FIRST DAY

MONDAY—MORNING SESSION

REPORT ON GUMS IN FOOD

A SPECTROPHOTOMETRIC METHOD FOR THE DETECTION OF CERTAIN STABILIZERS IN SOFT CURD CHEESES

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

Smith and Rogers[1] and Mathers and Beck[2] have used the absorbance of furfuraldehyde at 277.5 mμ to determine pentosans. Since the products of hydrolysis of gums include sugars which may be converted to furfuraldehyde, it was decided to utilize the absorbance of furfuraldehyde in the detection of added gums in cheese. Preliminary tests showed that if locust bean, arabic, and tragacanth gums were hydrolyzed and the hydrolyzed material distilled, the distillates showed the typical ultraviolet absorption curve of furfuraldehyde. Similar absorption curves for karaya showed maximum absorbance at 280 mμ.

Accordingly, a number of samples of soft curd cheese of countrywide origin, reported to be free from added stabilizer, were tested. They were first carried through the procedure to determine the absorbance at 277.5 mμ. Then definite amounts of various stabilizing agents were added to see if the stabilizing agent could be detected by the ultraviolet absorbance of furfuraldehyde. The following method was finally evolved:

METHOD

REAGENTS

(a) *Potassium aluminum sulfate solution.*—5% w/v.

(b) *Alcohol.*—80%.

(c) *Hydrochloric acid.*—12% by weight. To 1 vol. of HCl add 2 vols. of H_2O. Titrate against std alkali and adjust to proper concn.

APPARATUS

(a) *500 ml round-bottom Pyrex distilling flask.*—With 2 vertical short necks, one at center and one at side, both with std taper joints.

(b) *Steam generator.*—All glass, consisting of 2 l round-bottom Pyrex flask with std taper joints, equipped with safety tube and delivery tube connecting with (c) by means of a spherical joint.

(c) *Steam inlet tube.*—To fit center neck of flask (a) and extending to ¼" of bottom of flask. Lower end terminates in bulb with several small holes.

---

[1] *Anal. Chem.*, 25, 931 (1953).
[2] *This Journal*, 37, 861 (1954).

SEPARATION OF GUMS

Transfer 10 g cheese to a 250 ml Pyrex centrifuge bottle with a few ml H_2O. Add warm H_2O (ca 40°C.) to make total vol. of 100 ml. While swirling, add 3 ml NH_4OH, stopper bottle, and shake until curd is dissolved. Then add 4.5 ml glacial acetic acid, a little at a time, shaking the bottle after each addn. Shake well; then heat bottle in boiling H_2O in beaker for 15 min. (With a normal cheese these amounts of ammonia and acetic acid should produce a *p*H of ca 4.75 and there should appear a marked sepn of casein in the bottle.)

While bottle is still hot, centrifuge at 1200 rpm for ca 15 min. Decant supernatant liquid thru a funnel with loose pledget of cotton in stem into another 250 ml Pyrex centrifuge bottle. Lay first bottle on side for a few min. and then drain remaining few drops of liquid into funnel. When filtration has ceased, pull up pledget of cotton and squeeze out any liquid by pressing cotton against side of funnel. Check *p*H of filtrate, preferably with a *p*H meter. Adjust to ca *p*H 4.75 with acetic acid, if necessary. Heat bottle in boiling H_2O for 15 min., centrifuge at 1200 rpm, and decant thru pledget of cotton into a 600 ml beaker. Drain bottle as before and squeeze out cotton. Add 500 ml 95% alcohol, while stirring, to the filtrate in beaker; then add 4 or 5 drops of the 5% potassium aluminum sulfate soln (reagent **a**) and let stand 4 hrs or overnight. If a ppt does not form soon, or if supernatant liquid is milky, add more reagent (**a**), stir, and let settle.

Decant as much as possible of the supernatant liquid, transfer remaining contents of beaker to a centrifuge bottle, and centrifuge at 2000 rpm. Decant supernatant liquid and drain by inverting bottle. Add 50 ml 80% alcohol (reagent (**b**)), 1 drop acetic acid, and 3–4 drops reagent (**a**) to bottle; stopper; and shake to disperse ppt. Use shaking machine if necessary. Centrifuge at 2000 rpm, decant supernatant liquid, and drain. Again add 50 ml 80% alcohol, 1 drop acetic acid, and 3–4 drops reagent (**a**). Stopper bottle and shake as before. Centrifuge again, decant off alcohol, and drain bottle by inverting it.

(If at any time supernatant liquid cannot be decanted without pouring off some of the ppt, shake contents of bottle and centrifuge again for a longer time.)

Add 40 ml hot H_2O (ca 80°C.) and 3 drops NH_4OH to alcohol ppt in bottle, stopper, and shake until ppt dissolves. Add ca 7 drops acetic acid and shake well. Then add 200 ml 95% alcohol and 3–4 drops reagent (**a**), and mix thoroly. Allow to stand until ppt settles out; then centrifuge at 2000 rpm. (If supernatant liquid is milky, add more reagent (**a**), shake, and let settle.) Decant supernatant liquid and wash twice with 50 ml 80% alcohol by means of the centrifuge, adding 1 drop acetic acid and 3–4 drops reagent (**a**) to the first wash but only 3–4 drops reagent (**a**) to the second wash.

DETECTION OF GUM

Drain alcohol ppt thoroly by inverting bottle; then dry in air oven at 80°C. Cool bottle, and pulverize dried ppt with a stirring rod. Transfer the finely pulverized material by means of a funnel to the 500 ml round-bottom, 2-neck flask. With 90 ml of the 12% HCl, used portion-wise, transfer remaining portion of dried material adhering to centrifuge bottle. Use policeman and shake bottle to facilitate transfer. Rinse funnel with 10 ml H_2O and collect rinsings in centrifuge bottle, shake bottle, and reserve rinsings.

Connect the 2-neck flask to a Friedrichs condenser and set flask in a hot-cone electric heater connected to a variable transformer. Stopper outside neck of flask with glass stopper, bring contents of flask to boiling, and reflux 15 min. Remove flask from heater, allow to cool, remove condenser, and rinse it with the 10 ml rinse water reserved in the centrifuge bottle by rotating the condenser back and forth on its side. Drain rinse water into flask.

Connect the 2-neck flask to the steam generator and to a condenser. Allow steam to condense in the flask until the boiling point is reached; then keep vol. of liquid constant by means of a small flame. Collect the first 100 ml of distillate in a 100 ml vol. flask. The rate of distn in the apparatus should be regulated ahead of time so that 100 ml will distil over in 30 ± 2 min.

Mix the contents of the 100 ml flask, and read the absorbance in a 1 cm quartz cell in a spectrophotometer at 277.5 mμ against a blank of H_2O which has been distd thru the apparatus. An absorbance in excess of 0.25 indicates the presence of gum tragacanth, gum arabic, gum karaya, pectin, algin, propylene glycol alginate, or perhaps a gum not commonly used. This method does not detect the presence of carob bean gum, guar gum, Irish moss, carboxymethylcellulose, or gelatin.

