19.37	6	19.8
			20.2
3	20.52		
	20.60	Associate Referee	19.85
			20.35
4	19.5		
	19.7	Ave.	19.81

DISCUSSION

Dihydrocodeinone may be determined by the usual alkaloidal assay. The results of the collaborative samples are in good agreement, and no comments or criticisms were received regarding the proposed method.

* For report of Subcommittee B and action of the Association, see *This Journal,* 31, 46 (1948).

RECOMMENDATIONS*

It is recommended that the method for the determination of dihydrocodeinone be adopted as official (first action) and the subject be closed.

ACKNOWLEDGMENT

The Associate Referee wishes to thank the following persons, all members of the Food and Drug Administration, for their cooperation in this collaborative work: George E. Keppel, Minneapolis, Minn., H. P. Bennett Kansas City, Mo., Urban Oakdale and Wm. F. Kunke, Chicago, Ill., James H. McGee, St. Louis, Mo., and Rupert Hyatt, Cincinnati, Ohio.

No report was given on beutacaine sulfate; or on spectrophotometric methods.

REPORT ON THIOURACIL IN TABLET MIXTURE

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

A study was undertaken to propose a determination for thiouracil in a tablet mixture.

Thiouracil powder was obtained with the cooperation of the manufacturer. When assayed, this sample was found to contain 98.7 per cent of the theoretical amount of nitrogen and 99.8 per cent of the theoretical amount of sulfur. The melting point of thiouracil (317°C. dec) cannot be used as a criterion of purity for routine laboratory work, because it is above the flash point of the common melting-point bath mixtures.

Thiouracil brominates very readily. Use of this fact was made in adapting a bromination procedure for the assay as reported in the literature.[1] Preliminary experiments served to indicate that the ease with which thiouracil reacts with bromine makes the assay an empirical method in which some conditions have to be specified. Several trial runs showed that about 0.125 g thiouracil and 25 ml of 0.5 *N* potassium bromide-bromate was a practicable combination. Using dried thiouracil powder, two additional sets of experiments were made by varying (Table 1) the bromination time and (Table 2) the quantity of thiouracil.

Experiment (A)

Conditions kept constant: 3 ml of 5% NaOH; 25 ml of 0.5 *N* KBr-$KBrO_3$; 10 ml of 10% HCl; 10 ml of 15% KI soln; reaction time with KI soln was exactly 5 minutes; titrated without the aid of starch.

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* For report of Subcommittee B and action of the Association, see *This Journal*, **31**, 46 (1948).

[1] *J. Am. Med. Ass.*, **133**, No. 9, 619 (1947).

TABLE 1.—*Results of varying time*

COLLABORATOR	WEIGHT OF THIOURACIL	EXACT BROMINATION TIME	CALCULATED THIOURACIL	PER CENT OF THEORETICAL
	gm	*min*	*gm*	*per cent*
1	0.1256	6	0.1221	97.2
2	0.1262	8	0.1244	98.6
3	0.1258	10	0.1238	98.4
4	0.1275	12	0.1264	99.1
5	0.1266	14	0.1269	100.2
6	0.1262	16	0.1270	100.6

Experiment (B)

Conditions kept constant: 3 ml of 5% NaOH; 25 ml 0.5 *N* KBr-$KBrO_3$; 10 ml of 10% HCl; bromination time was exactly 15 minutes; 10 ml of 15% KI soln; reaction time with KI soln was exactly 5 minutes; titrated without the aid of starch.

TABLE 2.—*Results with varying quantities of thiouracil*

	WEIGHT OF THIOURACIL	CALCULATED THIOURACIL	PER CENT OF THEORETICAL
	gm	*gm*	*per cent*
1	0.1000	0.0991	99.1
2	0.1104	0.1096	99.3
3	0.1206	0.1171	97.1
4	0.1300	0.1252	96.3
5	0.1406	0.1353	96.2
6	0.1505	0.1433	95.2

The foregoing results, as might be predicted, prove that the quantity of thiouracil combining with bromine increases as (1) the reaction time increases (Experiment A), and (2) the available amount of bromine increases (Experiment B).

A collaborative sample was prepared to resemble a typical tablet granulation. This was made from dried thiouracil, potato starch, talc U.S.P., lactose U.S.P., and stearic acid U.S.P., theoretically containing 50.00 per cent of thiouracil.

The collaborators, who are members of the Food and Drug Administration, were instructed to use a 0.5 g sample and to report the weight of sample taken. Their results are contained in Table 3.

COMMENTS

A. L. Diamond.—The only inconvenience found in the method was the length of time required to filter the gelatinous soln of thiouracil and alkali.

M. A. Braun.—I noticed that on addition of the NaOH the starch in the sample swelled and made filtration extremely difficult, making it necessary to change to a coarser filter paper. Even then the filtration was slow, and the filtrate was cloudy. Perhaps adding some water simultaneously with the NaOH would help some to prevent swelling of the starch.

TABLE 3.—*Thiouracil in tablet mixture No. 1*

COLLABORATOR	WEIGHT OF SAMPLE	PER CENT OF THIOURACIL FOUND	PER CENT OF THEORETICAL
	g		
A. L. Diamond, New York	0.5000	50.72	101.4
	0.5000	50.54	101.1
M. A. Braun, St. Louis	0.5024	51.1	102.2
E. H. Grant, Boston	0.5000	49.65	99.3
H. F. O'Keefe, Chicago	0.5006	51.17	102.3
	0.5008	50.71	101.4
Associate Referee	0.5056	49.84	99.7
	0.5029	49.59	99.2
	0.5026	49.34	98.7
Per Cent Average Recovery		50.29	100.6

TABLE 4.—*Thiouracil in tablet mixture No. 2*

COLLABORATOR	WEIGHT OF SAMPLE	PER CENT THIOURACIL FOUND	PER CENT OF THEORETICAL
	g		
M. A. Braun, St. Louis	0.5030	50.55	101.1
	0.5074	50.56	101.1
C. F. Bruening, Baltimore		52.05	104.1
		52.20	104.4
A. L. Diamond, New York	0.5000	51.35	102.7
	0.5000	51.30	102.6
H. F. O'Keefe, Chicago	0.5013	50.67	101.3
	0.4997	50.07	100.1
Associate Referee	0.5039	50.45	100.9
	0.5025	50.83	101.7
	0.5002	50.54	101.1
Per Cent Average Recovery		50.96	101.9

E. H. Grant.—By diluting almost to volume before adding the alkali and choosing a thin filter paper, I had no trouble with gel formation.

H. F. O'Keefe.—I took the first end point reached in the titration but noted that the iodine color returned to the material in a very short time.

DISCUSSION

Most of the collaborators experienced difficulty in filtering the alkaline solution of the tablet mixture. The Associate Referee washed the tablet powder down the sides of the volumetric flask with distilled water before adding the alkali and had no trouble with the analysis. She believed that the collaborators, in following the first set of directions, had weighed the dry powder into a volumetric flask and then had added alkali. Consequently, a second collaborative mixture, identical in composition with the first one, was prepared and sent to the original collaborators, together with directions which were modified ("Add 50 ml of distilled water and . . . ") by adding water to the weighed sample. Details of the method are published in *This Journal*, **31**, 117 (1948).

Collaborators' data are in Table 4.

COMMENTS

M. A. Braun.—No difficulties were encountered in following your procedure this time.

C. F. Bruening.—Starch was used as an indicator and it was noted that the blue color returned approximately one minute after the initial end point was obtained.

H. F. O'Keefe.—Your method was followed exactly as written but again I took the first end point reached, as the iodine color soon returned to the solution.

DISCUSSION

The collaborative work evidences that the empirical method studied in this paper provides a satisfactory assay for thiouracil in tablet mixtures.

It is recommended* that the method be adopted as tentative and the subject be closed.

REPORT ON PHENOLPHTHALEIN IN CHOCOLATE PREPARATIONS

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

A study was made to determine whether Hubacher's[1] modifications of the official method for phenolphthalein in chocolate preparations[2] should be incorporated into the A.O.A.C. method.

The A.O.A.C. method is based on the report of Palkin.[3] The collaborative sample prepared by Palkin consisted of $33\frac{1}{3}$ per cent of phenolphthalein and $66\frac{2}{3}$ per cent of cocoa. Some of the commercial preparations on the market contain about 10 per cent of phenolphthalein.

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* For report of Subcommittee B and action of the Association, see *This Journal*, **31**, 46 (1948).

[1] M. L. Hubacher, *This Journal*, **26**, 194 (1943).

[2] *Methods of Analysis, A.O.A.C.*, 6th Ed., sec. 39.168, p. 719.

[3] S. Palkin, *This Journal*, **8**, 541 (1924–25).

When a mixture containing 10 per cent phenolphthalein and 90 per cent of chocolate was analyzed by the A.O.A.C. method, low recoveries were obtained. The large-sized sample necessary for analysis of a 10 per cent phenolphthalein mixture results in considerable fat, which slows subsequent filtrations and entraps phenolphthalein. When the sample was analyzed by Hubacher's method, good recoveries were obtained and the time for analysis shortened. When this method was tried on a mixture of 10 per cent phenolphthalein and 90 per cent breakfast cocoa (high fat cocoa), recoveries greater than 100 per cent were obtained. When Hubacher's method was modified to include digestion of the tetraiodophenolphthalein precipitate on steam bath and washing with petroleum ether, recoveries approaching the theoretical amount were obtained.

It is recommended* that the subject be continued and that a collaborative study of the method be made.

REPORT ON MISCELLANEOUS DRUGS

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

RECOMMENDATIONS†

Compound Ointment of Benzoic Acid.—A report was submitted by the Associate Referee and he recommends that the method be adopted as tentative and the subject closed. The Referee concurs.

Iodine.—A re-investigation of the official method for Iodine (*Methods of Analysis*, 6th Ed. **39.202**). A report was received and the Associate Referee recommends that samples be submitted for collaborative study by the present official method and by the proposed modification of the official method. The Referee concurs.

Separation of Bromides, Chlorides, and Iodides.—The Associate Referee submitted a report covering a study by himself and Miss McMullen on Iodide, Bromide, and Chloride in Drugs, and the authors recommend:

(1) That the revised acetone method for chloride in the presence of bromide and iodide be subjected to further collaborative study.

(2) That the volumetric method for iodide and bromide be studied in comparison with the aeration absorption method and that one or both methods be submitted to collaborators.

(3) That the modified open Carius method as published (*This Journal*, **25**, 836, 1942) be adopted as a tentative method for total halides, with the notation, "Not applicable to many organic halogen compounds." Reagent **(c)** should be deleted from the list of reagents.

The Referee concurs.

The Associate Referee has requested that he be relieved of this assign-

* For report of Subcommittee B and action of the Association, see *This Journal*, **31**, 46 (1948).
† For report of Subcommittee B and action of the Association, see *This Journal*, **31**, 47 (1948).

ment and recommends that the subject be reassigned. The Referee concurs.

Preservatives and Bacteriostatic Agents in Ampul Solutions.—The Associate Referee has made a progress report and recommends that the subject be continued. Referee concurs in this recommendation.

Determination of Glycols and Related Products.—No report. The Associate Referee recommends continuance for another year. The Referee concurs.

Chemical Methods for the estimation of Estrone and Estradiol.—The Associate Referee reports that no samples were submitted for collaborative study and recommends that the subject be continued. The Referee concurs.

Chromatographic Separation of Drugs.—The Associate Referee reports that some preliminary work has been done, but not enough for a report. He recommends that the subject be continued. The Referee concurs.

Microchemical Tests for Alkaloids and Synthetics.—The Associate Referee has submitted a progress report in which he indicates that he has worked up considerable data on various compounds for inclusion in determinative tables for identification purposes and he recommends that the work be continued. He also recommends that the general title of the project be changed to "Microscopical Tests for Alkaloids and Synthetics" since this would include tests for both of a chemical microscopic nature and also crystallographic microscopic tests. The eferee concurs in both recommendations.

Study of the Ethanolamine Method for Mercurials, (including Phenyl Mercuric Acetate and Phenyl Mercuric Iodide).—The Associate Referee reports that work is under way and recommends that the subject be continued. The Referee concurs.

Phosphorus, Calcium, and Iron in Vitamin Preparations.—The Associate Referee reported that he has completed the preliminary work and is ready to submit sample for collaborative study, and recommends continuance. The Referee concurs.

Alkali Metals.—The Associate Referee has recommended that the subject be continued. The Referee concurs.

Organic Iodides.—No report received. The Referee recommends continuance.

Use of Superheated Steam in the Separation of Drugs.—No report. The Associate Referee has resigned from the Food and Drug Administration. The Referee recommends that the subject be dropped.

No report on microscopical tests for alkaloids and synthetics or on mercury compounds.

REPORT ON SEPARATION OF BROMIDES, CHLORIDES, AND IODIDES IN DRUGS

By NEWELL E. FREEMAN (*Associate Referee*) and BEULAH V. MCMULLEN, (Food and Drug Administration, Federal Security Agency, Atlanta, Ga.)

At the 1941 meeting (*This Journal*, **25**, 833) the Associate Referee reported on suggested methods for:

(1) Ashing with fixatives to eliminate interference of organic material. (Applicable in absence of iodide.)

(2) The "Open Carius" method for total halides.

(3) The recovery of halides as the sodium salts from the mixed silver precipitate. (Applicable in the presence of iodide.)

(4) The determination of iodide by an iron-aeration-KI absorption procedure.

(5) The determination of bromide by a ceric sulphate-aeration-KI absorption procedure.

Samples were prepared and sent out for collaborative study of points (2) and (3) above. These contained one, two, or three halides in the presence of sugar, glycerol, alcohol, and various organic drugs frequently associated with the halides in proprietary and official preparations. The collaborators were asked to determine total halide and to recover the halide from the silver salts by the methods given in the previous report. (*loc. cit.*, p. 836). In order to check the recovery of halides they were also asked to "Adjust the volume of the filtrate obtained by the hydroxylamine procedure to about 150 ml, acidify with ca 2 ml HNO_3 and add a slight excess of the $AgNO_3$ solution. Boil to agglomerate the silver halide, filter through a tared Gooch crucible and wash thoroughly with hot H_2O. Dry as before and weigh." Largely because of pressure of the war effort only a few results were obtained. These results are shown in Table 1.

The agreement between analysts on these five samples using the "Open Carius" method appears to be remarkably good and would indicate that the procedure, as outlined, is satisfactory for the determination of the three halides under consideration, either alone or in combination and in almost any mixture with organic substances where the halide itself is in the inorganic form. The hydroxylamine procedure appears to be quite satisfactory. The slight excess of silver salt recovered over that initially found, which is apparent in the majority of cases, may possibly be due to contamination by the reagents used. No blanks were run to correct for any halides present in the reagents. It is not clear why sample No. 5 shows a much poorer recovery than the others. All three halogens were present in this sample with only sugar and glycerine as organic matter. The fact that this precipitate was larger than any of the others, with the accompanying difficulty of obtaining complete disintegration and reduction of the precipitate, may be the explanation, in part, at least. It is believed that this procedure is sufficiently reliable to warrant its use in

TABLE 1.—*Weights silver halide obtained by "Open Carius" and by hydroxylamine recovery procedure*

METHOD	IRUENING	SCHIFFMAN	HAYNES	O'KEEFE	CONWAY
			Sample #1		
"Open Carius" AgX/20 ml (1)	.5536 .5530	.5525 .5537	.5549	.5540	.5529
Hydroxylamine Recovery g. AgX (2)	.5596 .5505	— .5528	.5510		
			Sample #2		
1	.1104 .1106	.1128 .1116	.1127 .1118	.1110	.1115
2	.1117	.1148 .1133	.1170 .1122		
			Sample #3		
1	.1549 .1558	.1525 .1528	— —	.1536	.1526
2	.1542 .1576	.1528 .1522	— —		
			Sample #4		
1	.2127 .2122	.2119 .2118	.2120 —	.2110	.2122
2	2112 .2116	.2130 .2122	.2028 —		
			Sample #5		
1	.6549 .6543	.6528	.6552 .6561	.6519	.6529
2	.6485 —	.6488	.6437 .6543		

those cases where the presence of iodide in the sample makes ashing procedures inapplicable.

DETERMINATION OF CHLORIDE IN THE PRESENCE OF LARGE AMOUNTS OF BROMIDE AND/OR IODIDE

Since the most usual case of mixed halides encountered in drug analysis is the presence of a small amount of chloride which enters the product as

an impurity of the therapeutic agent or other ingredients, a method for this determination seemed desirable. This is particularly true with products containing large doses of bromides, since USP bromides may contain as much as 1 or 2 per cent of chloride (Cl^-), and the best reagent quality may contain as much as 0.2–0.5 per cent.

After preliminary trials, the method reported by McAlpine[1] (*cf.* Berg,[2]) was sent out, substantially as published, to a few collaborators with samples of USP and ACS bromides and iodides. The results reported are tabulated below:

TABLE 2.—*Chloride in presence of bromide and iodide*

COLLABORATOR	SAMPLE #1	SAMPLE #2	SAMPLE #3	SAMPLE #4
		(*Mg chloride/3 gm*)		(*Mg Cl/20 ml*)
Banes, Chicago	37.2			
	38.6	11.0		71.6
	38.1	17.1		72.8
Shuman, Phila.	25.2	10.8	1.5	49.6
	24.5	8.2	1.3	36.6
McClellan, N. O.	33.6	20.9	.12	57.2
	27.3	22.8	.27	82.0
	63.9	48.6		43.4
	31.2	71.7		49.4

In view of the difficulties encountered by some of the analysts and the erratic results obtained, modification of the method was attempted. This method depends on the reaction between acetone and free halogen to form halo-acetone according to the sense of the following series of equations:

(1) $$10X^- + 2(MnO_4)^- + 16H^+ \rightarrow 5X_2 + 2Mn^{++} + 8H_2O$$

(2) $$X_2 \rightleftarrows X^- + X^+$$

(3) $$X^+ + CH_3COCH_3 \rightleftarrows C_3H_5OX + H^+$$

Since only half of the free halogen formed by reaction (1) is utilizable as X^+, more oxidant must be added so that from this standpoint equations (1) and (2) might be combined as:

(4) $$X^- + 2 \oplus \cdots X^+$$

Under the conditions here used Cl^- (chloride) is not affected and neither X^+ nor halo-acetone reacts with silver nitrate.

[1] Mc Alpine, *J. Am. Chem. Soc.*, **51**, 1065 (1929).
[2] Berg, *Z. Anal. Chem.*, **62**, 342 (1926).

The principal difficulty with the procedure, as sent out for study, appears to be due to the reversibility of reactions (2) and (3), and to the fact that the excess of hydrogen peroxide added to destroy the excess potassium permanganate slowly reduces the X^+ to X^-. I^+ appears to be the more susceptible to this reduction. Decreased temperature and low concentration of both hydrogen peroxide and X^+ retard this reduction.

It has been found that, in the presence of ether, the chloride of silver is sufficiently agglomerated to permit filtration within one minute after the addition of the silver nitrate.

Consideration of equation (3) leads to the conclusion that a large excess of acetone and a low acidity would tend to retard the reverse reaction and so decrease the concentration of the X^+. However, too low an acidity slows up the potassium permanganate oxidation so that a practical balance must be drawn between these two desiderata. McAlpine (*loc. cit.*) calls for a solution 11 *N* with respect to sulfuric acid. Decreasing the amount of sulfuric acid so that the solution is 1.25–1.5 molar in this respect, gives much more satisfactory results. It not only retards the precipitation of silver bromide and silver iodide; but also greatly increases the oxidizing efficiency of the potassium permanganate with iodide and decreases the precipitation of manganese dioxide.

The details of the following method, which is the one now being used, have been worked out from the foregoing considerations; and it is believed that satisfactory results will be obtained by its use.

METHOD

CAUTION: The halogen acetone compounds produced in this procedure are extremely irritating to the eyes and respiratory tract. All operations should be performed under hood with good draft and care taken to avoid getting these vapors into the eyes or inhaling them.

Select a sample containing preferably not more than 1.5 milli-moles of Cl^- and not more than 5 g total halides. If organic matter is present, ash at dull red heat with an excess of calcium acetate.

Place sample in a 200–300 ml flask, add water to make a volume of ca 50 ml, 10 ml $MnSO_4$ soln (5 g/100 ml), and 20 ml dil. H_2SO_4(1+1). Add in small portions and with swirling to permit solution and reaction, an amount of solid $KMnO_4$ equal to 50–100 mg less than the quantity calculated to be necessary to convert all the Br^- and I^- to the free halogen; or, make a trial titration with 5% $KMnO_4$ soln and determine the amount of solid $KMnO_4$ to be used from this result. Cover the flask with a watch-glass, heat to boiling, and simmer slowly until most of the free halogen has been volitalized and only a light yellow color remains. Cool the soln, add ca 10 ml of acetone (free of reducing substances), dilute to 100 ml, and place in an ice-water bath. Add, dropwise, 5% $KMnO_4$ soln until the color of the soln changes thru amber to a deep wine or coffee color. Allow to remain in the ice bath about 15 min., adding more $KMnO_4$ from time to time, as the color fades.

Add 1% H_2O_2 soln, dropwise, slowly and with swirling to decolorize the soln and to dissolve any MnO_2 but avoiding any appreciable excess and keeping the liquid as cold as possible.

At once add 10 ml dil. HNO_3 (1+1), 10 ml ether, and then $AgNO_3$ soln (ca 0.1 *N*) with swirling until the opalescence decreases and the AgCl agglomerates, and

then about 5 ml more (a total of 10–20 ml). Swirl ca 1 min and immediately filter thru a tared Gooch crucible. Wash the flask and ppt thoroly with water, then with a little alcohol, and finally with ether. Dry at 110°C., cool, and weigh.

$$1 \text{ g } AgCl = 0.2474 \text{ g } Cl^-$$

NOTE 1: A blank should be determined using all reagents, except the solid $KMnO_4$, in the amount used in the sample determination unless previous blanks on the same lot of chemicals have shown their chloride content to be negligible.

NOTE 2: If no opalescence appears in the filtrate within a half hour, add to it a few drops of $AgNO_3$. If a precipitate then forms, repeat the determination using more $AgNO_3$ or a smaller sample.

The following table gives the results obtained by the authors on the collaborative samples, using three modifications of this method, including the one just given in detail.

TABLE 3.—*Mg of chloride per 3 g sample*

SAMPLE NUMBER	METHOD AS SENT TO COLLABORATORS	SAME, BUT FILTERED AT ONCE	METHOD AS REPORTED HEREIN	(BVMc)
(1) USPNaBr	22.8	29.7	27.3	22.3
	23.5	22.3		
(2) ACS	5.4			
KBr	5.2	5.8	6.8	18.2
Reagent KI		0.8	0.1	
		2.9	0.3	0.2
			0.2	
(4) (0.2 g KI; 0.1 g NaCl)	69.8	90.6	69.6	72.5
	61.2			75.1

These results indicate a need for some further study of the procedure, especially where iodide is concerned.

Using the method here given, filtrates containing mono-brom-acetone in the presence of excesses of both silver nitrate and hydrogen peroxide remained perfectly clear for 2–3 hours. Those containing the iodo-compound precipitated much sooner, but even these did not show even a slight opalescence until about 20 minutes after filtration.

PRELIMINARY STUDIES OF A RAPID, VOLUMETRIC METHOD FOR THE DETERMINATION OF IODIDE AND BROMIDE IN THE PRESENCE OF CHLORIDE

The aeration methods previously reported upon seem to give satisfactory results, but they are time-consuming and require an all-glass aeration and absorption apparatus which may not be readily available to all analysts. Therefore the search for a rapid, simple method was continued. Various authors have studied volumetric applications of the oxidation of

the halides to the +1 valent form (see equation 4 above). Among these, Andrews[3] used potassium iodate in strong hydrochloric acid solution to form iodine monochloride. Willard and Fenwick[4] used potassium permanganate in the presence of hydrocyanic acid to oxidize bromide to bromine cyanide with electrometric determination of the end point. Kolthoff[5] and Lang[6], using potassium iodate as oxidant, and Berg[7], using potassium bromate, have studied this halogen-cyanide reaction extensively. Since it appeared most promising, this reaction was selected for study in the present investigation. The method depends upon the oxidation of iodide and bromide in the presence of hydrocyanic acid according to equation (4) above and the following reaction:

$$X^+ + HCN \rightleftarrows XCN + H^+ \tag{5.}$$

During the oxidation, when both X^- and X^+ ions are present, equation 2 becomes evident. This, and the fact that Br^- is not effected as long as I^- is present, are utilized for the titration of I^-. As in the Andrews method (*loc. cit.*) chloroform (or in this case starch) is added at the beginning of the titration and the oxidant is added up to a disappearance of the iodine color.

The halogen cyanides appear to be more stable and the X^+ ions less readily reduced than is the case with the acetone compounds. Lang, Kolthoff, and Berg (*loc. cit.*) mention a large number of reducing agents which may be used to remove excess oxidant from the reaction mixture without affecting the halogen cyanide. These include hydrazine, phenol, aniline, oxalate, and ferrous ions.

In order to complete the oxidation of Br^- to bromine cyanide, a slight excess of oxidant is required, as well as a somewhat increased acidity. Since no visual indicator for this equivalence point has been found, the removal of the excess oxidant becomes essential. Then, when all the I^- and Br^- have been converted into the corresponding cyanides, the reverse of reactions (5) and (2) are utilized by adding potassium iodide and titrating the liberated iodine with standard thiosulfate.

$$2XCN + 2I^- + 2H^+ \rightarrow I_2 + 2X^- + 2HCN \tag{6.}$$

Chloride requires a very large excess of oxidant and a very high acid concentration before it is oxidized to cyanogen chloride and therefore does not interfere with the determination of iodide and bromide.

The principal difficulty found in applying the methods of Lang and Berg lay in the removal of the excess $(IO_3)^-$ or $(BrO_3)^-$ without some of the halogen from these compounds being captured, *en passent*, by the hydrocyanic acid. Using various reducing agents recommended by the

[3] Andrews, *J. Am. Chem. Soc.*, **25**, 756 (1903); *cf.* USP XIII, 273.
[4] Willard and Fenwick, *Ibid.*, **45**, 623 (1923).
[5] Kolthoff, *Mikrochemie*, **3**, 75 (1925).
[6] Lang, *Z. anor. allgem. Chem.*, **122**, 332 (1922); **142**, 229, 279 (1925); **144**, 75 (1925).
[7] Berg, *Z. Anal. Chem.*, **69**, 1, 369 (1926).

aforementioned authors, residual oxidation blanks varying between .05 and .3 ml. of 0.1 *N* thiosulfate per ml of *M*/40 oxidant were obtained. Ferrous salts were found to be unsatisfactory for the purpose, not because they reduced the X^+ ions, but because the ferric ion formed in reducing the excess oxidant, itself oxidized some of the I^- to I_2 in the highly acid solutions used (ca 2*N*).

Cerate oxidation was tried in order to eliminate halogen-containing oxidants and permanganate, which is notorious for its action on chloride. Cerate was found to be quite satisfactory after some trials and adjustments were made. The sulfuric acid solution of the USP (ca *M*/2 in sulfuric acid) reacted much too slowly with Br^- and this was still true when the sulfuric acid contents of both standard and solution being oxidized were increased to ca 2.5 *M*. However, in nitric acid solutions, 1.5–2 *M* in nitric acid, bromide was completely oxidized at a temperature of ca 45°C in 15–30 minutes. Nitric acid solutions of this strength have no effect on I^- even over considerable periods of time, provided that no nitrous acid or oxides of nitrogen are present. This can be accomplished by boiling the nitric acid down with a little water and then diluting with an equal volume of water.

Potassium iodide solutions titrated to iodine cyanide with cerate and then treated with an excess of the oxidant at 45°C for 30 minutes showed no appreciable increase in oxidizing value after reduction of the excess cerate.

0.3 g of sodium chloride treated in the same way showed a titration of only 0.1 ml N/10; 3 g sodium chloride in the presence of 0.1 g potassium bromide showed no appreciable difference in titration from 0.1 g potassium bromide treated in the same way with no Cl^- present.

In order to check the effect of different reducing agents on the halogen cyanide, a stock solution of bromine cyanide was prepared by oxidizing a quantity of reagent potassium bromide with the least possible excess of potassium permanganate and removing this excess with a minimum amount of hydrogen peroxide. After standing overnight, excesses of the reducing agents were added to identical portions of this stock solution, and the resulting mixtures were allowed to stand 15–30 minutes before adding potassium iodide and titrating. Among the substances tried were hydroxylamine, hydrazine, oxalate, hydrogen peroxide, and ferrous sulfate. The aromatic amines and phenols were not tried because of the colors produced by their oxidation. Of these, hydroxylamine proved far the most satisfactory. Two millimoles of this reagent added to the titration mixture containing bromine cyanide, produced, after 30 minutes, a decrease in titration of only 0.2 ml 0.1 *N* from that obtained on a similar solution to which no reducing agent had been added.

Hydroxylamine is also a convenient reducing agent to use in other phases of this investigation since an excess of this substance can readily

be eliminated, without affecting the state of other components, by boiling with excess sodium hydroxide, especially in the presence of a little alcohol. For example, 10 millimoles of hydroxylamine (as the sulfate) were added to a sodium carbonate solution of ca 0.2 g potassium iodide and a drop or two of bromine water. The solution was boiled for a few minutes and then 2 ml of saturated sodium hydroxide solution and 2 ml of alcohol were added and the boiling was continued for 10 minutes. After cooling and acidifying, an amount of free iodine equivalent to 0.25 ml 0.1 *N* was found. This small blank can probably be eliminated by protecting the alkaline solution from oxidation by the air.

This fact forms the connecting link between the hydroxylamine recovery procedure referred to in the first part of this paper, and the volumetric method here considered. It is thus possible to use the open Carius silver halide isolation and the hydroxylamine recovery procedure as preliminary preparations for the volumetric determination of iodide and bromide, in those cases where the presence of both iodide and organic matter make them necessary. They should also be used in the presence of iron, sulfur compounds, arsenic, or other inorganic oxidizing or reducing substances.

The method in outline form is as follows:

The cerate soln contains 63.5 g of ceric ammonium sulfate $(NH_4)_4Ce(SO_4)_4 \cdot 2H_2O$ dissolved in 200 ml dilute HNO_3(1+1) and enough water to make 1 liter.

The sample should contain from 0.5–2 millimoles of either I^- or Br^- and, preferably, not more than 2 millimoles total.

Iodine present.—In a volume of ca 70 ml, add 10 ml KCN soln (6.5 g/100 ml) and enough dilute nitric acid (1+1) to equal 20 ml per 100 ml. Add starch and titrate with standard cerate soln to the disappearance of the starch-iodine color.

Bromide present.—Use the soln titrated for iodide or add potassium cyanide and dilute nitric acid as in the preceding paragraph. Add the cerate soln in slight excess (the yellow color of the cerate is evident) and warm at 45°C. for 30 min. Cool, discharge the excess cerate with $NH_2OH \cdot H_2SO_4$ soln (8.2 g/100 ml). Add potassium iodide and titrate with 0.1 *N* sodium thiosulfate, adding starch near the end point if necessary.

The difference between the thiosulfate titration and the cerate titration for iodide is that due to bromide.

SUMMARY

Very satisfactory results are reported of the collaborative study of the modified open Carius method for total halides and of the hydroxylamine procedure for the recovery of the halides from the mixed silver precipitate. (Both previously published, *This Journal*, **25**, 836, 1942.)

A method for the determination of small amounts of chloride in the presence of large quantities of iodide and bromide was submitted to collaborative study. The results were very erratic and erroneous, but the method has now been revised and the procedure reported herein is believed to be reliable.

A rapid volumetric method for the determination of iodide and bromide in the presence of chloride has been studied on a preliminary basis and appears to hold much promise.

RECOMMENDATIONS*

It is recommended—

(1) That the revised acetone method for chloride in the presence of bromide and iodide be subjected to further collaborative study.

(2) That the volumetric method for iodide and bromide be studied in comparison with the aeration absorption method and that one or both methods be submitted to collaborators.

(3) That the modified open Carius method as published (*This Journal*, **25**, 836, 1942) be adopted as a tentative method for total halides, with the notation, "Not applicable to many organic halogen compounds."

Reagent (C) should be deleted from the list of reagents.

No report was given on organic iodides.

REPORT ON COMPOUND OINTMENT OF BENZOIC ACID[1]

By WILLIAM F. KUNKE (Food and Drug Administration, Federal Security Agency, Chicago, Ill.), *Associate Referee*

Collaborative work was undertaken this year as recommended by Subcommittee B, *This Journal*, **28**, 52 (1945). None has been done previously.

A brief review of the previous work by the Associate Referee has been reported. *This Journal*, **28**, 723 (1945). This included an experimental study of the quantitative bromination of salicylic acid, which was done because the literature is surprisingly meager in giving thorough and critical investigations. The most important experiments are given in Table 1, to show what the optimum conditions are, and how certain deviations from those conditions affect the quantitative results.

By the proposed method[2] the benzoic and salicylic acids are extracted together and titrated with 0.1 *N* sodium hydroxide; and after evaporation of the solvent (alcohol) the salicylic acid is determined bromometrically (benzoic acid does not consume bromine) and the benzoic acid is calculated by difference.

COLLABORATIVE SAMPLES

The benzoic acid and salicylic acid were separately and accurately weighed directly in a small vial and the approximately equivalent molten

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* For report of Subcommittee B and action of the Association, see *This Journal*, **31**, 47 (1948).

[1] National Formulary VIII: Ointment of Benzoic and Salicylic Acids.

[2] The details of the method are published in *This Journal*, **31**, 117 (1948).

TABLE 1.—*Bromination of salicyclic acid**

DETERMINATIONS	Br_2 IN EXCESS	HCl	REACTION PERIOD	SALICYLIC	
				MIN.	MAX.
	per cent	*ml*	*min.*	*per cent*	*per cent*
20	25	5	30	99.8	100.2
3	25	5	60	100.0	100.3
6	25	10	30	99.4	99.8
4	25	10	45	98.5	99.0
7	25	5	150	101.3	101.7
11	100	5	30	100.1	101.0
4	25	20	30	92.4	97.3
1	25	5	10	97.9	—
2	25	10	10	98.6	98.8
2	25	20	10	88.4	92.8
1	1.3	5	30	97.3	—
1	1.3	12.5	60	95.0	—
2	1.3	5.0	1	76.8	91.5

* Total volume in each determination 100 ml.

quantity of the (previously thoroughly mixed) ointment base was added. The collaborator was requested to use the entire contents of one vial for a determination. The preparation of the collaborative samples in this manner avoided the possibility of non-uniformity of a sample sufficiently large for all the collaborative work. It is well-known that an ointment of uniform consistency is not readily prepared by hand; furthermore, an increase in temperature will cause partial segregation.

According to the National Formulary VIII the ointment contains 12 per cent of benzoic acid and 6 per cent of salicylic acid incorporated in a white petrolatum and wool-fat base.

TABLE 2.—*Collaborative results*

ANALYST	BENZOIC ACID		SALICYLIC ACID	
	gram	*per cent*	*gram*	*per cent*
Conroy	0.2962	99.3	0.1589	99.5
	0.2952	99.0	0.1593	99.8
O'Keefe	0.2926	98.1	0.1580	99.0
	0.2924	98.1	0.1588	99.5
McNair	0.2911	97.6	0.1606	100.5
	0.2904	97.4	0.1614	101.0
	0.2906	97.4	0.1592	99.7
Fine	0.2895	97.1	0.1617	101.2
	0.2932	98.0	0.1604	100.4
Hyatt	0.289	97.0	0.157	98.3
	0.292	98.0	0.157	98.3

RECOMMENDATIONS*

It is recommended—

(1) That the proposed method be adopted as tentative.

(2) That no further work be done.

No reports were given on alkali metals or on glycols and related compounds.

REPORT ON PRESERVATIVES AND BACTERIOSTATIC AGENTS IN AMPUL SOLUTIONS

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

The recommendations of the previous report[1] on this topic were followed in that attempts were made to find some qualitative method for the detection of the mercurial type of preservative. In place of having individual tests for the several preservatives, the problem resolved itself into the detection of a small amount of organic mercury. One method was tried with considerable promise, using dithizone as the ultimate reagent for mercury. However, this method was not studied objectively enough to justify its incorporation into this report.

It is recommended* by the Associate Referee that the work be continued.

No report was given on phosphorus, calcium, and iron in vitamin preparations; see report of Referee on miscellaneous drugs.

REPORT ON IODINE

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

The present official method for iodine[2] gives low results on samples which contain small amounts of organic material. The loss of iodine occurs in such samples at the point where the procedure directs " . . . Neutralize filtrate with sulfuric acid (1+1), make alkaline again with 4 per cent sodium hydroxide solution, and add 1 ml. in excess . . . ." It was noted that free iodine was liberated as the sulfuric acid was added to the

* For the report of Subcommittee B and action of the Association, see *This Journal*, 31, 47 (1948).

[1] *This Journal*, 30, 486 (1947).

[2] *Methods of Analysis*, 6th Ed., sec. 39.202, page 728.

strongly alkaline solution. It was evident that during the charring some iodate must have been formed which reacted with the remaining iodide to liberate iodine.

EXPERIMENTAL

The Associate Referee tried two different procedures in order to obtain higher recoveries. The first was to omit the step beginning with " . . . Neutralize filtrate with sulfuric acid (1+1) . . . " and proceed with the oxidation with potassium permanganate in the strongly alkaline solution, instead of in the slightly alkaline solution specified. A potassium iodide solution of known concentration was assayed by the method as written and by the modification above. Recoveries were 92.8 and 94.5 per cent, respectively.

The second procedure was to add organic material (sodium benzoate) to the potassium iodide solution before charring. The solution, plus organic material, was assayed by the method as written and by the modification above. Recoveries were 98.4 and 96.3 per cent respectively.

All of the above determinations were charred in a furnace simultaneously at 500°C. and for the same length of time.

The following additional experiments were conducted:

(1) A known amount of potassium iodide and 3 g of potassium hydroxide were dissolved in sufficient water to equal the volume normally obtained in the filtrate from the charred mixture; and then the procedure specified in the official method was followed. Recovery was 99.95%.

(2) A known amount of potassium iodide and 3 g of potassium hydroxide were charred and the procedure specified in the official method was followed. The alkaline filtrate was chilled in an ice-bath and the sulfuric acid was added very slowly. Iodine color was noted in the soln on the addition of sulfuric acid. Recovery was 96.63%.

(3) A known amount of potassium iodide, 1 g of sodium benzoate, and 3 g of potassium hydroxide were charred at the same time as in (2) and the procedure specified in the official method was followed. There was no evidence of iodine color on the addition of the sulfuric acid. Recovery was 99.40%.

DISCUSSION

These experiments demonstrate that the low results obtained by the present official method on samples which contain small amounts of organic material can be corrected. The best way to correct these low recoveries seems to be by the addition of organic material to the sample before charring, which prevents the formation of iodate during the charring, with resultant loss of iodine when sulfuric acid is added. Direct oxidation in strongly alkaline solution results in only partial improvement of the recovery on samples of this nature.

It is recommended* that samples be submitted for collaborative study with instructions to make determinations by the present official method

* For report of Subcommittee B and action of the Association, see *This Journal*, 31, 47 (1948).

and by the proposed modified official method (addition of organic material before charring).

No reports were given on superheated steam in separation of drugs, on estrone and estradiol, or on chromatographic separation of drugs.

There was no report on drug bioassays, or an any of the included subjects.

REPORT ON SPECTROGRAPHIC METHODS

By W. T. MATHIS (Agricultural Experiment Station, New Haven, Conn.), *Referee*

No attempt has been made so far to enlist collaboration on spectrographic methods for the reason that, with the exception of analysis of plant materials, the work in our laboratory has been mainly exploratory, in an effort to find the most promising possibilities for use of the spectrograph on general analytical problems.

Some merely interesting, and some very good, results were obtained on many materials, such as ammonium acetate soil extracts for major and minor constituents; spray residue extracts for lead, copper, mercury; urine for lead; and biological and miscellaneous samples for semi-quantitative estimation of poisonous metals, etc. Much additional work will be required, however, to bring some of the procedures up to the point where they are ready for collaborative trial.

It would seem that some standardized procedure of electrode preparation, excitation, etc., could be made to cover a large portion of such analyses; and in this connection present quantitative technique employed in our laboratory is outlined and suggested for consideration. This technique, applicable to the determination of both major and minor constituents, was selected on the basis of better uniformity of results over other schemes that were tried.

Craters 5 mm in diameter and 6 mm deep are drilled in purified $\frac{1}{4}''$ graphite electrodes and are packed with the graphite removed in the drilling process. Solutions to be analyzed are added to the electrodes and allowed to soak into the packed carbon and dried in an oven.

Alternative preparation, when salts are extremely high, is to treat a weighed portion of the carbon drillings with a definite amount of the sample solution. The mixture is dried, mixed thoroughly, and a portion packed into the electrode crater.

High voltage A.C. spark, with inductance, is used for excitation, with the opposing electrode pointed. During a sparking time of 25 seconds the

impregnated carbon is sprayed from the crater, the particles burning in such a way that distillation variables encountered with D.C. arc excitation are apparently eliminated.

Cobalt is used as an internal standard and all intensity ratios are based on a single cobalt line, 3044 Å. On this basis the technique becomes applicable to many types of agricultural materials and in many cases the same analysis scale values may be used.

As some analyses are on the basis of "available" constituents, and some are on surface extracts, etc., we have followed wet preparation of all samples for the sake of uniformity in leading up to what we like to visualize as a reasonably "universal technique." Sample preparation in general is as follows:

Plant materials are ashed, dissolved in dilute hydrochloric acid, and evaporated to dryness.

Soil extracts are evaporated to dryness, etc.

Aliquots of spray residue extracts are evaporated to dryness.

Miscellaneous materials are treated appropriately and their solutions evaporated to dryness.

The salt residues from the above treatments are dissolved by warming with definite volumes of acid-cobalt solution, containing 150 ml of hydrochloric acid and 200 mg of cobalt per liter. Tenth milliliter portions are added to the prepared electrodes in duplicate and dried at 130° C.

Obviously, desired concentrations may easily be obtained by varying either the amount of sample or the amount of acid-cobalt solution. Our analysis scales (in per cent) are based on 1 gram of sample in 5 ml acid-cobalt solution, the usual concentration for plant material. In using the same analysis scales for soil extracts, where concentration of 20 grams in 1 ml acid-cobalt solution is desirable, the 1.0 per cent value on the analysis scale becomes 0.01 per cent, etc.

The acid-cobalt solution may also contain sodium or lithium if desired, to overcome the effect on other elements of extreme variations in major constituents of samples. An alternative to the use of such salt buffers is to determine these effects from appropriate standards and use correspondingly corrected analysis scales for the varying ranges of major constituents. This latter scheme has worked to a reasonably satisfactory degree in our laboratory, and has certain practical advantages over the use of salt buffers, particularly with regard to plant materials.

During the past year several hundred pasture grass and tobacco samples were analyzed both spectrographically and chemically in our respective laboratories, with very gratifying agreement in results. The determinations involved were potassium, calcium, magnesium, phosphorus, and manganese, and in almost every instance of an occasional original disagreement a second chemical result checked the spectrograph. On many samples additional determinations of iron, aluminum, zinc, sodium, copper, and boron were made spectrographically but not chemically.