NOTES

1. The small sample simplifies operations and ensures better separation of protein and gum.

2. *p*H control with a *p*H meter and double precipitation in the boiling water bath reduce the absorbance blank appreciably.

3. Filtering at the end of each pptn thru cotton ensures removal of the fat from the 10 g sample without the special technique called for in the A.O.A.C. method.

4. The dried gum should be finely pulverized before the hydrolysis with 12% HCl is started.

5. The rate of distn is critical. Distns at a slow rate tend to increase the recovery of furfuraldehyde. A distn rate of 30 ± 2 min. is specified in the method.

RESULTS AND DISCUSSION

Fourteen samples of cottage cheese, reported to be free of stabilizers, were obtained from six widely separated states. When carried through the above procedure, these cheeses gave maximum blank absorbances ranging from 0.100 to 0.252 (av. = 0.178) in the range 277.5–282.5 mμ. In several duplicate determinations, absorbances varied as follows: 0.129 *vs.* 0.181; 0.134 *vs.* 0.190; 0.167 *vs.* 0.194; 0.150 *vs.* 0.228; 0.116 *vs.* 0.180; and 0.242 *vs.* 0.252.

Cheese from two samples showing blank absorbances of 0.100 and 0.116–0.180 was used as a base to which known increments of various gums were added. Results by the above procedure are summarized in Table 1. As is shown, pectin, gum tragacanth, gum arabic, and propylene glycol alginate are easily detected at a concentration as low as 0.05 per cent; algin and karaya at a concentration as low as 0.10 per cent. The results appear semiquantitative for all of these except karaya, where there is a falling off in recovery. Karaya is an insoluble gum and the drop in recovery is undoubtedly due to the fact that some of it is centrifuged off with the protein. It is seen that locust bean, guar gum, Irish moss, carboxymethylcellulose, and gelatin are not detected at the concentrations studied.

It may be of interest to note that maximum absorbance readings of the distillates of cheeses treated with pectin, algin, tragacanth, propylene glycol alginate, and locust bean consistently occur at 277.5 mμ, while those for karaya occur at 280 mμ.

TABLE 1.—*Relation between added gum and absorbance at 277.5 mμ*

GUM	PER CENT ADDED GUM					
	0.05	0.10	0.15	0.20	0.25	0.30
Locust bean	0.199	0.159	0.205	0.302	0.245	0.252
Karaya	0.225	0.335	0.426	0.466	0.480	0.497
Tragacanth	0.620	1.03	1.51	1.85	—	—
Arabic	0.380	0.648	0.961	1.26	1.63	1.85
Guar	0.195	0.181	0.195	0.168	—	0.168
Irish moss	0.141	0.212	0.215	0.224	—	0.257
Pectin	0.628	1.00	1.56	2.10 (*est.*)	—	—
Propylene glycol alginate	0.353	0.695	0.940	1.19	1.59	1.83
Carboxymethylcellulose	0.152	0.121	0.185	0.117	—	0.152
Algin	0.231	0.359	0.488	0.673	0.799	0.910
Gelatin	0.153	0.100	—	0.141	—	0.118

This year the Associate Referee on gums in frozen desserts submitted a method for collaborative study. Locust bean and gum tragacanth were determined satisfactorily. However, his collaborators had trouble in detecting gum karaya and carboxymethylcellulose, and he intends to study the method further.

The Associate Referee on gums in cheese spreads has developed a method which uses dioxane as a solvent for fat and moisture, and formic acid-alcohol solution as a solvent for protein. He recommends that it be submitted to collaborative study.

No formal report was received from the Associate Referee on gums in catsup and related products, or from the Associate Referee on gums in dressings for foods.

To date no Associate Referee on gums in cacao products has been appointed.

RECOMMENDATIONS

It is recommended*—

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

(2) That studies on the detection of alginates and other gums in cheese and cheese spreads be continued.

(3) That study on the methods for detection of gums and alginates in chocolate milk be extended to other dairy drinks and fountain materials, and that an Associate Referee be appointed for this purpose.

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

* For report of Subcommittee C and action of the Association, see *This Journal*, 38, 75 (1955).

(5) That work be continued on the detection of alginates and other stabilizing agents in dressings for food and that a new Associate Referee be appointed.

(6) That the spectrophotometric method for the detection of stabilizers in soft curd cheeses proposed by the Referee be submitted to collaborative study.

(7) That the official method for gums in soft curd cheese be clarified by adding below the title the following in parentheses: "(Not applicable to the detection of alginates)."

REPORT ON GUMS IN CHEESE SPREADS

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

An attempt was made to shorten and simplify the procedure for gums in cheese spreads. The use of dioxane as a dual solvent for fat and moisture was investigated and was found to offer promise in de-fatting cheese spreads without causing troublesome emulsions. The use of formic acid-alcohol solutions as a protein solvent was also investigated and was found to materially simplify the isolation of a gum from the non-fat ingredients permitted in the standard. A tentative procedure involving the use of these reagents has been prepared and has worked acceptably in the hands of the Associate Referee.

It is recommended* that this method be investigated further and subjected to collaborative study.

No reports were received on catsup and related tomato products, or dressings for foods.

REPORT ON EGGS AND EGG PRODUCTS

By F. J. McNall (Food and Drug Administration, Department of Health, Education, and Welfare, Cincinnati 2, Ohio), *Referee*

AMMONIA NITROGEN

Subcommittee C recommended that work on the determination of ammonia nitrogen in eggs be continued along the lines indicated by the

* For report of Subcommittee C and action of the Association, see *This Journal*, 38, 75 (1955).

Associate Referee, *This Journal*, **34**, 346 (1951). No report has been received from the Associate Referee on this subject for several years.

LIPOIDS AND LIPOID PHOSPHORIC ACID

The method for lipoids and lipoid phosphorus, **16.10–16.12**, was made first action in 1950. This method has been in use for many years and as far as we know has never been criticized adversely. It is recommended* that the method be adopted as official.

REPORT ON FISH AND OTHER MARINE PRODUCTS

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

In recent years considerable work has been done on methods for the determination of total solids in fish and related marine products, particularly oysters. Certain methods used in the preparation of samples were also studied critically. As a consequence, it appeared that a number of changes should be made in Chapter 18 for the forthcoming (1955) revision of *Official Methods of Analysis*. These changes were suggested to the Editorial Board and since the changes were quite numerous the General Chairman decided that it might be advisable to prepare an entirely new draft of these methods. With the assistance of Associate Referee Henry M. Risley the Referee therefore drafted a revision of the first portion of Chapter 18. This revision was submitted to a number of regulatory chemists for critical comment and several very valuable suggestions were received. On the basis of these combined efforts the Referee has prepared revisions of methods **18.1–18.4** which will appear in the 8th edition of *Official Methods of Analysis*.

Changes were made only after considering a number of factors. In certain instances investigative work and collaborative studies were made by the Referee and associates. New techniques and new equipment necessitated other changes. Some methods have been rewritten for greater clarity. In order to facilitate review of the new draft and changes which have been incorporated, comments and references are listed below.

COMMENTS ON PROPOSED REVISION

18.1 PRELIMINARY TREATMENT AND PREPARATION OF SAMPLE

(a) *Fresh fish.*—This subsection has been rewritten for greater clarity and some of the directions have been made more general. The comminuting of samples by means of equipment such as the Waring blendor has also been given new status. Although the Waring blendor cannot be used on absolutely all types of products,

* For report of Subcommittee C and action of the Association, see *This Journal*, **38**, 74 (1955).

the experience of most analysts has been that with some manipulation a surprisingly large number of samples can be handled by the use of this type of equipment.

(b) *Canned fish and other canned marine products.*—The heading of this subsection has been made more inclusive.

(c) *Canned marine products packed in oil.*—The heading of this subsection has been made more inclusive.

(d) *Fish packed in salt or brine.*—This subsection has been moved from the end of section 18.1 in order to place it next to the other fish subsections.

(e) *Dried smoked or dried salt fish.*—This subsection was also moved forward ahead of the shellfish subsection.

(f) *Shellfish other than oysters, clams, and scallops.*—Only minor changes were made in the wording of this subsection.

(g) *Shell oysters, shell clams, and scallops.*—Only minor changes were made in the wording of this subsection.

(h) *Shucked oysters, clams, or scallops.*—The greatest changes were made in this subsection. In the first place it was believed that the first paragraph under this subsection did not belong under "*Preparation of Sample*" but was in fact a volume determination. Consequently, with minor changes in wording, this entire paragraph was moved out of this section and placed into a new section numbered **18.2,** *Volume Determination.* That portion of the procedure in the Seventh Edition which directs that the liquid be separated from the oysters and analyzed separately in cases where the drained liquid is more than 10% has been entirely deleted. The use of the blendor in the preparation of oyster samples has made this portion of the procedure obsolete (1, 2). The last paragraph of this subsection with directions for preparing oyster samples has been deleted as obsolete for the same reason. Since collaborative work has been done on oysters these shellfish were distinguished from clams or scallops.

18.2 VOLUME DETERMINATION

(Shucked Oysters, Clams, or Scallops)

As mentioned previously, the volume determination of shucked oysters, clams, or scallops was given a separate section. Since raw oysters are now often marketed in containers smaller than 1 pint, the use of calibrated glass cylinders was authorized.

DRAINED LIQUID

(Shucked Oysters)

18.3 APPARATUS

The wording of the directions in portions of this section was changed to conform with the wording given in the standards for oysters (3). After considerable deliberation it was decided to change the wording specifying the perforations of the skimmer. Under the present directions which specify holes "at least ¼ inch in diameter and not more than 1¼ inch apart," it would be possible to have holes an inch in diameter. The size of the holes and distance between them has consequently been limited.

18.4 DETERMINATION

An addition was made to this section and a temperature at which the determination is to be made has been prescribed. It is highly desirable that the determination be made at a uniform temperature. Baltimore District of the Food and Drug Administration has done a great deal of work on oysters (4). The present work and recent oyster work at most Districts has been done at the suggested temperature and it is believed that this temperature requirement should be included in the directions.

TOTAL SOLIDS—FIRST ACTION

18.5 (All Marine Products Except Raw Oysters)

Collaborative work in recent years and studies just completed by the Associate Referee have shown that this method is applicable to all marine products. The use of this method on raw oysters has not been sanctioned since a specific method is available for oysters.

18.6 (Raw Oysters Only)

This section has been designated as applicable to oysters only since clams and scallops are better done by method **18.5.** The principal reason for leaving this section in *Official Methods of Analysis* is to make it available for those laboratories which do not have forced draft ovens.

18.7 Alternate Forced Draft Oven Method for Raw Oysters

This method is the result of extensive work by Associate Referee Traynor on the determination of solids in oysters (5).

OTHER WORK

During the past year the Associate Referee on total solids and ether extract in fish and other marine products continued his study of methods for the determination of total solids. Subcommittee C recommended (*a*) That the forced draft air oven method as adopted for oysters be studied for other molluscs, such as scallops, clams, and mussels, and for crustaceans, such as lobsters and crabs; (*b*) That the forced draft air oven method as adopted for fish be studied for crustaceans, such as lobsters and crabs.

The Associate Referee made these studies and submitted a report of the results. They showed that the general method for total solids in fish and other marine products as now written is satisfactory. As was suspected, the oyster method is not applicable to most other marine products.

Subcommittee C also recommended that collaborative work on the rapid method for ether extract in canned fish, described by the Associate Referee, be extended to fish other than salmon. Only a limited amount of work was done by the Associate Referee on this method. However, it was definitely determined that the method is not applicable to other than canned fish.

Due to the press of regulatory activities the Associate Referee on total solids in oysters was unable this year to continue his investigation of methods for total solids in oysters.

RECOMMENDATIONS

It is recommended*—

(1) That the first portion of Chapter 18, beginning with **18.1,** "Preliminary Treatment and Preparation of Sample," and continuing to but

* For report of Subcommittee C and action of the Association, see *This Journal,* 38, 74, 75 (1955).

not including section **18.5** (*Official Methods of Analysis*, 7th Ed.) be revised as described in this report.

(2) That collaborative work on the method for total solids in fish and other marine products except raw oysters be continued next year with the view that it may be adopted as official.

(3) That attempts to apply the forced draft air oven method for oysters to other marine products be discontinued.

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

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

REFERENCES

(1) PRICE, DOUGLAS D., *This Journal*, **35**, 218 (1952).
(2) ———, and TRAYNOR, JOHN P., *ibid.*, **36**, 608 (1953).
(3) *S.R.A., F.D.C. No. 2*, **36.10(c)(2)**.
(4) DAUGHTERS, G. T., and HOSHALL, E. M., *This Journal*, **36**, 947 (1953).
(5) TRAYNOR, JOHN P., *ibid.*, **37**, 607 (1954).

REPORT ON TOTAL SOLIDS AND ETHER EXTRACT IN FISH AND OTHER MARINE PRODUCTS

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

TOTAL SOLIDS

A method for the determination of total solids in fish and other marine products (except raw oysters) has been adopted as first action (*This Journal*, **37**, 70, 602 (1954)). During the past year this method was applied to crustaceans such as canned lobsters and canned crabs, as recommended by Subcommittee C; to molluscs such as raw scallops, raw clams, and canned mussels, and to canned fish such as tuna and sardines. Drying times of 1.5 and 2 hours in the forced draft oven were compared with the 1 hour time specified in the method to determine if additional drying time was necessary. The method gave good results with these additional types of fish products (Table 1). The Associate Referee believes that with further collaborative work on different fish products the method can be adopted as official. The alternate use of the forced draft oven, when available, will save considerable time over the air oven. No need is seen for increasing the drying time beyond 1 hour.