As a typical example of uniformity between duplicate electrodes as run by this technique, we cite the following analyses of twelve tobacco samples recently run in our laboratory:

TABLE 1.—*Results on 12 tobacco samples*

	K	Ca	Mg	P	Mn	Fe
Percentage Range	4.04–5.54	3.50–4.15	.43–.80	.29–.40	.012–.027	.051–.139
Maximum deviation from average	.26	.18	.04	.015	.001	.005
in % of amount present	4.7	4.7	8.3	4.1	3.7	5.1
Average deviation from average	.10	.05	.015	.004	.0002	.0026
in % of average amount present	2.2	1.3	2.6	1.2	1.1	2.6

It would appear that this accuracy of *duplication* is reasonably within the limits of the average routine chemical work. The actual correctness of results, however, depends upon the standardization for the particular material.

This standardization for all types of plant material is suggested as an important subject for collaborative study, particularly if a technique is used which may be applied to extracts and materials in general. The information gained will then assume a general significance which will be very helpful in working with miscellaneous agricultural materials. It is probable that the following subjects, or at least some phases of them, will fit very nicely into this picture:

Soil extracts for available constituents.
Spray residues.
Foods and biological materials.
Fertilizer materials.

RECOMMENDATIONS*

It is recommended—

(1) That analysis of proper samples of various types of plant materials be made by collaborators using their own techniques, so that the most promising procedures may be indicated for further study and trial by all collaborators.

(2) That any subjects or determinations of particular interest, to which the spectrograph might be applied, be suggested for study.

No report was given on radioactivity (quantum counter).

* For report of Subcommittee B and action of the Association, see *This Journal*, 31, 45 (1948).

TUESDAY—AFTERNOON SESSION

REPORT ON MICROCHEMICAL METHODS

By C. O. Willits (*Referee*) and C. L. Ogg (Eastern Regional Research Laboratory* Philadelphia 18, Pa.)

There has been no report on microchemical methods since the one made in 1941 by the late E. P. Clark. Inasmuch as the recognition of microanalytical methods has steadily increased it is desirable that referee work leading to the standardization of these methods be resumed.

Since about forty quantitative microchemical methods are now in use, it seemed too arbitrary for the Referees to select the methods for study or to designate the methods to be studied first. Few, if any, of the laboratories equipped to perform microanalyses employ all the micro methods. It was not known what determinations each laboratory was making or was prepared to make, nor was it known which laboratories would be willing to participate in collaborative studies on microchemical methods. Consequently, a questionnaire was sent to microchemists throughout the country asking for their opinions on the following questions: (1) Should standardization of microchemical methods be attempted? (2) Which determinations should be standardized? (3) Which determinations should receive first attention? Also in the questionnaire were the questions: (1) Would you participate in collaborative studies? (2) What determinations are you performing in your laboratory?

More than seventy-three replies to the questionnaire have been received, and returns are still not complete. The replies have clearly shown that collaborative work on standardization of micro methods is endorsed by the microanalysts throughout the country. Of the seventy-three microchemists who replied, none were opposed to the studies, and only three were indifferent. Many not only answered the questionnaire but also endorsed the proposed work. The response to the question regarding willingness to cooperate in these studies has also been gratifying. Forty-seven expressed a desire to participate, and most of the twenty-six unable to collaborate stated that they were not able to do so because of the nature of their present work or lack of equipment or personnel.

To determine the geographic location of the microanalytical laboratories in this country, the replies received from the East, Midwest, and West were tabulated, with the following results: 70 per cent are in the East, 20 per cent in the Midwest, and 10 per cent on the West Coast.

The replies to the question regarding methods that should be standardized mentioned eighteen in addition to the eighteen listed in the questionnaire, making a total of thirty-six suggested. The ten determinations that

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

received the most votes for standardization were: Carbon and hydrogen (37), sulfur (37), Kjeldahl nitrogen (36), molecular weight (36), Dumas nitrogen (35), chlorine (30), bromine (27), iodine (26), acyl group (25), alkoxyl group (25). These were closely followed by phosphorus, hydroxyl group, neutralization equivalent, and oxygen. The remainder received 18 or fewer votes. A large percentage of the chemists who regularly perform a determination recommended standardization of the method.

The selections of methods to be studied first, second, third, and fourth were weighted on a point system by multiplying each first choice by 4, second by 3, third by 2, and fourth by 1. The ten determinations which received the largest number of points were: Carbon and hydrogen (114), Dumas nitrogen (69), Kjeldahl nitrogen (56), sulfur (39), chlorine (33), molecular weight (31), oxygen (19), bromine (13), iodine (12), and acyl groups (11).

In view of these replies to the questionnaire, the Referee and Associate Referee recommend† that collaborative studies be initiated on the methods for the determination of carbon and hydrogen, and the Dumas and Kjeldahl methods, for the determination of nitrogen.

REPORT ON STANDARD SOLUTIONS

By H. G. Underwood (Food and Drug Administration, Federal Security Agency, Chicago, Ill.), *Referee*

Standard Sodium Thiosulfate Solutions.—The method for the standardization of sodium thiosulfate solutions was adopted as official, first action, in 1945, on the basis of excellent collaborative results, and after the Associate Referee had demonstrated that the solutions are stable for long periods of time under practical conditions. It was decided in 1945 that further studies were unnecessary. The method has been in use for a sufficient period of time to warrant its adoption as official, final action.

Thiocyanate Solutions.—No formal report has been received on this subject in view of the recommendation last year that the study be closed. The method should be adopted as official, final action.

Potassium Permanganate Solutions.—After the Referee on synthetic drugs observed that the official A.O.A.C. procedure for standardizing potassium permanganate solutions yielded higher results than the procedure recommended by the National Bureau of Standards, this subject was reopened. In 1944, Mr. Duggan found that the titers obtained by the A.O.A.C. procedure were 0.2–0.4 per cent higher than were obtained by that recommended by the Bureau of Standards. During the past year Mr. Duggan and two collaborators confirmed this difference and found that the results obtained by the Bureau of Standards procedure compare

† For report of Subcommittee C and action of the Association, see *This Journal*, 31, 53 (1948).

favorably with those obtained by direct comparison with Bureau of Standards arsenious oxide, and by indirect comparison with Bureau of Standards potassium dichromate. Although until this year Mr. Duggan did not favor changing the A.O.A.C. method, he is now convinced that the method which is now official should be replaced by that recommended by the Bureau of Standards. On the basis of the additional data, the method recommended by the Bureau of Standards should be adopted as official, first action.

Bromide-Bromate Solutions.—Mr. Van Dame submitted to collaborative study the procedure reported in 1946 for the standardization of bromide-bromate solutions against Bureau of Standards arsenious oxide. The maximum variation from the average of 35 determinations made by seven collaborators was 2 parts in 1000, and that in only 2 determinations. On the basis of these results, the method should be adopted as official, first action, and the study closed.

Titanium Trichloride Solutions.—Miss Breit has continued the study of the three methods of standardization of titanium trichloride solutions reported on last year. On the basis of the results obtained by Miss Breit and one collaborator in the same laboratory, and because of the simpler procedure, Miss Breit favors the method of standardization by comparison with Bureau of Standards potassium dichromate, using diphenylamine as indicator; she recommends that the method be adopted as tentative. However, since titanium trichloride solutions are not stable and are difficult to handle, it is believed that collaborative studies should be made in more than one laboratory before adopting any one of the methods as tentative.

Potassium Dichromate Solutions.—No report was received on the subject. This study was recently re-assigned to Mr. McClellan of the Food and Drug Administration, New Orleans, La.

Standard Buffer Solutions.—Dr. Manov reports the preparation of larger quantities of pure buffer salts for certification by the National Bureau of Standards. He proposes to submit samples of standard buffer salts and unknown buffer solutions to a few laboratories for collaborative *p*H measurements. This subject should be continued.

RECOMMENDATIONS*

It is recommended—

(1) That the method for the preparation and standardization of sodium thiosulfate solutions described in *This Journal*, **25**, 659 (1942) be adopted as official, final action;

(2) That the method recommended by the National Bureau of Standards for the standardization of potassium permanganate solutions be

* For report of Subcommittee A and action of the Association, see *This Journal*, 31, 44 (1948).

adopted as official, first action, and that it replace the official method appearing in the Sixth Edition of the Methods of Analysis, p. 807.

(3) That the method for the preparation and standardization of bromide-bromate solutions be adopted as official, first action;

(4) That the methods for standardizing titanium trichloride solutions be studied further;

(5) That the study of the preparation and standardization of potassium dichromate solutions be continued;

(6) That the studies on buffer solutions be continued;

(7) That the method for the preparation and standardization of thiocyanate solutions be adopted as official, final action.

No report was made on potassium dichromate solutions, or on thiocyanate solutions.

REPORT ON STANDARDIZATION OF POTASSIUM PERMANGANATE SOLUTIONS

By R. E. DUGGAN (Food and Drug Administration, Federal Security Agency, New Orleans, La.), *Associate Referee*

In previous reports (1, 2) on the comparison of the official A.O.A.C. procedure (3) and the procedure of Fowler and Bright (4), recommended by the National Bureau of Standards, for the standardization of potassium permanganate solutions using sodium oxalate as the primary standard, the Associate Referee has stated that the titers obtained by the A.O.A.C. procedure are 0.2–0.4 per cent higher than titers obtained by the Fowler and Bright procedure, which, for the sake of convenience, will be referred to as the National Bureau of Standards procedure. The two procedures are as follows:

PROCEDURE I—A.O.A.C.

Weigh 0.25–0.30 g of Bureau of Standards $Na_2C_2O_4$ in sufficient H_2O to make soln ca 0.1 *N*. Add 15 ml of 4 N H_2SO_4 for each 50 ml of soln. Heat of 75–85° and titrate with the $KMnO_4$ soln, maintaining this temp. thruout titration. Add the $KMnO_4$ slowly, especially at beginning, and wait each time until soln becomes colorless. Continue titration to end point, with continuous stirring. Correct for excess of $KMnO_4$ used for end point by matching color in another beaker containing same quantity of acid and hot H_2O.

PROCEDURE II—N.B.S.

Transfer 0.3 g of sodium oxalate (dried at 105°C.) to a 600-ml beaker. Add 250 ml of diluted sulphuric acid (5+95) previously boiled for 10–15 min. and then cooled to 27 ± 3°C.

Stir until the oxalate has dissolved. Add 39 to 40 ml of 0.1 *N* potassium permanganate at a rate of 25 to 35 ml per min. while stirring slowly. Let stand until the pink color disappears (about 45 seconds). Heat to 55 to 60°C., and complete the

titration by adding permanganate until a faint pink color persists for 30 seconds. Add the last 0.5 to 1 ml dropwise with particular care to allow each drop to become decolorized before the next is introduced.

Determine the excess of permanganate required to impart a pink color to the soln. This can be done by matching the color by adding permanganate to the same volume of the boiled and cooled diluted sulphuric acid at 55 to 60°C. This correction usually amounts to 0.03 to 0.05 ml.

EXPERIMENTAL

A solution of approximately 0.1 *N* potassium permanganate, prepared according to the A.O.A.C. method, was standardized to National Bureau of Standards, Standard Sample No. 40C, sodium oxalate, by both of the above procedures. The same solution was then standardized to arsenicus oxide, and indirectly to potassium dichromate, by the following two procedures:

PROCEDURE III (5)

Aliquots of a standard soln of National Bureau of Standards Arsenious Oxide, Standard Sample No. 83, prepared according to the official A.O.A.C. procedure, (6) were diluted with 100 ml water and made ca *N* with hydrochloric acid. After the addition of 1 g of sodium chloride and 1 drop of M/400 potassium iodate soln (used as a catalyst), the mixture was titrated with the potassium permanganate to the first appearance of color. No difficulties were encountered in the titration. No perceptible lag was noted as the end point was approached, and a very sharp end point was obtained.

PROCEDURE IV (7,8)

Approximately 0.1 *N* sodium thiosulfate soln was standardized according to the A.O.A.C. procedure (9) using N.B.S. standard sample No. 136 potassium dichromate. Aliquots of the potassium permanganate soln were pipetted into a soln of 2 g of potassium iodide dissolved in 10 ml of 10% sulfuric acid. The soln was gently swirled during the addition of the permanganate soln, then diluted with 200 ml water. The released iodine was titrated immediately with the sodium thiosulfate soln, using starch as an indicator toward the end of the titration. Although this is an indirect method, it was found to be a very rapid determination and capable of giving very precise results.

Table 1 presents the results of the experiments outlined above, as well as the normality obtained for the permanganate solution by other collaborators using the N.B.S. procedure.

Although only purely volumetric methods were used in these experiments, the findings confirm those obtained by Fowler and Bright potentiometrically. The above results show that the titer obtained by the official A.O.A.C. procedure, using sodium oxalate as a primary standard, is higher than that obtained by procedures based on other primary standards. These results also show that the titer obtained by use of the N.B.S. procedure recommended by Fowler and Bright, yields normalities identical with those obtained by procedures based on other primary standards.

In addition, the results presented in the tabulation indicate that dupli-

cate normalities can be obtained by different chemists using the N.B.S. procedure. The Associate Referee does not believe that additional collaborative determinations are necessary. No additional work on this topic is being recommended at this time.

It is recommended* that the N.B.S. procedure (Procedure II in this report) be adopted as official, first action, and be substituted for the procedure currently designated as official for the standardization of potassium permanganate solutions.

TABLE 1.—*Apparent normality of potassium permanganate solution*
(Procedures I–IV)

COLLABORATOR	I	II	III	IV
	A.O.A.C.	N.B.S.	ARSENIOUS OXIDE	POTASSIUM DICHROMATE
G. McClellan	0.1073	0.1070	0.1070	0.1071
	0.1073	0.1071	0.1070	0.1071
				0.1071
Av.	0.1074	0.1071	0.1070	0.1071
R. E. Duggan	0.1074	0.1071	0.1072	0.1071
	0.1074	0.1073	0.1070	0.1070
	0.1075	0.1071	0.1071	0.1071
	0.1074	0.1070	0.1073	0.1071
	0.1074	0.1070	0.1071	0.1070
				0.1070
Av.	0.1074	0.1071	0.1071	0.1071
E. C. Deal		0.1070		
		0.1071		
Av.		0.1071		

ACKNOWLEDGMENTS

The Associate Referee expresses the greatest appreciation for the invaluable suggestions and collaborative aid of G. McClellan and E. C. Deal of the New Orleans Station.

REFERENCES

(1) *This Journal*, **28**, 596 (1945).
(2) DUGGAN, R. E., *Ibid.*, **30**, 499 (1947).
(3) *Methods of Analysis, A.O.A.C.*, 6th Ed., **43.18**, p. 807.
(4) FOWLER and BRIGHT, R. P. 843, J. Res., N. Bur. Standards, **15** (1935).

* For report of Subcommittee B and action by the Association, see *This Journal*, **31**, 44 (1948). The details of the method as adopted are given on page 118, *loc. cit.*

(5) KOLTHOFF and FURMAN, Vol. Anal., *II*, p. 286 (1929).
(6) *Methods of Analysis, A.O.A.C.*, **43.20**, p. 808.
(7) KOLTHOFF and SANDELL, "Text Book of Quantitative Inorganic Analysis," p. 596. The Macmillan Co., N. Y. (1936).
(8) HILLEBRAND and LUNDELL, "Applied Inorganic Analysis," p. 153, John Wiley & Sons, N. Y. (1929).
(9) *Methods of Analysis, A.O.A.C.*, 6th Ed., **43.29**, p. 810.

REPORT ON BUFFER SOLUTIONS

BY GEORGE G. MANOV (National Bureau of Standards, Department of Commerce, Washington, D. C.), *Associate Referee*

There was reported last year the preparation of moderate quantities of very pure buffer salts, the directions for their use, and the *p*H values of their solutions. Announcement was made of the availability of these buffers, with the result that the first lots were exhausted much sooner than could have been anticipated.

Operations were therefore renewed in 1947 on a scale ten times that previously employed. Specifications were prepared from which bids could be obtained from commercial sources on high-purity, 250-pound lots of each of the salts. The resulting purchases were carefully sampled and analyzed for homogeneity and *p*H value. Certification of the phosphate buffer is still in progress, but it is believed that there will soon be on hand sufficient quantities of the pure buffer salts to meet all reasonable demands for several years. The solid buffers can be used to prepare solutions having *p*H values at 25°C of 4.01, 6.86, and 9.18.

With this phase of the work virtually completed, it is hoped that attention may now be turned toward collaborative work on *p*H measurements. It is planned to furnish two standard samples of pure buffer salts and two solutions whose *p*H values are unknown to the collaborator. It is hoped by this means (a) to enable the analyst to determine whether his *p*H meter is in proper operating condition and to take corrective action if necessary, and (b) obtain information concerning the accuracy with which *p*H measurements can be made by different laboratories. This information should make it possible for other groups in the Association to determine the extent to which differences in *p*H obtained by various laboratories are significant, and whether they should be made the basis of rejection of purchases or of possible court action by regulatory agencies.

It is recommended* that the study on buffer solutions be continued.

* For report of Subcommittee A and action of the Association, see *This Journal*, 31, 44 (1948).

REPORT ON BROMIDE-BROMATE SOLUTION

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

The collaborative work on standard bromide-bromate solutions recommended in the previous report on this subject (*This Journal*, **30**, 502 (1947) has been completed.

Details of the method for the preparation and standardization of bromide-bromate solutions are published in *This Journal*, **31**, 119 (1948), under "Changes in Methods of Analysis."

Collaborators were sent a standard arsenite solution whose normality at 20°C. was 0.11259 and an unknown standard bromide-bromate solution. They were asked to standardize the bromide-bromate solution against the standard arsenite solution and also against a standard arsenite solution which they prepared. There follows the report of the collaborators. All normalities were corrected to 20°C.

COLLABORATOR	NORMALITY OF BROMIDE BROMATE SOLUTION AT 20°C.	
	USING As_2O_3 SENT BY ASSOCIATE REFEREE	USING COLLABORATORS As_2O_3
1	0.1086, 0.1087, 0.1088	0.1088, 0.1088, 0.1087
2	0.1087, 0.1087, 0.1087	0.1088, 0.1089, 0.1089
3	0.1086, 0.1086, 1.1086	0.1087, 0.1087
4	0.1088, 0.1087	0.1086, 0.1086
5	0.1087, 0.1087, 0.1087	0.1086, 0.1087, 0.1087
6	0.1088, 0.1088, 0.1087	0.1088, 0.1087, 0.1088
7	0.1087, 0.1087	
Average of all determinations = 0.1087		

A standard bromide-bromate solution was standardized at various times over a year's period by the Associate Referee to determine its stability. The normality varied only 1 part in 1000.

CONCLUSIONS

The results on the standardizations by collaborators are in good agreement. The maximum variation from the average normality being only 2 parts in 1000 and that in only 2 of the 35 titrations made. The average variation is less than 1 part in 1000. The solution prepared as described and kept in the dark is stable and maintains its normality for a year or longer.

ACKNOWLEDGMENT

The Associate Referee wishes to thank the following members of the United States Food and Drug Administration for their cooperation as collaborators: John F. Weeks, Jr., New Orleans, Louisiana; Floyd E. Yarnell, Kansas City, Missouri; Gloria Getchell, Joyce M. Merting, and

William Horwitz, Minneapolis, Minnesota; Daniel Banes, Chicago, Illinois; James H. McGee, St. Louis, Missouri; and Sam D. Fine, Cincinnati, Ohio.

RECOMMENDATIONS*

It is recommended that the method of preparation and standardization described in this report be adopted as official, first action, and that the subject be closed.

REPORT ON STANDARDIZATION OF TITANIUM TRICHLORIDE

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

The work of the Associate Referee was concerned with a further study on the preparation and standardization of titanium trichloride using three following methods:

(1) Standardization of titanium trichloride using Method II, *Methods of Analysis, A.O.A.C.*, 6th Ed. (1945), **21.37** (p. 290).

(2) *Ibid.*, substituting potassium dichromate for potassium permanganate.

(3) Standardization of titanium trichloride using potassium dichromate and diphenylamine indicator.

PREPARATION OF SOLUTIONS

Titanium trichloride.—A 15–16 per cent solution of technical grade titanium trichloride was thoroughly mixed and transferred to separate 50 ml. glass-stoppered flasks, which were then sealed with paraffin. This was done to insure the use of a uniform solution in the event samples were submitted for collaborative work; however, on the basis of results obtained in the standardization it was considered inadvisable to send out collaborative samples at this time. To the titanium trichloride contained in one flask (the weight of the chloride being obtained by difference) were added ca 40 ml of hydrochloric acid and the mixture diluted at 20°C to 500 ml with recently boiled distilled water. The solution was placed in a dark container with hydrogen atmosphere provision[1] and allowed to stand at least two days to permit the absorption of residual oxygen. All connections of the apparatus were sealed with lacquer.

REAGENTS

Diphenylamine indicator.—One g of diphenylamine was dissolved in 100 ml of sulfuric acid.

Potassium dichromate.—A standard soln was prepared by using about 5 g of

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* For report of Subcommittee A and action of the Association, see *This Journal*, **31**, 44 (1948).

[1] *Methods of Analysis, A.O.A.C.*, 6th Ed. (1945), p. 289, Fig. 27.

U. S. Bureau of Standards potassium dichromate previously dried at 120°C. The normality was calculated at 20°C.

Potassium permanganate.—A standard solution was prepared using the official A.O.A.C. method.

PROCEDURE

1. *Methods of Analysis, A.O.A.C.*, 6th Ed. (1945), **21.37,** Method II.
2. *Ibid.*, substituting 0.1 *N* $K_2Cr_2O_7$ for 0.1 *N* $KMnO_4$.
3. Into a 500 ml flask put 50 ml of recently boiled H_2O and 25 ml of 40% (by weight) H_2SO_4. Pass a strong stream of CO_2 into the soln and add 40 ml of 0.1 *N* $K_2Cr_2O_7$. Then titrate the dichromate with $TiCl_3$ without interrupting the flow of CO_2. At the appearance of a green color add a few drops of diphenylamine indicator and continue the titration until the purple color just disappears. (In several of the titrations the indicator was added prior to the addition of $TiCl_3$. The results seemed to indicate that the diphenylamine may be added at any time)

In the results given in Table 1, the titanium trichloride was prepared by J. E. Breit using 69.1260 g (ca 50 ml) per 500 ml. It was allowed to stand 90 hours before being used for the standardization. For the results

TABLE 1.—*Results—three methods*

COLLABORATOR	METHOD	ML $KMnO_4$	ML $TiCl_3$	NORMALITY $TiCl_3$
Breit	1	40.00 (*N* 0.1038)	28.08	0.1479
Van Dame	1	40.00 ml $K_2Cr_2O_7$	28.02	0.1482
Breit	2	40.00 (*N* 0.1000)	26.90	0.1487
Breit	2	40.00	26.95	0.1484
Breit	3	40.00	26.55	0.1507
Breit	3	40.00	26.60	0.1504
Van Dame	2	40.00 (*N* 0.1014)	27.25	0.1489
Van Dame	2	40.00	27.28	0.1487
Van Dame	3	40.00	26.83	0.1512
Van Dame	3	40.00	26.85	0.1511

TABLE 2.—*Results—potassium dichromate*

COLLABORATOR	METHOD	ML $K_2Cr_2O_7$	ML $TiCl_3$	NORMALITY $TiCl_3$
Breit	2	40.00 (*N* 0.1000)	25.85	0.1547
Breit	2	40.00	25.83	0.1548
Breit	3	40.00	25.45	0.1572
Breit	3	40.00	25.42	0.1573
Van Dame	2	40.00 (*N* 0.1014)	26.10	0.1554
Van Dame	2	40.00	26.15	0.1551
Van Dame	3	40.00	25.84	0.1570
Van Dame	3	40.00	25.86	0.1569

obtained for Table 2, the titanium trichloride was prepared by H. C. Van Dame, using 70.3002 g per 500 ml. This solution was made 65 hours prior to use. For the determinations each analyst made and used his own 0.1 *N* potassium dichromate.

In Table 3 are given the results of a series of titrations made to determine the stability of titanium trichloride. This solution was prepared on

TABLE 3.—*Results on stability*

METHOD	DATE	ML $KMnO_4$ (*N* 0.1049)	ML $TiCl_3$	NORMALITY $TiCl_3$
1	8/ 5/47	30.00	32.75	0.0961
1	8/ 5/47	30.00	32.75	0.0961
1	8/ 6/47	30.00	32.75	0.0961
1	8/ 6/47	30.00	32.78	0.0960
1	8/ 8/47	30.05	32.80	0.0961
1	8/ 8/47	30.05	32.80	0.0961
1	8/15/47	30.00	32.83	0.0959
1	8/15/47	30.00	32.85	0.0958
		ml $K_2Cr_2O_7$ (*N* 0.1036)		
2	8/ 5/47	30.00	32.32	0.0962
2	8/ 5/47	30.00	32.28	0.0963
2	8/ 6/47	30.00	32.30	0.0962
		ml $KMnO_4$ (*N* 0.1049)		
2	8/ 6/47	32.00	34.50	0.0961
2	8/ 8/47	30.00	32.30	0.0962
2	8/ 8/47	30.00	32.30	0.0962
2	8/15/47	30.00	32.40	0.0959
2	8/15/47	30.00	32.35	0.0961
2	8/29/47	30.00	32.53	0.0955
2	8/29/47	30.00	32.55	0.0955
3	8/ 5/47	30.03	31.80	0.0978
3	8/ 5/47	30.00	31.78	0.0978
3	8/ 6/47	30.02	31.82	0.0977
3	8/ 6/47	30.15	31.95	0.0978
3	8/ 8/47	30.00	31.78	0.0978
3	8/ 8/47	30.00	31.80	0.0977
3	8/15/47	30.00	31.85	0.0976
3	8/15/47	30.00	31.83	0.0975
3	8/29/47	30.00	32.00	0.0971
3	8/29/47	30.00	32.05	0.0970

August 4, 1947, by adding 90 ml of hydrochloric acid to ca 80 ml of titanium trichloride and diluting with water to 1200 ml. In the titrations, 50 ml of ordinary 10 per cent sulfuric acid were substituted for the 50 ml of recently boiled water and 25 ml of 40 per cent sulfuric acid.

On the basis of results obtained it seemed inadvisable to send out, at the present time, samples for collaborative work; however, to obtain some data on the reliability and accuracy of Method 3, four chemists of the Food and Drug Administration, Cincinnati, Ohio, standardized the same

titanium trichloride solution, each using a standard potassium dichromate solution he himself had prepared. Results are given in Table 4. The titanium trichloride solution stood for 48 hours before being used and was prepared by the same method given in obtaining the results in Table 3.

TABLE 4.—*Comparable results, Method 3*

COLLABORATOR	ML N 0.1 $K_2Cr_2O_7$ (F=1.0025)	ML $TiCl_3$	NORMALITY $TiCl_3$
R. Hyatt	30.00	30.75	0.0978
R. Hyatt	30.00	30.72	0.0979
F. J. McNall	30.00	30.85	0.0975
F. J. McNall	30.00	30.90	0.0974
H. C. Van Dame	30.00	30.75	0.0978
H. C. Van Dame	30.00	30.75	0.0978
J. E. Breit	30.00	30.70	0.0980
J. E. Breit	30.00	30.70	0.0980

CONCLUSIONS

(1) That a solution of titanium trichloride is not stable.

(2) Method 3 gives a higher normality to a solution of titanium trichloride than Methods 1 and 2; however, results are consistent and can be duplicated by different chemists. Moreover, it involves the use of only two outside reagents, a primary standard, potassium permanganate, and an indicator, diphenylamine.

RECOMMENDATIONS*

It is recommended—

(1) That further study be made on the stability of titanium trichloride.

(2) That collaborative work be done on the standardization of titanium trichloride.

REPORT ON COSMETICS

By G. R. CLARK (Food and Drug Administration, Federal Security Agency, Washington, D.C.), *Referee*

The Referee makes the following recommendations:†

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

Alkalies in Cuticle Removers
Cosmetic Powders

* For report of Subcommittee B and action of the Association, see *This Journal*, 31, 44 (1948).
† For report of Subcommittee B and action of the Association, see *This Journal*, 31, 47 (1948).

Cosmetic Skin Lotions
Deodorants and Antiperspirants
Depilatories
Hair Dyes and Rinses
Hair Straighteners
Mercury Salts in Cosmetics

The Referee concurs in the following recommendations:†

The recommendation of Charles F. Bruening, Associate Referee on cosmetic creams, that the proposed periodate and acidimetric methods be adopted as tentative for the determination of glycerol, and that the study be continued.

The recommendation of J. F. Weeks that the study of moisture in cosmetics be continued.

The recommendation of C. R. Joiner that study of the problem of pyrogallol in hair dyes be continued.

The recommendation of Paul W. Jewel that study of mascaras, eyebrow pencils, and eye shadows be continued.

REPORT ON PYROGALLOL IN HAIR DYES

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

In a report last year (1) the Associate Referee recommended that further work be done on the extraction of pyrogallol from henna powder-pyrogallol mixtures. An outline of a method used for separating the pyrogallol from the henna was given. The final determination of the pyrogallol was made by the colorimetric method which has been adopted as tentative (2). The major difficulties encountered were incomplete recovery of pyrogallol from mixtures several months old and high blanks on henna powder.

A series of six samples were prepared and analyzed over a period of four to five months. Two samples were made from each of two different lots of henna, and for the other two, filter cel was substituted for the henna powder. Pyrogallol was mixed with filter cel in order to compare its stability when mixed with an inert material with its stability in henna mixtures. Copper sulfate and burnt sienna were added to one of the henna samples in order to prepare a mixture representative of one type of commercial preparation. Lawsone,[1] or 2-hydroxy-1, 4-naphthoquinone, the active constituent of henna for hair-dyeing purposes (3), was added to the extent of about one per cent to one of each of the henna and filter cel samples. If lawsone were the material in the henna which reacts with pyrogallol, then the addition of more lawsone should increase the rate of loss of pyro-

[1] The lawsone was synthesized from β-naphthol in four steps as given in *Organic Syntheses*, 11, 12 (1931); 21, 56 and 91 (1941); Collective Volume I, 411 (1941).

gallol from the mixture. Sixty-mesh pyrogallol was used in all samples, and all were mixed for six to eight hours.

At the end of five months, analysis of two of the samples made from one lot of henna showed a loss of approximately five per cent of the pyrogallol content. One of these samples contained added lawsone, and there was no significant difference in the loss from the two. The other two henna samples showed no significant loss of pyrogallol after four months. The two filter cel samples, after five months standing, showed approximately 30 and 40 per cent losses of pyrogallol, with the sample containing the lawsone having the smaller loss. These two samples had turned noticeably gray within one week after they were prepared, and the gray color developed more slowly in the one containing the lawsone.

Analysis of a two-year old henna-pyrogallol mixture showed a loss of about 20 per cent of the original pyrogallol content.

The analyzing of all these samples at intervals will be continued, and a complete tabulation of the results will be given in next year's report.

An attempt was made to prove that some of the pyrogallol had undergone a reaction, and had not, through a physical change, become merely difficult to extract. Absorption curves of ethyl acetate extracts of a two-year old henna-pyrogallol mixture and the henna from which the mixture had been prepared were made with a Beckman spectrophotometer. The two curves were parallel and almost identical except in the range from 450 to 500 millimicrons, where there was a pronounced divergence, with the sample extract showing the greater absorption. Further work will have to be done before any conclusions can be reached.

The problem of lowering the blank is an important one since there is no simple method of evaluating it for any given sample. The variability of the blanks on different samples of henna (0.20 to 0.36 per cent) precludes the possibility of including in the method an arbitrary blank correction.

Substituting mixtures of ethyl acetate and anhydrous ether in varying proportions for the ethyl acetate used in the initial extraction lowered the blanks roughly in proportion to the percentage of ether used. The addition of 15 per cent ether by volume resulted in a 60 per cent reduction in the blank, but it also slowed up the rate of extraction of the pyrogallol to such an extent as to make it impractical to use in routine determinations, and possibly reduced the total amoung of pyrogallol recoverable. An eight-hour extraction gave 75 per cent of the recovery obtained with ethyl acetate alone.

In the method as previously outlined (1), the ethyl acetate extract was evaporated to dryness, and the residue was taken up in water and given further treatment. When the dry residue was treated with anhydrous ether, the ether filtered and evaporated to dryness and this residue taken up in water, the blanks were reduced to about 10 to 35 per cent of their

former values. Blanks obtained by this method were low enough to be disregarded, but the additional treatment caused a loss of approximately 10 to 15 per cent of the pyrogallol present. Boiling the ether with the residue increased the recovery of pyrogallol only slightly.

By substituting ethyl acetate for the anhydrous ether in the treatment of the residue as given above, the blanks were reduced to 50 to 70 per cent of their former values. In a limited number of determinations, this additional treatment with ethyl acetate caused no loss of pyrogallol. The maximum blank obtained on three different henna samples was 0.20 per cent.

DISCUSSION

Dry pyrogallol, protected from light, is very stable. However, an aqueous solution in the presence of air begins to develop a yellow-brown color almost immediately. In solution pyrogallol undergoes many different types of oxidation reactions, such as: 1. To purpurogallin by sodium nitrite in acetic acid solution (4). 2. By hydrogen peroxide to purpurogallin in the presence of low concentrations of tungstic, molybdic, or vanadic acids (5). 3. In barium hydroxide solution to hexahydroxybiphenyl (6). 4. In ammoniacal silver nitrate solution to oxalic and acetic acids (7). In view of the reactive nature of pyrogallol, it is possible that, when mixed with powdered henna, it might slowly react with one or more of the constituents of the henna. Cox (3) reports that a typical specimen of henna contains, among other things: Moisture, 8.4%; sugars, as dextrose, 11.3%; gallic acid, 6.0%; hydroxynaphthoquinone (Lawsone), 1.0%. This moisture content might be sufficient to catalyze a slow oxidation reaction of added pyrogallol.

The low recoveries of pyrogallol obtained when anhydrous ether was used as a solvent might have been caused by the presence of peroxides in the ether which oxidized some of the pyrogallol. None of the ether used was tested for peroxides.

Since gallic and tannic acids both develop a blue-violet color with ferruous tartrate (8), the presence of either one in henna could account for the high blanks reported above. Alcohol, acetone, and water extracts gave very high blanks, while ether extracts were practically zero. These facts, when considered with the published solubilities of gallic acid and tannin in these solvents, indicate that tannic acid, and not gallic acid, is present in henna. However, this is contrary to the findings of Cox (3), who reported a complete absence of tannins in all samples of henna tested by him.

SUMMARY AND RECOMMENDATIONS*

Results of some investigational work indicate that pyrogallol might undergo a reaction on long standing when mixed with henna powder. The high blank obtained from an ethyl acetate extract of henna is

* For report of Subcommittee B and action of the Association, see *This Journal*, 31, 47 (1948).

caused by either tannic or gallic acid in the henna. Methods involving the use of anhydrous ether as a solvent result in lower blanks, but also give low recoveries of pyrogallol. One method by which the blank is materially decreased without lowering the recovery of pyrogallol is given.

It is recommended that the analyses of the series of samples reported on above be continued, and that the details of the method of extraction and subsequent treatment of the extract that gave the best results be worked out and the method submitted to collaborative study.

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REPORT ON COSMETIC CREAMS

DETERMINATION OF GLYCEROL IN VANISHING CREAMS

By CHARLES F. BRUENING (Food and Drug Administration, Federal Security Agency, Baltimore, Md.), *Associate Referee*

In the Associate Referee's report of last year,[1] a collaborative study was made of the determination of glycerol in vanishing cream, using the Malaprade periodate oxidation method. Glycerol, after isolation by an extraction procedure and oxidation with potassium periodate, was determined by two methods. In the first method the amount of periodate consumed was iodometrically determined; and in the second, the amount of formic acid obtained in the oxidation was determined by titrating with alkali using methyl red indicator and applying a correction for the acidity of the potassium periodate solution used. Nine collaborators who analyzed a vanishing cream containing a known amount of glycerol by the periodate consumption method obtained an average recovery of 100% glycerol with all results falling in the range $100\% \pm 2.3\%$. Using the formic acid titration method and applying the correction the same collaborators obtained an average recovery of glycerol of 96.4% with a range of $96.4\% \pm 3.4\%$. The periodate consumption method was concluded to be satisfactory, but the formic acid titration method was considered unsatisfactory because of the low yield and difficulties encountered in determining the

[1] *This Journal*, **30**, 507 (1947).

end points in both the formic acid titration and the acidity correction for the potassium periodate solution.

A study of the formic acid method was made by Newburger and Bruening[2] and a satisfactory modified titration method for determining glycerol developed. In this method the excess of periodate, which interferes with the formic acid titration, was destroyed by using propylene glycol, and bromcresol purple indicator was substituted for methyl red. It was also shown that no correction need be made for the acidity of the potassium periodate solution. This modified method appeared promising and was submitted to collaborative study. A sample of vanishing cream of the following composition was sent to collaborators.

COMPOSITION OF VANISHING CREAM

	per cent
Stearic Acid	20.93
Water	67.83
Glycerol	10.09
Potassium Hydroxide	1.05
Propyl p-Hydroxy Benzoate	.10

The method used by the collaborators is given in detail in *This Journal*, **31**, 72 (1948).

With this vanishing cream it was suggested to the collaborators that it was convenient to use a 2 gm sample and then take a 50 ml aliquot for oxidation from the 250 ml flask. This would provide about 40 mg of glycerol. Generally, the aliquot selected should contain 30 to 40 mg of glycerol so that appropriate titrations would result in the formic acid titration method and glycerol can be determined, if desired, on the same aliquot using the periodate consumption method described in the last year's report. The suggestion was also made for this individual sample, since only formic acid titrations were to be made, that a 110 ml volumetric flask be substituted for the 100 ml flask in the oxidation, so as to permit the removal of two 50 ml aliquots for the acid titration.

As indicated in last year's report, the potassium periodate solution is subject to decomposition on standing and decreases in strength. The dry reagent grade salt is stable, and freshly prepared solutions can be used without standardizing if only the formic acid titration is desired. If, however, the periodate solution has been allowed to stand for some time, it is necessary to standardize it as described in the periodate consumption method, in order to assure that sufficient periodate is present to completely oxidize all the glycerol contained in the aliquot. Alternatively, a preliminary test may be made to assure that excess periodate remains in the oxidation mixture by adding to a test portion an excess of sodium bicarbonate and a small amount of potassium iodide. The liberation of iodine indicates excess periodate.

[2] *Ibid.*, **30**, 651 (1947).

TABLE 1.—*Recovery of glycerol in vanishing cream*
(10.09% Glycerol)

COLLABORATOR	GLYCEROL FOUND	GLYCEROL RECOVERY
	per cent	*Average Per cent*
1	9.87	98.0
	9.90	
2	9.96	98.8
	9.97	
3	9.94	98.1
	9.86	
4	9.66	
	9.71	96.4
	9.81	
5	9.8	97.6
	9.9	
6	9.77	97.1
	9.82	
7	9.86	97.4
	9.79	
8	9.99	99.1
	10.01	
Average	9.87	97.8

COLLABORATORS

1. Meyer Matluck, U. S. Food and Drug Administration, Boston, Mass.
2. Sylvan H. Newburger, Cosmetic Division, U. S. Food and Drug Administration, Baltimore, Md.
3. Shirley M. Walden, U. S. Food and Drug Administration, Baltimore, Md.
4. A. T. Schramm, National Aniline Division, Allied Chemical and Dye Corporation, Buffalo, N. Y.
5. Harry Isacoff, U. S. Food and Drug Administration, New York, N. Y.
6. Edward C. Fearns, Lever Brothers Company, Cambridge, Mass.
7. Louis B. Dobie, Bristol-Myers Company, Hillside, N. J.
8. Charles F. Bruening, U. S. Food and Drug Administration, Baltimore, Md.

The results obtained by the collaborators are given in Table 1. The average recovery was 97.8% with a range of 97.8% ± 1.4%. No collaborator reported having any difficulties with the method.

When the results obtained by this method are compared with those of the formic acid method studied last year, in which the average recovery was 96.4%, with a range of 96.4% ± 3.4%, it can be readily seen that the

present method is more accurate and precise. An outstanding advantage of the present method over the past one is that the color change at the end point, using bromcresol purple, is much more abrupt than the change using methyl red. In addition, when the excess of potassium periodate is removed with propylene glycol, titration difficulties caused by this compound are obviated and no acidity correction is needed for the periodate solution. It is believed that some unavoidable losses occurred in the isolation procedure which would account for a recovery somewhat less than theory. Possibly, if the initial addition of water of 25–50 ml were increased to 50–100 ml, a better recovery of glycerol may result.

ACKNOWLEDGMENT

The Associate Referee expresses his gratitude to the collaborators for their generous effort.

RECOMMENDATIONS*

It is recommended—

(1) That the periodate consumption method for the determination of glycerol in vanishing cream reported last year be made tentative.

(2) That the formic acid titration method studied in this report be made tentative.

(3) That the subject of glycerol in vanishing cream be closed and study continued on other cosmetic cream problems.

No reports were made on alkalies in cuticle removers, cosmetic powders, cosmetic skin lotions, deodorants and antiperspirants, depilatories, hair dyes and rinses, or hair straighteners.

REPORT ON MOISTURE IN COSMETICS

By JOHN F. WEEKS, JR. (Food and Drug Administration, Federal Security Agency, New Orleans, La.), *Associate Referee*

MOISTURE IN COSMETIC CREAMS

This phase of the general subject was continued. The work consisted of preparing three new cold creams of the non-mineral fat type, bearing a minimum, average, and maximum amount of water. These, together with a two-year old specimen of the same type, were subjected to drying in a forced draft oven at 100 degrees C., room pressure (about 770 mm Hg.) under varying time conditions. The samples were reduced to the consistency of soft mayonnaise (when necessary) by heating in a water bath, shaken well, about 2 g weighed out into a 7½ cm covered butter-dish, dried one-half hour, then at 15-minute intervals to constant weight.

* For report of Subcommittee B and action of the Association, see *This Journal*, **31**, 47 (1948).

Duplicate determinations showed a precision variation of 0.01–0.07%, and the accuracy varied from 0.15% to 0.26% in excess of the calculated added water.

These, together with two mineral oil type of cold creams, and three vanishing creams bearing glycerine and glycerine substitutes, were assayed by a refinement of the conventional moisture distillation method. Two size receivers, 25 ml readable to 0.05 ml, and 5 ml readable to 0.01 ml, were employed. Since no higher "reading" sensitivity was obtainable with 25 ml receiver, it was soon abandoned. The samples were mixed as before, and the soft mass sucked up into roughly calibrated tubes, immediately blown into glass-stoppered distillation flasks and weighed to the nearest milligram. A few glass beads, a small lump of rosin, and the proper amount of varsol were added and the flask connected to the remainder of the apparatus. The condenser and receiver had previously been cleaned by filling completely with cleaning solution which stood overnight in the apparatus. Distillation was allowed to proceed at the maximum safe rate, until no further increase in the water volume was noticeable in fifteen minutes heating. The flask was then immediately removed from the hot plate, and the condenser washed down with 2 ml of 0.2% Aerosol O. T. dissolved in Varsol. This operation was sufficient to sweep down completely the water entrained in the condenser. No significant flattening of the meniscus curve was observable. The trap graduate was brought to room temperature in a water bath, and read to the nearest 0.01 ml (5 ml receiver), or 0.05 ml (25 ml receiver). Flushing with water, alcohol, and ether, and drying with an induced current of air will ready it for the next determination in about five minutes.