A method for determining total solids in raw oysters in a forced draft oven has been adopted as first action (*This Journal*, **37**, 70, 607 (1954)). During the past year this method was studied for other fish products.

TABLE 1.—*Per cent total solids in various fish products; comparison of methods*

PRODUCT	METHOD FOR FISH PRODUCTS				METHOD FOR RAW OYSTERS		
	FORCED DRAFT OVEN			AIR OVEN	FORCED DRAFT OVEN		
	1 HR	1.5 HRS	2 HRS	4 HRS	1.5 HRS	2 HRS	2.5 HRS
Frozen Japanese scallops	15.12	15.09	—	15.07	15.32	15.18	15.14
	15.09	15.06	—	15.05	15.35	15.19	15.14
Same as above, re-frozen 2 weeks and thawed	15.28	15.23	15.20	15.03	15.40	15.32	15.26
	15.25	15.19	15.17	15.12	16.13	15.46	15.37
Fresh Littleneck clams	20.24	20.21	20.14	20.12	28.13	23.44	22.27
	20.30	20.27	20.19	20.14	25.74	22.81	21.75
Canned smoked Holland mussels in oil	51.31	51.11	51.13	50.39	56.58	54.79	53.62
	51.09	50.96	50.99	51.02	56.47	54.23	53.03
Canned Canadian lobsters	17.12	17.06	17.05	17.65	25.52	21.16	19.25
	17.17	17.13	17.12	17.35	22.16	19.21	18.23
Canned King crabmeat	23.71	23.70	—	23.72	23.96	23.84	—
	23.76	23.75	—	23.77	23.89	23.79	—
Canned Dungeness crabmeat	24.28	24.25	24.21	24.34	24.59	24.45	24.43
	24.28	24.24	24.21	24.36	24.56	24.44	24.42
Canned grated tuna in oil	48.86	48.83	48.84	48.96	52.69	51.23	50.58
	48.90	48.87	48.87	48.80	51.81	50.48	49.90
Same after 2 days in refrigerator	48.60	48.57	48.57	48.97	50.57	49.61	49.23
	47.61	47.56	47.56	48.45	51.90	50.40	49.73
Canned solid pack tuna in brine	26.57	26.49	26.39	—	30.31	29.12	28.52
	26.42	26.40	26.33	26.44	30.06	28.88	28.32
Same after 2 days in refrigerator	26.36	26.35	26.31	26.31	28.90	28.20	27.82
	26.35	26.32	26.28	26.30	30.17	28.73	28.11
Canned Maine sardines in soybean oil	38.53	38.52	38.48	38.92	39.78	39.12	39.09
	38.44	38.43	38.38	38.67	39.02	38.92	38.91

The results are also listed in Table 1. Since the results did not usually agree with those obtained by the general method, drying times of 2 hours and 2.5 hours were also tried. However, the method for total solids in oysters appears to be unsuitable for the most part when applied to other fishery products.

ETHER EXTRACT

Last year a rapid method for the determination of ether extract in canned salmon was described (*This Journal*, **37**, 70, 602 (1954)) and the results of a collaborative study on two samples of canned salmon were reported. Subcommittee C recommended that collaborative work be extended to fish other than salmon. Lack of time prevented such collaborative work, but further study of the method was made during the year. The results are reported in Table 2. The A.O.A.C. method referred to in the table is found in *Official Methods of Analysis*, 7th Ed., 1950, **18.9** and **18.10.**

TABLE 2.—*Per cent ether extract in various fish products; comparison of methods*

PRODUCT	A.O.A.C. METHOD	RAPID METHOD
Fresh halibut	0.73	0.07
	0.62	0.13
Fresh salmon	4.87	1.55
	4.69	1.14
Fresh clams	1.29	0.97
	1.09	0.85
Canned grated tuna in oil	25.44	25.39
	25.43	25.06
Same as above, re-examined after 4 days in refrigerator	25.14	25.88
	24.53	26.23
Canned solid pack tuna in brine	1.10	1.32
	1.12	0.71
Same as above, re-examined after 2 days in refrigerator	0.87	0.96
	0.87	1.01
Canned Canadian lobster	0.90	1.04
	1.22	1.46
Canned smoked Holland mussels in oil	15.39	12.45
	15.12	12.66
Canned Maine sardines in soybean oil	16.46	16.95
	16.53	16.84

The method was tried on raw halibut and salmon without success; apparently raw fish needs the acid hydrolysis treatment to release the fat. Raw clams gave somewhat better results, possibly because they could be ground finely in the Waring blendor and more completely extracted by the ether. In most instances, however, the method probably will not work on raw fish.

Results on the canned fish products were fairly consistent with those obtained on canned salmon. If time permits, collaborative samples with a wide range of fat and moisture content will be sent out next year. This method gives considerable promise as a rapid sorting method if it can be applied to a variety of canned fish products.

RECOMMENDATIONS

It is recommended*—

(1) That collaborative work on the method for total solids in fish and other marine products (except raw oysters) be continued with the view that it may be adopted as official next year.

(2) That no further work be done on applying the method for total solids in oysters to other fishery products.

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

No report was received on salt and solids in oysters.

REPORT ON MEAT AND MEAT PRODUCTS

By R. M. MEHURIN (Meat Inspection Branch, Agricultural Research Service, U. S. Department of Agriculture, Washington 25, D. C.), *Referee*

The extensive collaborative work directed by the Associate Referee on moisture and fat in meat products appears to show that in some ways petroleum ether makes a more desirable fat extractant than the ethyl ether now used. The modified Babcock method described by the Associate Referee should be useful to food inspectors who require rapid approximate determinations of fat in meat products.

The Associate Referee on starch in meat products has submitted another excellent report. The average results from seventeen collaborators warrant further investigation of the anthrone method with special emphasis on the cause of the significant percentage of wide fluctuations.

* For report of Subcommittee C and action of the Association, see *This Journal*, **38**, 74, 75 (1955).

The Associate Referee on serological tests for the identification of meats made no report this year but he has developed a serological method which incorporates the tested procedures of well-known control laboratories. Collaborative work on this method is expected to be undertaken during the coming year.

The Referee was unable to secure, as authorized last year, an Associate Referee for the determination of lactose in the presence of maltose. This work is important because of the increasing use of dried corn sirup in meat products.

No reports were received from the Associate Referee on the chemical detection of horse meat, or on the determination of creatin in meat products.

RECOMMENDATIONS

It is recommended*—

(1) That when the determination of fat but not moisture is desired, the drying period be shortened to 6 hours at 100–102° or 1.5 hours at 125°, and therefore that method **23.6** be amended accordingly; that, if desired, the residue from the moisture determination, **23.2,** be used for the fat determination, and the use of petroleum ether be authorized as an alternate solvent for extraction of fat; and that therefore method **23.6** be further amended by the addition of the following: "using petroleum ether, **10.69,** if desired."

(2) That work be continued on the determination of starch in meat products.

(3) That collaborative work be carried out on the serological method for horse meat.

(4) That work, including spectrophotometric procedures, be continued on the chemical detection of horse meat.

(5) That work, including a study of the apparatus involved, be continued on the determination of fat in meat products.

(6) That work be continued on the determination of lactose in the presence of maltose.

* For report of Subcommittee C and action of the Association, see *This Journal,* **38,** 75, 76 (1955).

REPORT ON STARCH IN MEAT PRODUCTS

THE DETERMINATION OF STARCH IN MEAT PRODUCTS WITH THE ANTHRONE REAGENT

By F. J. STEVENS* and Ross A. CHAPMAN, *Associate Referee* (Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Canada)

A method for the determination of starch in meat products has been proposed by Stevens and Chapman (1). This procedure is based on the extraction of soluble sugars in the presence of a precipitate of zinc ferrocyanide and the estimation of the starch, after hydrolysis, with the use of a modified Fehling's reagent. It appeared that this method could be improved by employing a colorimetric technique for the determination of the starch. Preliminary experiments with the anthrone reagent as described by Dreywood (2) and adapted by Morris (3) gave promising results. Therefore this procedure was investigated with a view to adapting it to the determination of starch in meat products. In order to employ the anthrone reagent for this purpose it was necessary to develop a procedure whereby it would be possible to remove soluble sugars, solubilize the starch, filter off the residue of protein and fat, and finally determine the starch in the filtrate. Optimum conditions for each of these steps were established by the following experiments.

EXPERIMENTAL

Fairbairn (4) has described a modified reagent which consists of 0.1 per cent anthrone in 72 per cent *v/v* sulfuric acid. He stated that this reagent was much lighter in color than the usual reagents prepared with concentrated acid and that it gave satisfactory results after standing at room temperature for one month. The stability of the reagent was confirmed in this laboratory. However, when an attempt was made to increase the concentration of anthrone to 0.2 per cent, the concentration of acid was found to be very critical. In some instances the anthrone precipitated out when the reagent was added to 2 ml of the carbohydrate solution. It was noted that this difficulty could be avoided by increasing the concentration to 75 ml of concentrated sulfuric acid plus 25 ml of water. A reagent consisting of 0.2 per cent anthrone in this concentration of acid was found to be the most satisfactory and was adopted for further experiments.

Koehler (5) has reported that maximum color formation is obtained with *d*-glucose after it is heated for 8 to 11 minutes at 100°C. with the anthrone reagent. In order to confirm this point, samples of dextrose, wheat flour, potato starch, and wheat starch were heated for 1 hour with

* Present address: Rath Packing Co., Waterloo, Iowa.

0.5 *N* sulfuric acid. The absorbance obtained by heating the resulting solutions in the presence of the anthrone reagent for 5 to 15 minutes at 100°C. is shown in Table 1. These data indicate that under the conditions employed, optimum values are obtained by heating for 9 to 11 minutes. A period of 10 minutes was adopted for further work.

TABLE 1.—*Effect of heating on the absorbance produced with various materials*

TIME OF HEATING, MINUTES	ABSORBANCE			
	DEXTROSE	WHEAT FLOUR	POTATO STARCH	WHEAT STARCH
5	0.495	0.361	0.421	0.343
8	0.528	0.427	0.538	0.537
9	0.537	0.431	0.539	0.556
10	0.536	0.440	0.531	0.556
11	0.539	0.427	0.533	0.548
12	0.533	0.426	0.511	0.504
15	0.495	0.381	0.445	0.456

In order to determine the levels of soluble carbohydrates which could be extracted by this procedure, skim milk powder, dextrose, sucrose, and dextrin were added to meat samples at levels from 3 to 20 per cent. These samples were then carried through the entire procedure with the results shown in Table 2. It is apparent from these data that the carbohydrates present in all of these materials can be extracted at levels up to 12 per cent without serious error. At levels of 15 to 20 per cent a significant amount of skim milk and dextrin remains in the sample. However, the extraction procedure would appear to be satisfactory for commercial meat products.

TABLE 2.—*Apparent starch content of meat samples containing skim milk powder, dextrose, sucrose, and dextrin*

PER CENT ADDED	APPARENT STARCH, PER CENT			
	SKIM MILK POWDER	DEXTROSE	SUCROSE	DEXTRIN
3	0.03	0.03	0.02	0.09
6	0.09	0.00	0.03	0.12
9	0.11	0.00	0.07	0.25
12	0.23	0.05	0.16	0.29
15	0.41	0.20	0.25	0.71
20	0.62	0.30	0.22	1.05

In order to determine the optimum conditions for the solubilization of the starch, wheat flour and potato starch were added to meat samples and these were treated with varying concentrations of sulfuric acid for

15 to 90 minutes. The results are given in Table 3. These data indicate that the time of heating and the concentration of the acid are not highly critical and that satisfactory results could be obtained with a concentration of 0.5 *N* sulfuric acid and a heating period of 60 minutes. Samples of ground beef and pork without added carbohydrate were carried through

TABLE 3.—*Effect of concentration of sulfuric acid and period of heating on recovery of starch*

PERIOD OF HEATING, MINUTES	STARCH, PER CENT					
	WHEAT FLOUR			POTATO STARCH		
	0.25 *N*[a]	0.50 *N*	1.00 *N*	0.25 *N*	0.50 *N*	1.00 *N*
15	3.15	3.17	4.17	3.40	3.30	3.67
30	4.27	4.15	4.27	3.65	3.67	3.71
45	4.35	4.39	4.50	3.67	3.77	3.78
60	4.33	4.41	4.47	3.75	3.79	3.76
75	4.32	4.41	4.47	3.72	3.81	3.79
90	4.35	4.41	4.63	3.81	3.79	3.70

[a] Concentration of sulfuric acid employed.

the entire procedure and all gave values of approximately 0.1 per cent apparent starch. Therefore it was necessary to make a correction for this amount in all determinations.

On the basis of the foregoing experiments the following method was adopted:

METHOD

REAGENTS

(a) *Zinc acetate soln.*—Dissolve 6 g $Zn(C_2H_3O_2)_2.2H_2O$ in H_2O and dil. to 50 ml. (Prep. fresh each day).

(b) *Potassium ferrocyanide soln.*—Dissolve 3 g $K_4Fe(CN)_6.3H_2O$ in H_2O and dil. to 50 ml. (Prep. fresh each day).

(c) *Anthrone soln.*—To a cooled mixt. of 75 ml concd H_2SO_4 and 25 ml H_2O add 0.2 g anthrone. Prep. at least 4 hrs before use and store in dark place.

(d) *Standard dextrose.*—Dissolve 0.20 g pure dextrose in H_2O and dil. to 200 ml. Dil. 10 ml of this soln to 250 ml. (2 ml of this final soln = 4% dextrose in 5 g meat sample).