The product, Varsol, is a standardized petroleum fraction manufactured by the Standard Oil Company. The specimen used in this work had an initial boiling point of 82 degrees C., 2% distilled at 123 degrees C., and 99% at 191 degrees C. (about 770 mm pressure).

Values on the cold creams were excellent, approaching the mathematical theory of 0.2% in the case of the 24% moisture sample, and the mineral-oil type creams gave results 0.1–0.7% of the calculated added water.

As might be expected, the accuracy of the vanishing cream assays varied by almost inverse proportion to the quantity and volatility of their glycerine or glycol constituent. It is projected that the distillation may be carried out as above, the water layer made to volume, and the glycol constituent determined by conventional methods, to give the water by difference.

It is suggested that cold creams showing less than one-half of one per cent of non-saponifiable fat be assayed for moisture by the drying method given, pending collaborative work.

It is hoped that others will have occasion to check moisture by the re-

fined distillation method in the immediate future. Unless boil-over occurs, the original trap and condenser maybe repeatedly used without meniscus distortion. A 5 or 10 ml *modified* Bidwell & Sterling trap (Ace Glass Co.) is recommended.

Preparation and distribution of collaborative samples of nonmineral and mineral fat type cold creams is projected for the coming year. It is recommended* that the study of moisture be continued.

REPORT ON MASCARAS, EYEBROW PENCILS, AND EYE SHADOWS

By PAUL W. JEWEL (Chief Chemist, Max Factor & Co., Hollywood, Calif.), *Associate Referee*

ANALYSIS OF MASCARA

Additional surveys have been made of various brands of mascara offered for sale, and this survey indicates that more and more brands are shifting from the conventional formulation, using triethanolamine soaps, to that using potassium or sodium soaps. This makes it impossible to devise a general method which will be applicable to all types.

The method previously described[1] is satisfactory for the type which is based on mineral soaps. However, a new method developed during the past year must be used for those which contain triethanolamine soaps. This method will be described in detail in the experimental portion of this report.

ANALYSIS OF EYEBROW PENCILS

Methods for the analysis of products of this type are still far from complete. The same technique used for mascaras containing triethanolamine soaps may be used but the extraction time must be prolonged by some five or six hours.

ANALYSIS OF EYE SHADOWS

A tentative method is offered for this type of preparation for the first time. Eye shadows are very simple preparations which give little difficulty. They consist of mixtures of oils and waxes into which is milled sufficient pigments of the proper shade. The base is mostly mineral oil with small amounts of paraffin or ceresin wax, and possibly small amounts of lanolin. The pigments are usually titanium dioxide, ultramarine blue, iron oxide browns, carbon black, and in some cases chromium oxide green.

EXPERIMENTAL METHODS

MASCARA

Place a small piece of mascara in a beaker, add 10 ml chloroform, and boil for a

* For report of Subcommittee B and action of the Association, see *This Journal*, **31**, 48 (1948).
[1] *This Journal*, **29**, 32 (1946).

few minutes. If the mass disintegrates and appears to dissolve completely except for the pigments, it may be safely assumed that this is one in which the soaps are based on triethanolamine. If the mass dissolves slightly or not at all, the reverse is probably true.

If the soaps present are formed from mineral alkalis, proceed with the method previously described.[1]

If the soaps are formed from triethanolamine proceed according to the following method:

Total Base.—Weigh about three grams of mascara, wrap tightly in Munktell 00 filter paper, turning the ends under and tying with a thread to hold the paper in place. Place the wrapped sample in an extraction thimble, and extract in a Soxhlet extractor for eight hours with chloroform. Tare the extraction flask and wrap the extraction chamber with a towel so as to keep the sovent in the extraction chamber, as nearly as possible, at the boiling point. When extraction is complete, evaporate the solvent and weigh. The weight of the extract will be the total base in the mascara.

Triethanolamine.—To the base contained in the extraction flask add 25 ml of 0 50 *N* sulfuric acid, heat until the waxes melt, and allow to cool with occasional shaking. As soon as the mixture has cooled to room temperature, filter into a titration flask washing the residue and filter well with distilled water. Do not attempt to remove the solid waxes from the flask. Titrate the excess acid in the filtrate with 0.50 *N* sodium hydroxide using methyl red as an indicator. Each ml of 0.50 *N* acid used is equivalent to 0.07143 grams of triethanolamine.

Acid Number of Base.—After removal of the triethanolamine transfer any of the base which has come over onto the filter back to the flask. Pour several small portions of boiling absolute alcohol through the filter into the flask. The total should not exceed 25 ml. Heat to dissolve the waxes and titrate with 0.50 *N* alcoholic potassium hydroxide using phenolphthalein as an indicator. Multiply the ml of normal alkali used by 56.1 and divide by the sample weight to determine the acid number.

Arsenic and Lead.—The method previously described[1] is adequate. The digestion may be done on the extracted pigments if desired, or a sample, one–two grams, of the original material may be used.

EYEBROW PENCILS

Total Base.—Proceed as described under Mascara except that the extraction should be continued for 16 hours.

Arsenic and Lead.—Proceed as directed under Mascara.

EYE SHADOWS

Total Base—Weigh a sample, ca 5.0000 grams, and place in a 250 ml beaker. Add 50 ml petroleum ether and heat to gentle boiling for five minutes. Allow the pigments to settle and decant through a dry filter into a tared dish. Evaporate the solvent and weigh. Repeat the extraction until no further increase in weight is noted, finally bringing all of the pigments onto the filter.

Arsenic and Lead.—Place the filter paper with the extracted pigments in a Kjeldahl flask and proceed as directed under Mascara.

RECOMMENDATIONS*

It is recommended that work be continued on this project and that sam-

* For report of Subcommittee B, and action of the Association, see *This Journal*, 31, 48 (1948).

ples be submitted for collaborative study as soon as it appears that the methods are sufficiently workable to make such a study feasible.

No report was made on mercury salts in cosmetics.

REPORT ON COAL-TAR COLORS

By G. R. CLARK (Food and Drug Administration, Federal Security Agency, Washington, D.C.), *Referee*

The Referee makes the following recommendations:*

That the proposed method for the determination of aniline in coal-tar colors, recommended as tentative by the Associate Referee, be adopted as official, first action, and that the study of volatile amine intermediates be continued.

The Referee concurs in the following recommendations:

The recommendation of K. A. Freeman that the proposed titanium trichloride titration method be adopted as tentative for the determination of pure dye in D&C Red No. 7 and D&C Red No. 10 and lakes of these colors, and that study of lakes and pigments be continued.

The recommendation of K. A. Freeman that studies of the following topics be continued: Identification of certified coal-tar dyes, non-volatile unsulfonated amine intermediates in coal-tar colors, sulfonated amine intermediates in coal-tar colors, sulfonated phenolic intermediates, intermediates derived from phthalic acid, and halogens in halogenated fluoresceins.

The recommendation of W. C. Bainbridge that the study of mixtures of coal-tar colors for drug and cosmetic use be continued.

The recommendation of Mr. L. Koch that study of subsidiary dyes in D&C colors be continued.

The Referee further recommends:

That the following topics be reassigned to Associate Referees as listed:

Halogens in Halogenated Fluoresceins.—Nathan Gordon, Food and Drug Administration, Washington, D. C.

Nonvolatile Unsulfonated Amine Intermediates in Coal-tar Colors.—L. S. Harrow, Food and Drug Administration, Washington, D. C.

Sulfonated Amine Intermediates.—Nathan Ettelstein, Food and Drug Administration, Washington, D. C.

Intermediates Derived from Phthalic Acid.—Charles Graichen, Food and Drug Administration, Washington, D. C.

Lead in Coal-tar Colors. Nathan Ettelstein, Food and Drug Administration, Washington, D. C.

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* For report of Subcommittee B and action of the Association, see *This Journal*, 31, 48 (1948).

That an attempt be made to obtain another Associate Referee to study sulfonated phenolic intermediates.

That the following new topics be added and the Associate Referees appointed as follows:

Lead in Coal-Tar Colors.—Nathan Ettelstein, Food and Drug Administration, Washington, D. C.

Lead in Lakes of Coal-tar Colors.—L. S. Harrow, Food and Drug Administration, Washington, D. C.

Subsidiary Dyes in FD&C Colors.—M. Dolinsky, Food and Drug Administration, Washington, D. C.

Hygroscopic Properties of Coal-tar Colors.—Charles Stein, Food and Drug Administration, Washington, D. C.

That study be continued on topics for which no report was received, as follows:

Acetates, carbonates, halides, and sulfates in certified coal-tar colors.
Buffers and solvents in titanium trichloride titrations.
Ether extract in coal-tar colors.
Unsulfonated phenolic intermediates in coal-tar colors.

No report was given on acetates carbonates, halides, and sulfates in certified coal-tar colors or on buffers and solvents in titanium chloride titrations.

REPORT ON ETHER EXTRACT IN COAL-TAR COLORS

By S. S. Forrest (Cosmetic Division, Food and Drug Administration, Federal Security Agency, Washington, D. C.), *Associate Referee*

Since little work has been done on ether extracts from fluorescein colors, it was decided to investigate this problem. D&C Orange No. 5 was chosen for this study since it is one of the fluorescein colors most frequently submitted for certification.

A method which appeared to give good results was submitted to collaborative study. Samples with the method were sent to various collaborators of whom four replied in time for this report. Listed in the order received, they were:

Ansbacher-Siegle Corporation, H. Holtzman reporting.
H. Kohnstamm and Company, Inc., Samuel Zuckerman reporting.
Bates Chemical Company, Inc., C. O. Beecher reporting.
Wm. J. Stange Company, W. H. Kretlow reporting.

METHOD

Dissolve 5 g of the color in 200 ml of 2% NaOH, place in a continuous extractor and extract with 100 ml of ethyl ether for 3–4 hours. Transfer the extract to a

separatory funnel, wash with successive 10 ml portions of ca 0.1 *N* NaOH until the washings are colorless, then with 10 ml portions of distilled water until the washings are neutral to litmus paper. Decant the ether into a weighed evaporatng dish, allow to evaporate at about 40°C., dry over calcium chloride, and weigh. The increase in weight represents "ether extract."

As may be seen from the table, the results are widely divergent. It appears therefore that the method should be studied further.

TABLE 1.—*Collaborative results: ether extracts from D&C Orange No. 5*

COLLABORATOR	
1	0.19
	0.15
2	0.050
	0.048
	0.040
3	0.08
	0.108
	0.036
4	0.13
	0.10
Associate Referee	0.040
	0.048

RECOMMENDATION*

It is recommended that the topic be continued.

No report was given on identification of 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*

IODINE

A rapid method for the determination of iodine in soluble iodides and in organic compounds has been reported by Clark and Jones.[1] The method involves oxidation of the iodine to iodate in an acid-permanganate medium, removal of excess permanganate and manganese dioxide, addition of

* For report of Subcommittee B and action of the Association, see *This Journal*, **31**, 48 (1948).
[1] *This Journal*, **25**, 755 (1942)

potassium iodide, and titration of the liberated iodine with standard thiosulfate solution.

A collaborative study of this method has been undertaken. Samples of FD&C Red No. 3 and D&C Red No. 3, Aluminum Lake, were submitted with the method to the following collaborators listed alphabetically:

Bates Chemical Company, Inc., C. O. Beecher reporting.
Max Factor, Inc., P. W. Jewel reporting.
Hilton-Davis Chemical Company, Anna Bartruff reporting.
H. Kohnstamm and Company, Inc., I. Hanig reporting.
National Aniline Division, Allied Chemical and Dye Corporation, A. T. Schramm reporting.
Wm. J. Stange Company, W. H. Kretlow reporting.
Cosmetic Division, Food and Drug Administration, L. S. Harrow and C. Stein reporting.

In some cases, two or more reports were received from the same laboratory, making a total of twelve reports. Six collaborators reported no difficulty with the method, while the others experienced various degrees of difficulty. The principal recommendations and comments were:

1. The addition of the dilute potassium permanganate solution dropwise, instead of in 1 ml portions to reduce incidence of brown color formation.

Analyses were conducted following this recommendation. No difference in results were found.

2. Difficulty in obtaining the permanent pink after filtration, and the appearance of a brown coloration during filtration, even after repeated treatment with additional $KMnO_4$.

These difficulties appear to be due to the addition of excess potassium nitrite solution.

3. Difficulty in ridding the solution of manganese dioxide and in filtering.

The method emphasizes that no attempt should be made to destroy the remaining particles of manganese dioxide. Filtration is used to remove these particles of manganese dioxide from the solution.

4. More explicit description of the proper addition of potassium nitrite was requested by two collaborators. This step is the only truly critical point in the determination.

A revised procedure is being prepared to include a more detailed description of this step.

5. After the addition of sulfamic acid, the statement "swirl the contents until the evolution of gas ceases" has been pointed out to be meaningless, as only a very small volume of gas will be produced.

Accordingly, the revised procedure will not contain this statement.

All results, listed in the order they were received, are shown in Table 1.

The results of the collaborators and of the Associate Referee are in fair agreement.

CHLORINE

A method for the determination of chlorine and bromine in the presence of each other has been reported by Clark and Jones.[2]

[2] *This Journal*, 26, 316 (1943).

The chloride and bromide ions are determined in the presence of each other by precipitating the combined silver halides from one protion of the sample and determining the bromide in another portion. Thus bromine is determined directly and chlorine by difference.

The bromine determination depends upon oxidation with an excess of permanganate in a phosphoric acid solution containing cyanide. Under these conditions the bromide is quantitatively converted to bromine cyanide while the chloride is not oxidized. After the excess permanganate

TABLE 1.—*Collaborative results of iodine in halogenated fluoresceins*

COLLABORATOR	FD&C RED NO. 3 FOUND	D&C RED NO. 3, AL. LAKE FOUND
	per cent	*per cent*
Associate Referee	50.0	9.3
2	49.5	9.3
3	50.5	9.3
4	49.6	9.3
5	49.0	9.1
6	50.0	9.4
7	49.6	9.6
8	48.5	9.0
9	50.9	9.2
10	50.1	9.4
11	49.3	—
12	—	9.2
Average per cent	49.7	9.3
Average deviation	±0.5	±0.1

is reduced with ferrous ammonium sulfate, potassium iodide is added, and the liberated iodine titrated with thiosulfate.

The method for determining bromine is official, first action, A.O.A.C. **21.53,** Sixth Edition.

Samples of D&C Red No. 27 and directions for analysis were submitted to the following collaborators:

Max Factor, P. W. Jewel reporting.

H. Kohnstamm and Company, Inc., R. C. Cooney and Virginia Schmuckle reporting.

Wm. J. Stange Company, W. H. Kretlow reporting.

Cosmetic Division, Food and Drug Administration, R. N. Sclar reporting.

The sample submitted was a commercial product. In some cases, two reports were received from the same laboratory.

A total of six reports containing analytical results have been received.

One collaborator reported that his results for bromine were too variable to warrant continuation of the analysis for chlorine.

Another collaborator experienced difficulty duplicating results when

separate gravimetric samples were employed. However, he reported good checks upon using aliquot portions of a 1% solution.

All results, listed in the order they were received, are shown in Table 2.

TABLE 2.—*Collaborative results for chlorine and bromine in halogenated fluoresceins*

COLLABORATOR	D&C RED NO. 27	
	CHLORINE	BROMINE
	Per cent	*Per cent*
Associate Referee	17.0	37.8
2	20.3	37.6
3	—	37.2
4	15.8	37.7
5	17.6	37.5
6	—	—

The results of the collaborators and of the Associate Referee for bromine are in fair agreement. The results for chlorine are poor and in disagreement.

RECOMMENDATIONS*

It is recommended that—

(1) The iodine method be revised to include a more detailed explanation of the potassium nitrite addition.

(2) The revised method be submitted to collaborators for investigation.

(3) More work be done on the chlorine method.

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*

The methods most commonly used for the quantitative determination of free volatile amine intermediates in coal-tar colors involve colorimetric procedures. However, visual colorimetric determinations are subject to personal errors because of the variation in visual perception.

The method proposed involves separation of the intermediate by steam distillation, followed by diazotization, and coupling with an appropriate phenolic derivative. The resulting dye is titrated with standard titanium trichloride solution. The weight of amine intermediate may then be readily computed. The equipment used is normally found in all color laborato-

* For report of Subcommittee B and action of the Association, see *This Journal*, **31**, 48 (1948).

ries, and is inexpensive and readily replaced. Details of the method are given in *This Journal*, **31**, 81 (1948), under "Changes in Methods of Analysis."

In experiments conducted by the Associate Referee, recoveries of known amounts of aniline averaged 97 per cent. In these experiments, the method was applied to recoveries of added aniline from D&C Black No. 1, D&C Red No. 17, D&C Red No. 31, D&C Red No. 33, and Ext. D&C Yellow No. 3, Lake. Other members of the Color Certification Section also obtained good results in trials of the proposed method.

COLLABORATIVE RESULTS

Samples of D&C Orange No. 3 and FD&C Yellow No. 3 containing known amounts of aniline were prepared and distributed to collaborators. The results obtained are shown in Table 1.

TABLE 1.—*Collaborative results*

	FD&C YELLOW NO. 3	D&C ORANGE NO. 3
	per cent	*per cent*
Aniline added	0.20	0.20
Collaborator No. 1	0.090	0.086
	0.086	0.074
Collaborator No. 2	0.19	0.20
	0.19	0.19
Collaborator No. 3	0.1916	0.1916
	0.1930	0.1890
Collaborator No. 4	0.20	0.30
	0.20	
Associate Referee	0.20	0.20
	0.20	0.20

With one exception, the collaborators obtained results agreeing very closely with the actual amount of aniline present.

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

Ansbacher-Siegle Corporation, H. Holtzman reporting.
Harmon Color Works, Inc., V. C. Vesce (per J. W. Ingram) reporting.
H. Kohnstamm and Company, Inc., L. Koch reporting.
Wm. J. Stange Company, W. H. Kretlow reporting.

FURTHER STUDIES

Since the method was submitted to collaborative study, investigations have been made to determine its applicability to other aromatic amine

intermediates that are volatile with steam. Good recoveries have been obtained for p-toluidine by the Associate Referee.

RECOMMENDATIONS*

It is recommended—

(1) That the method for volatile amine intermediates be adopted as tentative for aniline.

(2) That the method be further studied to determine its applicability to volatile amine intermediates other than aniline.

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*

D&C Black No. 1 (also known as Napthol Blue Black, C. I. 246) is one of the colors certifiable by the Food and Drug Administration for coloring drugs and cosmetics.[1] The free intermediate, p-nitroaniline, is permitted in a certified batch of this color to the extent of not more than 0.2 per cent.

The method proposed herein consists of extracting the p-nitroaniline from the color with ether and titrating the nitro group with titanium trichloride.[2]

METHOD

DETERMINATION

Place a 10 g sample of D&C Black No. 1 in a Soxhlet extraction thimble. Extract with ether until the flushings are colorless. (This should take at least three hours.) Transfer the extract to a 500 ml wide-mouth Erlenmeyer flask. Rinse the flask with two 10 ml portions of ether and add these to the main extract. Add 50 ml of water to the extracts and evaporate on a steam bath until all the ether is driven off, using a gentle air blast to hasten the process. Remove the flask from the steam bath, cool to room temp, and add ca 15 g of sodium tartrate. Heat the soln to boiling and titrate with standard 0.1 *N* titanium trichloride under a stream of carbon dioxide to the disappearance of the 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 the end of the titration to serve as an indicator.

RESULTS

A sample of D&C Black No. 1 was purified by repeated extraction with ether until no more intermediate was shown by this method. Known quantities of p-nitroaniline were added to 10 gm. portions of the color and tested for recovery. Results obtained with the method are shown in Table 1.

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* For report of Subcommittee B and action of the Association, see *This Journal*, 31, 48 (1948).

[1] Food and Drug Administration, S.R.A., F.D.C. 3 (1940).

[2] KNECHT and HIBBERT, "New Reduction Methods in Volumetric Analysis," Longmans, 1918.

TABLE 1.—*Recovery of p-nitroaniline from D&C Black No. 1*

p-NITROANILINE ADDED	p-NITROANILINE ADDED	p-NITROANILINE RECOVERED	RECOVERY
per cent	*gram*	*gram*	*per cent*
	0.00	0.00	
0.2	0.0199	0.0189	94.8
	0.0200	0.0190	95.0
	0.0200	0.0195	97.1
0.4	0.0400	0.0378	94.4
	0.0403	0.0399	99.0
	0.0404	0.0404	100.0
0.6	0.0606	0.0603	99.6
	0.0600	0.0593	98.8
	0.0610	0.0608	99.7

Further investigations are being conducted to determine the applicability of the method to 2-4-dinitroaniline, 2-nitro-p-anisidine, and 3-nitro-p-toluidine in colors in which they may be encountered.

SUMMARY

A titrametric method has been presented for the quantitative determination of p-nitroaniline in D&C Black No. 1. The intermediate is removed from the color by ether extraction and quantitatively estimated by titration with titanium trichloride. Recoveries of over 95 per cent have been obtained when the method was used to determine 0.2 to 0.6 per cent of the intermediate.

No reports were given for sulfonated phenolic intermediates, intermediates derived from phthalic acid, or mixtures of coal-tar colors for drug and cosmetic use.

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 A.O.A.C. has adopted as tentative a titration procedure for D&C Red No. 31 and D&C Red No. 8. (1) At the annual meeting in October 1946 a report was presented further showing the accuracy of the method. (2) Since that time pure D&C Red No. 7 has been prepared, analyzed by various methods, and the results compared with the titration method. The results agreed within ± 0.8 per cent.

It appeared, therefore, that the titration procedure could be applied to D&C Red No. 7. Clark (3) has shown the method to be applicable to D&C Red No. 10.

Accordingly, samples of D&C Red No. 7, Calcium Lake, D&C Red No. 8, Sodium Lake, D&C Red No. 10, Sodium Lake, and D&C Red No. 31, Calcium Lake, were submitted to various laboratories for collaborative study. Samples with directions were sent to the following, listed alphabetically:

Ansbacher-Siegle Corporation, H. Holtzman reporting.

Calco Chemical Division, American Cyanamid Company, Mrs. E. Z. Montgomery reporting.

Harmon Color Works, Inc., Vincent C. Vesce reporting (per J. W. Ingram).

Hilton-Davis Chemical Company, Anna Bartruff reporting.

H. Kohnstamm & Company, Inc., Eugene F. Wojt reporting.

National Aniline Division, Allied Chemical & Dye Corporation, A. T. Schramm reporting.

Wm. J. Stange Company, W. H. Kretlow reporting.

U. S. Food & Drug Administration, Cosmetic Division, Lee S. Harrow, S. S. Forrest, and Charles Stein reporting.

The results are listed in Table 1 in the order in which they were received.

An inspection of the table discloses that the results of each collaborator are consistent. In cases where the collaborators results are high for one dye, they are generally high for all four dyes. Where they are low for one they are generally low for all four. It is the opinion of the Referee that this is due to variation in the standard titanium trichloride solutions used rather than to failure of the method. The A.O.A.C. Book of Methods describes two tentative methods for standardizing titanium trichloride solutions. Method I uses Bureau of Standards ingot iron as a primary standard while Method II uses standard potassium permanganate solution. Our experience in the color certification laboratory has shown that Method I gives unsatisfactory results because the Bureau of Standards iron is not pure, nor is the purity known. The Bureau of Standards itself points out this fact. If, therefore, the collaborators standardized their titanium trichloride solutions with iron, the results would vary with the purity of the iron used for standardization. Titanium trichloride is standardized by the color certification laboratory by Method II except that Bureau of Standards potassium dichromate is used instead of potassium permanganate. This has been found to be more convenient and equally accurate, and should have been specified in the submitted method.

The collaborators encountered some difficulty with the method for all colors except D&C Red No. 7. D&C Red No. 10 appeared to give the most trouble since three collaborators each reported some difficulty.

Their comments were:

"End point of titration was variable in the shade of the reduction product."

" . . . We found the titration to be sensitive to small differences in acidity, a

TABLE 1.—*Collaborative results*

COLOR	COLLABORATOR	PURE DYE	AVERAGE VALUE	STANDARD DEVIATION
		per cent	*per cent*	*per cent*
D&C Red No. 7	1	24.7		
Calcium Lake	2	26.6		
	3	24.4		
	4	26.6		
	5	25.7		
	6	25.8		
	7	25.9		
	8	25.5		
	9	25.6		
	Referee	25.5	25.6	±0.7
D&C Red No. 8	1	73.8		
Sodium Lake	2	73.8		
	3	73.2		
	4	76.7		
	5	74.4		
	6	75.8		
	7	75.9		
	8	73.2		
	9	73.2		
	Referee	73.6	74.4	±1.1
D&C Red No. 10	1	92.4		
Sodium Lake	2	92.3		
	3	90.0		
	4	91.7		
	5	92.0		
	6	92.2		
	7	92.2		
	8	90.9		
	9	92.3		
	Referee	91.2	91.7	±0.7
D&C Red No. 31	1	*		
Calcium Lake	2	44.8		
	3	44.3		
	4	47.5		
	5	45.4		
	6	45.2		
	7	43.6		
	8	42.9		
	9	45.3		
	Referee	44.2	44.9	±1.2

* This collaborator reported inability to obtain a satisfactory end point.

slightly more alkaline condition producing a cloudy soln with an orange, instead of a yellow end point. In such conditions low results were obtained, whereas under the proper condition there was no difficulty in obtaining a clear soln, yellow end point, and good duplicability."

"It was observed that the D&C Red No. 10 decomposed when allowed to stand in contact with conc. H_2SO_4 for a prolonged period of time."

In the case of D&C Red No. 8 two collaborators reported a slow fading of the end point but were able to obtain reasonably good checks.

D&C Red No. 31 also gave some trouble, one collaborator observing that duplicability was poorest with this color. Another reported "Sodium bitartrate precipitated the dye, making the titration impossible. Alcohol did not remedy the situation."

One collaborator suggested a back titration procedure to avoid uncertainty as to the true end point, particularly in those cases where the end point is a gradual change from orange to yellow. This suggestion was studied by the Associate Referee and found to be sound. Reserve samples of each color submitted to collaborative study were analyzed by the back titration procedure. The results were the same but in some cases the end points were easier to see. Therefore, the method is revised to include the back titration procedure at the option of the analyst.

RECOMMENDATIONS*

It is recommended that—

(1) The method be adopted as tentative for D&C Red No.'s 7 and 10 and their lakes.

(2) The following be inserted; "If the end point is indistinct, overtitrate by ca 0.5 ml of the titanium trichloride solution and back-titrate with standard indicator solution substracting the indicator blank from the standard titanium trichloride solution used."

(3) The study of Lakes and Pigments be continued.

REFERENCES

(1) *Methods of Analysis, A.O.A.C.*, 6th Ed., **21.41(d)**, p. 291.
(2) FREEMAN, KENNETH A., *This Journal*, **30**, 520 (1947).
(3) CLARK, G. R., *Ibid.*, **28**, 761 (1945).

REPORT ON SPECTROPHOTOMETRIC TESTING

ANALYSIS OF COAL-TAR COLORS
EXT. D&C ORANGE NO. 1

By RACHEL N. SCLAR (Cosmetic Division, Food and Drug Administration, Federal Security Agency, Washington, D. C.), *Associate Referee*

Spectrophotometric analysis of oil-soluble dyes certifiable under the

* For report of Subcommittee B and action of the Association, see *This Journal*, **31**, 48 (1948).

Coal-Tar Color Regulations[1] is extended in this report to Ext. D&C Orange No. 1 (Hansa Orange). Previous reports gave spectrophotometric data for Ext. D&C Yellow No. 5[2] and for D&C Red No. 35[3] and D&C Red No. 36.[3]

Agreement with Beer's law, location of the absorption peak, and the extinction ratio were determined for solutions of this dye.

Data were obtained with a General Electric recording spectrophotometer equipped with an automatic slit adjustment for an 8 millimicron wave-length band.

EXPERIMENTAL

Preparation of Standard Dyestuff.—3 nitro-4-aminoanisole (m.p. 123°) was diazotized and coupled with aceto-aceto-ortho-toluidide (m.p. 103°) in alkaline solution. The product was washed thoroughly with hot water,

TABLE 1.—*Extinction values of solutions of Ext. D&C Orange No. 1 in U.S.P. chloroform*

Typical Data

CURVE NO. (CHART 1)	CONCENTRATION	EXTINCTION			$E_{432\ m\mu}$ CONCENTRATION	$\frac{E_{420\ m\mu}}{E_{450\ m\mu}}$
		420 mμ	432 mμ	450 mμ		
	mg./liter					
1	3.17	.150	.158	.136	.0498	1.103
2	6.33	.300	.316	.272	.0499	1.103
3	12.66	.598	.632	.544	.0499	1.099
4	25.33	1.196	1.264	1.086	.0499	1.101
					Av. .0499	Av. 1.10

dried and recrystallized from benzene; it melted at 210°C (on Fisher Block). A portion of this product recrystallized from chloroform showed the same melting point.

A sample of the dye, made commercially but with a special effort to obtain purity, melted at 207.5°C. Spectrophotometric examination of this material showed the extinction per milligram/liter to be lower than that of the material prepared in this laboratory. When the commercial sample was washed with hot water and recrystallized first from benzene, then from chloroform, the melting point and extinction per milligram/liter were found to agree with those of the laboratory sample. The laboratory sample was therefore considered sufficiently pure to serve as a standard.

Preparation of Solutions.—A 25.33 mg portion of dye weighed on a

[1] U. S. Food and Drug Administration, Service and Regulatory Announcements, F.D.C. 3.
[2] G. R. Clark and S. H. Newberger, *This Journal*, **27**, 576 (1944).
[3] Rachel N. Sclar, *Ibid.*, **30**, 522 (1947).

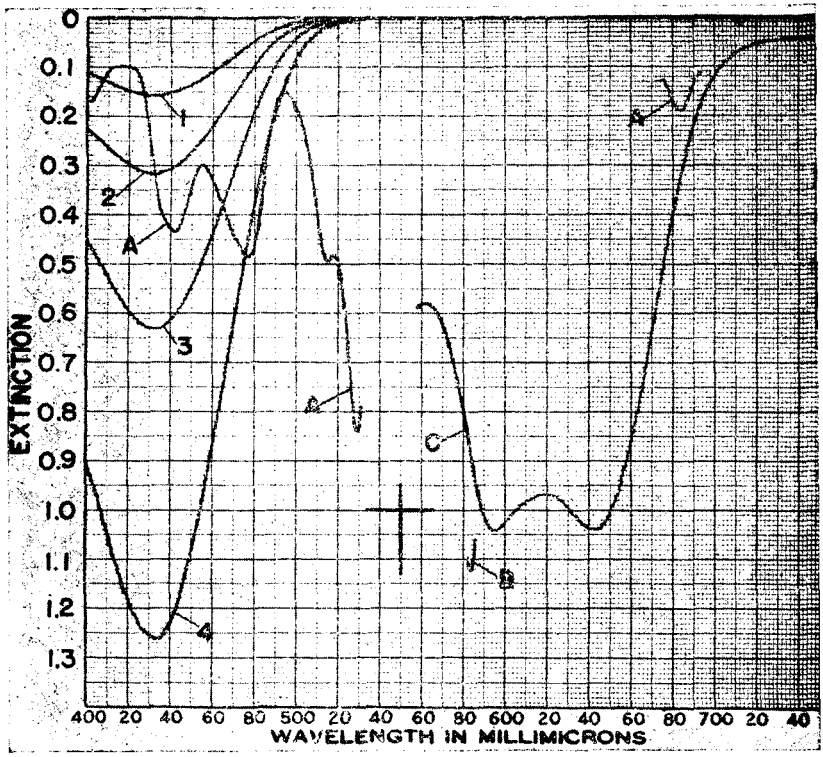

CHART 1.—Pure Ext. D&C Orange No. 1 in chloroform.

Curve 1— 3.17 mg./liter.
Curve 2— 6.33 mg./liter.
Curve 3—12.66 mg./liter.
Curve 4—25.33 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.

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 and aliquot portions diluted with chloroform to the concentrations shown in Table 1. 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, it was found that gentle warming on a

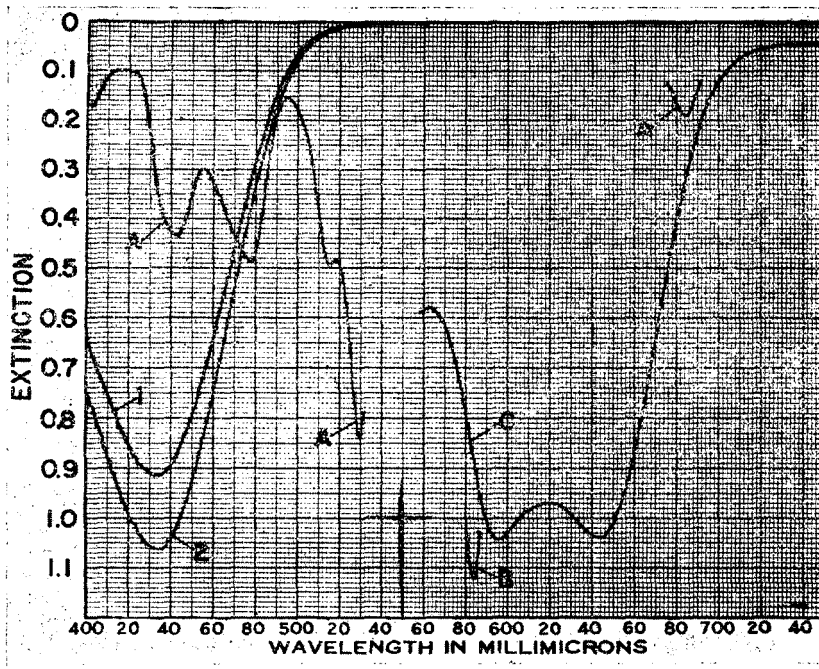

CHART 2.—Certified samples of Ext. D&C Orange No. 1.

Curve 1—Primary Color—18.63 mg./liter.
Curve 2—Lake —42.09 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.

water bath facilitated the solution of samples of the color. No adverse effects from this treatment were noted.

The stability of the color in chloroform solution was investigated by aging a diluted aliquot of a master solution for three days in the dark. When this solution was made to volume spectrophotometric examination showed no fading of color when compared with a diluted aliquot examined at the beginning of this period.

Spectrophotometric Data.—The extinction curves for the chloroform solutions of Ext. D&C Orange No. 1 are shown in Chart 1. They show an absorption peak at 432±2 mμ. (All wave lengths were corrected to ±2 mμ with the aid of didymium glasses tested by the National Bureau of

Standards; see footnote to Charts 1 and 2.) Average extinction per milligram/liter at this wave length was found to be .0499.

The ratio of extinction values at 420 mμ and 450 mμ was calculated This ratio E_{420} mμ/E_{450} mμ = 1.10 ± .005; see Table 1.

DISCUSSION

The ratios of extinction to concentration in Table 1 indicate that at 432 mμ, chloroform solutions of Ext. D&C Orange No. 1 containing 3.2 to 25.3 mg of color per liter obey Beer's law. The pure dye content of a sample of this color can therefore be determined from spectrophotometric data of its solution by comparison with the experimentally determined standard.

APPLICATION TO COMMERCIAL SAMPLES

Primary Colors and Lakes

One sample of certified Ext. D&C Orange No. 1 and one sample of Ext. D&C Orange No. 1, Lake (made by extending the dye on insoluble substrata) were analyzed spectrophotometrically. Weighed samples were warmed on the steam bath with U.S.P. chloroform to dissolve the color. It was necessary to filter the solution obtained from the lake through a fine sintered glass crucible to remove the substratum. The solutions were transferred to 100 ml flasks and made to volume at room temperature. Extinction measurements were made on suitably diluted aliquots. The curves are shown in Chart 2, and the data in Table 2.

TABLE 2.—*Analysis of certified samples of Ext. D&C Orange No. 1: a primary color and a lake*

SAMPLE NO.	M.P.	CONCENTRATION OF SAMPLE	E_{432} mμ	DYE[1] SPECTROPHOTOMETRICALLY	DYE FROM CHEMICAL ANALYSIS
	°C.	*mg./liter*		*per cent*	*per cent*
Primary Color	209	18.63	.916	98.5	98.5 (from N_2 content)
Lake[2]		42.09	1.064	50.7	51.0 (by extraction)[3]

[1] The dye content was calculated by using .0499 (Table 1) as the extinction value for 1 mg./liter of Ext. D&C Orange No. 1.
[2] Substratum—alumina and talc.
[3] The color was extracted from the lake with benzene, the solvent evaporated, and the extracted material weighed.

SUMMARY

Spectrophotometric data for chloroform solutions of purified Ext. D&C Orange No. 1 are presented. Beer's law is shown to be applicable. The absorption peak is at 432 ± 2 mμ; extinction per milligram/liter is .0499 at this wave length, and the extinction ratio E_{420}/E_{450} mμ = 1.10 ± .005.

Application is made of these data to the determination of pure dye in commercial samples of the color. Typical results are given.

REPORT ON SUBSIDIARY DYES IN D&C COLORS

D&C RED Nos. 6 or 7

By L. Koch (H. Kohnstamm & Co., Inc., Brooklyn, N. Y.),
Associate Referee

Previous investigation of this subject by the Associate Referee,[1] and by collaborators,[2] has indicated the feasability of estimating the percentage of 4-toluene-azo-2-naphthol-3-carboxylic acid, in D&C Red No. 6, by titration with titanium trichloride. Continued work on this topic unearthed the fact that the method was not applicable to rosinated colors, because the extender masked the true end point.

A reduction type of assay was therefore considered, based on a modification of a procedure outlined in a previous paper.[3] Three certified specimens of rosinated D&C Red No. 7, with and without adulteration, were thereupon submitted to the following collaborators; K. C. Johnson, E. I. duPont de Nemours & Co., Wilmington, Del.; R. Pasternack, Chas. Pfizer & Co., New York, N. Y.; and A. T. Schramm, National Aniline Division, Allied Chemical & Dye Corp., Buffalo, N. Y. Their results are outlined in Table 1, and the Associate Referee wishes to express his indebtedness to them.

METHODS

REAGENTS

Stannous chloride soln.—100 grams of $SnCl_2$ per 100 ml of conc. HCl soln.

Sodium hydroxide soln.—50%.

Hydrochloric acid.—Approximately 0.3 *N*.

Potassium bromide-bromate soln.—0.05 *N* containing 1.3920 grams C.P. $KBrO_3$ and 10 grams of C.P. KBr per liter.

Sodium thiosulfate soln.—12.5 grams $Na_2S_2O_3 \cdot 5H_2O$ per liter. Standardize against $KBrO_3$–KBr soln as follows: Place 100 ml of water, 25 ml conc. HCl, and 100 grams of ice into an iodination flask. Add 20 ml of $KBrO_3$–KBr soln from a buret as rapidly as possible, and let stand in an ice bath for 10 min. Continue as directed under Procedure. Calculate the value of the $Na_2S_2O_3$ soln in the terms of the $KBrO_3$-KBr.

Starch indicator.—0.5% soln.

PROCEDURE

Weigh 5.0 g of sample into a beaker, and add 50 ml of glacial acetic acid, 25 ml of 6 *N* HCl, and 5 ml of the $SnCl_2$ soln. Cover the beaker with a watch-glass, and boil gently until the volume is ca 40 ml. Dil. the reaction mixture with 60 ml of methyl cellosolve, and boil again to half volume. Dil. the reduction product with 200 ml of water, cool and transfer to a 500 ml volumetric flask. Filter off a 200 ml aliquot, using Whatman #2, and place it in a 500 ml extraction funnel. Add 100 ml of water, and make the sample alkaline with ca 35 ml of 50% NaOH soln. Extract the liberated amine with two 100 ml portions of ether (if a non-separable emulsion forms

[1] *This Journal*, **25**, 948 (1942).
[2] *Ibid.*, **28**, 763 (1945).
[3] Koch, *This Journal*, **29**, 237 (1946).

add a few ml of methyl cellosolve to the supernatant ether layer and swirl gently). Wash the combined ether extracts with one 75 ml and three 40 ml portions of water.

Remove the amine from the ether with four 30 ml portions of ca 0.3 *N* HCl, and finally with 30 ml of water, transferring the washings to a 500 ml iodination flask, marked at the 100 ml level. Boil to expel dissolved ether, and conc. to 100 ml. Cool, and add 25 ml concd. HCl and ca 100 g of crushed ice. Add the $KBrO_3$-KBr soln from a buret, while agitating the flask, until the soln remains yellow at least a half-minute. Then add ca 5 ml more. Stopper the flask, and let it stand in an ice bath for 10 min. Add 2–3 g KI, and titrate while cold with the $Na_2S_2O_3$ soln, using the starch indicator internally only near the end point.

1 ml of 0.05 *N* $KBrO_3$ soln is equal to 4.11 mg of subsidiary dye—Na
1 ml of 0.05 *N* $KBrO_3$ soln is equal to 4.07 mg of subsidiary dye—Ca/2

TABLE 1.—*Collaborative results (per cent)*

ANALYST	SAMPLE 1		SAMPLE 2		SAMPLE 3	
A	0.18		0.95		3.48	
	0.17		0.90	0.93	3.47	
	0.18	0.18	——		3.18	
	——				3.23	
					3.40	3.35
					——	
B	0.02		0.67		3.02	
	0.02	0.02[1]	0.50	0.59	3.17	3.10
	——		——		——	
C		0.24		1.00		3.28
Ass. Referee		0.29		0.98		3.47
Calc.		0.24[2]		1.22[3]		4.01[3]

[1] Omitted in calculating average of results for Sample No. 1.
[2] Average subsidiary dye content of Sample No. 1.
[3] Average of results for Sample No. 1 plus the percentage adulteration.

COMMENTS BY COLLABORATORS

A. T. Schramm.—(1) Allow the first ether extraction to stand approximately two hours to facilitate separation.

(2) Add glass beads to the beaker before reduction, and during the evaporation of the ether to reduce the amount of "bumping."

DISCUSSION

The consistent low results obtained by the collaborators and the Associate Referee indicated that the reduction did not yield a quantitative recovery of p-toluidine. Experiments were therefore run to ascertain the percentage recovery of amine by treating 1 gram of pure subsidiary dye according to the proposed method. The results are shown in Table 2 in terms of subsidiary dye. Because it was also possible that large quantities of D&C Red No. 7 might alter the findings, 1 gram of subsidiary dye and

4 grams of certified color were also reduced and assayed for p-toluidine. The recoveries are outlined in Table 3.

TABLE 2.—*Results in terms of subsidiary dye*

SUBSIDIARY DYE		
TAKEN	FOUND	RECOVERY
mg	*mg*	*mg*
76.8	65.5	85.3
48.0	40.3	84.0
19.2	17.5	91.1

TABLE 3.—*Results assayed for p-toluidine*

SUBSIDIARY DYE		
TAKEN	FOUND	RECOVERY
mg	*mg**	*per cent*
76.8	67.0	87.2
48.0	40.6	84.6
19.2	17.5	91.1

* Minus the subsidiary dye content of the D&C Red No. 7.

Conclusions, based on these tentative experiments, indicate that the reduction conditions of the method do not give quantitative yields of the expected product. If these findings can be confirmed by additional work, a correction factor should be applied to the results obtained by this method, or a variation of the procedure should be made in order to obtain more quantitative recoveries of the amine.

It is therefore recommended* that the study of this topic should be continued.

* For report of Subcommittee B and action by the Association, see *This Journal*, 31, 48 (1948).

WEDNESDAY—MORNING SESSION

REPORT ON FEEDING STUFFS

By L. S. WALKER (Agricultural Experiment Station, Burlington, Vt.), *Referee*

RECOMMENDATIONS*

(1) It is recommended that further study be made on the following:

Mineral mixed feeds (calcium and iodine)
Lactose in mixed feeds
Fat in fish meal
Adulteration of condensed milk products
Crude fat or ether extract
Activity of yeast
Microscopic examination of feeds
Fluorine
Protein evaluation in fish and animal products
Hydrocyanic acid glucosides
Sampling and analysis of condensed buttermilk
Tankage (hide, hoof, horn, and hair content)

(2) It is recommended that the methods for calcium and phosphorus and the acetone method for fat in fish meal, as outlined by the Associate Referees, be made tentative and work continued.

(3) It is recommended that the editorial changes in the method for crude fiber, as recommended by the Associate Referee, be made, and study continued.

(4) It is recommended that work on the following be discontinued:

Fat in cooked animal feeds containing cereals
Crude protein (catalysis)

No report was given on mineral mixed feeds (calcium and iodine); or lactose in mixed feeds.

REPORT ON FAT IN FISH MEAL

By MAURICE E. STANSBY (Fish and Wildlife Service, Department of the Interior, Seattle, Washington), *Associate Referee*

Tests carried on in laboratories of the Fish and Wildlife Service during the past ten years have shown that when the fat content of fish meal is determined by conventional methods, that is, by extraction with ethyl ether, the apparent fat content drops during even short storage periods; while after six months to a year values obtained are as low as 30 per cent of the original. It has also been shown that when acetone is substituted for ethyl ether much higher recoveries of the initial fat are obtained, although

* For report of Subcommittee A and action of the Association, see *This Journal*, 31, 41 (1948).

a small drop in the apparent fat content does take place after extended storage periods. More recent studies have shown that certain mixed solvents give somewhat higher results than is obtained with acetone, but other difficulties in technique have thus far prevented the use of such mixed solvents on a routine basis. Inasmuch as such extremely low values are obtained when using conventional methods, it seemed advisable to test the acetone-extraction method on a collaborative basis even though it has not been perfected. Accordingly, during the past year such a collaborative analysis has been conducted.

Before starting the collaborative analysis it was necessary to standardize on a procedure. In preliminary experiments carried out during the past years it had been customary to first extract the meal with acetone then to evaporate off the acetone and redissolve the fat in ethyl ether. This solution was then evaporated and the ethyl ether-soluble material weighed as fat. This purification step was carried out in the past to be certain that the acetone was not dissolving extraneous material and that the final reported value was true fat. Extensive experience with this procedure, however, showed that the gross unpurified acetone extract of old meals was never higher than the purified ether extract of the same meal in the fresh condition nor was it higher than the initial fat content as determined by standard A.O.A.C. procedure. Thus, once having shown that the acetone extract contained no significant amount of extraneous materials it was felt that for a routine analysis it would be unnecessary to carry out the so-called "purification" step, and that by merely weighing the crude acetone extract a minimum value would be obtained which in no case would be higher than the true fat content of the meal. Accordingly, no purification with ethyl ether was included in the final procedure as adopted for the collaborative analysis.

Another decision which had to be made was whether a second step, namely, hydrolysis of the extracted meal with acid followed by a second extraction, should be included in the standardized method. Previous tests had shown that a considerable amount of additional fat could be extracted from meals in this way, especially meals which had been stored for a considerable length of time. Such a hydrolysis and extraction procedure on fresh meals usually yielded about 1 per cent additional fat by this method. For very old meals a considerably larger proportion of the meal was not extracted by the initial solvent but could be obtained by this hydrolysis procedure. Accordingly, in order to determine more nearly the true fat content in older meals it was decided to include the acid hydrolysis in the standard procedure. By so doing, the initial fat content of fresh meals is increased by about 1 per cent over values which would have been obtained had this process not been included.

The acetone procedure for fat in fish meal, exactly as sent to collaborative laboratories for this analysis is published in *This Journal*, **31**, 98 (1948).

FAT DETERMINATION—A.O.A.C. METHOD

Determine oil content by the official A.O.A.C. method for grain and stock feeds, as described on page 408 of the sixth edition of *Methods of Analysis*, section **27.24** and **27.25,** first drying the meal as described under section **27.3.** It should be noted that in drying to constant weight, ordinarily, a truly constant weight will never be attained with fish meal, because loss in weight due to evaporation of moisture, and gain of weight due to oxidation of fish oils (even in vacuum oven), both occur simultaneously. Drying is therefore discontinued after the sample ceases to lose weight.

Average results obtained in the collaborative analysis are shown in Table 1, while individual determinations in triplicate, as reported by the different laboratories, are given in Table 2. The meal used in this col-

TABLE 1.—*Average values for fat in fish meal as reported by collaborative laboratories*

LABORATORY	ACETONE EXTRACTION METHOD			A.O.A.C. ETHYL ETHER METHOD
	INITIAL EXTRACT	ACID HYDROLYSIS EXTRACT	TOTAL EXTRACT	
	Per cent	*Per cent*	*Per cent*	*Per cent*
	Fat Content of Pilchard Meal Stored 7½ months			
A	11.15	2.57	13.72	5.25
B	13.32	2.57	15.67	4.92
C	11.9	3.7	15.6	5.8
D	11.0	2.1	13.1	4.57
E[1]	12.31	2.05	14.36	6.12
F	13.5	2.5	16.0	5.7
G	11.38	2.80	14.18	4.97
Average	12.08	2.58	14.66	5.33
	Initial fat content of freshly prepared meal			
	17.96	0.93	18.89	14.75[2]

[1] Soxhlet extraction equipment was used by Laboratory E.
[2] With hydrochloric acid digest extraction included, this value was 16.96 per cent.

laborative assay was an experimental pilchard meal prepared[1] in the pilot plant of the Seattle Fishery Technological Laboratory of the Fish and Wildlife Service, on September 25, 1946. The meal was stored in a paper bag at room temperature under conditions similar to those prevailing in the storage of commercial meals. A large sample was withdrawn during the latter part of April 1947, ground, and mixed thoroughly to insure homogeneity; and samples were placed in glass-stoppered paraffined

[1] The meal was prepared by a method somewhat similar to commercial wet process rendering but on a small scale. This involved cooking the pilchard with steam, pressing out oil in a hydraulic press, and drying the meal. The latter step differed from commercial practise in that a tunnel drier was employed using a blast of air at 150°F.

bottles and mailed to collaborative laboratories with instructions that all tests should be run during the ten-day period of May 10 to May 20. As shown in Table 1 the average fat content of the meal had fallen from the original 14.75 per cent in the freshly prepared meal (when determined by the ether extract method) to an apparent value of 14.66 per cent when determined by the acetone method, but to a value of 5.33 per cent when determined by the ether extract method (A.O.A.C.).

There was no outstanding difference in precision between the A.O.A.C. method and the acetone method either in regard to reproducibility between the triplicate samples run within the same laboratory or with

TABLE 2.—*Precision attained by individual laboratories on acetone and ethyl ether extraction of fish meal*

LABORATORY	FAT CONTENT OF MEAL			
	INITIAL EXTRACT	ACID HYDROLYSIS	TOTAL EXTRACT	A.O.A.C. ETHYL ETHER EXTRACT
Number	*Per cent*	*Per cent*	*Per cent*	*Per cent*
A	11.21; 11.27; 10.98	2.75; 2.32; 2.65	13.96; 13.59; 13.63	5.17; 5.27; 5.33
B	13.10; 13.18; 13.68	2.05; 2.77; 2.24	15.15; 15.95; 15.92	4.93; 4.90; 4.92
C	10.8; 12.0; 11.9	4.0; 3.5; 3.7	14.8; 15.5; 15.6	6.0; 5.7; 5.8
D	11.2; 10.6; 11.0	2.1; 2.1; 2.1	13.3; 12.7; 13.1	4.55; 4.70; 4.60
E	12.24; 12.17; 12.51	1.99; 2.08; 2.09	14.23; 14.25; 14.60	6.00; 6.20; 6.07
G	11.14; 11.48; 11.51	3.00; 2.72; 2.67	14.14; 14.20; 14.18	4.93; 4.98; 5.00

respect to differences between individual laboratories. As a rule, individual laboratories were able to check themselves among the triplicate determinations better than they checked each other and this was true with both the acetone extraction method and the A.O.A.C. method. While absolute differences were greater in the case of the acetone extraction method, relative per cent differences were about the same. Values about three times as high were obtained for the acetone extraction fat determinations as for the A.O.A.C. method, and differences between replicate samples and individual laboratories were likewise about three times as high. Thus, the relative difference was of the same order of magnitude for the two different methods. On the whole, the precision was not nearly as good as could be desired, and future studies should be carried out to determine the cause for such lack of precision, and, if possible, to improve upon the procedure in order to obtain better agreement.

In spite of the fact that the acetone extraction method does not give ideal and complete extraction of fat from old meals and precision is not all that could be expected, the results by this method are so far superior to those obtained by ethyl ether extraction that it is felt the acetone method should be adopted tentatively, pending improvements which may require

a considerable number of years to be attained. Accordingly, the following recommendations are made:

(1) It is recommended* that the acetone extraction procedure proposed be made tentative.

(2) That study be continued to improve the precision of the acetone extraction procedure.

(3) That studies be continued on the use of other solvents or mixtures of solvents which might eventually lead to a better procedure than is possible by the use of acetone.

LIST OF COLLABORATIVE LABORATORIES

Commonwealth of Virginia, Department of Agriculture and Immigration, Division of Chemistry, Richmond, Virginia

Maine Agricultural Experiment Station, Orono, Maine

State of California Department of Agriculture, Sacramento, California

State of Michigan Department of Agriculture, Lansing, Michigan

U. S. Department of the Interior, Fish and Wildlife Service, Fishery Technological Laboratory, Seattle, Washington

Wirthmore Research Laboratory, Malden, Massachusetts

No report was given on adulteration of condensed milk products.

REPORT ON FAT IN COOKED ANIMAL FEEDS CONTAINING CEREALS (ACID HYDROLYSIS)†

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

The Associate Referee has continued the investigation of the acid hydrolysis method for fat in cooked animal feeds containing cereals. Previously, it was shown (1, 2, 3) in individual and collaborative studies by the author that this method may give higher fat results than does direct ether extraction. Furthermore, it has been pointed out that extreme care must be exercised in order to obtain duplicable results by the acid hydrolysis method. Several other difficulties, such as emulsion formation, filtration of the sample, and separation of ether extract and hydrolysate, have been reported.

Schall and Thornton (4) have shown that not all the material extracted by this method is fat. The findings of the Associate Referee are in agreement with this report. It is apparent that the solvents used in the procedure may extract non-fatty materials. In view of the fact that this method is more tedious and requires greater skill of operation than the direct ether extraction method, your Associate Referee feels that the acid

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* For report of Subcommittee A and action of the Association, see *This Journal*, 31, 41 (1948).

† Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University, Department of Chemistry.

hydrolysis method would not receive widespread use, even tho the accuracy of the two methods were the same.

It is the opinion of the Associate Referee that the present acid hydrolysis method is not so reliable as the direct ether extraction method for the determination of fat in dog feeds. There are more difficulties encountered in the acid hydrolysis procedure. It is admittedly true that both methods determine ether extract and not true fat. It is recommended,† therefore, that the acid hydrolysis method be not adopted for the determination of fat in cooked animal feeds containing cereals and that further investigation of the method for this purpose be discontinued.

REFERENCES

(1) *This Journal*, **25**, 864, 1942.
(2) *Ibid.*, **26**, 340, 1943.
(3) *Ibid.*, **28**, 768, 1945.
(4) *Ibid.*, **26**, 404, 1943.

No report was made on crude fat or ether extract, or on activity of yeast.

REPORT ON MICROSCOPIC EXAMINATION OF FEEDS

By H. J. WITTEVEEN (Department of Agriculture, Dairy and Food, St. Paul, Minn.), *Associate Referee*

The last report on the microscopic examination of feeds to this Association was made by Mr. A. W. Creswell of the Ohio Department of Agriculture in 1944. Based on a survey made about that time, microscopic examination of feeds was started in several States in 1907. This work continued to expand so that by 1942, 24 States and the Dominion of Canada employed full or part time microscopists. Included in the survey was an outline of a procedure, together with the necessary equipment.

Early this year this study was resumed by sending letters to twenty-four feed control officials and chemists associated with feed manufacturing concerns requesting that an outline of their procedure be submitted, stating the preliminary steps and describing the characteristic appearance of each ingredient identified. Sixteen replies were received, five of which included procedures. In addition to the five who submitted procedures two stated that microscopic work was being done; procedures were not submitted but interesting and helpful comments were made.

The request for the description of the characteristic appearance of each ingredient identified resulted in a variety of comments. The majority stated that studying reference samples and acquiring experience was the

† For report of Subcommittee A and action of the Association, see *This Journal*, **31**, 41 (1948).

most satisfactory way of learning how to identify ingredients. Simplicity in procedure was expressed as an important factor particularly where many samples are examined and a limited amount of time can be devoted to each sample. Others submitted procedures and outlines describing the characteristic appearance of the various feed ingredients.

Of interest in the study of this problem is the work of the Check Sample Committee of the Association of the American Feed Control Officials. During the early part of the year a sample of feed was prepared containing ten ingredients common to most feeds, nine of which were included in a list of fifteen comparable to the ingredients listed on a tag or label. Portions of the sample were sent to forty-five collaborators, thirty-one of whom were control chemists, the other being chemists associated with feed manufacturing concerns. Each collaborator was supplied with a copy of the following procedure and a description of the characteristic appearance of each of the fifteen ingredients listed as stated in "Microscopical Examination of Feeds and Feedingstuffs" by B. H. Silberberg (1918), revised by Geo. L. Keenan (1940).

PROCEDURE FOR IDENTIFICATION OF FEED INGREDIENTS

Prepared by the A.A.F.C.O. Collaborative Check Sample Committee

As a preliminary to the actual identification of the numerous ingredients contained in present day mixed feeds, each analyst should provide himself with the proper equipment for the work, including a microscope, hand lenses, slides, cover glasses, needles, sieves, scalpels, solutions, and authentic type samples of the various ingredients commonly contained in mixed feeds. The following references, and others not mentioned, are also of great importance:

The Microscopy of Vegetable Foods, by A. L. Winton.
The Structure and Composition of Foods, by A. L. and K. B. Winton.
Elementary Chemical Microscopy, by Chamot.
Massachusetts Agricultural Experiment Bulletin 141.
Special Bulletin 120 Agricultural Experiment Station, East Lansing, Mich.
Bulletin 246 Vermont Agricultural Experiment Station, Burlington, Vermont.

The next step is for the analyst to thoroughly familiarize himself with the appearance of the various feed ingredients without magnification and with magnification of such degree as he feels will enable him to recognize numerous ingredients both singly and mixed with other ingredients. For practice, mixtures of various ingredients should be made up by the analyst and identification studied. Labels showing manufacturers' statements of ingredients contained are of assistance to the analyst in providing leads of what to look for. In cases where an ingredient is claimed and not possible of identification, a minor amount (one or two per cent) of the missing ingredient might be added from an authentic source, to determine if such small amounts can be identified in the mixture under examination.

The following is a modification of the preliminary procedures used in the Maryland and Minnesota Feed Control laboratories on mixed feeds (especially those containing added mineral constituents).

Sift a portion (ca 10 g) of the unground mixed feed on a 20-mesh sieve. The material remaining on the sieve is placed on a white paper or glass and examined under a strong light, with a hand lens or microscopically, or both. The portion which passes thru the sieve may be used to verify the first findings by examining under the microscope, using chloral hydrate soln (45 g chloral hydrate, 25 ml hydrochloric acid (1–8), with 10 ml of glycerine) as a clearing agent.

A second portion of 10 g of the unground feed is mixed with 50 ml of chloroform in a 100 ml beaker. After stirring with a spoon allow to settle briefly and skim off the floating portions containing most of the feed. Pour off the excess chloroform. After drying on a low-heat hot plate to evaporate the chloroform, examine the settlings which contain the mineral ingredients in fairly pure condition. Individual chemical tests should be made for chlorides, carbonates, sulphates, iron, copper, iodine, phosphates, and grit, as specified in A.A.F.C.O. Official Publication; the A.O.A.C. *Methods of Analysis* (6th Ed.); and other standard references.

These collaborators were asked to identify the ingredients listed and any other ingredients they might find.

Results Obtained in the Microscopic Examination of Prepared Feed

INGREDIENTS DECLARED	31 CONTROL CHEMISTS		14 INDUSTRIAL CHEMISTS	
	FOUND BY—	PER CENT	FOUND BY—	PER CENT
Ground Corn	31	100.0	14	100.0
Ground Oats	24	77.5	14	100.0
Corn Gluten Feed	23	74.2	13	92.9
Alfalfa Meal	23	74.2	10	71.5
Cottonseed Meal	25	80.6	10	71.5
Soybean Oil Meal	22	71.0	10	71.5
Linseed Meal	26	83.8	10	71.5
Wheat Bran	31	100.0	14	100.0
Iodized Salt	31	100.0	14	100.0
Defluorinated Phosphate	18	58.1	9	64.2
INGREDIENTS NOT DECLARED				
Calcium Carbonate	19	61.3	13	92.9
Brewers Grains	6	19.4	5	35.7
Weed and Grass Seeds	6	19.4	3	21.4
Ground Barley	12	38.7	7	50.0
Meat Scraps	10	32.2	5	35.7
Coconut Meal	5	16.1	4	28.6
Peanut Meal	6	19.4	3	21.4

The data in the above table indicate that five control chemists and one industrial chemist made perfect analyses. A high percentage of perfect analyses would be unlikely inasmuch as feeds are impure products, small amounts of ingredients being present as impurities or as generally occur-

ring with a product of similar nature, such as oats and barley, bran and middlings, etc.

In this connection familiarity with macroscopic characteristics might be helpful in avoiding identification of ingredients present as impurities or accompanying ingredients of a similar nature such as those just mentioned. To identify ingredients from their macroscopic appearance would also reduce the amount of time required for each sample.

Thus far little consideration has been given to the identification of mineral ingredients and whole weed seeds in feeding stuffs. Further study including these phases of the work would be desirable.

It is recommended* therefore that the study of microscopic examination of feeds be continued.

No report was given on fluorine in feeding stuffs.

MINERAL CONSTITUENTS OF MIXED FEEDS

By J. L. St. John (*Associate Referee*) and Edith Eng Huey
(Division of Chemistry, Agricultural Experiment Stations, and State Chemist's Laboratory, Pullman, Washington)

Following last year's report (*This Journal*, **30**, 606 (1947)), additional samples of the identical feed samples previously utilized were sent to a second group of collaborators. The methods used are described in the previous report.

Since only summarized averages were reported last year, the results obtained by individual collaborators for both 1946 and 1947 are included in Table 1.

The results of the two years' work are so nearly the same that the conclusions of last year are not changed. The results obtained by the nitric-perchloric acid method of sample preparation are essentially the same as those given by the A.O.A.C. method **12.5.** It thus appears that these two methods of sample preparation for calcium and phosphorus in mixed feeds may be used interchangeably. The nitric-perchloric acid method of sample preparation is therefore recommended† as tentative. The details of the procedure are published in *This Journal*, **31**, 98 (1948).

Precautions regarding the use of perchloric acid to guard against the danger of an explosion, which has been feared by analysts, are described in the previous paper, (*This Journal, loc. cit.*) and in *Ind. Eng. Chem. Anal. Ed.*, **13**, 48; **14**, 301; **16**, 630. It is emphasized that all of the easily oxidizable organic matter should be oxidized with nitric acid before the perchloric acid is added.

* For report of Subcommittee A and action of the Association, see *This Journal*, 31, 41 (1948).
† For report of Subcommittee A and action of the Association, see *This Journal*, 31, 41 (1948).

TABLE 1.—*Comparison of results by two methods*

COLLABORATORS	CALCIUM IN FEEDS						PHOSPHORUS IN FEEDS					
	SAMPLE 1		SAMPLE 2		SAMPLE 3		SAMPLE		SAMPLE 2		SAMPLE 3	
	A.*	B.	A.	B.	A.	B.	A.	B.	A.	B.	A.	B.
	Per Cent		*Per Cent*		*Per Cent*		*Per Cent*		*Per Cent*		*Per Cent*	
Bartlett, L. E.	1.96	2.01	2.25	2.22	2.06	2.11	0.924	0.956	1.076	1.096	1.124	1.120
Beaver, B. F.	1.97	1.97	2.22	2.17	2.04	2.01	0.777	0.670	0.945	0.914	0.975	0.970
Despaul, J. E.	1.91	1.98	2.09	2.22	1.99	2.01	0.795	0.805	0.935	0.915	0.965	0.943
Fuqua, V. L.	1.75	1.66	1.94	1.94	1.84	1.75	0.76	0.57	0.91	0.75	0.95	0.76
Haskins, A. L.	1.96	2.05	2.20	2.20	2.06	2.08	0.76	0.79	0.91	0.89	0.95	0.93
Hayes, C. M.	2.01	2.02	2.21	2.26	2.11	2.08	0.911	0.862	1.018	1.058	1.054	1.112
Jarvis, N. D.	2.32	1.90	2.49	2.15	2.22	1.97	0.97	0.83	0.93	0.92	1.16	0.94
Kinney, C. N.			1.99	2.18	1.99	2.11			0.95	0.79	1.02	0.83
Koskoski, F. J.	2.01	2.03	2.11	2.19	2.05	2.07	0.81	0.78	0.94	0.95	0.99	0.96
Struve, O. I.	1.98	1.95	2.18	2.16	2.02	2.06	0.79	0.79	0.97	0.95	0.97	0.97
Huey, E. E.	2.02	1.98	2.21	2.06	2.05	1.97	0.82	0.83	0.80	0.94	0.83	1.00
Randall, F. E.	2.05	1.91	2.23	2.10	2.11	1.97	.81	.82	.93	.94	1.00	1.00
Midkiff, V. C.	2.00	2.00	2.17	2.17	2.06	2.10	.79	.78	.93	.93	.97	.97
Hunter, W. L.	1.99	1.98	2.19	2.17	2.05	2.04	.803	.796	.950	.940	.988	.969
Geagley, W. C.	1.96	1.96	2.17	2.19	2.05	2.05	.78	.79	.91	.92	.94	.95
Fritz, J. C.	2.00	2.00	2.28	2.27	2.14	2.13	0.87	.88	1.02	1.02	1.06	1.07
Overholser, J. S.	1.96	1.96	2.20	2.15	2.03	2.05	.81	.81	.94	.94	.97	1.01
Fudge, J. F.	2.28	2.82	2.47	2.93	2.32	2.75	1.34	.89	1.57	1.19	1.65	1.17
Elmslie, W. P.	2.12	2.01	2.27	2.22	2.08	2.09	.78	.79	.95	.94	.98	.98
Merrill, E. C.	2.22	2.41	2.49	2.60	2.19	2.39	0.71	0.76	0.83	0.90	0.86	0.99
Ingram, W. J.	2.00	2.02	2.17	2.16	2.08	2.03	0.84		0.97		1.01	
Average	2.02	2.03	2.22	2.22	2.07	2.09	0.84	0.80	0.96	0.95	1.02	0.98

* A—A.O.A.C. Methods of Analysis (6th Ed., p. 415 (27.47), p. 127 (12.37 (b).
B = Nitric-perchloric Method (*Ind. Eng. Chem. Anal. Ed.*, 7, p. 167 (1935).

REPORT ON CRUDE FIBER

By WILLIAM L. HUNTER (State of California, Department of Agriculture, Sacramento 14, Calif.), *Associate Referee*

The 1946 report on the determination of crude fiber contained a preliminary discussion of a survey of the method as applied by the several participants in the collaborative sample of the Association of American Feed Control Officials. It was observed that the present procedure was but rarely followed in all details.

The diversity of techniques reported is extremely disconcerting in the instance of a method which is as openly arbitrary and empirical as that for crude fiber. This is especially true in some cases where the variations depart from the official method almost completely. It seems necessary to warn all laboratories interested in crude fiber that departure from the official method in any respect places them in a vulnerable position in an action based on crude fiber analyses. It may be argued that the results obtained by a variation of the method do not represent crude fiber content in a legalistic sense and are, therefore, immaterial and irrelevant. This possibility is not too far-fetched since crude fiber is not a specific chemical substance, but rather the result of an arbitrary physico-chemical test. It is the residue, the insoluble material remaining after a series of carefully prescribed operations. Liberties taken with the procedure may alter the results and defeat agreement between laboratories. Strict adherence to the established method cannot be too strongly emphasized. It should be followed in all respects.

While insisting upon adherence to the official method, it appears that some revisions of this procedure are in order for the purposes of clarity, preciseness, and emphasis of detail. The use of Erlenmeyer flasks, which are now prescribed for the digestion, has given way to the use of beakers almost unanimously.

The present allowance for final filtration through either alundum crucibles or filter cloth is in an opposite category. Study of results of laboratories using these has indicated that they may be involved in unsatisfactory results. The alundum crucibles are difficult to wash free of the alkaline extract, and the use of cloth involves excessive handling. Revisions are recommended which may be regarded as editorial.[1]

It is recommended*—

(a) That study of the crude fiber determination be continued to allow for investigation of possible revisions as well as those necessary in the case of materials which are high in mineral.

[1] The changes suggested have been published (*This Journal*, **31**, 99 (1948).
* For report of Subcommittee A and action of the Association, see *This Journal*, **31**, 41 (1948).

REPORT ON CRUDE PROTEIN IN FEEDING STUFFS (CATALYSTS)

By RODNEY C. BERRY (State Div. of Chemistry, Department of Agriculture, Richmond, Va.), *Associate Referee*

According to the recommendation of the Association at the 1946 meeting and under the approval of the General Referee on Feeding Stuffs, a collaborative study on Crude Protein in high concentrate feeding stuffs, with special reference to catalysts, was inaugurated during the year.

Samples of cottonseed meal, fish meal, and meat scrap were submitted to 15 collaborators (13 of whom completed the work, with one too late to tabulate) who were instructed to make analyses in triplicate according to four methods, *viz:* (1) *Methods of Analysis*, sec. **2.24** using both cupric sulfate and mercuric oxide (2) sec. **2.25** cupric sulfate only (3) sec. **2.26,** using mercuric acid only, and (4) using selenized granules along with approximately 0.7 grams of mercuric oxide, 30 ml concentrated sulfuric acid and 10 grams of potassium sulfate. Selenized granules were furnished by the Referee.

Criticisms of the methods were requested and the collaborators were very generous with their cooperation; some of them also submitted results based on their own methods or other variations in procedure.

COLLABORATORS

(A) Walker, L. S., Chemist-in-charge, State Agricultural College, Burlington, Vermont.

(B) Randle, Stacy B., New Jersey Agri. Experiment Sta., New Brunswick, N. J.

(C) Smith, John B., R. I. State College, Kingston, R. I.

(D) Marshall, Chas., Plant Production Div., Dept. of Agri., Ottawa, Canada.

(E) Hunter, Wm. L., State Dept. of Agri., Sacramento 14, California.

(F) Geagley, W. C., Michigan Dept. of Agriculture, Lansing, Michigan.

(G) Randall, Fred, Cooperative G. L. F. Mills, Inc., Buffalo, New York.

(H) Etheredge, M. P., State College, Miss.

(I) Bides, P. R., Auburn, Alabama, 515 Dexter Avenue.

(J) Koskoski, Frank J., N. Y. State Agri. Exp. Sta., Geneva, New York.

(K) Burns, Loren V., M. F. A. Milling Co., Springfield, Missouri.

(L) Berry, Rodney C., 1123 State Office Building, Richmond, Virginia. Shuey, Phillip McG., c/o Shuey & Co., 115 E. Bay St., Savannah, Georgia.

RESULTS AND DISCUSSION

A complete tabulation of results is submitted in the tables along with criticisms of the methods.

The average clearing time for all four methods on all samples was shorter when selenium was used, varying from 12 to 50 minutes. The use of mercury alone (method 3) was preferred by the majority of collaborators. Some analysts prefer selenium-oxychloride alone and selenium and copper sulphate for their routine analyses.

TABLE 1.—*Collaborative results on crude protein in feeding stuffs*

COLLABORATOR	SAMPLE 1. C. S. MEAL				SAMPLE 2. MEAT SCRAP				SAMPLE 3. FISH MEAL			
	METHOD				METHOD				METHOD			
	Cu-Hg	Cu	Hg	Se-Hg	Cu-Hg	Cu	Hg	Se-Hg	Cu-Hg	Cu	Hg	Se-Hg
	1	2	3	4	1	2	3	4	1	2	3	4
A	35.86	36.01	36.60	36.07	46.92	47.38	47.90	47.95	58.84	58.56	60.19	60.59
B	36.69	36.94	37.38	37.06	48.31	47.46	49.13	49.09	60.46	60.52	61.29	61.42
C	37.31	36.64	37.54	36.84	48.58	48.66	49.56	49.11	60.76	61.88	61.85	61.30
D	36.10	35.50	36.50	36.30	47.90	46.40	47.70	47.80	59.80	58.10	59.60	59.70
E	36.6	37.4	37.6	37.5	48.4	48.8	48.8	48.6	60.6	61.0	61.4	61.4
F	35.9	36.48	36.96	35.79	47.52	47.34	48.48	47.54	59.38	60.34	60.67	59.75
G	36.85	36.95	36.92	37.15	48.05	48.25	48.92	48.82	60.48	60.58	61.15	61.32
H	36.73	37.21	37.23	37.11	48.48	48.67	48.81	48.67	60.56	61.40	61.29	61.25
I	36.66	37.19	37.19	37.19	48.54	48.54	48.75	48.85	60.21	60.62	61.66	61.04
J	36.50	37.50	37.30	36.90	48.60	49.00	48.60	48.30	61.20	59.30	61.20	61.40
K	35.74	36.56	36.53	36.58	47.17	48.37	48.24	48.33	59.51	60.69	60.59	60.60
L	37.88	38.25	38.38	38.13	49.13	49.06	49.50	49.38	62.25	62.44	61.75	61.56
Av.	36.24	36.88	37.17	36.88	48.13	48.16	48.70	48.53	60.33	60.45	61.05	60.94
Av. Total		36.88				48.38				60.70		
Dev. from Av.	−.64	+.005	+.297	+.005	−.25	−.22	+.32	+.156	−.363	−.15	+.35	+.244
Diff. High & Low	1.09	2.75	1.88	2.34	2.21	2.66	1.86	2.84	3.41	4.34	2.25	1.86
#3 over #1			+.937				+.57				+.713	
No. High	none	1	9	2	1	3	7	2	none	4	3	5
No. Low	9	2	none	1	7	4	none	1	9	3	none	none

The percentage recovery of protein as compared with the average of all four methods seems to be in the order: mercury, selenium and mercury, copper, copper and mercury, with the greatest difference on the sample

TABLE 2.—*Collaborative comments on crude protein in feeding stuffs*

	#1—C.S. MEAL METHOD				#2—MEAT SCRAP METHOD				#3—FISH MEAL METHOD				
	1	2	3	4	1	2	3	4	1	2	3	4	ANALYST
Clearing time in Minutes	100	80	60	45	45	50	35	30	50	60	40	30	(A) E. F. Boyce
	75	80	60	50	75	80	85	50	85	90	70	50	(B) Ralph L. Willis
	No significant difference. Prefers Method #3												(C) R. W. Gilbert
	24 Choice #3 Also run by Se-Oxychloride—					Choice #1			Choice #1 preferred				(D) Dr. R. Payfer
	No comments.												(E) V. E. Entwistle C. A. Luman
	25 24 17 14 Clearing effect due to the ratio of acid and K_2SO_4												(F) Percy O'Meara
	25 30 18 12 Slight preference for Selenium.												(G) F. E. Randall
	Prefers #3—Mercury												(H) A. G. McKee
	Method #4 is faster.												(I) P. R. Bidez
	19 35 25 13 17 19 22 13 15 21 17 13 Digests 3.5 hours. Also ran 3 additional methods. Prefers $CuSO_4$—Se.												(J) F. J. Kokoski
	20 30 15 15 #1—is violent on addition of water. #3—tends to crystallize.												(K) R. L. Matthews
	20 20 20 20 Same comments as above.												(L) W. J. Franklin

of cottonseed meal. There was only a slight difference in recovery between mercury alone and mercury and selenium, when applied to meat scrap and fish meal.

The digestion time varied from one hour after clearing to three hours.

There was considerable difference in results among collaborators on each method, with a high low ratio of 4.34% in method (2) on fish meal.

RECOMMENDATIONS*

In view of the results obtained by this study and also according to results obtained in previous work on nitrogen in soils, plant material, and fertilizers (see references), it is recommended that A.O.A.C. Method **2.26** for nitrogen be made preferential when applied to protein materials in feeding stuffs containing 30% or more, and that further work on catalysts in protein determination be discontinued.

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———, *This Journal*, **24**, 264–268 (1941).
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* For report of Subcommittee A and action of the Association, see *This Journal*, **31**, 41 (1948). Details of official changes are given on page 99 (*loc. cit.*).

No reports were given for protein evaluation in fish and animal products on hydrocyanic acid glucosides, or on sampling and analysis of condensed buttermilk.

APPOINTMENTS

K. G. Clarke, Division of Fertilizer and Agricultural Lime, Department of Agriculture, Beltsville, Md., has been appointed as Associate Referee on Inert Materials (Fertilizers).

CONTRIBUTED PAPERS

THE PRESENT STATUS OF THE ANTIMONY TRICHLORIDE (BLUE-COLOR) PROCEDURE FOR THE ESTIMATION OF VITAMIN A IN MARGARINE[1]

By E. E. Rice,* E. Primm,† A. I. Coombes‡

When the program of enrichment of margarine with vitamin A was initiated several years ago, the search for a rapid, precise, and accurate method of analysis for this vitamin was intensified. From the practical viewpoint of control laboratories, an acceptable method must be capable of yielding accurate and reproducible results in the hands of skilled technicians. It should be adaptable to different types of margarines without extensive modifications, yet simple enough to permit workers in different laboratories to obtain concordant values.

In general, there are three types of methods which can be used for the determination of vitamin A: biological, spectrophotometric, and colorimetric.

1. *Biological assays for vitamin A*, while fundamentally essential to prove the validity of any chemical or physical method, are obviously unsuited for the routine testing of large numbers of samples, being slow, expensive, and lacking in precision.

2. *Spectrophotometric methods*, under certain conditions, can be used to advantage. In general, there are three approaches, each of which has certain limitations.

(a) The vitamin A content may be determined from the difference in optical density at 328 mμ between the unfortified margarine oil and oils separated from the melted, fortified margarine. This method is restricted to cases where the unfortified oils can be obtained, since oil mixtures differ sufficiently to necessitate a blank for each run. However, when the unfortified oils are available, the method is rapid and precise.

(b) The sample may be saponified and the vitamin A content determined from the difference between the optical densities of the unsaponifiable fraction before and after irradiation with ultraviolet light to destroy the vitamin (1). This method is much more complicated than **(a)**, requiring careful control of analytical conditions and the use of especially purified solvents. Furthermore, the unsaponifiable materials other than vitamin A must be stable to ultraviolet irradiation.

(c) The sample may be saponified and purified by chromatography before the determination of the optical density at several wave lengths in the 300–350 mμ region. There are several such methods, each of which requires rather complicated manipulations. For example, that of Wilkie and DeWitt (2) involves calculations based on the measurement of vitamin A at an optical density other than that of the maximum. On a theoretical basis, this latter point is sufficient to cause most analysts

[1] This paper is a report of the work of the Subcommittee on Vitamin A, National Association of Margarine Manufacturers.

* Swift & Company, Chicago, Ill.
† John F. Jelke Company.
‡ Wilson & Co., Inc.

to pause before undertaking the method. In practice, the scheme has proved successful in the hands of some analysts, but has failed completely for others. A relatively new method has been advocated by Awapara, Mattson, Mehl, and Deuel (3). With this technique, the optical density of a benzene solution of the unsaponifiable fraction of a margarine is measured before and after specific adsorption of vitamin A on Floridin, and the vitamin A in the sample is calculated from the difference between the observed optical densities.

3. *Chemical methods* are generally based upon the development and measurement of the blue color formed by treatment of vitamin A with antimony trichloride. Most of these methods are complicated by the need for preparing an extract of the unsaponifiable material in margarine. While vitamin A is ordinarily the only substance occurring in margarine which yields the blue color with antimony trichloride, other substances sometimes affect the rate of development and the intensity of the color, thus requiring the use of correction factors. With care, the method yields reproducible values which check biological assays within the limits of error of the latter method.

In the hope that a unified program might bring about more rapid advances than individual efforts, a number of investigators interested in vitamin A methods met in 1944 and thoroughly discussed the problems involved and the best plan for solving them. This meeting initiated a series of collaborative studies of the various methods and modifications.

Initially the blue-color method, essentially as applied by Dann and Evelyn (4), was most commonly used and seemed most promising for development. However, there was no agreement regarding the exact conditions necessary for best results, and little data regarding the precision and accuracy of the method when applied to margarine. Accordingly, replicate samples of several margarines, and of the oils and vitamin concentrates going into them, were distributed for analysis by methods in use by the various laboratories. These investigations were directed toward determinations of the conditions necessary for most efficient and effective application of the various operations involved in blue-color methods. After consideration of the data obtained in two preliminary collaborative studies, a blue-color procedure was developed embodying the combined experience of the group. In a third and fourth collaboration, this method has been compared with two modifications of the spectrophotometric technique and its application to a variety of margarines has been studied. The following report presents the data obtained in these studies and some of the viewpoints of the collaborators.

METHODS

(1) *Distribution of Samples.*[1]—Samples of the oils to be used in the manufacture of margarine were removed from the mixing vats prior to addition of vitamin A concentrates. The manufacturing processes, including enrichment with known quantities of vitamin A, were then com-

[1] By John F. Jelke Company, Armour and Company, Durkee Famous Foods, Kraft Foods Company, Swift and Company, and Mrs. Tucker's Foods, Inc.

pleted and fifty one-pound prints of each margarine were taken directly from the packaging lines. Two prints, 100 ml of the mixed unfortified oils, and several capsules of a high-potency vitamin A ester concentrate[2] for use as a standard were shipped to:

Sadie Brenner, QM Food & Container Institute for the Armed Forces
Francis W. Chornock and Eldon E. Rice, Swift & Company
A. Irving Coombes, Wilson & Co., Inc.
H. J. Deuel, Jr., J. W. Mehl, and J. Awapara, University of Southern California
Stephen Fein, Monica Cunningham, and Delores Gehrs, Kraft Foods Company
Carl S. Ferguson, Massachusetts Dept. of Public Health
C. Gilmore, Durkee Famous Foods
J. A. Mathews, Distillation Products, Inc.
Kenneth Morgareidge, Nopco Chemical Company, Inc.
Ralph H. Neal, The Best Foods, Inc.
Bernard Oser, Food Research Laboratories, Inc.
Elizabeth Primm, John F. Jelke Company
John F. Roland, The Armour Laboratories
Lawrence Rosner and Gerda L. Siegel, Laboratory of Vitamin Technology
Arthur H. Seibert, Shilstone Testing Laboratory
Harold K. Steele, The Fleischmann Laboratories
Fred W. Wharton, Mrs. Tucker's Foods, Inc.

(The above alphabetical arrangement does not correspond with the numerical order in the tables; these numbers have been assigned in the order in which the reports were received in the third collaboration).

(2) *Analytical Procedures.*—Each collaborator was requested to assay the margarine in duplicate by one or more of the following methods.

A. Blue Color Method[3]

(1) Quarter a print, mix thoroly, and weigh a 10.0-g portion into a 250–350 ml flask.

(2) Add 50 ml of alcoholic alkali (10 g of KOH per 100 ml of soln; specially denatured No. 30 alcohol is satisfactory) and reflux for 15 mins.

(3) Cool, add 100–150 ml of water, and extract successively with 125-, 100-, and 75-ml portions of peroxide-free diethyl ether.

(4) Pool the extracts and wash once with 100 ml of water, once with 50 ml of 0.5 *N* KOH or NaOH, and then with successive 75- to 100-ml portions of water until free from alkali (wash soln colorless to phenolphthalein).

(5) Allow to stand 15 min., drain out the last droplets of water, and shake the ether extract with 3–5 g anhydrous sodium sulfate. Filter and wash the extraction vessel and sodium sulfate twice with 25-ml portions of ether.

(6) Remove the ether by evaporation *in vacuo* or in an atmosphere of nitrogen.

(7) *Immediately*, dissolve the unsaponifiable material in chloroform and dilute to a volume such that the expected vitamin concentration is in the proper range for the instrument to be used in measuring the intensity of the blue color. This concentration is usually 5–15 units per ml.

(8) Place 1 ml of this solution in a cuvette, add 1 ml of chloroform, then add rapidly 10 ml of antimony trichloride reagent (25 g reagent grade $SbCl_3$ dissolved in 100 ml $CHCl_3$) and *immediately* determine the per cent transmission at 620 mμ.

Determine M, the U.S.P. units of vitamin A represented by this transmission

[2] Obtained from Distillation Products, Inc.
[3] This method is essentially that published by the Association of Vitamin Chemists, Inc. (5), which includes more detail regarding preparation of reagents and suggestions for avoiding or correcting difficulties.

reading, from a reference curve correlating per cent transmission with various known quantities of the vitamin prepared by direct dilution with chloroform of a weighed quantity of U.S.P. Vitamin A Reference Standard.[4] M, multiplied by the dilution factor (total volume of chloroform solution of margarine unsaponifiables divided by the weight of margarine), gives the potency of the margarine in terms of units per gram.

As a check upon the accuracy of this value it is desirable to make an additional reading and an increment calculation.

(9) To another 1-ml portion of the soln of unsaponifiables add 1 ml of a chloroform soln containing S, a known number of units of vitamin A. (Prepared by dilution of the U.S.P. Reference Standard.) For greatest precision, S should be approximately equal to M. Add rapidly 10 ml of reagent and *immediately* determine the per cent transmission at 620 mμ. Determine I, the units of vitamin A represented by this transmission reading, from the reference curve.

It is now possible to use an increment technique for determining the vitamin A potency of the margarine by relating M and I to the known value of S. This relation may be stated:

$$\text{Units per ml of dilution} = M \div \frac{(I-M)}{S}$$

This equation introduces [(I−M)/S] as a correction factor to compensate for any inhibition of blue color formation by impurities in the sample under test. In effect, [(I−M)/S] is the ratio between the recovery of a known quantity of vitamin A in the presence of possible interferences and the known value, S.

In place of M and I, their corresponding per cents transmission may be converted to optical densities (optical density = 2 − log per cent transmission) and substituted in the above equation. S, in this case, is still expressed in units of vitamin A per ml.