PROCEDURE

Weigh 5 g finely comminuted and thoroly mixed sample into a 250 ml heat-resistant centrifuge bottle. Add 150 ml H_2O, 5 ml of the zinc acetate soln, and 5 ml of the potassium ferrocyanide soln. Allow to stand for 15 minutes with occasional stirring; then centrifuge for 5 min. at 1500 rpm. Decant supernatant liquid thru a Whatman No. 3 filter paper in a conical funnel, employing light suction. To the residue in the centrifuge bottle add 50 ml of a soln contg 1 ml of the zinc acetate soln and 1 ml of the potassium ferrocyanide soln per 200 ml. Allow to stand for 5 min. and stir several times during that period. Decant suspended sample thru

same filter paper. Repeat this last extn with an addnl 25 ml of the zinc acetate-potassium ferrocyanide washing soln. Filter to dryness before adding each portion of washing soln.

Transfer residue and filter paper to original centrifuge bottle and add 100 ml 0.5 *N* H_2SO_4. Suspend bottle in boiling H_2O bath, so that the H_2O level in the bath is at the level of the soln within the centrifuge bottle. Do not reflux. Heat for 1 hr, maintaining the H_2O level of the bath at its original position, stirring the contents of the bottle occasionally.

Cool immediately. Transfer contents of the centrifuge bottle to a 200 ml phosphoric acid flask or a 200 ml erlenmeyer marked at a vol of 200 ml. Rinse centrifuge bottle with small portions of H_2O, leaving undigested filter paper in the centrifuge bottle. Make to vol. with H_2O, stopper, and shake. Filter thru Whatman No. 1 filter paper. Pipet 10 ml of the filtrate into a 250 ml vol. flask. Make up to vol. with H_2O, stopper, and shake.

DETERMINATION OF CARBOHYDRATES

Pipet 2 ml of the dil. filtrate into a colorimeter tube previously rinsed with alcohol (traces of dust or dirt in the colorimeter tube may give erroneous results). Place tube in a beaker of cold H_2O and add slowly, with continuous agitation, 10 ml of the anthrone reagent from a pipet or buret. Cool tube for at least 5 min. in cold H_2O; then immerse for exactly 10 min. in a boiling H_2O bath. Cool again for at least 5 min. Prep. a std, using 2 ml of the dil. std dextrose soln in place of the dil. filtrate. Similarly prep. a blank, using 2 ml distd H_2O.

Measure the absorbance of the sample and std solns in an Evelyn colorimeter or similar instrument at a wavelength of 620 mμ, employing the blank soln as a reference.

CALCULATIONS

Per cent starch $= (A/B \times 4 \times 0.9) - 0.1$, where A = absorbance of sample soln; B = absorbance of std dextrose soln; 4 = 2 ml of the dil. std dextrose soln representing 4% dextrose in a 5 g meat sample; 0.9 = factor for conversion of dextrose to starch; and 0.1 = average meat blank.

It was considered that the glycogen or dextrose present in liver might be determined as starch by this procedure. Therefore determinations were conducted on beef, pork, and calf livers by both the anthrone procedure and the Fehling method (1) as previously described. The results are given in Table 4. The values obtained by the Fehling procedure indicate that after the solubilization with sulfuric acid, reducing sugars are present in some livers in significant amounts. In most cases the values by the two methods are in satisfactory agreement, but in a few samples the results by the anthrone procedure are significantly higher. It is believed that the higher values are caused by the reaction of the anthrone reagent with small amounts of free amino acids present in the liver. It is known that certain amino acids react with anthrone to give a color similar to that given by carbohydrates. These data do indicate, however, that the presence of an appreciable amount of liver in a meat product could result in a significant error in the value for starch.

To determine the recovery of starch added to meat products, a number of materials likely to be employed as meat binders were added to ground

TABLE 4.—*Apparent starch content of liver*

SAMPLE		APPARENT STARCH, PER CENT	
		ANTHRONE METHOD	FEHLING'S METHOD
Beef liver	1	0.04	0.05
	2	1.35	0.09
	3	0.32	0.02
	4	0.34	0.07
	5	2.90	2.61
Pork liver	1	0.00	0.04
	2	0.07	0.03
	3	0.09	0.00
	4	0.54	0.15
	5	0.15	0.00
Calf liver	1	0.04	0.04
	2	0.45	0.00
	3	1.00	0.25
	4	5.15	5.00
	5	0.21	0.03

pork at levels of 1, 3, 5, and 7 per cent. These samples were then carried through the entire procedure, with the results shown in Table 5. These recoveries are considered reasonably satisfactory. It would also appear that interferences due to non-sugar reducing substances previously encountered with the Fehling method (1) had been eliminated.

In a previous collaborative study (1) it was suggested that processes involved in the preparation of cooked or partially cooked meat products might alter the starch in such a manner that it would be washed out during the extraction procedure. To check this point, wheat flour, potato starch, wheat starch, and a commercial sausage binder were added to fresh ground pork at a level of 4 per cent. These samples were then sealed in cans and immersed in a boiling water bath for 1 hour and for 5

TABLE 5.—*Recovery of starch from carbohydrate materials added to meat*

MATERIAL	STARCH FOUND, PER CENT				AVERAGE RECOVERY,[a] PER CENT
	1% ADDED	3% ADDED	5% ADDED	7% ADDED	
Potato starch	0.81	2.38	4.14	5.70	95.5
Wheat starch	0.77	2.28	4.12	5.59	94.9
Wheat flour	0.67	2.01	3.24	4.70	95.1
Corn starch	0.79	2.39	4.19	5.91	94.9
Soluble starch	0.79	2.31	3.91	5.54	95.5

[a] The recoveries were calculated by comparing the values obtained on the meat samples to the results given on the pure carbohydrate materials by the anthrone method, omitting the extraction procedure.

hours. The starch content was then determined by the anthrone method and by a previous A.O.A.C. procedure (6) which was based on saponification of the sample followed by precipitation of the starch with alcohol. The results are shown in Table 6. It is evident from these data that there is a slight but definite decrease of from 2 to 7 per cent in the starch values on heating. The major change appeared to take place during the first hour of heating and the additional 4 hours had little further effect. The results given by the A.O.A.C. procedure (6) on the sample containing the commercial meat binder were significantly lower than the values obtained by the anthrone method. This discrepancy was attributed to the use of a modified starch in the meat binder which was not completely precipitated by alcohol.[c]

TABLE 6.—*Effect of processing on recovery of starch from meat samples*

CARBOHYDRATE MATERIAL	METHOD	PER CENT STARCH		
		0[a]	1 HOUR	5 HOURS
Wheat flour	A[b]	2.93	2.81	2.80
	B[c]	3.10	2.94	2.88
Potato starch	A	3.60	3.45	3.46
	B	3.51	3.51	3.47
Wheat starch	A	3.30	3.14	3.13
	B	3.38	3.26	3.14
Commercial sausage binder	A	1.53	1.51	—
	B	1.13	1.07	1.07

[a] Period of heating in boiling water.
[b] Anthrone procedure.
[c] A.O.A.C. procedure.