Such calculations do not take into account the color of the test solution or of colors formed from non-vitamin A materials upon treatment with the reagent. Ordinarily, corrections for these extraneous colors are unnecessary. However, in order to make certain that such colors would not affect precision in the third collaboration, each collaborator was requested to prepare a blank by saponifying 8.1 g[5] of the unfortified oil and to determine the blank B. (B is again expressed as units of vitamin A per ml from the standard curve, or as optical density calculated from the observed per cent transmission.) Determination of the concentration of vitamin A in the solution of the unsaponifiable fraction of the margarine was than made from the formula:

$$\text{Units per ml of dilution} = (M-B) \div \frac{(I-M)}{S}$$

B. Blue Color Method on Whole Margarine Oils (no saponification).

Melt slowly a portion of the margarine and allow the oil to separate. Filter through

[4] This reference curve should be checked at frequent intervals to minimize variations due to changes in reagents and manipulation. At the time of the collaboration this standard was not available and oil of high potency, supplied by Distillation Products, Inc., was used as a reference.

[5] Margarines contain approximately 81 per cent of oil mixtures.

rapid paper. Using this clear oil and the untreated unfortified oil (or $CHCl_3$ dilutions of them), determine values corresponding to M, B, S, and I, and calculate the potency as indicated in A.

C. Spectrophotometric Method on Whole Margarine Oils.

Prepare oil from the margarine as in Section B. Dilute with cyclohexane as required and determine the optical density at 325–8 mμ, using a similar concentration of the unfortified oil as a blank. Calculate potency from the formula (O.D./C×2000 ×454×0.81 = units per pound of margarine, where O.D. = observed optical density, C = concentration of oil in grams per 100 ml of soln, and 2000 is the factor for converting O.D./C into units of vitamin A. (Using the new U.S.P. Vitamin A Reference Standard, the conversion factor is 1894).

D. The Method of Wilkie and DeWitt (2, 8).

E. The Method of Awapara, Mattson, Mehl, and Deuel (3).

In the third collaborative study of vitamin A methods, each member of the committee was supplied with two pounds of product from a single manufacturer, and with a sample of the unfortified oils from which the margarine had been made. Each analyst was requested to assay the samples by as many of the methods as possible. The values obtained are summarized in Table 1.

TABLE 1.—*Vitamin A analyses reported for a margarine sample—third collaboration*

(Thousands of units per pound)

LAB. NO.	DATE OF ASSAY 1946	SPECTROPHOTOMETRIC TECHNIQUE*	BLUE COLOR TECHNIQUES*†			
		UNFORTIFIED OIL *vs.* OIL SEPARATED FROM MARGARINE	VALUES OBTAINED DIRECTLY FROM CALIBRATION CURVE	VALUES OBTAINED BY INCREMENT TECHNIQUE	VALUES FOR UNSAPONIFIED OIL, BY INCREMENT TECHNIQUE	BLANK VALUES, FROM CALIBRATION CURVE
1	2–5		14.3 E	16.1		
	2.7		17.2 E	19.7		
	Avg.	17.8 B	15.7	17.9		0.5
2	2–6		17.3 E	18.1		
	2.6		16.5 E	17.5		
	Avg.	17.4 B	16.9	17.7	18.6	.5
3	2–6		15.6 E	15.3	Impossible (values?)	.0
4	2–8		14.5 E	15.4		
	3–27		15.5 E	16.5		
	Avg.		15.0	15.9		.5

TABLE 1.—(*continued*)

LAB. NO.	DATE OF ASSAY 1946	SPECTROPHOTOMETRIC TECHNIQUE*	BLUE COLOR TECHNIQUES*†			
		UNFORTIFIED OIL *vs.* OIL SEPARATED FROM MARGARINE	VALUES OBTAINED DIRECTLY FROM CALIBRATION CURVE	VALUES OBTAINED BY INCREMENT TECHNIQUE	VALUES FOR UNSAPONIFIED OIL, BY INCREMENT TECHNIQUE	BLANK VALUES, FROM CALIBRATION CURVE
5	2–8		16.4 C	16.1		
	2–8		17.4 C	15.9		
	Avg.	17.4 B	16.9	16.0	16.2	.6
6	2–16	16.3 G	16.3 E	17.4	17.8	.3
7	2–26		15.1 E	16.6		
	2–26		15.0 E	16.2		
	Avg.		15.0	16.4	—	.8
8	2–27		16.7 E	16.4	—	.3
9	3–4	15.5 C	19.0 C	18.2	—	1.6
10	2–20		16.6 E	17.8		
	3–4		14.8 E	15.4		
	3–6		15.4 E	15.7		
	Avg.	16.8 B	15.6	16.3	15.4	—
11	3–8		17.5 C			
	3–8		17.6 C			
	Avg.		17.5			
12	3–10	20.3 B	17.7 E	18.1		.5
13	2–18	16.4 B				
14	2–13		15.4 E	15.0		
	Avg.	17.2 (16.8)	16.4	16.7	17.0	.5
Standard Deviation		1.4 .78	1.2	1.1		
Coefficient of Variation		8% 5%	7%	7%		

* The types of instruments used in determining the vitamin content of the various solutions are indicated by the letters B, C, E, and G, these referring to the Beckman spectrophotometer, the Coleman spectrophotometer, the Evelyn photoelectric colorimeter, and the Gaertner spectrograph, respectively.

† Each value recorded in the table is the average of duplicate analyses. In no case did these duplicates differ by more than 6% of their mean, and in most cases the differences were less than 4%.

After meeting and discussing these data, the Committee arranged for a fourth collaboration. Six different manufacturers sent samples to the committee members, who were asked to assay each sample by the blue color method and to report values representing direct readings from a standard curve, and also values representing the calculation $M \div (I-M)/S$. The Floridin technique (3) and modification of the glycerol dichlorhydrin method (6) were also to be used where possible. The manufacturers were requested to supply products with a vitamin A content either slightly higher or slightly lower than their normal production to provide a range of expected potencies. Several types of oils and vitamin concentrates were used in preparing the margarines, the combinations being:

Manufacturer I, soybean oil plus vitamin A esters
Manufacturer II, mixed vegetable oils, plus vitamin A esters
Manufacturer III, mixed vegetable oils plus vitamin A alcohol
Manufacturer IV, 75 per cent oleo oil and 25 per cent vegetable oils plus vitamin A esters
Manufacturer V, mixed vegetable oils, rich in cottonseed oil, plus vitamin A esters
Manufacturer VI, mixed vegetable oils, plus vitamin A esters.

Data obtained in this fourth collaboration appear in Tables 2 and 3.

DISCUSSION

The transient nature of the blue color and the unequal rates of fading of the color in standard and test solutions, coupled with differences in techniques for making the galvanometer readings, preclude expectation of perfect checks between laboratories or even within a single laboratory. In view of these difficulties, the variations in values reported by blue-color methods (Tables 1 and 2) are not surprising, even though they do leave much to be desired insofar as precision is concerned.

Because the analytical manipulations are few and simple, greater precision might be expected when values are obtained by the spectrophotometric technique in which oil from fortified margarine is compared with the unfortified oil blend from which the margarine was made. Actually, the values found by this technique were disappointing, their range being even greater than that for the blue-color assays (column 3, Table 1). It is of interest, however, that the low value obtained by this method (15.5 by Laboratory 9) was obtained with a Coleman spectrophotometer with readings from a standard curve; and the high value (20.3 by Laboratory 12) was obtained with a Beckman spectrophotometer by separate readings of E1%/1 cm for blank oil and fortified oil. In the other laboratories, where Beckman or Gaertner instruments were used and in which method C was followed, the agreement was much better, the values ranging from 16.3 to 17.8.

Blue-color values found by interpolating galvanometer readings upon recently checked calibration curves usually agreed reasonably well with

TABLE 2.—*Blue color analyses reported for various margarines—fourth collaboration*
(Thousands of units per pound)

LAB. NO.	SOURCE OF SAMPLE											
	MANUFACTURER I		MANUFACTURER II		MANUFACTURER III		MANUFACTURER IV		MANUFACTURER V		MANUFACTURER VI	
	A[1]	B[2]	A[1]	B[2]	A[1]	B[2]	A[1]	B[2]	A[1]	B[2]	A[1]	B[2]
(Fortification Level)	21.1	21.1	17.0	17.0	18.8	18.8	19.2	19.2	18.0	18.0	16.5	16.5
1	17.9	20.7*	13.8	15.6	16.4	18.0	16.2	17.7	15.9	17.2	14.7	16.0
2	19.6	22.7	15.8	17.8	18.4	21.3	17.1	20.4	17.4	20.1	16.2	18.4
3	17.6	20.1*	13.6	15.3*	16.0*	17.6*	16.0*	17.9*	15.7	17.0*	14.4	16.5*
5	18.9	18.1	15.5	15.7	17.6*	18.0	18.0*	17.9	17.0	18.1	16.1	16.4
6	19.3	19.9	15.1	15.6	17.4	17.8	17.6	18.0	16.8	17.1	15.6	16.0
7	18.3	19.8	14.3	15.4	16.5	18.2	16.4	17.9	16.4	17.7	14.7	15.8
10	18.7	21.2	13.8	15.5	16.2	17.9	16.2	17.0	14.6	15.2	13.9	15.0
11	18.7	17.9*	15.1	15.1	17.6	18.2*	17.6	16.7	17.1*	17.4*	15.9	16.3*
13	17.7	18.1*	14.1	15.3	15.6	16.7	17.0*	17.1*	15.6	15.9*	14.1	14.1
14	18.7	20.5	14.7	15.3	17.2	17.9	17.6	18.5	16.9	18.4	15.7	16.3*
15	21.7	22.5	16.9	16.7	19.6	20.1*	19.5*	20.0*	18.7*	19.9*	14.7	16.6
16†	(17.8)	(17.3)	(13.4)	(13.1)	(14.3)	(13.1)	(15.5)	(17.8*)	(15.9)	(17.1*)	(13.6)	(14.1)
17	18.2		12.6		15.8		13.8		14.5		14.5	
Avg.	18.7	20.1	14.6	15.8	17.0	18.3	16.9	18.1	16.4	17.6	15.0	16.1
Standard dev.	1.2	1.2	1.2	0.6	1.1	1.4	1.4	1.1	1.1	1.4	1.1	1.1
Coef. of var.	6.3	7.9	8.1	7.0	6.6	7.4	8.5	5.6	5.9	7.6	6.9	6.8

[1] Values obtained by interpolation of values upon a previously prepared reference curve.
[2] Values obtained by an increment calculation.
* Poor checks: spread of 1000 units per pound or more.
† Not included in averages because subsequent data show instruments used to be in need of adjustment.

those found by an increment technique (Table 1, columns 4 and 5; and Table 2 columns A and B). However, in general, the increment calculations indicate a vitamin A content 4 to 8 per cent greater than do calculations based on readings from a calibration curve; this despite the fact that the former entails a correction for the blank while the latter does not. Occasional assays have been reported in which the increment technique results in values as much as 20 per cent greater than those read from reference curves. This trend has been observed previously for a number

TABLE 3.—*Per cent recovery of vitamin A added to unsaponifiable material of margarines as determined by blue color analyses**

LAB. NO.	FOURTH COLLABORATION — MANUFACTURER NO.— 1	2	3	4	5	6	LABORATORY MEAN	STANDARD DEVIATION
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		
1	88	88	90	90	92	90	89.7	1.5
3	88	89	91	89	92	87	89.3	1.9
4	98	92	94	99	98	100	96.8	3.1
5	104	99	98	100	94	100	99.2	3.3
6	97	97	97	98	98	97	97.3	.2
7	92	93	91	92	92	93	92.2	.2
10	88	89	91	95	97	93	93.2	3.5
11	105	99	97	105	99	98	100.5	3.6
14	94	93	90	94	91	94	92.7	1.7
15	96	101	98	98	94	89	96.0	4.2
16	102	102	110	87	93	97	98.5	8.0
Mean	95.6	94.7	95.1	95.1	94.6	94.4		
Standard Deviation	6.3	5.0	5.9	5.3	2.9	4.3		

* These figures represent the per cent recovery of a known amount of vitamin A added to $CHCl_3$ solutions of the unsaponifiable of the various margarines. Each figure is the average of two values.

of types of margarine, and brings into question the use of a blank value in calculations.

The blanks used in obtaining the data shown in Table 1 were measured by assaying the unfortified oil blend from which the margarine was made. Theoretically, the use of such a blank is the most valid procedure. Practically, however, the unfortified oils are seldom available and blanks of this nature cannot be obtained. When the unfortified oils are available, calculations should be made by an increment technique to compensate for inhibitions of color development by materials present in the unsaponifiable fraction.

Another type of blank has been reported (7), this being a color-blank

which is estimated by diluting the test solution with chloroform instead of with reagent, and reading the per cent light transmission the same as for the sample. In either case the readings for the blank are small, and relatively small instrument errors cause large errors in the blank. Also the color-blank does not compensate for decreases or increases in color which may occur when reagent is used, but which do not occur in chloroform. The increment values shown in Table 1 were calculated from the formula $(M-B) \div [(I-M)/S] =$ units per ml of dilution; those in Table 2 were calculated from the formula $M \div [(I-M)/S]$ units per ml of dilution.

The existence of an inhibitory effect is amply demonstrated by the data in Table 3, which show recoveries averaging 94 to 96 per cent of vitamin A added to the unsaponifiable materials of the fortified margarines of both collaborations. In many cases the decrease in vitamin A potency caused by this inhibition is almost the same as the value for the unfortified oils; thus, direct readings from a calibration curve check closely with calculations by the more complicated increment technique. This observation has led many analysts to question the validity of a blank correction. In the case of the sample used in collaboration No. 3 it is rather apparent that subtraction of blanks from the values reported by calculations from a calibration curve (Table 1, Column 4) would result in values in poorer agreement with those obtained by the several other methods. This has been observed in previous collaborations and in many observations reported in committee meetings. In fact, it has been so general as to lead to the tacit assumption by some analysts that the blank compensates for inhibition of color formation, and that values may be calculated directly from a calibration curve without consideration of the blank. For routine work with margarine or oils of known composition, this technique reduces manipulative error and greatly simplifies calculations. With unknown materials the assays should always be checked by recovery experiments, and increment calculations must be made if the recoveries are lower than 95 per cent. It should be realized that the use of readings from a curve without the use of a proper blank is not a scientifically sound method. since it involves compensating errors.

While the increment technique is theoretically the most sound, it exaggerates deviations in blue-color readings, since an error made in M also results in an erroneous value for $I-M$. Thus, if the value for M is 1 per cent low, $I-M$ will be about 1 per cent high; and since M is divided by $I-M$, the answer will be approximately 2 per cent low instead of the 1 per cent of the initial value. Likewise, each increment calculation involves two values, M and I, and hence doubles the chance for error. Nevertheless the presence of inhibitory substances in many margarine extracts is sufficient to warrant the use of increment calculations. It should be noted that "increment results" in Table 1 are rather uniformly higher than those read from a curve. Random error would of course produce an equal number of high and low values.

The data presented in Table 2 give an erroneous impression of the precision of the methods so far as individual laboratories are concerned. By careful inspection of the data it may be seen that certain laboratories reported values which are consistently higher (or lower) than the mean. Thus, the values of Laboratories 2 and 15 are 5 to 15 per cent higher than the means for the various samples, while those for Laboratories 16 and 17 are low by similar amounts. Other laboratories (notably Nos. 6, 7, and 14) report values consistently near the means. This indicates good precision within the individual laboratories, but failure of all laboratories to obtain the same level of values. The experiences of several laboratories indicated the quality of the antimony trichloride used in preparing the reagent to be a factor influencing these interlaboratory variations.

The extension of blue-color assays by increment technique to include unsaponified oils (Method B) seems an interesting possibility. In the third collaboration, four laboratories were able to get reasonable values (Column 6, Table 1). Only one reported failure of this method. In the fourth collaboration, five laboratories reported values by this technique in agreement with the regular blue-color assays.

Considerable difficulty was reported in conducting assays according to the procedure of Wilkie and DeWitt. In the third collaboration, eight laboratories attempted the procedure. Three failed completely and the other five reported erratic values. Data for four (Nos. 1, 2, 10, 13) have been reported in detail by Wilkie (8) as Nos. 2, 7, 19, and 4, respectively. Laboratory 5, in a number of attempts, obtained but one value of 18,000 units per pound. Even those laboratories reporting values have had difficulties in obtaining consistent adsorption on the chromatographic columns as discussed by Wilkie (8). In general, the opinion of many analysts working with this latter method has been that it is too long and the technique is too involved for satisfactory analyses. Two types of correction factors are involved: (1) the use of a factor to correct for incomplete recoveries, and (2) the use of optical densities determined at 340 mμ rather than at 325–30 mμ. Failure of the method when optical densities are measured at the point of maximum absorption for vitamin A constitutes a serious fundamental drawback. This becomes of special significance when attempts to recover vitamin A by recognized procedures fail. Three laboratories conducting analyses by the chromatographic adsorption technique also conducted blue-color assays of solutions which had been chromatographed. The blue-color values were only 10,000–12,000 units per pound (60–75 per cent of the amounts found by the regular blue-color procedure). In fact, in repeated attempts, one laboratory was unable to get blue-color assays of more than 10,000–12,000 units per pound when solutions of unsaponifiables were prepared according to the saponification and extraction technique recommended for the chromatographic procedure. Despite this, following adsorption and elution, values of 15,000–17,000 units were indicated by measurements at 340 mμ and calculations

according to directions for the chromatographic method. This seems to indicate measurement of a modified form of vitamin A by the latter technique, and raises the question as to whether the method might measure this form of the vitamin if it occurred naturally. More data of this type are needed before the merits of chromatography can be evaluated fully.

In the fourth collaboration several laboratories attempted to apply the method of Awapara, Mattson, Mehl, and Deuel (3). This method recommends saponification of the sample, extraction of the vitamin A and part of the other unsaponifiables with Skellysolve A, washing of the extract with water, evaporation of the solvent followed by solution of the residue in benzene, and determination of the vitamin content from the optical density of the benzene solution before and after treatment with specially-prepared Floridin. The Floridin quantitatively adsorbs vitamin A and the difference in the optical density at 328 mμ between untreated and Floridin-treated extracts should be a measure of the vitamin content of the solution. Success of the method depends upon adjustment of extraction and adsorption techniques so that all of the vitamin is extracted from the saponification mixture and so that vitamin A is the only material absorbing light at 328 mμ which is retained by the Floridin column. In general, those laboratories using this technique have obtained high values for vitamin A, presumably because of the removal of materials other than vitamin A from the benzene extract by the Floridin. Refinements of the technique which are being developed by its proponents may eliminate this fault.

Modifications of the technique of Sobel and Werbin (6) have also failed for the several laboratories attempting to devise techniques for the adaptation of glycerol dichlorohydrin to margarine assays. The most common criticisms have been that the rate of color formation is different in margarine extracts than in standard solutions, and that the rate of fading is much more rapid for extracts than for standards.

SUMMARY AND CONCLUSIONS

Results have been presented of two collaborative studies in which replicate samples of margarine have been assayed by seventeen laboratories. In general, there has been better agreement among the values reported by blue-color analyses than among those obtained by the spectrophotometric methods now available. Direct calculation of the potency of the sample from values found by interpolation of the observed transmission upon a calibration curve agreed well with those obtained by a calculation involving a correction for observed recoveries of vitamin A, *i.e.*, by an increment type calculation. The coefficient of variation for blue-color values reported from thirteen laboratories is 7 per cent, with mean values of 16,425 units per pound (readings from a calibration curve) and 16,724 units per pound (increment calculations).

At present, most of the analysts who participated in this collaborative work believe the blue-color technique as outlined for increment calculation to be the most reliable general method available for margarine, but that, in most cases, readings may be made directly from a calibration curve.

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ISOLATION AND SPECTROPHOTOMETRIC CHARACTERIZATION OF FOUR CAROTENE ISOMERS

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The presence of neo-beta-carotene U and neo-beta-carotene B has been demonstrated in "pure" carotene extracts from plant sources (1, 10). Since these isomeric pigments have reduced nutritional value (3, 4), it is evident that physiological activity as determined by beta-carotene analysis may be in considerable error if the relative amounts of the stereoisomers present are not taken into account. Quantitative spectral data for these isomers are meager. The scarcity of such information on neo-beta-carotenes U and B made it desirable to prepare these materials in pure crystalline form for use as standards in the determination of the individual stereoisomers after separating them from one another by the flowing chromatographic technique (2).

In a recent A.O.A.C. report on carotene (11), the Associate Referee recommended that the spectrophotometric method for carotene be tentatively discontinued and that further work be done on the absorption

TABLE 1.—*Specific absorption coefficients at highest maximum for all-trans-alpha- and all-trans-beta-carotene (taken from literature)*

SOLVENT	ALL-TRANS-ALPHA CAROTENE		ALL-TRANS-BETA CAROTENE		REF.
	WAVE LENGTH mμ	SPECIFIC ABSORPTION COEFFICIENT	WAVE LENGTH mμ	SPECIFIC ABSORPTION COEFFICIENT	
Petroleum ether, b.p. 63–70°C (Skelly Oil Co.)	—	—	450	238	(15)
Heptane	448	—	455	240	(18)
Petroleum ether (40–60°C.)	—	—	450	243	(15)
Petroleum ether (40–55°C.)	—	—	447	245	(17)
Iso-octane	444	252	450	245	(14)
Petroleum ether	445	255	450	249	(13)
Petroleum ether (60–80°C.)	—	—	452	250	(8)
Hexane	—	—	453	253	(5)
Petroleum ether, 63–70°C (Skelly Oil Co.)	—	—	450	253	(19)
Petroleum ether (55–65°C.)	445	279	450	254	(21)
Hexane (65–67°C.)	446	272	450	258	(24)
Hexane	—	—	450	259	(19)
Petroleum ether (62–65°C.)	445–6	272	452	259	(23)
Hexane	—	—	453	261	(5)
Hexane	—	270	450	276	(20)
Petroleum benzine	—	—	450	280	(7)
Hexane	—	273	—	302	(12)

coefficients, since carotene content of plant extracts did not agree at the three wave lengths prescribed by the method. A study of the literature revealed a wide variation in the specific absorption coefficients for all-trans-alpha and all-trans-beta-carotene (Table 1). In the present investigation these isomers were prepared in as pure a state as possible. The methods of isolation and purification and the specific absorption coefficients of these four carotene isomers are given. The effects of several hydrocarbon solvents upon specific absorption coefficients have been evaluated.

EXPERIMENTAL

Materials:

Adsorbent.—The adsorbent used to separate alpha- and beta-carotene from one another was a 1-to-1 mixture of Micron brand* magnesia (Westvaco Chlorine Products Co. No. 2642) and Dicalite,* (Dicalite Co. No. 4200): a diatomaceous earth (22). This mixed adsorbent was also employed for concentrating dilute solutions in preference to evaporation procedures.

* The mention of these products does not imply that they are endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.

The adsorbent used to separate the beta-carotene stereoisomers was Shell brand* lime, chemical hydrate, 325 mesh, as recommended by Polgar and Zechmeister (16) and used by us in previous work (2).

Carotene.—A commercial crystalline carotene (90% beta, 10% alpha) was employed as the starting material for the preparation of purified isomers. Isomerized carotene solutions were prepared by adding iodine (equivalent to 2% of the weight of the dissolved carotene) to the solution of purified beta-carotene in hexane about one hour before the solution was added to the chromatographic column.

Drying Agent.—Anhydrous crystalline sodium sulfate was used in all cases to dry the petroleum ether solutions prior to chromatographic adsorption.

Solvents.—For the development of the chromatograms on magnesia-Dicalite columns, 2% by volume of reagent-grade acetone in commercial hexane (Phillips Petroleum Co.)* was used.

For the development of the isomerized beta-carotene chromatogram on hydrated lime columns, either a 2% solution (by volume) of acetone in hexane or a 1½% solution (by volume) of para-cresyl methyl ether in hexane was used (2).

Other solvents used in this work were:

Methyl and ethyl alcohols, refluxed over magnesium and redistilled prior to use.

Chloroform, shaken with concentrated sulfuric acid, washed, dried, and redistilled.

Benzene, dried over sodium and redistilled.

Reagent-grade acetone, used without redistillation.

A low-boiling fraction of petroleum ether (37° to 43°C.), obtained by fractionation of a low-boiling commercial solvent.

The following materials, without any pretreatment, were used as solvents for carotene in the determination of absorption coefficients: iso-octane (Rohm and Haas),* commercial hexane (Phillips Petroleum Co.), and three petroleum ethers having boiling point ranges as indicated in Table 3 (Skelly Oil Co.).*

For comparison with Zscheile's work, a special hexane fraction was prepared as described in his report (24) by treating the commercial hexane exhaustively with alkaline permanganate (about 3 weeks) and redistilling. Only the portion which distilled between 65° and 67°C. was used.

Chromatographic Apparatus:

The chromatographic tubes used were similar to those described by Zechmeister (22). No. VI tubes were used for the separation of isomers, No. II tubes for the concentration of solutions on magnesia-Dicalite, and No. I tubes for the analytical determination of the purity of the various preparations.

Spectrophotometric Measurements:

A Beckman quartz spectrophotometer, Model DU,* was used for absorption spectra measurements. The band width over the region 400 mμ–500 mμ was substantially constant at 0.5 mμ. In the region 320 mμ–400 mμ, the band width was less than 1.0 mμ, being about 0.6 mμ at 340

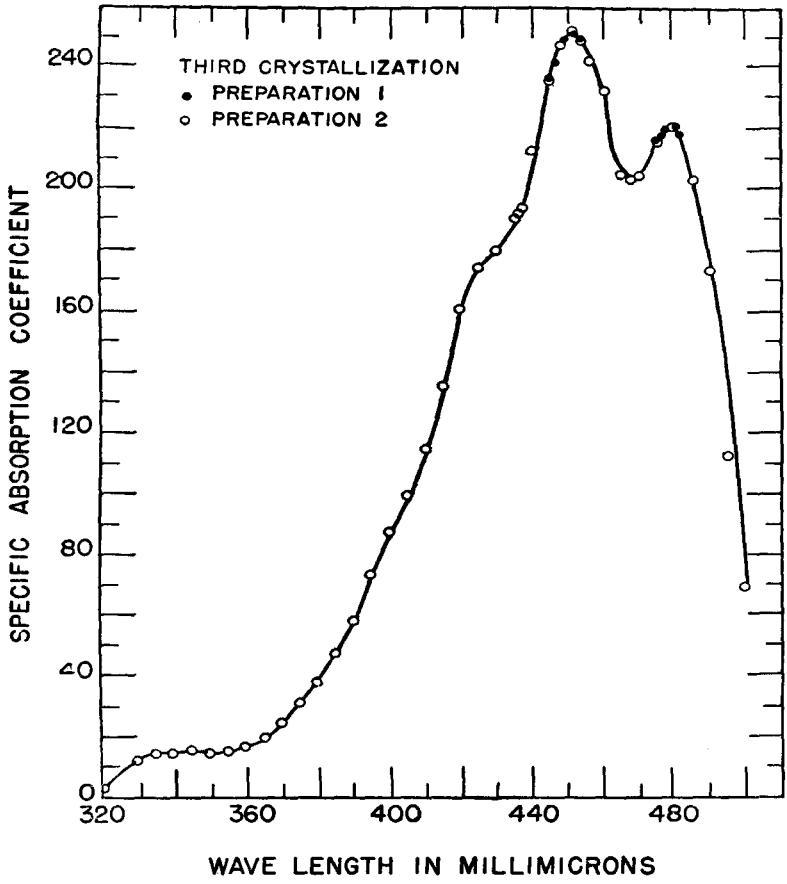

FIG 1.—Absorption spectrum of all-trans-beta-carotene in iso-octane.

mμ. The absorption coefficients obtained with narrower spectral bands (0.3 mμ) in the region 400 mμ–500 mμ were identical with those presented. The wave length scale, as calibrated against the mercury arc spectrum, is within 0.2 mμ in the region covered in this study. There were no observable changes of the specific absorption coefficients for the carotene solutions during the interval required for obtaining the spectral values.

Purification of Pigments:

All-trans-beta-carotene.—900 ml of a hexane solution containing 0.5 g of commercial carotene (90% beta, 10% alpha) was added to a No. VI chromatographic column packed to a height of 28 cm with the magnesia-Dicalite mixture. The column was developed with a 2% solution of acetone in hexane. After the alpha- and beta-carotene bands had become sufficiently separated, the column was extruded, the alpha- and beta-

TABLE 2.—*Wave length positions of maximum and minimum absorption, and corresponding specific absorption coefficient,*[1] *of carotene isomers in iso-octane*

	ISOMER			
	ALL-TRANS-ALPHA-CAROTENE	ALL-TRANS-BETA-CAROTENE	NEO-BETA-CAROTENE U	NEO-BETA-CAROTENE B
		Maximum		
Wave length, mμ	446	451	447	443
α-Preparation 1[1]	267–267	252–251	239–238	192–194
α-Preparation 2	267–269	252–251	237–237	187–189
Average	267	251	238	191
		Minimum		
Wave length, mμ	462	468	462	462
α-Preparation 1	184–184	203–203	184–185	149–151
α-Preparation 2	184–185	204–202	184–183	147–149
Average	184	203	184	149
		Maximum		
Wave length, mμ	474	479	474	470
α-Preparation 1	244–243	220–221	207–206	154–156
α-Preparation 2	245–245	220–221	205–206	150–150
Average	244	221	206	153

[1] Specific absorption coefficient: optical density referred to solution depth of 1 cm and concentration of 1 g/liter.

carotene bands were individually carved out, and the pigments eluted with a 10% solution of alcohol in hexane. The solution was filtered, the alcohol removed by a continuous washing device (6), and the solution dried with anhydrous sodium sulfate. The beta-carotene solution was subjected to two additional chromatographic separations on magnesia-Dicalite to remove the last traces of alpha-carotene. After elution of beta-carotene from the last column, alcohol equivalent to 5% by volume was added to the hexane solution of carotene (150 mg carotene in 300 ml) and the solution stored under nitrogen at −30°F. After several hours, the crystals that had settled were filtered off, washed with methyl alcohol, and dried under vacuum at room temperature for 2 hours. Chromatographic adsorption analysis of a solution of these crystals on hydrated lime

showed only one homogeneous zone of the all-trans-beta form with no evidence of the presence of alpha-carotene, oxidation products, or cis isomers of the beta-carotene. A portion of this material was used immediately for the determination of specific absorption coefficients, and the

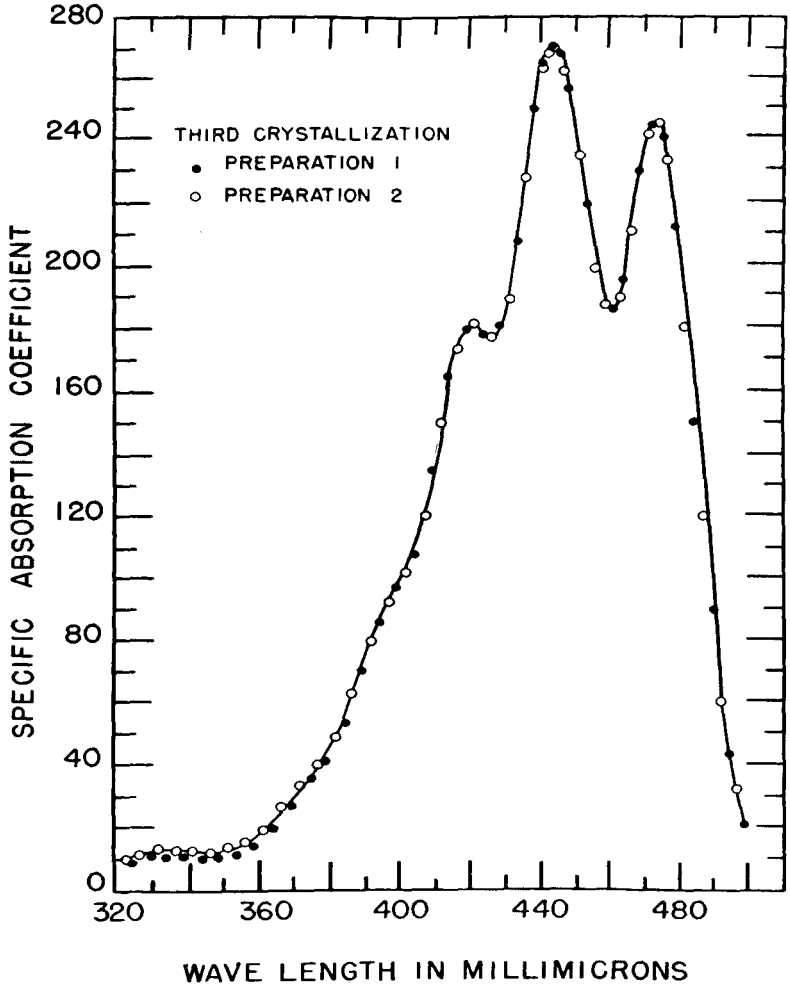

FIG. 2.—Absorption spectrum of all-trans-alpha-carotene in iso-octane.

remainder was dissolved in benzene and recrystallized with methyl alcohol. Specific absorption coefficients were again obtained after removal of the crystals by centrifugation, washing with methyl alcohol, and drying

as before. The material was subjected to two additional recrystallizations in the same manner, and specific absorption coefficients were obtained for each preparation. No increase in specific absorption coefficient was obtained after the first recrystallization (Figure 1 and Table 2).

All-trans-alpha-carotene.—The alpha-carotene was purified from the same source and with the same technique as described above for beta carotene (Figure 2, Table 2).

Neo-beta-carotene U.—Polgar and Zechmeister have described the preparation of this stereoisomer (16). An iodine-isomerized beta-carotene solution is chromatographed on a hydrated lime column, and after development with 2% acetone in petroleum ether, the homogeneous neo-beta-carotene U zone appears above and separated from the all-trans zone. It is carved out, eluted with ether, and the ether solution (which now amounts to several hundred ml) is evaporated to dryness. The pigment is then taken up in a minimum of benzene and the isomer is precipitated out upon addition of alcohol. When this procedure was followed in our laboratory, it was found that evaporation of the ether solution resulted in reisomerization of a portion of the neo-beta-carotene U isomer to the all-trans form even when precautions were taken to keep the solution cold and under an atmosphere of nitrogen at all times.

The procedure was therefore modified so that only one or two ml of solvent was evaporated. The purified isomer was eluted from the hydrated lime column with 20% alcohol in hexane instead of with ether. After the alcohol was removed by the continuous washing technique and dried, the pigment was readsorbed on a No. II column packed with the magnesia-Dicalite mixture. The minimum amount of adsorbent (1 g per 2.5 mg carotene) was used to retain all the carotene. After all of the pigment had been absorbed, 50 ml of the low-boiling fraction of petroleum ether (37°–43°C.) was drawn through the column. The pigment was then rapidly eluted with a solution containing equal volumes of acetone and ethyl alcohol. The eluate was washed free of acetone and alcohol and about 2 ml of the low-boiling petroleum ether (37°–43° C) remained, in which the carotene was now concentrated. This solution was filtered through an asbestos pad on a micro filter funnel into a 12-ml conical-type centrifuge tube and the solution was rapidly evaporated to dryness by bubbling a stream of nitrogen through it for 1 to 2 minutes. The pigment was taken up in a minimum of benzene and 3 to 4 volumes of methyl alcohol were added. When the solution was cooled in an ice-water bath, small needle-like crystals of neo-beta-carotene U precipitated out. The crystals were removed by centrifugation, washed several times with a minimum amount of methyl alcohol, and dried under vacuum at room temperature for one hour. A portion of this preparation was taken for absorption spectra determinations, and the remainder was recrystallized twice more. The absorption coefficients obtained from the three crystalline prepara-

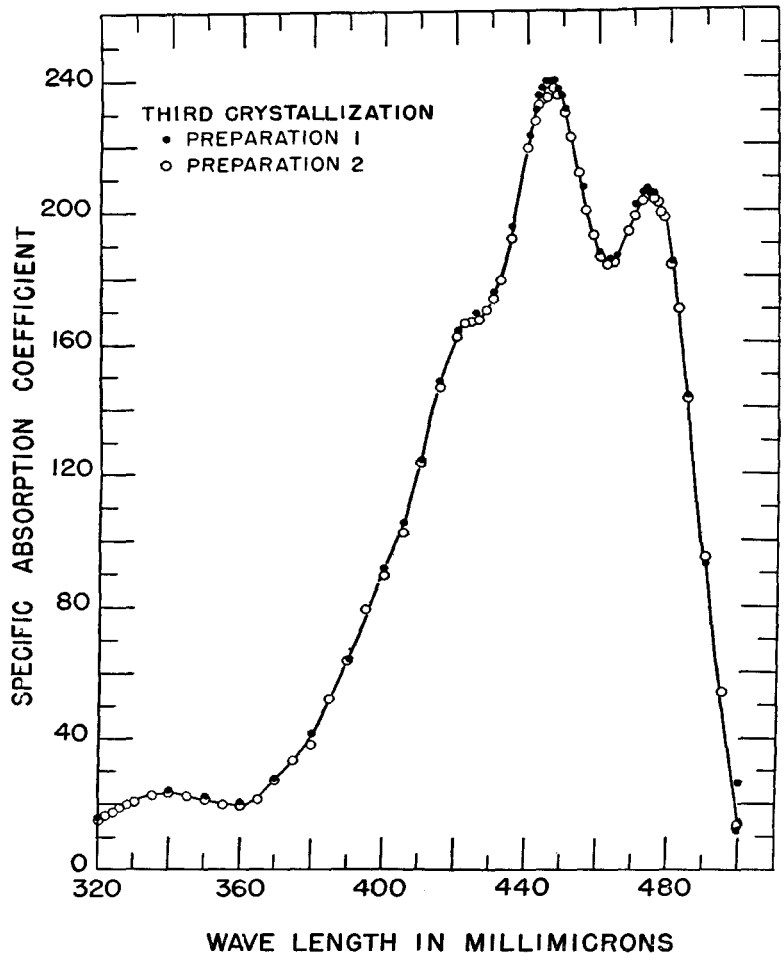

FIG. 3.—Absorption spectrum of neo-beta-carotene U in iso-octane.

tions agreed with each other within 2%. A second preparation prepared in the same way checked these values. Figure 3 presents the absorption spectra obtained for neo-beta-carotene U dissolved in iso-octane. Table 2 contains the specific absorption coefficients at the two maxima for this isomer.

Neo-beta-carotene B.—This isomer is much more unstable than is the neo-beta-carotene U, and thus is more difficult to prepare in the pure state. It is also more difficult to separate from the all-trans-beta-carotene and neo-beta-carotene E, which, are, respectively, just above and below it on the chromatographic column (16). In order to improve the separation

of the zones during the development of the chromatogram, 1½% para-cresyl methyl ether was used as a developer instead of the 2% acetone. This material has been shown to increase the separation of the zones during the development of an isomerized beta-carotene solution on a hydrated lime column (2).

When the procedure described above for the preparation of crystalline neo-beta-carotene U was employed for the preparation of the B isomer, it was unsuccessful. Instead of crystals, only a viscous dark liquid resulted.

Since considerable difficulty was encountered in the preparation of neo-beta-carotene B, the procedure that was finally adopted is given in detail: A solution of purified beta-carotene in hexane (0.5 mg per ml) was isomerized as described above (see under carotene). This isomerized carotene was then poured on a No. VI hydrated lime column packed to a height of 28 cm. For best separation, not more than 40 mg total carotene could be added to each column. In this work, 4 columns were developed at the same time with 1½% para-cresyl methyl ether in hexane until the lower edge of the neo-beta-carotene E zone reached the bottom of the column. The development was then discontinued, the columns extruded, and the portions of the lime containing the neo-beta-carotene B zone were rapidly carved out and placed together on an 8-inch-diameter Büchner funnel. The carotene was eluted from the lime with the following eluents, respectively: 20% ethyl alcohol in hexane, 80% ethyl alcohol in hexane, and finally 100% ethyl alcohol. The reason for the succession of solvents was to elute the pigment with a minimum of hexane and not precipitate the carotene on the lime. About 1½ to 2 liters of eluent were required at this stage.

The eluate was washed in a continuous washing device to remove the alcohol, and dried. To concentrate the dilute carotene solution in a small volume for rechromatographing, the solution was adsorbed on magnesia-Dicalite (1 g adsorbent to 2.5 mg carotene) in a No. II column. The adsorbed carotene was eluted with 10% acetone in hexane, and the acetone removed by continuous washing. The use of a magnesia-Dicalite column permitted a liter of dilute carotene solution to be concentrated rapidly to 50 to 75 ml with little or no reisomerization. The partially purified neo-beta-carotene B solution in hexane was rechromatographed on a No. VI lime column. After three such chromatographic adsorptions on lime, a homogeneous band of neo-beta-carotene B free of the all-trans isomer was obtained. The isomer thus obtained was adsorbed on magnesia-Dicalite as described above, and 50 ml of a low-boiling fraction of petroleum ether (37–43°C.) was drawn through the column to remove the hexane and para-cresyl methyl ether which might have interfered with crystallization. The neo-beta-carotene B was eluted with reagent acetone, the solution was transferred to a 125-ml separatory funnel, and the acetone removed by continuous washing. The resulting carotene solution, containing about 20 to 30 mg of carotene in 2 ml of the low-boiling petroleum ether, was

carefully filtered through an asbestos filter pad on a micro filter funnel directly into a conical centrifuge tube, and 8 ml of methyl alcohol were added. Crystals did not form at once, even when the liquid was cooled.

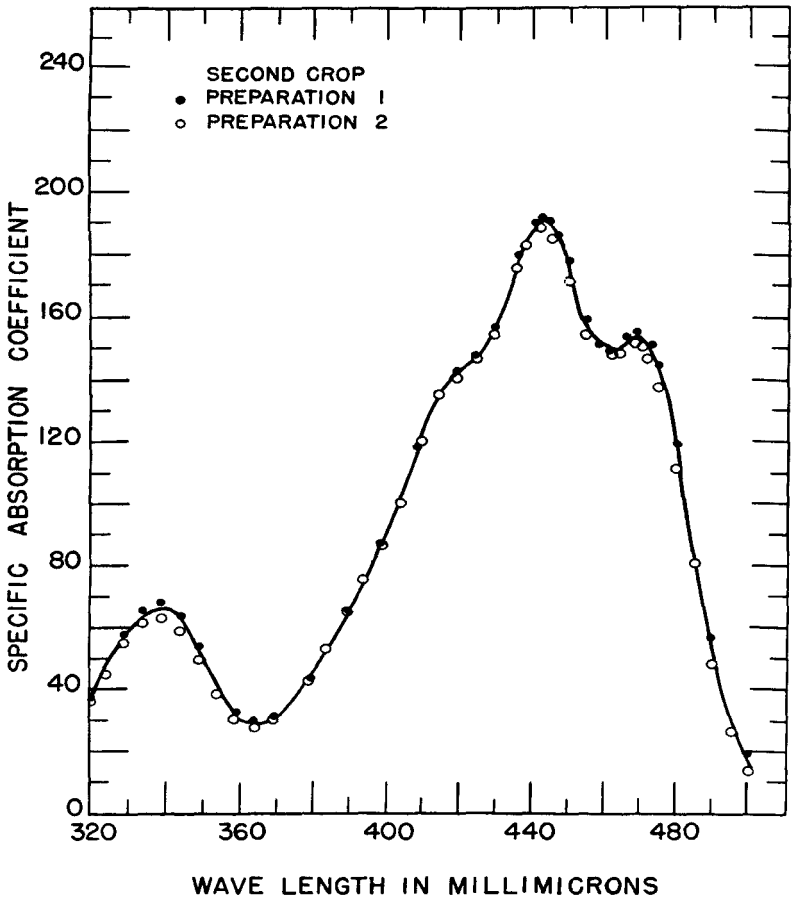

FIG. 4.—Absorption spectrum of neo-beta-carotene B in iso-octane.

However, after nitrogen (passed over hot, reduced copper to remove oxygen) had been bubbled through the liquid for several minutes, a crop of crystals in small spherulitic aggregates was formed. The crystals were removed by centrifugation, washed with redistilled methyl alcohol and dried under vacuum for one hour at room temperature. The mother liquor decanted from this first crop of crystals was stored for 2 hours at −30°F. under nitrogen, and a second crop of crystals was obtained. Again, the

crystals were removed from the mother liquor by centrifuging, and dried for one hour. A third crop of crystals was obtained by storing the mother liquor overnight at −30°F. under CO_2. Chromatographic analysis of these crystalline preparations on hydrated lime showed only a very slight trace of all-trans-beta-carotene. The specific absorption coefficients for the second and third crops of crystals were almost identical, and were higher

TABLE 3.—*The wave length of maximum absorption, and the specific absorption coefficient at this maximum, for all-trans-beta-carotene in various solvents*

SOLVENT	WAVE LENGTH mμ	SPECIFIC ABSORPTION COEFFICIENT
Iso-octane	451	251
Commercial hexane (65°–69°)[a]	451	251
Purified hexane B.P. 65°–67°	451	250
Petroleum ether B.P. 30°–60°[b]	449	253
Petroleum ether B.P. 30°–60°[b]	451	249
Petroleum ether B.P. 88°–99°[b]	453	247

[a] Phillips Petroleum Co.
[b] Skelly Oil Co.

than that for the first crop. This entire preparative procedure was repeated. The absorption spectra for both preparations are given in Figure 4. Specific absorption coefficients at the wave length of maximum absorption are given in Table 2.

DISCUSSION

Although all-trans-beta-carotene would seem to be readily purifiable by crystallization and chromatography, it has proved difficult for various investigators to obtain reproducible quantitative spectral absorption data even with related solvents (compare hexanes, Table 1). In order to evaluate the relative effect of solvent on the absorption coefficient, a single purified preparation of all-trans-beta-carotene was used. Portions of this material were dissolved in the various solvents, and data were obtained immediately with the same instrument. The data (Table 3) show that the different values reported in the literature may be attributed only partly to differences in solvent. However, since the specific solvent affects the absorption coefficient as well as the location of the absorption maxima, very consistent determinations by spectrophotometric measurements and published absorption coefficients can be obtained only if the same solvent is used in making the determination as was used for the constant. O'Connor and co-workers (14) discussed this problem and suggested iso-octane as a standard solvent. Iso-octane can be obtained in a very pure state, is readily available, and its cost is not excessive since it can be redistilled and reused without change in composi-

tion. Our results indicate that the absorption spectra of the various carotenes are very similar in iso-octane and hexane. This conclusion was reached also by Zscheile and co-workers for all-trans-alpha and all-trans-beta-carotene (24).

Some of the differences in the literature may have resulted from use of different instruments in making the spectrophotometric measurements. Instrumental differences are known to exist. To check our instrument, absorption coefficients were determined on a purified all-trans-beta-carotene preparation with two other instruments of the same make. The data thus obtained all agreed within 0.2 per cent. This agreement does not of course preclude the possibility that other instruments of different resolving power might give significantly different results.

Since no highly purified carotenes are commercially available, it is necessary for each laboratory to prepare its own carotene standards. Variations in spectral constants reported may be the result mainly of different methods of preparation, and it is here that improvements should be sought with the aim of developing uniform procedures. The values obtained in our laboratory for the specific absorption coefficients of all-trans-alpha- and all-trans-beta-carotene check closely with those reported by several other investigators (13, 19, 21). However, values several per cent higher have also been reported recently (5, 23, 24). It would seem reasonable to assume that the purer the preparation is, the higher the coefficient will be. It is difficult, however, to understand some of the extremely high values (Table 1; ref. 7, 12, 20).

Polgar and Zechmeister have described the preparation of crystalline neo-beta-carotene U (16), and have presented absorption constants for this isomer in hexane solution (23). No other quantitative data for this stereoisomer are available in the literature. In the present study the absorption spectrum of this isomer has been obtained in purified hexane, in iso-octane, and in two petroleum ethers (b.p. 63°–70°; 88°–98°, Skelly Oil Co.). Almost identical values were obtained in these solvents. However, our values are 5 to 6 per cent lower than those reported by Polgar and Zechmeister, and the value of the coefficient was not raised by repeated recrystallization of the carotene (Table 2).

Gillam and El Ridi (9) described the preparation of neo-beta-carotene B, which they termed pseudo-alpha-carotene. However, they did not obtain absorption coefficients for their preparation. Beadle and Zscheile (1) obtained quantitative absorption data for neo-beta-carotene B by an indirect method. They determined the absorption spectrum in hexane solution immediately after separating the isomer from the all-trans-beta-carotene by chromatography on an alumina column. The quantity of neo-beta-carotene B present in the hexane solution was then determined by evaporation of aliquots of the hexane solution to dryness and weighing the residual carotene. They explain that this procedure was used because of the great tendency for the stereoisomer to reisomerize back to the all-

trans form during its isolation and crystallization. It is probable that any neo-beta-carotene E that might have been present in their original extract would have been included together with the neo-beta-carotene B, since it would appear as a zone immediately below the neo-beta-B on the developing column (16). The average specific absorption coefficient for this isomer obtained in this laboratory with crystalline preparations was about 8 per cent lower than that reported by Beadle and Zscheile (1). The slight amount of reisomerization during the preparative procedure would tend to raise the value. Our lower value, therefore, can not be accounted for on this basis.

The specific absorption coefficients of the various isomers reported here are in each case averages from at least two preparations, obtained immediately in most cases, and never more than 24 hours after preparation. All preparations except that of neo-beta-carotene B showed but a single homogeneous band when examined chromatographically on hydrated lime. The neo-beta-carotene B showed a second extremely faint band, which was attributed to all-trans-beta-carotene. The methods employed for the separation and crystallization of the neo-beta-carotenes B and U in the preparative procedure are laborious, but seem to be satisfactory from the standpoint of purity of the resulting material.

ACKNOWLEDGMENTS

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SUMMARY

The following carotene isomers were isolated and purified by chromatographic and crystallization techniques: All-trans-alpha-carotene, all-trans-beta-carotene, neo-beta-carotene U, neo-beta-carotene B. The details of isolation and purification are described. The absorption spectrum of each was determined in iso-octane and in commercial hexane from 320 to 500 millimicrons by a photoelectric spectrophotometric method. The specific absorption values given are considered suitable as a basis of analysis for the individual isomers. The values thus obtained are compared with those obtained by other workers. Differences due to solvents are discussed.

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COMBINATION OF CATALYSTS IN THE DETERMINATION OF NITROGEN

III. MEAT PRODUCTS

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INTRODUCTION

The time required for the determination of nitrogen has been materially reduced since Kjeldahl (1) first proposed his original method. This reduction of time was first made possible by Wilfarth (2) with the introduction into the reaction mixture of a catalyst such as mercury or copper. Gunning (3) followed shortly with suggestions that potassium sulfate would increase the rate of digestion. Recently, the use of selenium has been advocated as a catalyst. Although Lauro (4) was the first to investigate the use of selenium, this substance has been combined with other catalysts by several investigators (5–14, incl.). Of these workers, several (9, 11, 12)

have found this element to reduce materially the amount of nitrogen obtained in the analysis. Sandstedt (14), also, has found with selenium some loss of nitrogen on long heating.

Folin and Wright (15) suggested the use of a mixture of phosphoric and sulfuric acids with potassium sulfate, a ten per cent solution of ferric chloride being used as a catalyst. Several other catalysts have been used, including phosphorus pentoxide and potassium permanganate (16), vanadium pentoxide (17), and perchloric acid (18, 19). These substances do not seem, however, to possess any great advantage over copper, mercury, and selenium.

Nearly all of the investigations in which selenium has been used for the determinations of nitrogen have been with cereal products. In this communication, there are reported the results obtained when selenium is used in combination with other catalysts in the determination of nitrogen in meat products. No experimental data are available on the efficiency of selenium when combined with copper, mercury, and strong hydrogen peroxide, outside of investigations (20, 21) reported from this laboratory.

METHODS AND PROCEDURE

The Gunning method, which has been adopted by the Association of Official and Agricultural Chemists (22), as an official method, was employed as the general basic procedure in the investigation. It is probable that none of the results reported in the tables represent the actual amount of nitrogen in the samples, since digestions were stopped immediately after the samples were clear.

Canned meat products and fresh meat were used. The canned meats were ground to a fine consistency, if not already in this condition. The samples were thoroughly mixed, and one-gram samples were weighed for analysis. The fresh meat samples were freed of all fat, ground, and dried for twelve to fifteen hours at 60°C. Each sample was then powdered in a mortar and dried for another twelve hours in a vacuum oven at 70°C. The percentages of nitrogen were calculated back to the original undried fresh meat after the amount of moisture present in each sample had been determined.

Ten grams of potassium sulfate and 20 cc of concentrated sulfuric acid were then added to each of the eight digestion flasks containing 1 gram samples of a given meat product with the following exceptions:

(1) In number (6), 4 g of potassium sulfate, 6 g of di-potassium phosphate, and 20 cc of sulfuric acid were added (23).

(2) In number (7), 15 cc of sulfuric acid and 5 cc of glacial phosphoric acid were added, the potassium sulfate being omitted.

The following catalysts then were added separately to one of the eight digestion flasks:

(1) Five-tenths g of mercuric oxide, with one cubic centimeter of 30 per cent hy-

TABLE 1.—*Determination of nitrogen in canned meat products*

CATALYST	POTTED MEAT		VIENNA SAUSAGE		DEVILED HAM		DEVILED MEAT		MEAT SPREAD	
	MINUTES FOR CLEARING	NITROGEN	MINUTES FOR CLEARING	NITROGEN	MINUTES FOR CLEARING	NITROGEN	MINUTES FOR CLEARING	NITROGEN	MINUTES FOR CLEARING	NITROGEN
		per cent		*per cent*		*per cent*		*per cent*		*per cent*
(1) $HgO+H_2O_2$	20	3.48	21	1.98	25	2.41	22	2.70	25	3.11
(2) $Se+H_2O_2$	20	3.42	13	2.07	24	2.45	20	2.70	22	3.02
(3) $CuSO_4+H_2O_2$	25	3.38	25	1.99	32	2.35	26	2.70	36	2.97
(4) $HgO+Se+CuSO_4$	16	3.44	13	2.00	21	2.30	16	2.73	15	3.12
(5) $HgO+Se+CuSO_4+H_2O_2$	15	3.42	12	1.96	18	2.40	15	2.72	16	3.05
(6) $HgO+Se+CuSO_4+H_2O_2+K_2HPO_4$	11	3.45	8	2.08	16	2.49	14	2.73	13	3.09
(7) $Se+CuSO_4+H_3PO_4$	19	3.43	19	2.00	24	2.43	16	2.70	19	2.96
(8) Control	90	3.45	75	1.98	90	2.41	115	2.71	120	3.07
Average		3.43		2.00		2.40		2.71		3.04

TABLE 2.—*Determination of nitrogen in fresh meats*

CATALYST	CHICKEN GIZZARD		CHICKEN		VEAL		BEEF		LAMB		PORK	
	MINUTES FOR CLEARING	NITROGEN	MINUTES FOR CLEARING	NITROGEN	MINUTES FOR CLEARING	NITROGEN	MINUTES FOR CLEARING	NITROGEN	MINUTES FOR CLEARING	NITROGEN	MINUTES FOR CLEARING	NITROGEN
		per cent		*per cent*		*per cent*		*per cent*		*per cent*		*per cent*
(1) $HgO+H_2O_2$	24	3.35	24	3.06	25	3.65	25	3.26	21	3.53	23	3.98
(2) $Se+H_2O_2$	24	3.29	22	3.07	24	3.62	24	3.25	22	3.43	25	3.95
(3) $CuSO_4+H_2O_2$	28	3.30	27	3.03	27	3.56	28	3.19	25	3.45	25	3.92
(4) $HgO+Se+CuSO_4$	14	3.32	13	3.06	14	3.62	16	3.26	15	3.48	15	3.97
(5) $HgO+Se+CuSO_4+H_2O_2$	14	3.33	12	3.07	13	3.58	15	3.25	13	3.48	14	3.97
(6) $HgO+Se+CuSO_4+H_2O_2+K_2HPO_4$	12	3.32	12	3.07	12	3.63	14	3.25	13	3.46	12	3.93
(7) $Se+CuSO_4+H_3PO_4$	23	3.30	20	3.07	15	3.60	17	3.19	14	3.42	16	3.92
(8) Control	90	3.32	145	3.07	100	3.61	105	3.25	120	3.46	120	3.95
Average		3.31		3.06		3.61		3.24		3.46		3.94

drogen peroxide added at the beginning of the digestion, and one cubic centimeter every five minutes thereafter, until the digestion mixture cleared as recommended by Poe and Dewey (24).

(2) Two-tenths g of selenium, with one cubic centimeter of hydrogen peroxide added every five minutes as in (1).

(3) One gram copper sulfate with one cubic centimeter of hydrogen peroxide added as in (1).

(4) Three-tenths g mercuric oxide, one-tenth g selenium, and five-tenths g copper sulfate.

(5) Three-tenths g mercuric oxide, one-tenth g selenium, five-tenths g copper sulfate and hydrogen peroxide added as in (1).

(6) The same catalysts as in (5) plus di-potassium hydrogen phosphate.

(7) One-tenth g selenium, and five-tenths g copper sulfate, plus phosphoric acid.

(8) A control with no catalysts added.

TABLE 3.—*Average results for clearing time and percentage of nitrogen of meat products*

CATALYST	CLEARING TIME—MINUTES			PER CENT NITROGEN	
	CANNED MEAT	FRESH MEAT	AVERAGE	CANNED MEAT	FRESH MEAT
(1) $HgO+H_2O_2$	22.6	23.7	23.2	2.74	3.47
(2) $Se+H_2O_2$	19.8	23.5	21.7	2.73	3.44
(3) $CuSO_4+H_2O_2$	28.8	26.7	27.8	2.68	3.41
(4) $HgO+Se+CuSO_4$	16.2	14.5	15.4	2.72	3.45
(5) $HgO+Se+CuSO_4+H_2O_2$	15.2	13.5	14.4	2.71	3.45
(6) $HgO+Se+CuSO_4+H_2O_2+K_2HPO_4$	12.4	12.5	12.5	2.77	3.44
(7) $Se+CuSO_4+H_3PO_4$	19.4	17.5	18.5	2.70	3.42
(8) Control	98.0	113.3	105.7	2.72	3.44

The amounts of nitrogen in different meat samples were determined under each of the eight conditions listed above. A blank determination was run on all reagents (1) to (8) inclusive. The percentages of nitrogen obtained and the time of clearing for the canned and fresh meats are listed in Tables 1 and 2. The average clearing times and percentages of nitrogen for all samples by each modification are given in Table 3.

The addition of potassium hydrogen phosphate in combination number 6 seemed to reduce the clearing time to a marked degree. An attempt was made to find the combination of potassium sulfate and di-potassium phosphate which would give the most rapid clearing of the digestion mixture. The catalysts used were three-tenths gram mercuric oxide, one-tenth gram selenium, five-tenths gram copper sulfate, with one cubic centimeter of 30 per cent hydrogen peroxide being added every five minutes. The amounts of potassium sulfate and di-potassium phosphate used and the corresponding times required for clearing are given in Table 4.

TABLE 4.—*Results obtained with varying amounts of potassium sulfate and di-potassium hydrogen phosphate**

DETERMINATION OF NITROGEN IN DRIED BEEF			
GRAMS K_2SO_4	GRAMS K_2HPO_4	MINUTES FOR CLEARING	PER CENT NITROGEN
10	0	16	3.19
8	2	17	3.26
6	4	16	3.22
5	5	14	3.15
4	6	13	3.21
4	10	14	3.20
2	8	13	3.25
10	6	18	3.18
Average			3.21

* Catalysts used were HgO, Se, $CuSO_4$, and H_2O_2.

DISCUSSION

A study of the tables shows a sharp decrease in clearing time when the three catalysts—mercuric oxide, selenium, and copper sulfate—are combined, as compared with the times when these catalysts were used independently. The addition of hydrogen peroxide to the preceding combination reduced the time still further, but probably not enough to justify the additional trouble and expense.

When, however, part of the potassium sulfate is replaced by di-potassium phosphate, with a retention of the same combination of catalysts—mercuric oxide, selenium, copper sulfate, and hydrogen peroxide—the clearing time is reduced considerably. In spite of the more rapid clearing time, the accuracy of the nitrogen determination does not seem to be seriously impaired.

The combination of copper sulfate and hydrogen peroxide, as shown by the tables, shows the greatest time for clearing and consistently gives low results for nitrogen content when the determination of nitrogen was made after the first clearing. This condition is covered by instruction in the official A.O.A.C. procedures (22) that mere clearing time is insufficient for complete digestion.

The combination of selenium and copper sulfate with phosphoric and sulfuric acids did not produce so satisfactory results as did the combination of selenium, copper sulfate, and mercuric oxide with potassium hydrogen phosphate and sulfuric acid.

CONCLUSIONS

1. Mercuric oxide, selenium, and copper sulfate, when used separately

with hydrogen peroxide, act about equally effectively in reducing the time necessary for clearing.

2. Copper sulfate used in combination with hydrogen peroxide gave slightly lower yields of nitrogen when determined at the time of clearing than were obtained with other catalysts.

3. A combination of mercuric oxide, selenium, and copper sulfate reduces the clearing time to approximately 60 per cent of the time required when either of these catalysts is used alone with hydrogen peroxide. Hydrogen peroxide added to the above mentioned combination has little effect in hastening the reaction further.

4. The addition of di-potassium hydrogen phosphate to the combination of mercuric oxide, selenium, copper sulfate, and hydrogen peroxide causes a reduction in clearing time of about 15 per cent over the combination without the potassium hydrogen phosphate.

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ANALYSIS OF SULFADIAZINE-SULFATHIAZOLE MIXTURES

A SPECTROPHOTOMETRIC METHOD

By DANIEL BANES (U. S. Food and Drug Administration, Federal Security Agency, Chicago, Ill.)*

Increasing numbers of preparations containing a mixture of sulfonamides have appeared in recent years. Since official methods of assay for the individual compounds have been restricted largely to nitrite titrations of the active amino group common to all of them, it has become necessary to devise new procedures for the control analysis of specific constituents. The sulfonamides absorb ultra-violet light between 220 and 300 mμ, and their characteristic spectra differ, depending upon the amide substituent. Mixtures may therefore be analyzed by optical density measurements under varied conditions of absorption.

A simple, rapid spectrophotometric method can be designed for the analysis of preparations containing sulfadiazine and sulfathiazole. Sulfadiazine in strongly acid solution shows a peak absorption at 239 mμ, the optical density diminishing from that point to about 275 mμ, and flattening to a plateau from 275 to 300 mμ. Sulfathiazole yields a more symmetrical curve with a well-defined maximum near 278 mμ and a minimum at 240 mμ. (See the accompanying graph.)

By "two-color" analysis, measuring the optical densities of standards and unknown solutions in 1 cm cells at 239 and 280 mμ, a set of two equations in two unknowns can be established for the estimation of sulfadiazine and sulfathiazole. (Both compounds obey Beer's Law at the two wave-lengths.) Let

K_1 = extinction coefficient for sulfadiazine at 239 mμ
K_2 = extinction coefficient for sulfathiazole at 239 mμ
K_3 = extinction coefficient for sulfadiazine at 280 mμ
K_4 = extinction coefficient for sulfathiazole at 280 mμ
C_N = concentration of sulfadiazine as mg/100 ml HCl (1+3)
C_L = concentration of sulfathiazole as mg/100 ml HCl (1+3)
E_{239} = optical density of the unknown solution at 239 mμ
E_{280} = optical density of the unknown solution at 280 mμ.

Then, since the optical density of a mixture is the sum of the constituent densities,

$$E_{239} \times 1000 = K_1C_N + K_2C_L \quad (1a)$$

$$E_{280} \times 1000 = K_3C_N + K_4C_L \quad (1b)$$

These equations can be solved explicitly for C_N and C_L as functions of E_{239} and E_{280}:

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$$C_N = \frac{1000(K_4 E_{239} - K_2 E_{280})}{K_1K_4 - K_2K_3} \quad (2a)$$

$$C_L = \frac{1000(K_1 E_{280} - K_3 E_{239})}{K_1K_4 - K_2K_3} \quad (2b)$$

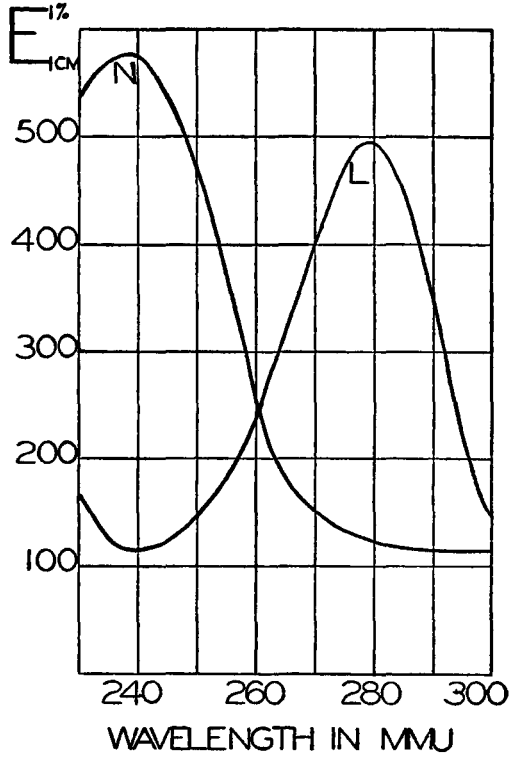

FIG. 1.—Absorption Spectra of Sulfonamides in 3N HCl.
N = Sulfadiazine
L = Sulfathiazole

PROPOSED SPECTROPHOTOMETRIC METHOD

Shake a convenient representative sample containing sulfadiazine and sulfathiazole with 200 ml HCl (1+3) in a 500 ml volumetric flask until the sulfonamides are completely dissolved. (For ointments transfer the sample to a 125-ml separatory funnel, dissolve in 50 ml of ethyl ether, and shake out with several 50 ml portions of HCl (1+3), collecting the aqueous layers in a 500 ml volumetric flask.) Dilute to the mark with the acid soln, mix thoroly and filter thru a dry folded filter paper if necessary. Transfer an aliquot containing 1–2 mg total sulfonamides to a 100 ml volumetric flask, and dilute to volume with HCl (1+3). Determine the optical densities of this solution at 239 and 280 mμ in the ultra-violet spectrophotometer, with distilled water as reference cell.

At the same time, using the same HCl (1+3) as diluent, prepare a soln containing 1.5 mg sulfadiazine of known purity per 100 ml and a soln containing 1.5 mg sulfathiazole of known purity per 100 ml. Measure the optical densities of these standards, and of the dilute HCl as correction blank, together with those of the unknown under identical conditions of wave length, slit-width, and cell thickness. From data on the standards compute the extinction coefficients and calculate the concentrations of sulfadiazine and sulfathiazole in the unknown soln from equations (2a) and (2b).

EXPERIMENTAL

Data obtained by the use of known 1.5 mg sulfonamide solutions yielded the following numerical values:

$K_1=555$; $K_2=106$; $K_3=103$; $K_4=469$. Substitution of these values in equations (2) gave:

$$C_N = 1.881\ E_{239} - 0.425\ E_{280} \qquad (3a)$$

$$C_L = 2.225\ E_{280} - 0.413\ E_{239} \qquad (3b)$$

Several samples containing varying proportions of sulfadiazine and sulfathiazole were analyzed according to the proposed method, using equations (3a) and (3b). Results of analysis are shown in Table 1. A commercial sample of tablets also assayed spectrophotometrically yielded the following results: Sulfadiazine, Declared 0.25 gm/tablet; Found 0.252 gm/tablet. Sulfathiazole, Declared 0.25 gm/tablet; Found 0.247 gm/tablet.

TABLE 1.—*Sulfadiazine-sulfathiazole mixtures*

PUT IN		RECOVERED			
SULFADIAZINE	SULFATHIAZOLE	SULFADIAZINE		SULFATHIAZOLE	
mg	*mg*	*mg*	*per cent*	*mg*	*per cent*
0.00	2.00	−0.011	—	2.021	101.0
0.25	1.75	0.248	99.3	1.764	100.8
0.50	1.50	0.495	99.1	1.519	101.3
0.75	1.25	0.739	98.2	1.274	101.9
1.00	1.00	0.995	99.5	1.026	102.6
1.25	0.75	1.234	98.7	0.776	103.4
1.50	0.50	1.506	100.4	0.524	104.8
1.75	0.25	1.775	101.4	0.256	102.5
2.00	0.00	2.043	102.1	0.003	—

CONCLUSIONS

A simple rapid method has been proposed for the analysis of mixtures containing sulfadiazine and sulfathiazole. The method is suitable for the assay of pharmaceuticals containing these sulfonamides.

POLYMORPHIC MODIFICATIONS OF SULFANILAMIDE

By MORRIS L. YAKOWITZ (U. S. Food and Drug Administration, San Francisco, Calif.)

Van Zyp (1) observed that sulfanilamide appears in several crystalline forms when crystallized from a drop of water and examined under the microscope.

Watanabe (2) made on X-ray diffraction study of sulfanilamide crystallized from various solvents and determined that sulfanilamide can crystallize in at least three polymorphic modifications, one of which is orthorhombic and the other two monoclinic.

Prien and Frondel (3) made a study of sulfanilamide deposited from urine and reported that it crystallizes in the orthorhombic system and has the refractive indexes, $\alpha = 1.550$, $\beta = 1.622$, and $\gamma = 1.80$.

Keenan (4), in his report on the refractive indexes of substances in the USP XII, gave the refractive indexes of sulfanilamide as $\alpha = 1.570$, $n_i = 1.677$, $\gamma = > 1.733$.

An examination of sulfanilamide crystallized from various solvents has confirmed Watanabe's discovery that sulfanilamide is polymorphic with at least three phases. The refractive indexes of the several forms with their probable crystal system are as follows:

DESIGNATION	LOWEST R.I. α	INTERMEDIATE R.I. β	HIGHEST R.I. γ	CRYSTAL SYSTEM
Modification I	1.547	1.622	>1.733	Orthorhombic
Modification II	1.558	1.675	>1.733	Monoclinic
Modification III	1.500	1.673	>1.733	Monoclinic

Modifications I and II can be obtained from water, ethyl alcohol, acetone, and n-propyl alcohol. Under some conditions of crystallization, the crystals formed from these solvents may be largely Modification I, while under different conditions of crystallization, Modification II will predominate.

Modification I tends to form as elongated laths giving a centered interference figure on the flattened side with β parallel to the long direction and α crosswise.

Modification II tends to form as equant blocks with an uncentered interference figure when lying in their preferred position. Such crystals give β and another intermediate R.I.

When either Modification I or II is heated, the crystals transform into Modification III at about 110°C. to 130C°. Modification III is the only stable phase above about 130°C. When large crystals of I or II are heated to about 130°C., they become chalky and fissured when they

transform into III. Small, thin crystals of I or II transform smoothly and completely into III when heated and exhibit the refractive indexes of III while retaining the outward appearance of I or II.

III is the phase which forms when molten sulfanilamide is cooled. III may be obtained from toluene solution in the form of elongated lath-like crystals.

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DETERMINATION OF THE NITROGEN CONTENT OF PROTEINS AND AMINO ACIDS CONTAINING REFRACTORY NITROGEN BY MICRO- AND MACROKJELDAHL METHODS

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The recent excellent reviews by Kirk (1) and Dunn and Rockland (2) forcibly called attention to the difficulties involved in the use of the Kjeldahl method to determine the total nitrogen content of proteins and amino acids containing refractory nitrogen. Of the various difficulties encountered in this method, analysts have probably given the most attention to the composition of the catalyst and the length of the digestion period. Chibnall, Rees, and Williams (3), Jonnard (4), Dunn and Rockland (2), and others have stressed the need for 6- to 8-hour digestion periods for proteins and amino acids. Many workers have used digestion catalysts that have been shown to be inferior to mercury in promoting conversion of the organic nitrogen to ammonia, and others, finding that tyrosine (5, 6) and tryptophan (5) were not completely decomposed with the usual digestion times, have assumed that proteins would also require extended digestion.

Success in determining the total nitrogen in tryptophan and in gramicidin (a polypeptide containing about 40% tryptophan) by minor modifications (7) of the A.O.A.C. tentative microkjeldahl method (8) requiring a total digestion time of approximately 80 minutes, prompted a study of the recovery of nitrogen from the other amino acids containing refractory nitrogen, and from typical proteins, by the official macro- and the tentative microkjeldahl methods. Accordingly, lysine (2, 3, 5, 9, 10), histidine (3, 10), tryptophan (5, 9), tyrosine (5, 6), and a group of crystallized or otherwise purified proteins selected for their high content of these amino acids, were analyzed by these methods. The data presented below show

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that the precision and accuracy of the methods are satisfactory for these materials.

EXPERIMENTAL

Methods:

Microkjeldahl.—The tentative microkjeldahl method (8) as modified by White and Secor (7) was used. In certain cases the digestion time was increased from the standard 80 minutes to 110 or 360 minutes. A Scientific Glass Apparatus Co.[2] No. M3074 microkjeldahl still was used.

Macrokjeldahl.—Samples containing 15 to 25 mg nitrogen were digested according to the Kjeldahl-Gunning-Arnold official method (mercuric oxide catalyst) (11) on an electrically heated rack for a period of two hours after becoming clear and colorless. The ammonia was distilled into 50 ml of 5% boric acid and titrated with 0.07 *N* sulfuric acid using methyl orange-xylene cyanole indicator (Eastman No. 2216).[2]

Equilibration and Moisture.—All samples were equilibrated several days at approximately 27° and 42% relative humidity, and subsequent weighings were made under these conditions. For moisture determinations, 50-mg portions of the equilibrated materials were heated 16 hours at $102 \pm 2°$ in air. Great care was taken to prevent the reabsorption of water by the dried sample. All results presented in the tables have been corrected for the moisture contents of the samples.

Materials:

Amino Acids.—Merck[2] L-lysine monohydrochloride, DL-lysine monohydrochloride, DL-tryptophan, L-histidine monohydrochloride monohydrate, and L-tyrosine were used without purification. As a basis for comparing the various times and methods of digestion, the amino acids were assumed to be pure.

Proteins.—The protein samples used were obtained as indicated under "Acknowledgments" and may be characterized as follows:

Bovine plasma albumin, crystalline, Armour[2] lot 46.

Casein I, prepared and reprecipitated according to Van Slyke and Baker (12).

Casein II, purified, nitrogen content 15.61% by the method of Chibnall *et al.* (3).

Chymotrypsinogen I, crystalline. A commercial preparation was recrystallized 7 times according to the procedure of Kunitz and Northrop (13) and dialyzed.

Chymotrypsinogen II, crystalline, dialyzed and crystallized from dilute alcohol.

β-Lactoglobulin, crystalline, recrystallized 4 times according to Palmer (14).

[2] The mention of this product does not imply that it is endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.

Lysozyme I, crystalline, crystallized and dialyzed according to Alderton and Fevold (15).

Lysozyme II, crystalline, crystallized 3 times at *p*H 4 by the method of Alderton and Fevold (15) and electrodialyzed.

Ovalbumin, crystalline, prepared and recrystallized 3 times by the method of Kekwick and Cannan (16).

Bovine methemoglobin, prepared according to Anson (17), nitrogen content 16.66% by the method of Chibnall *et al.* (3).

TABLE 1.—*Nitrogen recovery from amino acids*

AMINO ACID	N CONTENT (THEORETICAL) PER CENT	DIGESTION[1] TIME, MINUTES	NITROGEN RECOVERED[2] BY			
			MICRO METHOD[3]		MACRO METHOD[4]	
			PER CENT	PER CENT OF THEORETICAL	PER CENT	PER CENT OF THEORETICAL
DL-lysine · HCl	15.34	80	15.20	99.09		
		110	15.26	99.48	15.19	99.02
		360	15.38	100.26		
L-lysine · HCl	15.34	80	15.24	99.35		
		110	15.40	100.39	15.25	99.41
		360	15.41	100.46		
DL-tryptophan	13.72	80	13.52	98.54		
		110	13.66	99.56	13.71	99.92
		360	13.70	99.85		
L-histidine · HCl · H_2O	20.05	80	20.12	100.35		
		110	20.12	100.35	20.06	100.05
		360	20.14	100.45		
L-tyrosine	7.73	80	7.74	100.13		
		110	—	—	7.80	100.77
		360	7.74	100.13		

[1] Macro digestions were continued 120 minutes after the liquid cleared.
[2] Recoveries are the averages of 3 to 11 unselected replications of the stated conditions.
[3] Individual results differed from the means of their respective groups by an average of 0.025% *N* and 87% of them differed by 0.04% or less.
[4] Individual results differed from the means of their respective groups by an average of 0.067% *N* and 81% of them differed by 0.11% or less.

DISCUSSION OF RESULTS

Table 1 shows that lysine and tryptophan yielded 98.5 to 99.9 per cent of their nitrogen by the 80-minute micro and the macro digestions. Recovery of nitrogen from these acids by the micro method increased with time, but recoveries from histidine and tyrosine were complete after the 80-minute micro and the macro digestions.

Chibnal *et al.* (3) showed that recovery of nitrogen from lysine was more difficult than it was from β-lactoglobulin, a protein rich in lysine.

This is to be expected, since proteins rarely contain more than 10 to 12 per cent of lysine; therefore, the maximum amount of nitrogen that would not be recovered by methods used in this study would approximate only 0.02 to 0.04 per cent, an amount which is within the experimental error of the method. Likewise, proteins rich in tryptophan would appear to give quantitative recoveries more readily than does the pure amino acid.

TABLE 2.—*Nitrogen content of various proteins*

PROTEIN	MOISTURE	DIGESTION[1] TIME	NITROGEN CONTENT (MOISTURE-FREE BASIS)		
			MICRO METHOD	MACRO METHOD	LITERATURE OR OTHER ANALYSIS
	Per Cent	*Minutes*	*Per Cent*	*Per Cent*	*Per Cent*
Bovine methemoglobin	9.11	80	16.73 (16.71, 16.74, 16.75)	—	16.66[2]
		120			
		360	16.73 (16.71, 16.73, 16.75)		
Bovine plasma Albumin	8.58	80	16.08 (16.07, 16.09)	16.02 (15.96, 16.08)	16.07 (*18*)
		120			
		360	15.96 (15.93, 15.98)		
Casein I	7.65	80	15.44 (15.43, 15.44)	15.39 (15.32, 15.45)	15.61 (*3*)
		120			
		360	15.41 (15.38, 15.43)		
Casein II	8.59	80	15.72 (15.71, 15.73)	15.68 (15.55, 15.81)	15.61[2]
		120			
		360	15.74 (15.73, 15.74)		
Chymotrypsinogen I	8.78	80	16.47 (16.44, 16.49)	16.38 (16.37, 16.38)	15.87 (*19*)
		120			
		360	16.48 (16.47, 16.49)		
Chymotrypsinogen II	9.35	80	16.42 (16.40, 16.43, 16.44)	16.49 (16.47, 16.50)	15.87 (*19*)
		120			
		360	16.44 (16.41, 16.45, 16.45)		
β-Lactoglobulin	9.20	80	15.66 (15.64, 15.68)	15.70 (15.66, 15.73)	15.60±02 (*20*)
		120			
		360	15.65 (15.65, 15.65)		
Lysozyme I	9.75	80	18.60 (18.55, 18.64)	18.50 (18.49, 18.50)	—
		120			
		360	18.56 (18.55, 18.56)		
Lysozyme II	10.04	80	18.34 (18.31, 18.35, 18.37)	18.34 (18.32, 18.36)	—
		120			
		360	18.35 (18.34, 18.35, 18.36)		
Ovalbumin	8.06	80	15.72 (15.71, 15.73)	15.61 (15.59, 15.62)	15.68 (*3*)
		120			
		360	15.71 (15.68, 15.73)		

[1] Macro digestions continued 120 minutes after liquid cleared.
[2] Analysis of this sample by the method of Chibnall *et al.* (3).

Table 2 shows that none of the proteins studied had a higher apparent nitrogen content after prolonged digestion, the 80-minute procedure being sufficient in all cases to give values in agreement (except for chymotryp-

sinogen) with the generally accepted values in the literature or with those determined by Chibnall's method. The micro and macro methods employed in this study gave results that are in excellent agreement.

Chymotrypsinogen has been reported by Brand and Kassell (19) to contain 15.87% *N* (moisture-free basis) and 16.18% *N* after correction for the 1.94% sulfate content of the sample. Analysis showed that chymotrypsinogen I has only 0.04 per cent of ash (constant weight at 550° with no additives) and 0.75 per cent of sulfate (moisture-free basis). Its nitrogen content (moisture- and sulfate-free basis) is 16.59 per cent. Chymotrypsinogen I, crystallized 8 times and exhaustively dialyzed (*cf.* low sulfate content) and the chymotrypsinogen II prepared by Dr. Kunitz have the same apparent nitrogen content. It is therefore believed that the new value (16.59%) reported here more closely approximates the true nitrogen content of chymotrypsinogen. The difference between our result and that reported previously may lie in the difficulties encountered in determining the moisture content of proteins. Chibnall *et al.* (3) have discussed this point at length.

To determine whether hydrolysis of the protein before digestion would affect the recovery of nitrogen, 5–7 mg samples of bovine plasma albumin, casein I, β-lactoglobulin, and lysozyme I were heated 4 hours at 95 to 100° in open microkjeldahl flasks with 2 ml of 27 *N* sulfuric acid. Recoveries after 80- and 110-minute digestions (counting the time from the appearance of sulfuric fumes) were identical within experimental error, and the results were in agreement with those obtained on the unhydrolyzed samples.

It can be concluded from this study that the 6–8 hour digestions recommended by many other workers are not needed for the complete recovery of amino and heterocyclic nitrogen from amino acids and proteins. Therefore the time-saving methods used in this work may be safely employed for the determination of total nitrogen in proteins and amino acids. However, it should not be considered that these methods suffice for all nitrogenous compounds. For example, Ogg, Brand, and Willits (21) and Shirley and Becker (22), working on the micro and macro scales, respectively, and using mercury-selenium catalysts, have shown that digestion periods up to four hours are necessary to obtain complete recovery of nitrogen from certain heterocyclic nitrogenous compounds. We have confirmed these findings in our laboratory.

ACKNOWLEDGMENT

This study would not have been possible without the generous cooperation and counsel of those who furnished samples of pure proteins. We thank Moses Kunitz for the chymotrypsinogen II, E. F. Jansen for the chymotrypsinogen I and the β-lactoglobulin, G. Alderton for the lysozymes, H. S. Olcott and D. K. Mecham for the casein I and the ovalbumin,

Leo Kline for the bovine methemoglobin, and R. C. Warner for the casein II.

SUMMARY AND CONCLUSIONS

Amino acids containing refractory nitrogen and a group of purified proteins rich in these acids were analyzed by the tentative microkjeldahl method employing mercuric oxide catalyst with an 80-minute digestion and also by the Official Kjeldahl-Gunning-Arnold macro method. Recovery of nitrogen from the amino acids was at least 98.5% complete by both methods. Results obtained for the proteins were concordant and, except for chymotrypsinogen, were in agreement with the "best" values in the literature. The higher results obtained for chymotrypsinogen are discussed.

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MICRO AND SEMIMICRO DETERMINATION OF NITROGEN IN HETEROCYCLIC NITROGEN RING COMPOUNDS BY A KJELDAHL METHOD*

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Modifications in the Kjeldahl procedure to increase the range of its application have been numerous, and as Shirley and Becker (8) pointed out, conflicting claims have been made as to the correct procedure for the analysis of compounds containing refractory ring-type nitrogen. Clark (5) claimed that the semimicro adaptation of the Gunning-Arnold-Dyer modification was applicable to practically all animal and vegetable materials, purines, pyrimidines, and pyridine and quinoline derivatives.

The Clark modification, which is the tentative micro Kjeldahl method of the Association of Official Agricultural Chemists (2), uses mercuric oxide as catalyst with 1.5 ml concentrated sulfuric acid and 0.5 g potassium sulfate and a total digestion time of 80–85 minutes. However, Clark stated that some alkaloids and related compounds require a longer digestion time, and that atropine and quinine-type compounds require 2 hours' digestion. Acree (1), in doing collaborative work with this method, was able to obtain only one-fourth of the nitrogen in nicotinic acid with a 30-minute digestion and found that a 2-hour digestion was required for strychnine and quinine hydrobromides and atropine sulfate.

Belcher and Godbert (3) reported satisfactory nitrogen values for nicotinic acid, quinolinic acid, atropine sulfate, picolinic acid, etc., by a semimicro method employing mercuric sulfate and metallic selenium as catalysts and a total digestion time of 45 minutes. . . . However, they state that loss of nitrogen occurs when the digestion time exceeds 75 minutes. This loss probably is caused by the high concentration of selenium used in the digestion mixture since the concentration exceeds the limit found by Bradstreet (4). To avoid this loss an empirical digestion time which may vary with different compounds must be used. Miller and Houghton (6), using a micro method with mercuric oxide as catalyst, found that *l*-tryptophane (sample in solution) required a 6-hour digestion, and consequently they adopted this digestion time as standard for proteins and amino acids.

White and Secor (9) reported 98.5 to 100 per cent recovery of the nitrogen from *l*-tryptophane with Clark's method. Although their nitrogen values for *l*-tryptophane with an 85-minute digestion were close to theory, better values were obtained when the time was increased to 115 minutes.

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Previous to the studies reported in this paper, all compounds containing ring nitrogen were analyzed in this laboratory by the Dumas method. Since so much time is required to condition the Dumas apparatus and establish the blank when it is not in continuous use, and since a number of samples can be run simultaneously by the Kjeldahl method with a considerable saving in time, a reliable micro Kjeldahl procedure for the analysis of such materials is highly desirable. Consequently, a study of the Kjeldahl method was made in which various catalysts and combinations of catalysts were used and the time of digestion ranged from 1 to 6 hours. The semimicro procedure adopted is substantially the same as the macro procedure of Shirley and Becker, and the results obtained in the digestion-

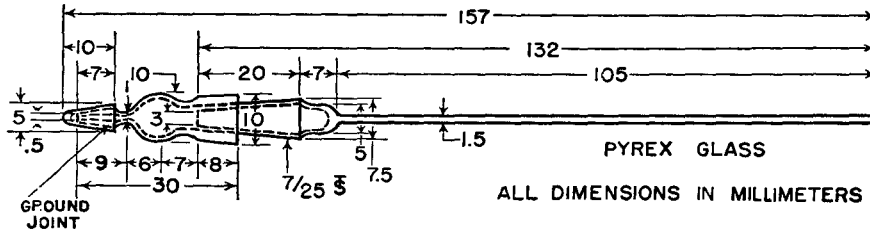

FIG. 1.—Semimicro Charging Tube.

time studies on nicotinic acid on the semi-micro and macro scale are relatively parallel. . . . Since the semimicro procedure and apparatus have been successfully applied to micro analyses, both methods are described.

APPARATUS

(1) *Digestion stand.*—6-unit, gas-heated, with glass manifold to allow for the removal of acid fumes by suction from a water aspirator.