COLLABORATIVE STUDY

The method was submitted to collaborative trial. Three samples of meat fillers, labeled A, B, and C and consisting respectively of skim milk powder, wheat flour, and potato starch, were sent to each analyst. Collaborators were instructed to obtain a sample of fresh ground pork and to add each of the fillers at levels of 3 and 6 per cent. The correct amount was added to each individual sample in order to avoid any possible error through improper mixing.

The results submitted by seventeen analysts are shown in Table 7. The values for added starch were again obtained by carrying out a determination on each filler without the addition of meat and eliminating the extraction procedure. The average recoveries which ranged from 93.9 to 96.3 per cent are considered quite satisfactory. The results submitted by collaborators 1 to 8 showed good agreement between duplicates and

TABLE 7.—*Collaborative results*

COLLABORATOR	SAMPLE					
	A		B		C	
	3%	6%	3%	6%	3%	6%
	Starch Added, *per cent*					
	0.0	0.0	2.04	4.08	2.58	5.16
	Starch Found, *per cent*					
1	0.14	0.28	2.05	4.22	2.54	4.91
	0.11	0.28	2.12	4.35	2.52	4.98
2	0.01	0.00	2.00	3.96	2.45	5.05
	0.02	0.00	1.98	3.85	2.47	5.18
3	0.05	0.22	2.04	4.02	2.57	5.01
	0.05	0.21	2.00	3.95	2.57	5.09
4	0.00	0.00	1.97	4.21	2.49	4.90
	0.00	0.00	1.71	4.04	2.31	4.86
5	0.00	0.00	1.87	4.00	2.57	4.98
	0.00	0.00	1.91	3.96	2.50	4.95
6	0.00	0.00	2.13	4.09	2.67	5.17
	0.00	0.00	2.03	4.03	2.63	5.21
7	0.01	0.08	2.06	4.01	2.66	5.09
	0.00	0.09	2.09	4.12	2.62	5.05
8	0.00	0.20	2.12	4.21	2.77	5.29
	0.04	0.12	2.03	4.29	2.66	4.99
9	0.12	0.00	2.03	3.99	2.60	5.04
	0.00	0.10	1.73	3.51	2.78	4.49
10	0.20	0.51	1.85	3.72	2.16	4.27
	0.17	0.44	1.45	3.73	2.37	5.62
11	0.00	0.00	1.70	3.56	2.30	4.66
	0.00	0.00	1.70	3.50	2.30	4.63
12	0.14	0.00	1.47	3.09	2.00	4.52
	0.00	0.00	1.49	3.09	2.04	4.28
13	0.02	0.21	2.03	4.07	2.70	4.45
	0.17	0.31	1.82	3.84	2.42	5.01
14	0.00	0.00	2.11	3.75	2.54	4.40
	0.00	0.00	2.11	3.70	2.47	4.36
15	0.00	0.00	1.43	3.10	2.46	6.62
	0.00	0.00	1.94	2.90	2.12	4.40
16	0.00	0.06	1.62	3.75	2.57	6.41
	0.00	0.00	1.68	3.85	2.55	6.22
17	0.00	0.00	2.59	3.87	2.33	4.52
	0.00	0.00	2.52	3.73	2.27	4.52
Average	0.04	0.09	1.92	3.83	2.47	4.97
Recovery, %			94.1	93.9	95.7	96.3

the correct relationship between the two levels of addition. However, the values found by collaborators 9 to 17 were in error in one or more instances. In some cases, such as collaborator 11, the standard solution appears to be at fault since all values are low by approximately the same proportion.

A number of collaborators submitted comments on the method. Several analysts found the procedure laborious and time-consuming. However, we feel that no simple method is likely to be developed for the separation of starch from such compounds as sucrose, dextrose, and lactose. Some difficulty was encountered in transferring the sample from the centrifuge bottle to the phosphoric acid flask, but the results did not indicate any significant loss due to this manipulation. Several analysts found it more desirable to develop the color in a 25 ml volumetric flask. This modification was particularly desirable in cases where the instrument used was equipped with expensive cuvettes. One laboratory reported that the meat blank continued to increase during the storage of the ground meat at refrigerator temperatures. This phenomenon, if confirmed, would invalidate the method since there would be no way of correcting for the blank on unknown samples. However, this same laboratory reported that the absorbance of the standard also increased from 0.375 to 0.620, a situation not encountered by any other collaborator. It is obvious that this point must be investigated.

SUMMARY

A method employing the anthrone reagent has been developed for the determination of starch in meat products. Recoveries of added starch of approximately 95 per cent have been obtained by this procedure. Losses of 2 to 7 per cent were encountered when samples were canned and placed in a boiling water bath for periods up to 5 hours. The glycogen or dextrose present in liver interferes in the procedure and therefore the method is not applicable to meat products containing a significant amount of liver. Satisfactory results were obtained in the presence of amounts up to 12 per cent of skim milk powder, dextrose, sucrose, and dextrin.

Satisfactory results were reported by approximately 50 per cent of the collaborators. However, erratic results were obtained by the remainder of the analysts. One laboratory reported that the meat blank increased significantly during storage at refrigerator temperatures. Therefore it is recommended* that this method be given further study.

ACKNOWLEDGMENT

The assistance of the following collaborators is gratefully acknowledged:

J. M. McCoy and H. R. Cook, U. S. Department of Agriculture, Agricultural Research Service, Meat Inspection Branch, Washington, D.C.

* For report of Subcommittee C and action of the Association, see *This Journal*, 38, 75, 76 (1955).

W. C. Owens and H. L. Medcraft, U. S. Department of Agriculture, Agricultural Research Service, Meat Inspection Branch, San Francisco, Calif.

G. L. McGinty and W. H. Hendricks, Sr., U. S. Department of Agriculture, Agricultural Research Service, Meat Inspection Branch, St. Louis, Mo.

F. B. Hilty, U. S. Department of Agriculture, Agricultural Research Service, Meat Inspection Branch, Omaha, Neb.

O. L. Bennett, U. S. Department of Agriculture, Agricultural Research Service, Meat Inspection Branch, Chicago, Ill.

H. O. Tomlinson, N. Bluman, and L. Thompson, Food and Drug Laboratories, Department of National Health and Welfare, Vancouver, B. C., Canada.

B. Rousseau, Food and Drug Laboratories, Department of National Health and Welfare, Montreal, Quebec, Canada.

J. F. Blanchard and J. B. Jones, Food and Drug Laboratories, Department of National Health and Welfare, Winnipeg, Manitoba, Canada.

G. E. Mack and E. W. Corck, Food and Drug Laboratories, Department of National Health and Welfare, Toronto, Ontario, Canada.

Andrew Hollett and J. C. Pottie, Food and Drug Laboratories, Department of National Health and Welfare, Halifax, Nova Scotia, Canada.