(2) *Kjeldahl flasks.*—30 ml Pyrex glass.

(3) *Distillation apparatus.*—Micro Kjeldahl apparatus, Scientific Glass Apparatus Co. Number M-3704.*

(4) *Steam generator.*—A two-liter, three-necked flask with a 24/40 outer joint on the center neck. The flask contains a heating element consisting of a coiled wire of 25-ohms resistance. The ends of the resistance wire are brazed to 14-gage copper leads which pass out through rubber stoppers in the two outer necks and are soldered to the wires of an insulated extension cord. The rate of steam evolution caused by passing a current through the resistance wire is controlled by a variable resistor of 5 ampere rating. With no heat loss between heating element and the water, the heating can be instantaneously controlled.

(5) *Burettes.*—10-ml graduated in 0.05 ml (semimicro) or 5 ml graduated in 0.01 ml (micro).

REAGENTS

(1) *Catalyst.*—Thoroly mix 150 g powdered potassium sulfate, 10 g of mercuric oxide, and 3 g of powdered metallic selenium.

(2) *Sodium hydroxide-sodium thiosulfate soln.*—Mix 100 ml of 50% sodium hydroxide and 25 ml of 8% sodium thiosulfate soln.

* The mention of commercial products does not imply that they are endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.

(3) *Indicator.*—Mix 100 ml of 0.2% methyl red dissolved in 95% ethanol with 50 ml of 0.2% methylene blue also dissolved in 95% ethanol.

(4) *Boric acid.*—Dissolve 4 g boric acid in 100 ml distilled water.

(5) *Standard acids.*—0.02 *N* hydrochloric acid (semimicro) of 0.01 *N* (micro).

PROCEDURE

Weigh a 15–30 mg sample (4–8 mg for the micro procedure) in a long-handled charging tube and transfer to a 30 ml Pyrex Kjeldahl flask. Add 0.65 –0.70 g of the catalyst 3–4 boiling chips, and 2 ml of concd sulfuric acid, rotating the flask as the acid is added so that any sample adhering to the lower portion of the neck will be rinsed into the bulb. Place the flask on the digesting apparatus, heat slowly until carbonization occurs or until all danger of frothing is past, and then adjust the heat so that the sulfuric acid distills ca two-thirds the way up the neck of the flask. Continue the digestion for 4 hours for materials containing ring nitrogen, or 1 hour for more easily digested material such as amines or amides. After the digestion is complete, cool the flask, add 3 ml of water, and again cool the flask to room temp. Condition the distillation apparatus by steaming it out for 2–3 min., and then rinse it with distilled water. Place 10 ml of a 4% boric acid absorbing soln and 3 drops of indicator in a freshly rinsed 125 ml Erlenmeyer flask and place this flask under the tube of the condenser so that the delivery tip is immersed in the soln. Transfer the digestion mixture to the distillation unit with both stopcocks open to prevent any sample from being drawn into the outer chamber. To insure quantitative transfer, rinse the flask with four 2 ml portions of distilled water, or until the rinsings remain yellow on addition of a drop of methyl orange indicator. Add 6 ml of the sodium hydroxide-sodium thiosulfate soln (1 ml in excess of that needed to neutralize the acid), close both stopcocks, and heat the water in the steam generator slowly until the displaced air has mixed the acid and alkali. If the generator is heated too rapidly, the violent reaction which will result from the sudden mixing of the acid and alkali may cause the sample and absorbing soln to be sucked back thru the apparatus. After the acid and alkali are thoroly mixed, turn the current on full to generate the steam at a rapid rate until it reaches the condenser, then reduce the rate so that the steam does not pass the upper half of the condenser. (This should allow 3–4 ml of distillate to be collected per min.) After it has distilled for 3 min., lower the receiving flask so that the delivery tip is above the soln and continue the distillation for 1 min. to rinse the inside of the condenser tube. Rinse the outside of the delivery tip with distd water, dilute the soln to 50 ± 5 ml, and titrate with the appropriate standard hydrochloric acid until the soln changes from green to gray or gray-purple. Run a blank determination to correct for nitrogen in the reagents. Use this blank value to correct the volume of acid used in titrating the sample and calculate the percentage of nitrogen.

(Ten milliliters of the 4% boric acid soln, after dilution to 50 ml, is slightly acid to the indicator, but in practice the ammonia from the reagents "blank" has always been sufficient to neutralize the boric acid.)

Hygroscopic solids are dried and weighed in long-handled charging tubes (7) (Figure 1). The tube and cap are made from a 7/25 inner and outer joint with 10 mm of the outer joint (large cap) cut off to reduce the weight. Sealed to the top of the large cap is a smaller inner joint with a capillary opening, which in turn is covered by a ground-glass cap. The sample is placed in the charging tube and dried under vacuum, with only the large cap attached. When the sample is dry, the vacuum is released by admitting dry air to the drying chamber. The charging tube is then

TABLE 1.—*Effect of catalyst and digestion time in the Kjeldahl analyses of nicotinic acid*
Nicotinic Acid, Theory = 11.38% *N*

CATALYST	PER CENT NITROGEN FOUND AFTER DIGESTION TIME OF:											
	1 HOUR			2 HOURS			3 HOURS			4 HOURS		
	Max.	*Min*	*Ave.*	*Max.*	*Min.*	*Ave.*	*Max.*	*Min.*	*Ave.*	*Max.*	*Min.*	*Ave.*
Hg (1 drop)				8.69	7.86	8.33						
HgO (40 mg)				9.30	8.40	8.85	9.91	8.66	9.54	11.26	10.78	11.00
HgO + Se[1]	7.40	5.68	6.54	10.16	9.36	9.74	11.39	10.33	11.08	11.39	11.28	11.36
$CuSO_4$[2] + Se				8.42	7.28	7.95						
HgO + $SeOCl_2$ (1 drop)	4.98	4.88	4.93	8.82	7.22	8.26	11.02	8.10	9.66	11.35	11.28	11.31
HgO + $SeOCl_2$ (2 drops)				11.12	8.01	10.04	10.76	10.39	10.58			
$CuSO_4$ + $SeOCl_2$ (1 drop)				7.54	6.11	6.58						
HgO + Se + $CuSO_4$	5.55	4.55	5.05	10.34	7.78	8.99				11.28	11.07	11.16
HgO + $CuSeO_3$ (5 mg)				8.42	7.28	7.95						
Clark's Method (5)	5.31	3.58	4.45									

[1] Selenium-coated granule.
[2] 0.15 g. $CuSO_4 \cdot 5H_2O$.

removed, and the capillary is immediately closed with the small cap and allowed to come to room temperature before weighing.

Moderately volatile or hydroscopic liquid samples are weighed in capsules made by sealing the tube of a 5/12 inner joint just below the joint. For weighing and introducing into the digestion flask the capsule is inserted loosely in a holder consisting of a 5/12 outer joint whose tube serves as a handle. After the capsule and holder are tared, the sample is introduced in the capsule with either a hypodermic syringe or a medicine dropper having a capillary tip, and the capsule is placed in the holder. The capsule with the sample is weighed and dropped into a digestion flask containing the acid and catalyst by gently tapping the holder against the neck of the flask. After digestion, the mixture is transferred to the distilling apparatus and the flask is rinsed as usual. The capsule, which is caught in the funnel, is removed with tweezers, held in an inverted position over the funnel, and rinsed with 2 ml of water from a hypodermic syringe. The needle is bent in a V-shape so that the rinse solution which drains down is transferred to the funnel. Very viscous liquids, gums and resins are introduced into the flask in porcelain boats. Hygroscopic samples are dried and weighed in weighing pigs.

RESULTS

Nicotinic acid was chosen for use in studies of catalyst and digestion time, since it contains a ring-type nitrogen which has proved difficult to obtain by the Kjeldahl method, is easily obtained in pure form, and is a non-hygroscopic solid. Several catalysts and combinations of catalysts were used, and the digestion time was varied from 1 to 4 hours. . . . The amount of catalyst in each combination was the same unless otherwise noted. Table 1 shows the results obtained with the various digesting conditions.

A 2-hour digestion period was used in comparing the relative effectiveness of the various catalysts and combinations, since it was not sufficiently long to give complete recovery with any of the catalysts. The results obtained under these conditions indicate the most effective catalyst to be a combination of mercuric oxide and selenium. Mercuric oxide and selenium oxychloride (2 drops) gave a higher nitrogen value in 2 hours than did the mercuric oxide and selenium, but as Bradstreet (4) has shown, the ratio of selenium to sulfuric acid was so high that loss of nitrogen was almost certain, and as expected, the values obtained after the 3-hour digestion were lower than those obtained with mercury and selenium. Increasing the amount of selenium increased the rate of conversion of nitrogen to ammonia, but apparently nitrogen was lost when the selenium content was raised much above 10 mg per ml of sulfuric acid. The results obtained for the 3-hour digestion with mercuric oxide and selenium show that in some cases theoretical values were obtained but the recoveries were not

TABLE 2.—*Kjeldahl nitrogen analyses of several nitrogenous compounds*

COMPOUND	CATALYST	DIGESTION TIME	NITROGEN FOUND		NITROGEN AVE.	NITROGEN THEORETICAL
		hours	*per cent*	*per cent*	*per cent*	*per cent*
N (α-pyridyl) nicotinamide	HgO+Se	3	20.80			
		3	20.85	20.67	20.77	21.10
		4	21.02	21.03		
		4	21.02	21.07	21.04	21.10
Nicotine	HgO+Se	1	13.74	14.79	14.27	17.27
		2	16.93	16.46	16.70	17.27
		3	17.22	17.23		
		3	17.31	17.19	17.24	17.27
		6	17.20	17.22	17.21	17.27
8-Hydroquinoline	HgO+Se	4	9.64	9.69	9.67	9.65
Tryptophane	HgO+$SeOCl_2$	4	13.74	13.72		
		4	13.75	13.77	13.75	13.72
Tryptophane	HgO+Se	1	13.17	13.05	13.11	13.72
		2	13.75	13.70	13.73	13.72
		4	13.65	13.66		
		4	13.64	13.69	13.67	13.72
Acetonicotyrine	HgO+$SeOCl_2$	4	11.34	12.88	12.11	13.73
		6	13.66	13.51	13.59	13.73
Nicotine picrate[1]	HgO+Se	4	18.11	18.05		
		4	17.89	18.16	18.05	18.06
Nicotine picrate[2]	HgO+Se	4	18.01	17.98	18.00	18.06
S-benzyl thiuronium chloride	HgO+Se	1	13.78	13.83		
		1	13.79	13.79	13.80	13.82
Acetanilide	HgO+Se	1	10.35	10.31	10.33	10.36

[1] Sample treated with salicylic acid and sodium thiosulfate. 3 ml. H_2SO_4 used.
[2] Sample digested for 2 hours with carbon prior to regular 4-hour digestion. 3 ml. H_2SO_4 used.

consistent, whereas with 4 hours' digestion concordant results were obtained which were close to the theoretical values. With the catalyst mixture recommended complete recovery of the nitrogen was obtained in 4 hours and no loss of nitrogen was detected with a 6-hour digestion. Consequently, a 4-hour digestion period was tentatively adopted as standard for all materials containing ring-type nitrogen. This digestion time is 1 hour longer than was indicated by Shirley and Becker's work (8), in which macro procedures were used. The difference may be due to the type of

heater used during the digestion, that is, electrical, versus gas heaters. Nitrogen was determined in several materials in addition to nicotinic acid by the method described to test its applicability to several types of compounds having ring nitrogen. The results are reported in Table 2.

All the compounds studied yielded their nitrogen after a 4-hour digestion with mercuric oxide and selenium except acetonicotyrine, which required a 6-hour digestion for the values to approach theory.

Nicotine, whose structure differs only slightly from that of nicotyrine, needed only a 3-hour digestion, thus indicating the difficulty that may be experienced in attempting to predict the digestion time required from the structure of the compound to be analyzed. Doubling the digestion time required for nicotine did not significantly lower the nitrogen values, proving that if loss of nitrogen does result from the use of selenium catalyst it is negligible. As would be expected, a 1-hour digestion was sufficient for acetanilide and S-benzyl thiuronium chloride, since they do not contain ring nitrogen. Tryptophane yielded its nitrogen after a 2-hour digestion. This is in agreement with the observations of White and Secor (9).

The results obtained show that in most cases a 4-hour digestion with mercuric oxide and selenium as catalysts is sufficiently long to obtain the nitrogen from heterocyclic nitrogen ring compounds. Apparently a few compounds require at least a 6-hour digestion. It is easy to detect these materials, however, since the incomplete 4-hour digestion usually is accompanied by discordant results.

SUMMARY

A semimicro Kjeldahl method is presented for the determination of nitrogen in compounds containing heterocyclic ring nitrogen. This method has proved successful in the analysis of micro-sized samples. With a mixture of mercury and selenium, the best catalyst found, a minimum digestion time of 4 hours is required. For compounds not containing ring nitrogen, the digestion time can be shortened to 1 hour.

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THE ANALYSIS OF LANOLIN-HYDROCARBON MIXTURES*

By S. H. NEWBURGER (Cosmetic Division, Food and Drug Administration, Federal Security Agency, Washington, D. C.)

The analysis of lanolin-hydrocarbon mixtures is of interest because hydrocarbons are often an adulterant in lanolin and because hydrocarbons are a constituent of lanolin absorption bases. In a recent article in *The Analyst*, Williams[1] states that he separates hydrocarbons from wool grease by chromatographing on an aluminum oxide column with petroleum benzin as the solvent. However, no data or experimental details were given. This investigation was undertaken to determine the conditions under which this separation could be effected.

METHOD

PREPARATION OF Al_2O_3 COLUMN

Prepare an activated Al_2O_3 column $9'' \times \frac{3}{4}''$ in the following manner: Tamp a $\frac{3}{4}''$ cotton plug into the bottom of the chromatograph tube, fill the tube with petroleum benzin, and pour enough Al_2O_3 ("Alorco" grade F-20 mesh 80–200) thru the petroleum benzin to form a column about $1\frac{1}{2}''$ high. Pack the Al_2O_3 by applying an air pressure of 5 or 6 pounds to the chromatograph tube. Then add successive increments of Al_2O_3 in this manner until the column totals a length of about 9". Finally, place another cotton plug on top of the column.

CHROMATOGRAPHIC PROCEDURE

Dissolve sample in 50 ml warm petroleum benzin (B.P. 30-75°C.), cool the soln to room temp., transfer to chromatograph tube with the aid of 25 ml of petroleum benzin, and allow the soln to flow by gravity thru the column at a rate of 3.3 to 6 ml per min. Follow this soln with 175 ml of petroleum benzin, combine the two filtrates and label them as filtrate No. 1. Put another 50 ml of petroleum benzin thru the column and label this filtrate as filtrate No. 2. Evaporate the filtrates on the steam bath, dry the residues in an oven at 100°C. for 10 min., cool in a vacuum desiccator, and weigh. Repeat drying in oven until weight is constant to 1 mg.

The first experiments tested the adsorbability of lanolin on the column. Samples of U.S.P. anhydrous lanolin varying from 2.5 to 6.6 grams were chromatographed. All samples larger than 3 grams gave an appreciable residue in both filtrates. Samples less than 3 grams gave a small residue in the first filtrate and a negligible residue in the second filtrate. The data are tabulated in Table 1.

As lanolin is a complex natural product of variable composition, 3 gram samples of several commercial products including one of hydrous lanolin were chromatographed. The results are presented in Table 2.

The retention of all the lanolin samples on the aluminum oxide was greater than 99 per cent.

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[1] Williams, K. A., *Analyst*, 71, 259 (1946).

A 5.230 gram sample of U.S.P. mineral oil was then dissolved in petroleum benzin and chromatographed by the procedure described for lanolin. The rate of flow of the solution was 4 ml per minute. About 99.9 per cent of the mineral oil came through in the first filtrate.

Mixtures of mineral oil with anhydrous lanolin (Product "a") and with

TABLE 1.—*The adsorption of lanolin on aluminum oxide*

SAMPLE U.S.P. ANHYDROUS LANOLIN PRODUCT "A"	RATE OF FLOW PETROLEUM BENZIN	RESIDUE FROM FIRST FILTRATE	RESIDUE FROM SECOND FILTRATE
gm	*ml per minute*	*gm*	*gm*
6.625	3.3	1.894	0.068
5.027	4.0	0.674	0.088
4.014	4.6	0.098	0.066
3.110	4.0	0.014 (0.5%)	0.000
3.024	5.5	0.014 (0.5%)	0.000
2.505	4.5	0.013 (0.5%)	0.000

TABLE 2.—*Variations in adsorbability of lanolin samples*

SAMPLE		RATE OF FLOW PETROLEUM BENZIN	RESIDUE FROM FIRST FILTRATE	RESIDUE FROM SECOND FILTRATE
	gms	*ml per minute*	*per cent*	*per cent*
Product "a" ..... (U.S.P. Anhydrous Lanolin)	3.024	5.5	0.5	0.0
Product "b" ..... (U.S.P. Anhydrous Lanolin)	2.998	3.3	0.3	0.0
Product "c" ..... (U.S.P. Anhydrous Lanolin)	2.999	4.0	0.8	0.0
Product "d" ..... (U.S.P. Hydrous Lanolin)	3.018	4.5	0.3	0.0

hydrous lanolin, the total weight of each mixed sample not exceeding 3 grams, were prepared and chromatographed. The data are given in Table 3.

The values for the unadsorbed material are greater than the percentages of mineral oil; this is to be expected as the lanolins have blanks of 0.5 and 0.3 per cent. If the lanolin blanks are subtracted, the percentages of mineral oil are in good agreement with the unadsorbed matter.

A sample of U.S.P. solid petrolatum was then chromatographed. Only 92.6 per cent came through in the first filtrate and an additional 1 per cent in the second filtrate (Table 4). Approximately the same behavior was

observed with a sample of vaseline. Neither of these samples had any appreciable volatile matter at 100°C. As shown in the table, a third filtrate of another 50 ml petroleum benzin was also collected.

Mineral oil is a distilled product and is often purified with fuming sulfuric acid. The end product is almost wholly saturated hydrocarbons.

TABLE 3.—*The chromatography of mixtures of mineral oil and lanolin*

SAMPLE		RATE OF FLOW PETROLEUM BENZIN	RESIDUE FROM FIRST FILTRATE	RESIDUE FROM SECOND FILTRATE	MINERAL OIL ADDED	UNADSORBED MATERIAL
	gm	*ml per minute*	*gm*	*gm*	*per cent*	*per cent*
U.S.P. Anhydrous lanolin....	2.733	3.5	0.110	0.000	3.4	3.9
U.S.P. Heavy mineral oil....	0.096					
Total.....	2.829					
U.S.P. Anhydrous lanolin....	2.637	4.0	0.311	0.001	10.2	10.6
U.S.P. Heavy mineral oil....	0.300					
Total.....	2.937					
U.S.P. Anhydrous lanolin....	2.250	3.5	0.732	0.000	24.2	24.7
U.S.P. Heavy mineral oil....	0.719					
Total.....	2.969					
U.S.P. Anhydrous lanolin....	1.453	4.5	1.532	0.002	51.3	51.4
U.S.P. Heavy mineral oil....	1.528					
Total.....	2.981					
U.S.P. Hydrous lanolin.....	2.506	4.5	0.296	0.000	10.4	10.6
U.S.P. Heavy mineral oil....	0.290					
Total.....	2.796					

TABLE 4.—*The chromatography of solid petrolatum*

SAMPLE		RATE OF FLOW PETROLEUM BENZIN	RESIDUE FROM FIRST FILTRATE	RESIDUE FROM SECOND FILTRATE	RESIDUE FROM THIRD FILTRATE
	gm	*ml per minute*	*per cent*	*per cent*	*per cent*
U.S.P. Solid petrolatum.	2.983	3.5	92.6	1.0	0.7
Vaseline.....	2.316	4.4	91.2	1.2	0.7

Petrolatum, on the other hand, is a residue and contains unsaturated hydrocarbons as well as their oxidation products. It is probably this material which is held on the column.

TABLE 5.—*The chromatography of mixtures of lanolin and solid petrolatum*

SAMPLE		RATE OF FLOW PETROLEUM BENZIN	RESIDUE FROM FIRST FILTRATE	RESIDUE FROM SECOND FILTRATE	PETROLATUM ADDED	UNADSORBED MATERIAL
	gm	*ml per min.*	*per cent*	*per cent*	*per cent*	*per cent*
U.S.P. Anhydrous lanolin	2.905	6.0	2.9	0.0	2.6	2.9
U.S.P. Solid petrolatum	0.077					
Total	2.982					
U.S.P. Anhydrous lanolin	2.660	4.5	9.8	0.1	10.1	9.9
U.S.P. Solid petrolatum	0.298					
Total	2.958					
U.S.P. Anhydrous lanolin	1.894	5.0	32.1	0.4	34.1	32.5
U.S.P. Solid petrolatum	0.982					
Total	2.876					
U.S.P. Anhydrous lanolin	1.414	4.0	48.8	0.5	52.2	49.3
U.S.P. Solid petrolatum	1.545					
Total	2.959					
U.S.P. Anhydrous lanolin	0.426	4.5	79.4	0.9	85.6	80.3
U.S.P. Solid petrolatum	2.524					
Total	2.950					

Mixtures of anhydrous lanolin (Product "a") and solid petrolatum were prepared and chromatographed. The data are presented in Table 5.

The values for the unadsorbed material are the sum of the residues from both filtrates. However, the residue from the second filtrate was never greater than 0.9 per cent and for mixtures containing less than 10 per cent petrolatum it was not significant. The unadsorbed material calculated as petrolatum recoveries does not give values as good as those obtained for mineral oil; however, if the 0.5 per cent blank is subtracted from the unadsorbed material recovered, the resulting values all fall within 1.3 per cent of the 93.6 per cent unadsorbed material expected from the petrolatum.

SUMMARY

Under the described experimental conditions hydrous and anhydrous lanolin are adsorbed on the aluminum oxide to an extent greater than 99 per cent. Hydrocarbons are not adsorbed at all. Advantage is taken of these phenomena to separate mineral oil and solid petrolatum from lanolin.

SPECTROPHOTOMETRIC TESTING OF D&C VIOLET NO. 2

(ALIZUROL PURPLE SS)

By MEYER DOLINSKY (Cosmetic Division, Food and Drug Administration, Federal Security Agency, Washngton, D. C.)

The pure dye content of samples of D&C Violet No. 2 is usually determined either by titration with titanium trichloride, or by calculation from the nitrogen content of the sample. Neither of these methods is satisfactory. The end point of the titration is difficult to detect, since the reduction products are also colored. Calculation of the pure color from the nitrogen content of the sample will give high results when nitrogen containing impurities are present.

It was therefore decided to investigate a spectrophotometric method for the determination, following the method of Clark and Newburger on the Spectrophotometric Analysis of Coal-Tar Colors.[1]

EXPERIMENTAL

Preparation of Standard Sample.—Quinizarin (1, 4-dihydroxyanthraquinone), m.p. 194°C. (literature 194°C.), was reduced to "leuco-quinizarin" by means of stannous chloride plus hydrochloric acid.[2] The product, after recrystallization from benzene, melted at 155°C. (literature 150°–155°C.). This was then condensed with p-toluidine,[3] m.p. 43° '. (literature 42.8°–45°C.), The dye, after repeated crystallization from glacial acetic acid, melted at 190°C. (literature 183°C.). A further recrystallization of a portion of the product from a chloroform-alcohol mixture showed no change in melting point or spectrophotometric characteristics.

Nitrogen determination by a semimicro Kjeldahl method gave a pure dye content for the product of 100.4 per cent; titration with titanium trichloride, using di-sodium citrate as a buffer, gave a pure dye content of 99.5 per cent.

Preparation of Solutions.—A 31.02 mg sample of dye, weighed on a semimicro balance, was dissolved in sufficient U.S.P. chloroform to make the volume exactly 100 ml. Aliquot portions of this solution were then diluted with chloroform to give the concentrations shown in Table 1.

The chloroform solution of D&C Violet No. 2 (Chart 1) shows a characteristic flattened absorption curve with a peak at 587 ± 2 mμ. The ratio of E_{550} mμ/E_{600} mμ was found to be 1.00 ± 0.02. Deviations from Beer's law at 587 mμ are less than 1 per cent.

All optical measurements were made with a General Electric recording spectrophotometer using an 8 mμ slit. Melting points were taken on a Fisher M. P. Block.

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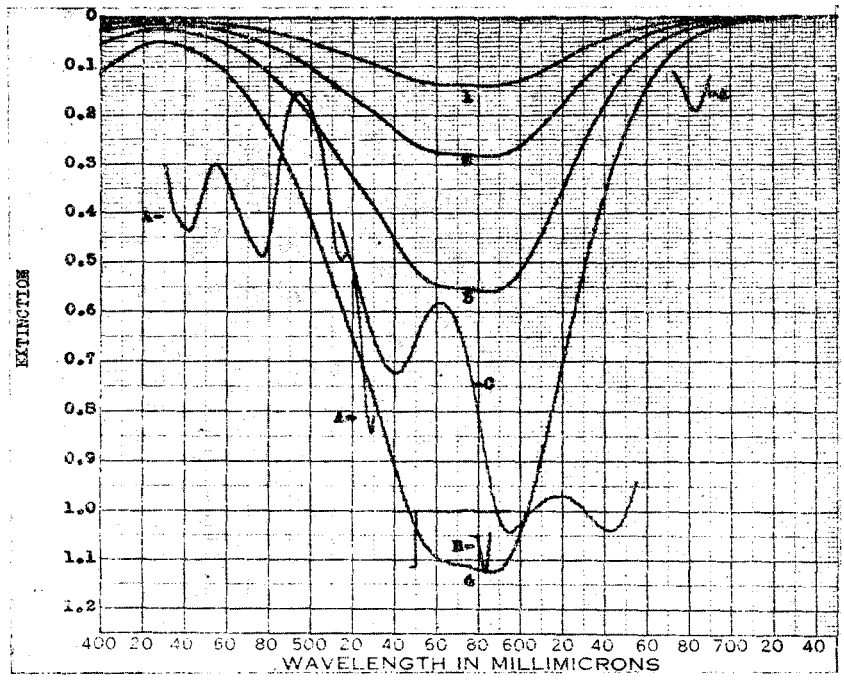


CHART 1.—Pure D&C Violet No. 2 in chloroform.
Curve 1— 3.88 mg./liter.
Curve 2— 7.76 mg./liter.
Curve 3—15.51 mg./liter.
Curve 4—31.02 mg./liter.
Cells—1 cm.
A—Corning Didymium Glass 512, 6.0 mm.
(Absorption peaks at 400.4, 441.4, 477.1, 529.0, and 684.8 mμ.)
B—Corning Didymium Glass 592, 4.02 mm.
(Absorption peak at 583.7 mμ.)
C—Signal Lunar White Glass H-6946236.

TABLE 1.—*Extinction values of chloroform solutions of purified D&C Violet No. 2*

CURVE NO. (CHART 1)	CONCENTRATION	$E_{557\ m\mu}$	$\frac{E_{557\ m\mu}}{\text{CONCENTRATION}}$
	mg/liter		
1	3.88	0.140	0.0361
2	7.76	0.282	0.0363
3	15.51	0.558	0.0360
4	31.02	1.123	0.0362
			Av. 0.0362

APPLICATION TO COMMERCIAL SAMPLES

A commercial sample of D&C Violet No. 2 was analyzed spectrophotometrically, following the procedure described for the standard sample. The curve is shown on Chart 2 and the data in Table 2.

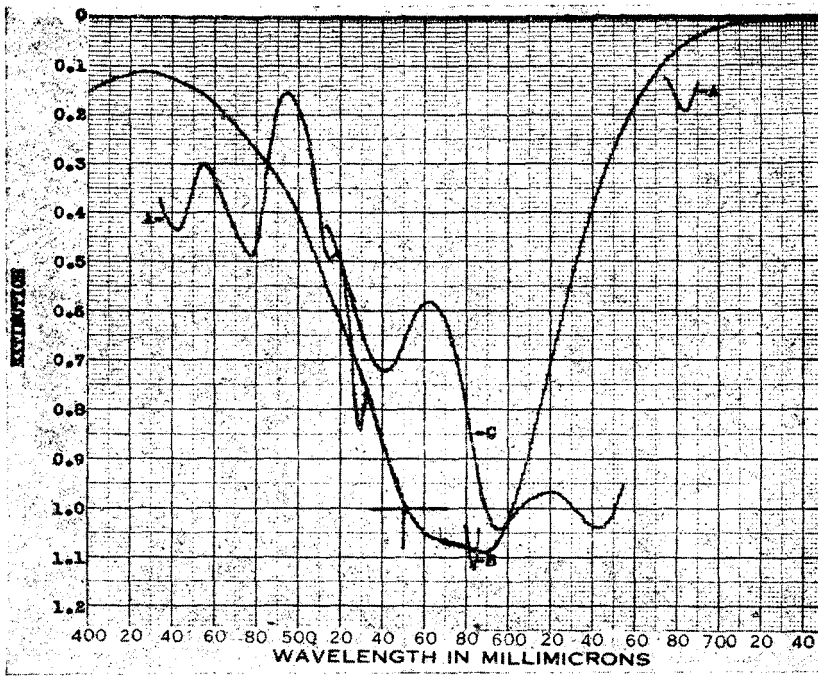


CHART 2.—Commercial Sample of D&C Violet No. 2 in chloroform.
Curve 1—31.90 mg./liter.
Cells—1 cm.
A—Corning Didymium Glass 512, 6.0 mm.
(Absorption peaks at 400.4, 441.4, 477.1, 529.0, and 684.8 mμ.)
B—Corning Didymium Glass 592, 4.02 mm.
(Absorption peak at 583.7 mμ.)
C—Signal Lunar White Glass H-6946236.

TABLE 2.—*Analysis of commercial sample of D&C Violet No. 2*

CONCENTRATION	E_{587} mμ (IN $CHCl_3$)	DYE SPECTROPHOTOMETRICALLY	DYE BY TITRATION WITH $TiCl_3$	DYE FROM N_2 CONTENT
mg/liter		*per cent*	*per cent*	*per cent*
31.90	1.089	94.3	93.4	98

A portion of this commercial sample after several recrystallizations from glacial acetic acid gave an extinction per milligram of 0.0362, corresponding to a pure dye content of 100 per cent by comparison with the laboratory sample.

SUMMARY

Spectrophotometric data for chloroform solutions of purified D&C Violet No. 2 are presented. Beer's law is shown to be applicable; the extinction per milligram/liter is 0.0362 ± 0.0002 at the absorption peak, 587 ± 2 mμ; the extinction ratio E_{550} mμ/E_{600} mμ is 1.00 ± 0.02.

Application is made of these data to the determination of pure dye in a commercial sample of the color.

DETERMINATION OF LEAD IN LAKES OF COAL-TAR COLORS

By LEE S. HARROW (Food and Drug Administration, Federal Security Agency, Washington, D. C.)

Studies of several methods for the determination of small amounts of lead in foods and biological materials have been reported by Wichmann *et al.* (1), Clifford (2), and others. The dithizone method for estimating lead in foods has been adopted as tentative in the "Official and Tentative Methods of Analysis," A.O.A.C., Sixth Edition (1945) **29.34.** Ettelstein (3) has reported the application of this method to the determination of lead in straight coal-tar colors. However, the determination of lead in lakes of coal-tar color offers additional problems. In the study to be described, separate methods were developed for aluminum lakes extended upon alumina, and for lakes containing barium and calcium.

ALUMINUM LAKES

Under the conditions usually employed in the extraction of lead as the dithizone complex from aqueous solutions, aluminum hydroxide is precipitated when considerable quantities of aluminum are present. Clifford (2) has shown that lead is occluded with the precipitate, and that the occluded lead is not recovered by the dithizone separation process even when a large amount of citric acid is used.

Table 1 is a tabulation of typical coal-tar color aluminum lakes showing the approximate percentages of aluminum oxide.

In order to eliminate these two possible sources of error, the *Methods of Analysis* (4) offers a means of separating the lead as lead sulfide from the interfering substances. The procedure consists essentially of precipitating the lead as lead sulfide at a *p*H of 3.0–3.4, filtering and dissolving the sulfides with hot concentrated nitric acid, then extracting the lead with di-

TABLE 1.—*Typical aluminum lakes*

COLOR LAKES	ALUMINUM OXIDE
	per cent
FD&C Blue No. 1	87
FD&C Red No. 1	58
FD&C Yellow No. 5	85
FD&C Yellow No. 6	83
D&C Green No. 1	88

thizone solution. This method was modified to make it applicable to aluminum lakes of coal-tar colors.

EXPERIMENTAL

APPARATUS

The apparatus for the operation as shown in Figure 1 consists of a precipitation tube fitted with various inlets to permit introduction of the sample, introduction of hydrogen sulfide, release of excess hydrogen sulfide, and transfer of the precipitated lead sulfide to the filter.

Careful preparation of the filter is essential in order to allow rapid filtration with complete retention of the precipitate. "Celite" or other similar filter aid placed on a fine porosity sintered glass filter gives satisfactory results. For quantities of lead between 20 and 300 micrograms, approximately 0.5 gram of the filter aid should be placed on the filter and washed thoroughly, first with concentrated nitric acid, then with water before use.

Particular attention should be paid to the cleanliness of the apparatus. In this work all glassware was washed successively with scouring powder, tap water, concentrated nitric acid, and distilled water. The precipitation apparatus was washed with concentrated nitric acid and water after each run.

REAGENTS

All reagents should be lead free (5). Any convenient source of H_2S may be used. The gas should be scrubbed first with (1:1) H_2SO_4 (v/v), then with distilled water, before being passed into the solution.

METHOD

Weigh 2 g of the sample into a 500 ml Kjeldahl digestion flask, add 10 ml of conc. H_2SO_4 and 10 ml of conc. HNO_3, and digest on a low flame until SO_3 fumes appear. Add successive 5 ml portions of conc. HNO_3 (waiting until SO_3 fumes appear before adding each succeeding portion) until all organic matter is in solution. Slowly introduce 5–10 ml of a (1:1) mixture of conc. HNO_3 and 60–70% $HClO_4$, and continue the digestion until the white ppt. formed showed the first signs of spattering. Allow the flask to cool and cautiously add 5 ml of H_2O and then a few drops of conc. NH_4OH. Swirl the flask vigorously and cool under running water. Add 20 ml of 50% (w/v) citric acid soln and adjust the *p*H to 3.0–3.4 (bromophenol blue) with conc. NH_4OH. Add 1 ml of $CuSO_4$ soln containing 1 mg Cu per ml and transfer the soln

to the pptn tube (B) of the sulfiding apparatus. Bubble H_2S thru the soln at a rate of ca 2 bubbles per sec. for 3–5 min. and filter the resulting suspension thru (C) at a rate of ca 1 drop per sec. When filtration is complete remove the receiver containing the filtrate and attach a suction test tube as shown in Figure 1 (E). Add 3 ml of hot conc. HNO_3 thru the separatory funnel (A) and draw thru the filter, followed with 2 ml of hot water. Detach the filter and pass an additional 3 ml of hot conc. HNO_3 thru the filter, wetting all sides. Again follow with 2 ml of hot water. If the filter is still colored with PbS, wash again with hot conc. HNO_3 and water. Wash the dissolved sulfides into the pptn tube (B), wetting all sides to take up any residual lead sulfide and then into a 50–100 ml glass-stoppered conical flask. Stopper and shake for a few sec., then remove the stopper and boil until the soln clears, to remove the last traces of H_2S and to coagulate any free sulfur present.

Transfer the soln to a 250 ml separatory funnel. Wash the flask with two 5 ml portions of distilled water and add the washings to the main soln. Add 10 ml of 50% (w/v) citric acid soln, 5 ml of 10% sodium cyanide soln, a few drops of hydroxylamine hydrochloride soln to prevent oxidation of the dithizone, adjust the *p*H to 8.5–9.5 (thymol blue) with conc. NH_4OH and proceed with the dithizone extraction and electrolysis as described in the A.O.A.C. Book of Methods (6).

RESULTS

The method was used to determine lead in a composite sample of D&C Blue No. 1, Aluminum Lake, containing approximately 88% of aluminum oxide. Quantities of lead ranging from 20 parts per million to 150 parts per million were added to 2 gram samples. Recoveries obtained are given in Table 2. As shown in Table 2, an average recovery of 99 per cent was obtained.

DISCUSSION

The success of the method depends for the most part on the complete solution of the digestion mixture upon neutralization. The following conditions were noted as being conducive to complete solution:

(1) The neutralization should be carried out slowly and with vigorous agitation to allow action of the ammonium hydroxide on the solid material. As the *p*H of 3.0 is approached, the solution should be allowed to heat up. Fifteen to twenty minutes is usually a sufficient time for the addition of the ammonium hydroxide if the solution has been adequately heated and swirled.

(2) If at the completion of the neutralization the solution contains solid material or is translucent, allow the solution to stand about 10 minutes. This usually brings about complete solution.

The color changes of bromophenol blue range from yellow at *p*H 3.0 through olive to purple at 3.4 or above. The *p*H range for the quantitative precipitation of lead sulfide without co-precipitation of ferrous sulfide is from 2.5–3.4 (orange-yellow with thymol blue to incipient purple or olive-green with bromophenol blue). Adjustments should be made only from the acid side to avoid any permanent precipitation of the hydroxides. (Addition of citric acid, cooling of the iron solution, and agitation should

prevent the precipitation of aluminum, iron, or alkaline earth hydroxides.) When enough iron is present to mask the color, final adjustments of the *p*H should be made on a spot plate.

Copper acts as a good collector of lead and also as a filter aid (2). (It

TABLE 2.—*Recovery of lead*

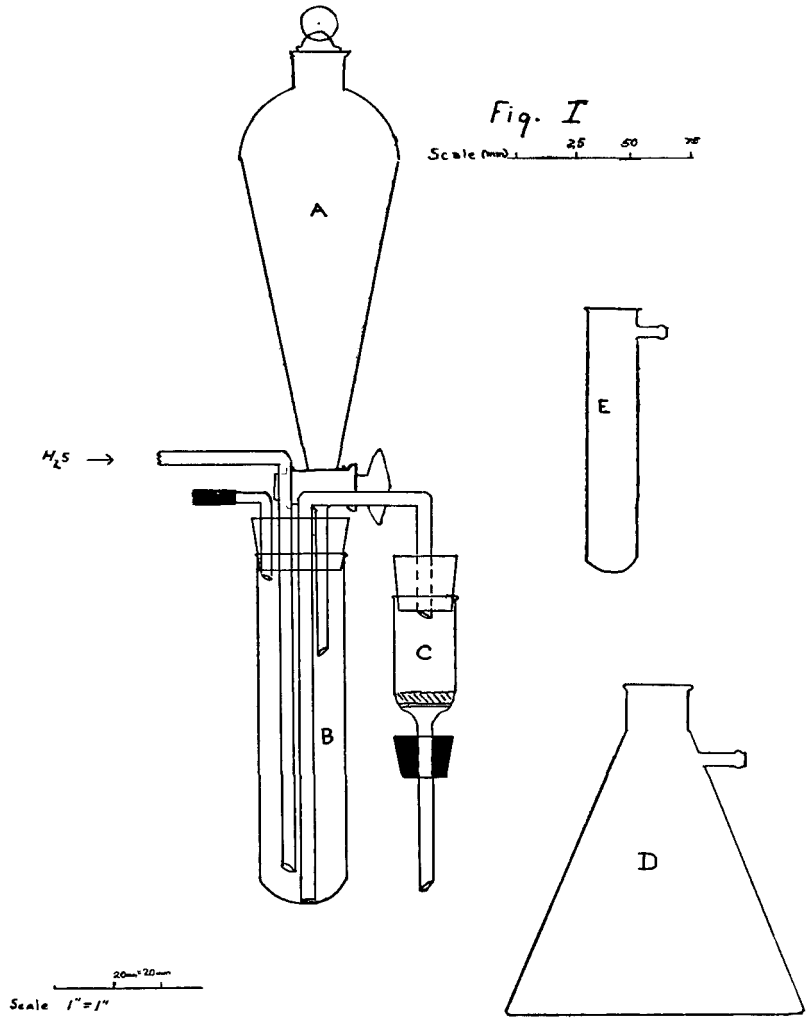
BLANK* MICROGRAMS Pb	Pb ADDED	EQUIVALENT TO PER CENT	Pb RECOVERED	NET RECOVERY	PER CENT	AVERAGE PER CENT
	micrograms		*micrograms*	*micrograms*		
20	40	0.0020	57	37	93	97
			59	39	98	
			58	38	95	
			59	39	98	
			60	40	100	
27	100	0.0050	124	97	97	99
			129	102	102	
			127	100	100	
			127	100	100	
			125	98	98	
29	160	0.0080	190	159	99	99
			193	162	101	
			190	159	99	
			188	160	100	
			186	158	99	
28	200	0.0100	227	200	100	97
			219	192	96	
			228	201	100	
			230	202	101	
			227	199	100	
28	300	0.0150	325	297	99	100
			328	300	100	
			329	301	100	
			327	299	100	
			335	308	103	
			328	300	100	

* The blank included the electrode blank, reagent blank, and dye without added lead.

has been found that for a quantity of lead ranging from 10 to 300 micrograms, 1–5 mg. should be added.)

CONCLUSION

The method described above appears to give accurate and reproducible results.



BARIUM, CALCIUM, AND STRONTIUM LAKES

The usual wet digestion methods cannot be used for the decomposition of alkaline earth lakes because of the insolubility of the digestion residue (calcium, barium, or strontium sulfates) in alkaline solution. Determination of lead in cosmetics by a dithizone extraction method following fusion with an excess of sodium and potassium carbonates has been described by

Kress (7). The method described herein represents a modification of this procedure applicable to the analysis of color lakes.

EXPERIMENTAL

REAGENTS

Sodium carbonate.—Lead free, analytical grade.
Potassium carbonate.—Lead free, analytical grade.
Sodium nitrate.—Lead free, analytical grade.
Sodium carbonate soln.—5% (w/v).
Hydrochloric acid.—(2:5) (v/v).

METHOD

Place 2 g of the lake, 4 g of Na_2CO_3, 6 g of K_2CO_3, and 0.5 g of $NaNO_2$ in a platinum crucible of suitable size. Mix thoroly. Heat carefully until the color is carbonized, then heat to about 850°C. and hold at that temp. for 15 minutes. If a controlled muffle furnace is available, it is only necessary to place the fusion mixture in the cold furnace and raise the temp. gradually to 850°C. over a two-hour period. Usually 15–30 min. heating at 850°C. is sufficient to complete the fusion.

When fusion is complete, allow the crucible and contents to cool below 100°C., then add 2 or 3 ml of water and heat over a low flame, using care to prevent spattering, until the contents can be separated from the crucible. Transfer the fused mixture to a 150 ml beaker with the aid of about 25 ml of hot water. Boil until the caked material is completely disintegrated, then filter thru a retentive filter paper. Wash the residue on the filter with two 15 ml portions of hot 5% Na_2CO_3 soln. Lead will be in both filtrate and residue. Transfer the filtrate to a separatory funnel and proceed to extract the lead from the filtrate as directed under aluminum lakes. Dissolve the residue on the filter in 10–20 ml of the hydrochloric acid soln, wash the filter with water and add washings to the soln. Boil the soln to expel carbon dioxide, then transfer to a separatory funnel and extract the lead as directed above. Combine with the chloroform extracts from the soluble portion of the fusion products and determine the total lead by the electrolytic method. (6)

RESULTS

The method described was used to test the recovery of lead ranging from 40 micrograms to 200 micrograms added to 2 gram samples of D&C Red No. 9, Barium Lake. The average recovery was 98 per cent. Results are shown in Table 3.

DISCUSSION

The use of sodium nitrate in the fusion mixture serves a dual purpose; the lead is kept oxidized, thus minimizing the possibility of loss of lead through volatilization, and the fusion temperature is lowered as a result of its flux properties.

CONCLUSION

The method described above appears to give accurate and reproducible results.

TABLE 3.—*Recovery of lead*

BLANK* MICROGRAMS Pb	Pb ADDED	EQUIVALENT TO PER CENT	NET Pb RECOVERED		AVERAGE RECOVERY
	micrograms		*micrograms*	*per cent*	*per cent*
40	40	0.0020	37	93	97
			36	90	
			39	97	
			42	106	
			39	98	
41	80	0.0040	76	96	97
			79	98	
			73	91	
			80	100	
			79	98	
41	100	0.0050	101	101	99
			100	100	
			96	96	
			100	100	
			98	98	
41	120	0.0060	115	96	98
			121	100	
			118	98	
			118	98	
41	200	0.0100	201	100	99
			201	100	
			198	99	
			197	98	
			200	98	

* The blank includes the electrode blank, reagent blank, and lead originally present in the lake.

REFERENCES

(1) WICHMANN, H.J., *et al.*, *This Journal*, **17**, 108 (1934).
(2) CLIFFORD, PAUL, *Ibid.*, **26**, 26 (1943).
(3) ETTELSTEIN, N., *Ibid.*, **26**, 552 (1947).
(4) *Methods of Analysis*, A.O.A.C., 6th Ed., **29.40** (1945), p. 460.
(5) *Ibid.*, **29.36**, p. 456.
(6) *Ibid.*, **29.39, 29.40, 29.41**, pp. 459–461.
(7) KRESS, "Proceedings of the Scientific Section of the Toilet Goods Association, Inc.," 4–20 (1945).

DETERMINATION OF ROTENONE IN SMALL SAMPLES

By MERRIAM A. JONES and CALEB PAGÁN[1]

The rotenone content of an individual root or part of a root is often required in a breeding and selection program or a physiological study. In such instances the quantity of plant material available for analysis is frequently so small that the regular gravimetric[2] or titrimetric methods[3] cannot be used.

Two modifications of these methods have been devised for the determination of rotenone in small samples. Since the methods involve less than one-tenth the amount of sample required by the official macro methods, they might properly be termed micro methods.

In devising the procedure it was desired that the new methods should involve the same principles as the official method so that the results obtained would be directly comparable. This end was accomplished by using a micro beaker to which was sealed a spout containing a sintered glass filter. Small samples can be handled in this device without involving the numerous transfers required by the macro methods. The micro beaker shown in Figure 1 is an inexpensive stock item in catalogs of laboratory apparatus. The procedure was so arranged that, after the extraction of the sample, either a gravimetric or titrimetric method could be used.

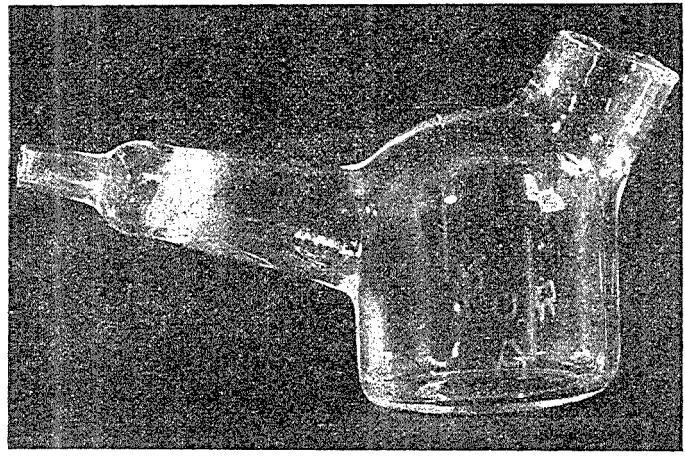


FIG. 1.—Micro beaker, for use in determination of small samples.

[1] Former chemist and present chemist, respectively, Federal Experiment Station in Puerto Rico, Mayagüez, P. R.

[2] A.O.A.C., *Official and Tentative Methods of Analysis*, Ed. 6 (1945).

[3] Jones, Howard A. "A Titrimetric Step in Determining Rotenone." *Ind. Eng. Chem., Anal. Ed.*, 10 684 (1938).

EXTRACTION OF SAMPLE

Weigh 2.000 gm of powdered root, ground to pass a 0.5 mm sieve and mix in a one-ounce screw-cap bottle with 0.66 gm of Norite-A decolorizing carbon. Add 20.0 ml of chloroform. Screw on the cap tightly and weigh to the nearest hundredth gram. (The inner surface of the cap of the screw-top bottle should be lined with cork or paper to prevent leakage and this material should be protected from the chloroform by aluminum foil.) Shake 4 hours, leave overnight, and shake 1 hour. Weigh the bottle, and replace any solvent lost by evaporation. Cool in ice bath for 30 min. and filter thru fast filter paper (11 cm). During filtration, cover the funnel with a beaker cover and plug the neck of the receiving flask with cotton to reduce loss by evaporation. Sample the filtrate after it has come to room temp.

GRAVIMETRIC PROCEDURE

Pipet 5.00 ml of the extract into a tared micro beaker (Fig. 1). Evaporate solvent on a steam bath while passing a slow current of air thru the beaker by means of suction on the filter spout. (The steam bath used consisted of a wide-mouth conical flask (250 ml), containing boiling chips or an anti-bump capillary.) Pipet another 5.00 ml of the extract into the beaker and evaporate as before. Add 2 ml of CCl_4 to the resinous residue, warm, and swirl. Evaporate solvent as before and repeat with another 2 ml of CCl_4. Cool and weigh to the nearest hundredth gram. (Weighings for the purpose of replacing evaporated solvents may be made on a torsion balance which allows for rapid weighing to .01 gram.) Multiply the weight of the residue in hundredths grams by 10 and add this number of ml of CCl_4 saturated with rotenone. Weigh beaker to .01 gram and warm to dissolve resin. When cool, reweigh, and replace with pure solvent any CCl_4 lost by evaporation. Cool in ice water and seed, if necessary, until solvate crystallizes. Keep at 0°C. for 4 hours or overnight. Filter by applying light suction to the spout. (For filtration an ordinary suction flask was used. A conical hole was cut in the rubber stopper so that the filter spout could be readily inserted and removed.) Add a volume of alcohol saturated with rotenone at room temp. equal to the volume of rotenone-saturated CCl_4 previously used. Weigh beaker to .01 gram, and warm to dissolve solvate in alcohol. Reweigh, and replace with pure solvent any alcohol lost by evaporation. Leave at room temp. 4 hours or overnight. Filter by gravity using a light suction to remove last of alcohol. Dry at 105° for 30 min., cool, and weigh to 0.1 mg. This weight minus the tare is multiplied by 100 to give the per cent rotenone in the root.

VOLUMETRIC PROCEDURE

Proceed as described under gravimetric procedure down to the point at which alcohol is added to the rotenone-CCl_4 complex. At this point, instead of adding alcohol, add 2 ml of acetone. Warm to dissolve precipitate in acetone, and evaporate acetone on steam bath. Add 0.5 ml of dichloroacetic acid and warm until soln is effected. Cool in ice-water for 2 min. while swirling. Add 10 drops of ice-water dropwise at the rate of 4 drops per min. while swirling the beaker. (The dropping pipet should consist of a glass tube drawn out so that about 40 drops equal 1 ml.) Seed with rotenone-dichloroacetic acid complex and add 5 drops of ice-water at 30-second intervals. Precipitation usually occurs within 5 min. If no precipitate forms, add ice-water dropwise at the rate of 4 drops per min., while swirling the beaker in the ice-water bath, until precipitation occurs. Swirl for several min. in the ice-water bath to complete the precipitation. Add 10 drops of ice-water at 10-sec. intervals while shaking. Add 10 drops rapidly while shaking, and then add 20 drops without shaking. Filter by tilting the beaker and applying gentle suction at the spout. Wash precipitate with 25 ml of water. Dissolve precipitate and wash out of device

with 5 ml of chloroform into a 50-ml beaker or conical flask. Add 10 ml of freshly boiled water, 3 drops 0.1 per cent phenolphthalein, and titrate with 0.01 *N* NaOH. Each ml of 0.1 *N* NaOH is equivalent to 3.94 mg of rotenone.

RESULTS AND DISCUSSION

The same precautions required in the official gravimetric method and its titrimetric modification should be observed in the micro methods. Two sources of possible error should be specially considered: (1) Incomplete crystallization of the solvate, and (2) loss of resin particles by entrainment when evaporating the chloroform. By following the method exactly as outlined crystallization of the CCl_4-rotenone complex should be obtained with minimum loss of resin by entrainment.

In the volumetric procedure it is essential that a crystalline rotenone-dichloroacetic acid precipitate be obtained. If an amorphous complex is obtained, excess acid will be retained by the precipitate introducing an

TABLE 1.—*Comparison of the results obtained by three methods in rotenone analysis of some derris and lonchocarpus root samples*

SAMPLE	ROTENONE		
	OFFICIAL MACRO	GRAVIMETRIC MICRO	VOLUMETRIC MICRO
	Per cent	*Per cent*	*Per cent*
Derris 1	1.8	1.85	1.90
		1.81	1.82
Derris 2	4.2	4.17	4.21
		4.12	4.32
Derris 3	4.7	4.62	4.76
		4.79	4.80
Derris 4	5.6	5.60	5.68
		5.58	5.66
Derris 5	5.9	5.82	5.86
		6.01	5.82
Derris 6	7.2	7.19	7.17
		7.25	7.23
Lonchocarpus 1	4.5	4.47	4.46
		4.52	4.50
Lonchocarpus 2	5.6	5.59	5.63
		5.55	5.61
Lonchocarpus 3	7.3	7.22	7.41
		7.30	7.19

error of unknown magnitude. For comparison of the results of the micro methods with the official macro method, a series of derris and lonchocarpus samples were chosen of low, medium, and high rotenone content. The results of the analyses by the three methods are shown in Table 1.

SUMMARY

Methods are described for the analysis of small samples of rotenone by a gravimetric and by a titrimetric procedure. The results compare favorably with those obtained by the official macro method.

DENSITY AND REFRACTIVE INDICES OF LACTOSE SOLUTIONS

By EMMA J. MCDONALD and ANNE L. TURCOTTE (National Bureau of Standards, Department of Commerce, Washington, D. C.)

I. INTRODUCTION

Lactose, the disaccharide commonly known as milk sugar, has for many years been of commercial importance. Until recently its principal use was in the manufacture of pharmaceuticals, food products, and in the preparation of modified diets; however, the penicillin industry now also requires large quantities of this sugar.[1]

In the present investigation measurements have been made to determine the densities and refractive indices of lactose solutions. The tables here presented are expected to be of use to the investigator working with the pure solutions, as well as to the analyst dealing with solutions in which the total solids may be calculated as lactose.

II. PREPARATION OF LACTOSE

The lactose used in this investigation was carefully prepared by repeated crystallizations of the commercial product. The procedure consisted in heating a 50-per cent solution in a water bath at 90°C., treating with decolorizing carbon, and filtering. The filtrate was then seeded with α-lactose hydrate crystals and stirred continuously while cooling in an ice bath. The resulting crystals, which were uniformly small and well formed, were purged on a centrifugal machine and washed consecutively with cold water and alcohol. After three crystallizations the ash content had decreased to .002 per cent and remained constant. The specific rotation of α-lactose hydrate in equilibrium solution is $+50.53°$. Thus the direct-reading of a 10 per cent solution would amount to $+30.35°$. The effect of the last traces of impurities on this reading is too slight to affect

[1] E. O. Whittier, *J. Dairy Science*, **27**, 505 (1944); a review, "Lactose and Its Utilization," contains many references to work on lactose.

the accuracy. Analysis by the method of Munson and Walker also showed the product to be unchanged within the accuracy of the method. Here, as in the case of specific rotation, small traces of impurities would not affect the results noticeably. We, therefore, believe that ash content is a more

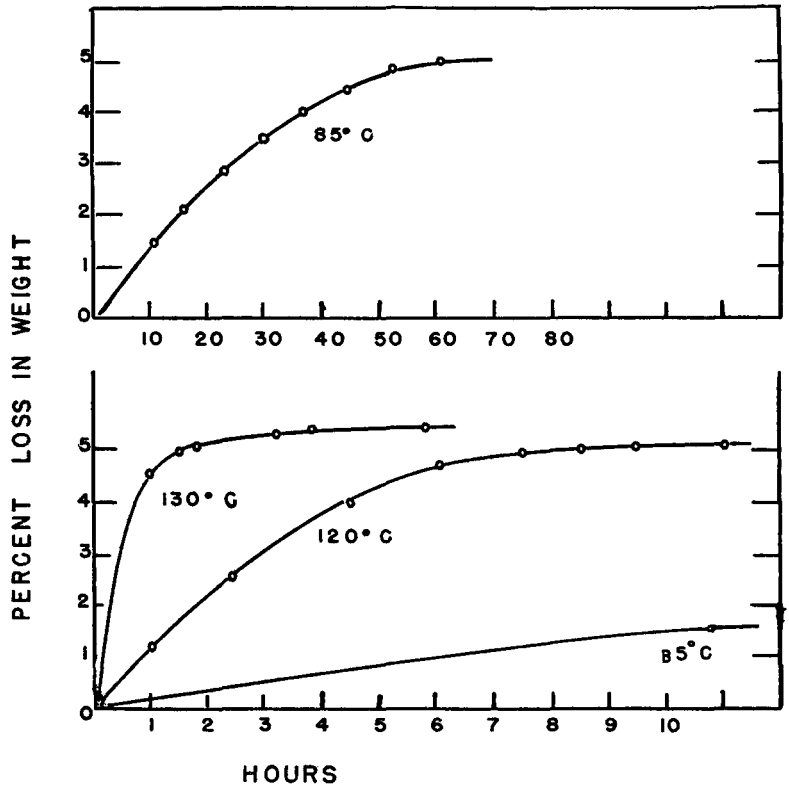


FIG. 1.—Dehydration of α-lactose hydrate at 85°, 120°, and 130°C. in vacuum.

reliable criterion for purity. The recrystallized product was dried in air and stored in a desiccator over commercial α-lactose hydrate. Under these conditions the vapor pressure was so maintained that loss of water of crystallization was prevented.

Lactose hydrate contains one molecule of water of crystallization, which amounts to 5 per cent of the total weight. Any change in the water of hydration would be reflected as an error in the percentage composition of the prepared solutions, and hence in the reported physical constants; we, therefore, carefully investigated the composition of the crystals as to moisture content and were assured that we were dealing with a hydrate

of uniform composition. Many moisture determinations were run in order to determine the most favorable conditions for this analysis.

The results obtained when the hydrate was dried in a vacuum at 85°, 120°, and 130°C. are shown in Figure 1. In all cases the loss in water was accompanied by some decomposition of the sugar as indicated by a yellowing of the sample. This discoloration was more pronounced in the recrystallized sugar than in the original commercial product. Because the ash con-

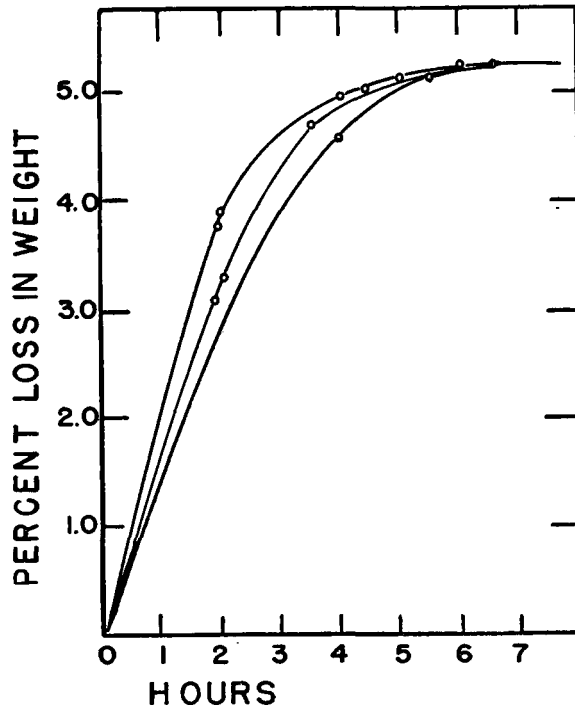


FIG. 2.—Dehydration of α-lactose hydrate at 120°C. in vacuum.

tent decreased on recrystallization from 0.0135 to 0.002 per cent, it is suggested that a buffering material is present in the commercial product and that this retards decomposition. The minimum discoloration during loss of water appeared to take place at 120°C., and, therefore, this temperature was subsequently used in determining the moisture content. The drying curves shown in Figure 2 are representative of those obtained during the progress of the investigation. Inspection of these curves shows the danger of using an arbitrary time when determining the moisture content of any given sample of α-lactose hydrate.

III. PHYSICAL MEASUREMENTS

The range in concentration where accurate density and refractive index measurements can be made is limited by the relatively low solubility of lactose and by the readiness with which supersaturated solutions tend to deposit crystals. At 20°C. a saturated solution of lactose contains 16 per cent of the sugar, a low value as compared with 49, 67, and 70, the respective percentages of saturated dextrose, sucrose, and levulose solutions at this temperature. Supersaturated solutions deposit lactose hydrate crystals with great ease, and thus limit the reliability of measurements made upon them. We have, therefore, carried out density and refractive index measurements with high precision in the range of unsaturation. The values reported for supersaturated solutions, although not obtained with the same precision, are suitable for many purposes and thus were included in this investigation.

1. *DENSITY OF LACTOSE SOLUTIONS*

Density measurements were made in a carefully calibrated flask. When filled to the lowest graduation at 20°C., this flask contained 126.333 ml. Its neck, 6 mm inside diameter, was graduated in 10 divisions at intervals of 0.02 ml. The volume reading could be estimated to one-tenth of one of these divisions, thus permitting a precision slightly greater than two units in the fifth place of density. The flask was recalibrated from time to time during the progress of the investigation, and the volume was found to be constant within the error of reading.

A quantity of α-lactose hydrate was introduced into the weighed flask and accurately weighed. The sugar was dissolved in distilled water, and air was removed from the solution by placing the slightly warmed flask in an environment of reduced pressure, care being taken to avoid spattering during this procedure. Water was then added to some point within the graduation, and the flask was rotated in such a way that the solution did not wet the upper part of the neck of the flask. After removing the water adhering to the flask above the solution by means of a current of dry air, the flask was placed in a thermostat and the final volume taken when the solution had come to temperature. Since the manipulation was time-consuming, mutarotation was completed before the final observation of volume.[2]

At the time of each weighing, the density of the air in the balance case was determined by weighing a glass bulb, whose true mass had been determined at this Bureau. All weights were converted to weight in vacuo, the value for the density of solid α-lactose hydrate being taken as 1.540 in accordance with the determinations recorded below.

The experimental data are assembled in Tables 1 and 2. From these ob-

[2] The procedure used in making the density determinations was essentially that described by Jackson and Matthews in their work on levulose. *NBS Jour. Research*, 8, 405 (1932) R P 426.

TABLE 1.—*Density of lactose solutions at 20.0°C.*

WEIGHT OF LACTOSE[1] (AIR BRASS WTS.)	WEIGHT OF LACTOSE (VACUUM)	WEIGHT OF SOLUTION (VACUUM)	VOLUME OF SOLUTION	CONCENTRATION OF LACTOSE	D_4^{20} OBSERVED	D_4^{20} CALCULATED BY FORMULA[2]	DEVIATION FROM FORMULA
g	*g*	*g*	*ml*	*per cent*			
2.2413	2.2427	126.9997	126.381	1.766	1.00490	1.00487	+.00003
2.7596	2.7613	127.1586	126.344	2.172	1.00644	1.00641	+.00003
5.8969	5.9004	128.3891	126.405	4.596	1.01570	1.01569	+.00001
10.9203	10.9268	130.2569	126.404	8.389	1.03048	1.03050	−.00002
15.8408	15.8502	132.1234	126.444	11.996	1.04492	1.04493	−.00001
17.1718	17.1903	132.6147	126.442	12.963	1.04882	1.04885	−.00003
23.5294	23.5441	134.9147	126.398	17.451	1.06738	1.06738	0
24.0298	24.0450	135.1012	126.402	17.798	1.06884	1.06884	0
25.3294	25.3444	135.6730	126.493	18.681	1.07257	1.07255	+.00002

[1] α-lactose hydrate.
[2] $D_4^{20} = .99823 + .003739p + .00001281p^2$.

TABLE 2.—*Density of lactose solutions at 25.0°C.*

WEIGHT OF LACTOSE[1] (AIR BRASS WTS.)	WEIGHT OF LACTOSE (VACUUM)	WEIGHT OF SOLUTION (VACUUM)	VOLUME OF SOLUTION	CONCENTRATION OF LACTOSE	D_4^{25} OBSERVED	D_4^{25} CALCULATED BY FORMULA[2]	DEVIATION FROM FORMULA
g	*g*	*g*	*ml*	*per cent*			
1.0643	1.0650	126.4207	126.390	0.843	1.00024	1.00021	+.00003
1.8585	1.8596	126.7549	126.432	1.467	1.00255	1.00255	0
5.2778	5.2810	128.0341	126.436	4.125	1.01264	1.01262	+.00002
8.1789	8.2838	129.1437	126.470	6.337	1.02114	1.02113	+.00001
10.2912	10.2974	129.9499	126.496	7.924	1.02730	1.02732	−.00002
15.1632	15.1723	131.7102	126.457	11.519	1.04154	1.04157	−.00003
20.1185	20.1306	133.5434	126.463	15.074	1.05599	1.05598	+.00001

[1] α-lactose hydrate.
[2] $D_4^{25} = .99707 + .0037175p + .00001263p^2$.

served values the following equations were calculated by the method of least squares. Here the density of a lactose solution is expressed as a function of the percentage (p), of α-lactose present.

(1) $$D_4^{20} = .99823 + .003739p + .00001281p^2$$

(2) $$D_4^{25} = .99707 + .003717p + .00001263p^2$$

The deviation of the observed values from those calculated by formula are given in the last column of each table. The observed values show a mean deviation of less than 3 in the fifth place at 20°C., and less than 2 at 25°C., from those calculated by formula. We, therefore, believe that the formulas are valid to a few units in the fifth decimal.

TABLE 3.—*Density of lactose solutions at 20° and 25°C.*

α LACTOSE HYDRATE	D_4^{20} [1]	D_4^{25} [2]	α LACTOSE HYDRATE	D_4^{20} [3]
per cent				
0	.99823	.99707	20	1.0782
1	1.00198	1.00080	22	1.0869
2	1.00576	1.00456	24	1.0956
3	1.00956	1.00834	26	1.1045
4	1.01339	1.01214	28	1.1136
5	1.01725	1.01597	30	1.1227
6	1.02113	1.01983	32	1.1320
7	1.02503	1.02371	34	1.1414
8	1.02896	1.02762	36	1.1509
9	1.03292	1.03155	38	1.1605
10	1.03690	1.03551	40	1.1702
11	1.04091	1.03949	42	1.1801
12	1.04494	1.04350	44	1.1900
13	1.04900	1.04753	46	1.2001
14	1.05309	1.05159	48	1.2104
15	1.05720	1.05568	50	1.2207
16	1.06133	1.05978		
17	1.06550	1.06392		
18	1.06968			

[1] $D_4^{20} = .99823 + .003739p + .00001281p^2$.
[2] $D_4^{25} = .99707 + .0037175p + .00001263p^2$.
[3] $D_4^{20} = .9982 + .00370p + .0000150p^2$.

The values now given in the literature for the density of lactose solutions have been determined in connection with specific rotation studies. The work of Schmoeger (1), as well as that of Fleischman and Weigner (2), will be considered because these investigators extended their observations to supersaturated solutions. Schmoeger reported values for the density of lactose solutions containing as much as 36 per cent of the sugar. His

values are given to the fourth decimal place. Fleischman and Weigner obtained data on solutions containing as much as 69 per cent of lactose. The latter authors report that great difficulty was encountered in preparing solutions of the higher concentrations and hence only few observations were made. We believe that the inclusion of density values on supersaturated solutions, although they cannot be obtained with great precision, may be of value to investigators working with this sugar. Therefore, we have applied the method of least squares to the data presented by these early investigators on supersaturated solutions for concentrations as high as 50 per cent and obtained the following equation relating the density to the percentage concentration.

$$D_4^{20} = .9982 + .00370p + .0000150p^2 \tag{3}$$

Tables 3 and 4 are working tables containing density values for unsaturated solutions and for saturated solutions of concentrations up to 50 per cent. The values for unsaturated solutions are based on equations (1) and (2) while those for the supersaturated solutions were calculated by use of equation (3).

TABLE 4.—*Density of lactose solutions at 20°C.*

ANHYDROUS LACTOSE	D_4^{20}	ANHYDROUS LACTOSE	D_4^{20}
per cent		*per cent*	
1	1.00218	18	1.07367
2	1.00616	20	1.0827
3	1.01016	22	1.0919
4	1.01420	24	1.1012
5	1.01824	26	1.1107
6	1.02236	28	1.1203
7	1.02647	30	1.1300
8	1.03062	32	1.1399
9	1.03480	34	1.1498
10	1.03901	36	1.1600
11	1.04324	38	1.1702
12	1.04750	40	1.1806
13	1.05179	42	1.1911
14	1.05611	44	1.2017
15	1.06046	46	1.2125
16	1.06484	48	1.2234
17	1.06924	50	1.2345

2. *DENSITY OF CRYSTALLINE LACTOSE*

The density of the crystalline α-lactose hydrate[3] was required in order to convert the weight in air to weight in vacuum.

[3] Previous values [3] Lichtenstein 1.543, Boedeker 1.5384, Filhol 1.534, Joule and Playfair 1.530, and Pionchon 1.525 [4] Hudson and Brown 1.54 (Sp. gr. α-lactose hydrate), 1.59 (sp. gr. β-lactose).

TABLE 5.—*Density of crystalline lactose*

WEIGHT PURE TOLUENE (VACUUM)	VOLUME TOLUENE	D_4^{20}[1] TOLUENE	WEIGHT α-LACTOSE HYDRATE (VACUUM)	WEIGHT TOLUENE (VACUUM)	TOTAL VOLUME	VOLUME TOLUENE	VOLUME α-LACTOSE HYDRATE	D_4^{20} α-LACTOSE HYDRATE
g	*ml*		*g*	*g*	*ml*	*ml*	*ml*	
86.6441	100.2355	0.86441	19.1054	98.5649	126.4265	114.0243	12.4022	1.5405
86.5196	100.0915	0.86441	19.1054	98.6121	126.4838	114.0789	12.4049	1.5401
86.6413	100.2285	0.86444	19.1054	98.5252	126.3883	113.9784	12.4099	1.5395
86.5947	100.1765	0.86442	20.0613	97.9627	126.3597	113.3277	13.0320	1.5394
86.5650	100.1425	0.86442						
	average	0.86442						
86.6176	100.1685	0.86472	10.2736	103.5757	126.4532	119.7836	6.6696	1.5404
86.6241	100.1805	0.86468	10.2736	103.5025	126.3692	119.6989	6.6703	1.5402
86.5739	100.1235	0.86467						
	average	0.86469					average	1.5400
			WEIGHT β-LACTOSE (VACUUM)				VOLUME β-LACTOSE	D_4^{20} β-LACTOSE
			g				ml	
		0.86469	22.8752	96.8837	126.4704	112.0794	14.3910	1.5895
		0.86469	22.8752	96.7750	126.3501	111.9537	14.3964	1.5890
		0.86469	22.8752	96.8459	126.4265	112.0357	14.3908	1.5896
							average	1.5893

[1] Samples of toluene from two different lots were used for these experiments. The density of toluene recorded in the determinations on β-lactose is the average value for the second lot of reagent.

The lactose was weighed in a calibrated flask, and the flask was filled with dry toluene saturated with lactose at 20°C. The trapped air was removed from the crystals by applying gentle suction while rotating the flask. All weights were converted to weight in vacuo. The results of these measurements are given in Table 5. The density of lactose hydrate was found to be 1.540. The density of commercial β-lactose, an anhydrous form that has found extensive commercial use because of its greater solubility, was found to be 1.589.

3. *REFRACTIVE INDICES*

The refractive indices of unsaturated solutions of lactose at 20°C. and 25°C. were determined by use of a carefully calibrated immersion refractometer under accurately controlled conditions of temperature. All measurements were made in a constant temperature room whose temperature was maintained within two degrees of that at which the water in the bath surrounding the instrument was held.

The instrument was inserted in a large glass tube, 83 mm in diameter and 350 mm in length. This contained sufficient water to surround the instrument cup containing the sugar solution. The glass tube with the suspended instrument was placed in a constant temperature water bath so that only a small part of it extended above the surface of the water. A thermometer was placed beside the instrument. Illumination was supplied by an electric bulb submerged in a glass tube similar to that containing the refractometer. The floor of the bath reflected the light into the instrument. Under these conditions a very sharp line characteristic of total refraction was observed, permitting a precision, and an estimated accuracy, of 0.1 to 0.2 of a scale division in the reading of the instrument.

The conversion tables supplied by the manufacturer are based on readings for distilled water. In accord with these tables the instrument was set at 14.50 at 20°C. and at 13.25 at 25°C. These readings are equivalent to indices of 1.33299 at 20°C. and 1.33252 at 25°C. Readings on distilled water were made frequently, thus insuring the setting of the instrument. The arbitrary scale readings were converted to refractive indices by use of the manufacturer's tables.

Equations relating the refractive index to the concentration were prepared by subjecting the observed data to the method of least squares.

$$(4) \qquad n_D^{20} = 1.33299 + .001409p + .00000498p^2$$

$$(5) \qquad n_D^{25} = 1.33251 + .001405p + .000004805p^2$$

Tables 6 and 7 contain the observed data as well as those calculated by formula.

The refractive indices of supersaturated solutions at 25°C., as well as those of all concentrations at 15°C., were determined with an Abbé refractometer. The scale of the instrument used permitted readings to a few

TABLE 6.—*Refractive indices of lactose solutions at 20.0°C.*

WEIGHT OF LACTOSE[1] (AIR BRASS WTS.)	WEIGHT OF LACTOSE (VACUUM)	WEIGHT OF SOLUTION (VACUUM)	CONCENTRATION OF LACTOSE	ZEISS IMMERSION SCALE READING t=20.0°C.	n_D^{20} OBSERVED	n_D^{20} CALCULATED BY FORMULA[2]	DEVIATION FROM FORMULA
g	*g*	*g*	*per cent*				
2.2413	2.2427	126.9997	1.766	21.00	1.33551	1.33549	+.00002
3.3207	3.3227	127.3975	2.608	24.12	1.33671	1.33669	+.00002
5.2365	5.2397	101.7278	5.151	33.74	1.34038	1.34038	0
7.3119	7.3162	102.5546	7.134	41.47	1.34330	1.34329	+.00001
15.1730	15.1819	131.8051	11.518	59.10	1.34988	1.34988	0
15.2437	15.2525	131.8654	11.567	59.30	1.34995	1.34996	−.00001
22.2235	22.2367	134.4964	16.533	80.41	1.35765	1.35764	+.00001

[1] α-lactose hydrate.
[2] $n_D^{20} = 1.33299 + .001409p + .00000498p^2$.

TABLE 7.—*Refractive indices of lactose solutions at 25.0°C.*

WEIGHT OF LACTOSE[1] (AIR BRASS WTS.)	WEIGHT OF LACTOSE (VACUUM)	WEIGHT OF SOLUTION (VACUUM)	CONCENTRATION OF LACTOSE	ZEISS IMMERSION SCALE READING to 25.0°C.	n_D^{25} OBSERVED	n_D^{25} CALCULATED BY FORMULA[2]	DEVIATION FROM FORMULA
g	*g*	*g*	*per cent*				
1.0643	1.0650	126.4207	0.843	16.32	1.33371	1.33370	+.00001
2.5473	2.5498	127.0832	2.006	20.54	1.33534	1.33535	−.00001
5.2778	5.2810	128.0341	4.125	28.54	1.33841	1.33839	+.00002
8.1789	8.1838	129.1437	6.337	36.90	1.34158	1.34160	−.00002
10.2912	10.2974	129.9499	7.924	43.20	1.34396	1.34395	+.00001
15.1632	15.1723	131.7102	11.519	57.63	1.34933	1.34933	0
20.1185	20.1306	133.5434	15.074	72.46	1.35478	1.35478	0

[1] α-lactose hydrate.
[2] $n_D^{25} = 1.33251 + .001405p + .000004805p^2$.

TABLE 8.—*Refractive indices of lactose solutions at 25°C.*

WEIGHT OF LACTOSE[1] (AIR BRASS WTS.)	WEIGHT OF LACTOSE (VACUUM)	WEIGHT OF SOLUTION (VACUUM)	CONCENTRATION OF LACTOSE	n_D^{25} OBSERVED ABBÉ	n_D^{25} CALCULATED BY FORMULA[2]	DEVIATION FROM FORMULA
g	*g*	*g*	*per cent*			
10.9856	10.9923	53.7058	20.468	1.3634	1.3634	.0000
14.0010	14.0095	54.9307	25.504	1.3720	1.3719	+.0001
17.1285	17.1389	56.6435	30.257	1.3800	1.3800	.0000
20.2416	20.3139	56.8220	35.750	1.3899	1.3900	−.0001

[1] α-lactose hydrate.
[2] $n_D^{25} = 1.3325 + .001384p + .00000624p^2$.

TABLE 9.—*Refractive indices of lactose solutions at 15°C.*

WEIGHT OF LACTOSE[1] (AIR BRASS WTS.)	WEIGHT OF LACTOSE (VACUUM)	WEIGHT OF SOLUTION (VACUUM)	CONCENTRATION OF LACTOSE	n_D^{15} OBSERVED ABBÉ	n_D^{1} CALCULATED BY FORMULA[2]	DEVIATION FROM FORMULA
g	*g*	*g*	*per cent*			
2.6839	2.6855	50.7500	5.292	1.3410	1.3410	.0000
13.1331	13.1412	131.1152	10.023	1.3480	1.3480	.0000
26.3086	26.3248	136.0419	19.351	1.3629	1.3627	+.0002
33.1227	33.1431	138.4840	23.933	1.3701	1.3703	−.0002
39.6613	39.6857	141.0027	28.145	1.3772	1.3774	−.0002
52.8430	52.8755	145.7118	36.288	1.3919	1.3917	+.0002

[1] α-lactose hydrate.
[2] $n_D^{15} = 1.3334 + .001412p + .00000537p^2$.

units in the fourth decimal place. This was the limiting factor in the precision of the readings since they were all made under carefully controlled conditions. Equations 6 and 7 are based upon these data.

$$(6) \qquad n_D^{25} = 1.3325 + .001384p + .00000624p^2$$

$$(7) \qquad n_D^{15} = 1.3334 + .001412p + .00000537p^2.$$

The observed data for indices of refraction, and also the values calculated by the formulas, are given in Tables 8 and 9. Table 10 is included as a working table. It has been prepared by use of the proper formula for each temperature.

TABLE 10.—*Refractive indices of lactose solutions*

LACTOSE HYDRATE	n_D^{25}	n_D^{20}	n_D^{15}	$\Delta n/\Delta t$
per cent				
1	1.33392	1.33440	1.3348	.000096
2	1.33534	1.33583	1.3362	.000098
3	1.33677	1.33726	1.3376	.000098
4	1.33821	1.33871	1.3391	.000100
5	1.33966	1.34016	1.3406	.000100
6	1.34111	1.34162	1.3421	.000102
7	1.34258	1.34310	1.3436	.000104
8	1.34406	1.34458	1.3451	.000104
9	1.34554	1.34607	1.3466	.000106
10	1.34704	1.34758	1.3481	.000108
11	1.34855	1.34909	1.3496	.000108
12	1.35006	1.35062	1.3511	.000112
13	1.35159	1.35215	1.3526	.000112
14	1.35312	1.35369	1.3542	.000116
15	1.35467	1.35524	1.3558	.000116
16	1.35622	1.35681	1.3573	.000118
18	1.3594		1.3605	.00011
20	1.3627		1.3637	.00010
22	1.3660		1.3670	.00010
24	1.3693		1.3703	.00010
26	1.3727		1.3737	.00010
28	1.3762		1.3770	.00008
30	1.3797		1.3805	.00008
32	1.3832		1.3839	.00007
34	1.3868		1.3875	.00007
36	1.3904		1.3910	.00006

IV. SUMMARY

Density and refractive index values are reported for lactose solutions over the range of unsaturation with a precision of 3 or 4 in the fifth decimal place at 20°C. and at 25°C. For solutions of higher concentrations, up to 50

per cent, the corresponding values are reported at 25°C., and refractive indices alone at 15° C.

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BOOK REVIEW

Dynamic Aspects of Biochemistry. By ERNEST BALDWIN. xviii+457 pages. The Macmillan Co., 60 Fifth Ave., New York, N. Y., 1947. Price $4.00.

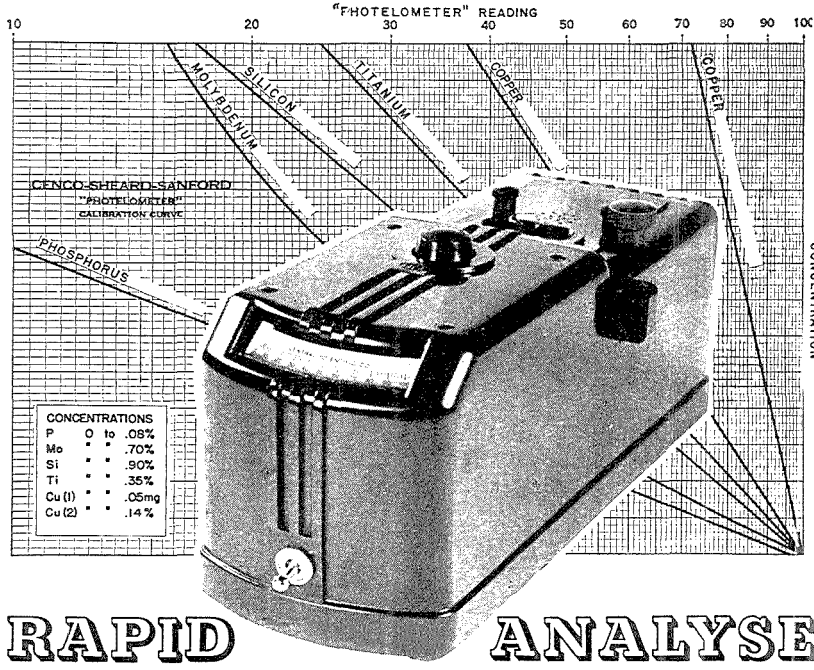
The chemical reactions accompanying biological processes are rapidly becoming better understood; the individual steps which make up the mechanism of each are being discovered at an increasing pace; the chemical agents so characteristic and necessary for these life processes are assigned the name enzymes. In this book, "Dynamic Aspects of Biochemistry," some of the various threads, which, when woven together make up a living organism, are systematized to the extent that present knowledge allows.

This book is designed to cover a phase of biochemistry in a form suitable for textbook use in an advanced college course. The author is an experienced teacher and has used some innovations, particularly in writing equations, which give "a distinctly pictorial representation of chemical events." The book is divided into two parts: Part I. Enzymes; Part II. Metabolism. In Part I, after an introduction on "The General Behaviour and Properties of Enzymes" and on "The Nature of the Catalytic Process," some individual enzymes are discussed briefly by types as follows: (1) Hydrolases and Phosphorylases: Included in these are proteases, carbohydrases, lipases, and esterases. (2) Oxidizing Enzymes: Among these are certain well known oxidases, including catalases, the cytochrome oxidases, and those enzymes which oxidize compounds of the phenolic type. The dehydrogenases are also classified as oxidizing enzymes. (3) Other Enzymes: Adding, Transferring, and Isomerizing Enzymes. The individual enzymes are discussed briefly along with the fundamental reactions which each catalyzes.

In Part II, after a general discussion of "Methods Employed in the Investigation of Intermediary Metabolism" and "Food, Digestion, and Absorption," the metabolism of nitrogenous compounds, especially the proteins and amino acids, is described at length. Then follows a similar treatment of carbohydrates including both the aerobic and anaerobic systems. Finally the enzymatic transformations concerned with the metabolism of fats are discussed.

At the time when many regulatory chemists were students, so little was understood about the intricate and still complex mechanisms of food metabolism and of biological reactions in general, that the subject, if mentioned at all, was beclouded with mystery. To one who reads this book, much of this mystery is resolved into a series of definite chemical reactions in which both inorganic and organic chemistry play their parts. The problems of life processes are so complex that only a beginning toward their complete elucidation is made in this book. However, as in so many other cases, once the foundations are laid and the plan becomes more legible, then progress on the building as a whole becomes much more rapid. For those who would like to know more about the chemical dynamics of life processes this book can be recommended. It is particularly free of errors, and the style is much more readily understandable than one might expect in such a complex field.

W. I. PATTERSON



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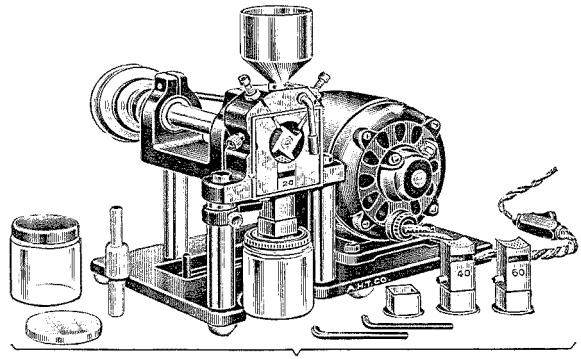

4276-M.

WILEY LABORATORY MILL, Intermediate Model. A rugged, compact, motor-driven mill, as suggested by the U. S. Bureau of Plant Industry for the rapid milling of homogeneous samples of desiccated plant tissue but since found suitable for a great variety of commercial materials, including roots, seed, grain, dried animal products, etc.

With polished chamber 40 mm diameter × 22 mm deep, two stationary blades and rotor with four cutting edges which revolve at high speed to produce a shearing action which prevents loss of moisture from heat. Will prepare a 20 gram, 40 mesh sample of ½-inch pieces of soybean stems in 5 to 7 minutes.

4276-M. Wiley Laboratory Mill, Intermediate Model, as above described, with hopper, three delivery tubes with sieve tops of 20, 40 and 60 mesh, respectively, each with receiver for collecting small samples, and other accessories as shown in illustration. With ½ h.p. continuous duty motor for 115 volts, 60 cycles, a.c. $213.50

4276-M2. Ditto, but with motor for 115 volts, 50 to 60 cycles, a.c. $215.60
4276-N. Ditto, but with motor for 115 volts, d.c. $231.20
4276-P. Ditto, but with motor for 220 volts, 60 cycles, a.c. $217.65

Copy of Sup. 110, giving more detailed description of above and other models of the Wiley Mill, sent upon request.

ARTHUR H. THOMAS COMPANY

RETAIL—WHOLESALE—EXPORT

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