C. W. Everson, Thomas Keyahian, and D. M. Doty, American Meat Institution Foundation, University of Chicago, Chicago, Ill.

R. B. Carson, Chemistry Division, Science Service, Department of Agriculture, Ottawa, Ontario, Canada.

H. Nordin, Canada Packers Limited, Toronto, Ontario, Canada.

J. R. Kinosian, State of California, Department of Public Health, Los Angeles, Calif.

Flo H. Ward, Army Medical Service Graduate School, Walter Reed Army Medical Center, Washington, D. C.

REFERENCES

(1) STEVENS, F. J., and CHAPMAN, R. A., *This Journal*, **36**, 292 (1953).
(2) DREYWOOD, R., *Ind. Eng. Chem., Anal. Ed.*, **18**, 499 (1946).
(3) MORRIS, D. L., *Science*, **107**, 254 (1948).
(4) FAIRBAIRN, N. J., *Chemistry & Industry*, **No. 4**, 86 (1953).
(5) KOEHLER, L. H., *Anal. Chem.*, **24**, 1576 (1952).
(6) *Official Methods of Analysis*, 6th Ed., Association of Official Agricultural Chemists, Box 540, Benjamin Franklin Station, Wash. 4, D. C., 1945, **28.19.**

REPORT ON THE DETERMINATION OF FAT IN MEAT PRODUCTS

By E. S. WINDHAM (Veterinary Division, Army Medical Service Graduate School, Walter Reed Army Medical Center, Washington, D. C.), *Associate Referee*

This year a collaborative study was conducted of some variations of the ether extraction method, **23.6,** for determining fat in meat. A rapid screening method employing a modification of the Babcock test for fat in dairy products was included in the study.

Some chemists consider that the drying conditions as now specified

for meat samples are too harsh. They fear the possibility of excessively oxidizing the fat, which may render the sample partly impervious to ether and increase the solubility of non-fat material in the ether. Therefore, a mild drying method was used in addition to those specified in 23.6.

An earlier report[1] gave results of a study of the use of petroleum ether instead of anhydrous ethyl ether as the fat extractant. Since the results indicated certain advantages in its use this variation was included in the present collaborative study.

The Associate Referee has developed an apparently satisfactory screening test along the lines of the Babcock test for milk. Information and ideas gleaned from many sources have been used in developing the final form of this method, which has been found so promising that it was included in the study.

COLLABORATIVE STUDY

The following instructions were issued to collaborating laboratories:

Determine fat in quadruplicate by each of the following methods and report individual results.

Determine fat on 4 or more samples of each of as many products as are available and which you normally run for fat.

If overnight extraction is impossible, please report extraction times.

If other than Soxhlet extractors are used, please describe them.

Comments on any difficulties and your preferences would be appreciated.

METHOD 1

Weigh ca 4 g freshly prepd sample into extn thimble. Dry ca 6 hrs at 100–102°C. Ext. 16–18 hrs in Soxhlet extractor, using absolute or dehydrated ethyl ether. Evap. solvent, dry fat to constant weight at 100–102°C. (usually 1.5–2 hrs), cool, and weigh. Wash out fat, using petr. ether. Dry flask in oven, cool, and weigh. Report any noticeable amount of material, other than fat, in the fat or inside the rinsed flask.

METHOD 2

Proceed as in Method 1 except use petr. ether, boiling range 30–60°C., instead of ethyl ether as the extn solvent.

METHOD 3

Proceed as for Method 1 except dry the sample as in 23.6 (16–18 hrs at 101–102°C. or 2–3 hrs at ca 125°C.)

METHOD 4

APPARATUS

(a) *Bottles.*—Paley 20% ice cream and 50% cheeese, 9 g Babcock test bottles with rubber stoppers.

(b) *Centrifuge.*—2 or 4 place hand Babcock.

(c) *Balance.*—With 4.5 and 9 g weights, accurate to ±0.05 g (or Babcock cream balance).

(d) *Dividers (calipers).*—For reading test.

---

[1] E. S. WINDHAM, *This Journal*, 36, 288 (1953).

REAGENTS

(a) *Sulfuric acid.*—1.83 specific gravity, commercial.
(b) *Reading oil.*—"Glymol" or red colored mineral oil.

DETERMINATION

Expected fat content	*Bottle size*	*Sample size*	*Original water*
Less than 15%	20%	9 g	9 ml
15–40%	50%	9 g	9 ml
More than 40%	50%	4.5 g	13.5 ml
High starch products	20%	4.5 g	13.5 ml

Weigh sample of proper size into Paley bottle (above table). Stopper lower opening tightly. Add appropriate vol. of cold H_2O. Do not mix. Add ca 18 ml H_2SO_4 in 2 portions, mixing after first portion. Mix continually until all meat is dissolved as shown by no particles in fat layer on 10 sec. standing (ca 3 min. for most samples.) Centrifuge at Babcock speed for 2 min. Add hot H_2O (ca 60°C.) to near top of graduations. Centrifuge 1 min. Read immediately, using Glymol for 50% bottles, but not for 20% bottles. Multiply readings by 2 if 4.5 g sample was used. Read only to nearest scale division.

NOTES

1. The bottles may be tempered in a water bath at 55–60°C. if desired.
2. Adjustment of the height of the fat column may be made by adjusting the rubber stopper.
3. No column should have over one scale division of sediment present. Repeat test with more care in the digestion if sediment interferes with reading. Too weak acid or insufficient acid tends to give excessive sediment.

RESULTS

Seven collaborators reported results. One collaborator omitted method 3 from his study and his results are not included in the comparisons of the four methods. In Table 1 are reported the averages of quadruplicate results by each method and the range in per cent fat. A number of products of a wide range of fat content were analyzed as well as samples containing cereal and milk powder. There were no great differences among the extraction procedures (methods 1, 2, 3). The Babcock procedure (method 4) gave results which were considered satisfactory for a screening method. For 60 samples the average fat was 24.12 per cent as compared to 24.21, 24.26, and 24.11 per cent, respectively, for the other methods. The average range was 0.64 per cent as compared to 0.51, 0.44, and 0.54 per cent, respectively, for the extraction procedures.

Viewed from other angles the results by method 4 were much less satisfactory. The range of values found on a sample was 1 per cent or more on 15 samples out of 60 as compared to 6, 4, and 5, respectively, out of 60 by the other methods. For the total of 72 samples by all collaborators, results by the Babcock procedure exceeded those by method 2 by 1 per cent or more 12 times, and fell at least 1 per cent lower than method 2 eight times.

Although the differences were slight among methods 1, 2, and 3, method 2 was reported as consistently giving a clearer fat with less foreign material suspended in the extract or adhering to the flask. Method 3 was reported the worst in this respect and frequently yielded a darkened fat as well. The average range among quadruplicate determinations was also slightly lower by method 2.

DISCUSSION

The requirement in Federal and Military specifications for meat products that petroleum ether (boiling range 30–60°C.) be used exclusively as the fat solvent appears to be justified, as the results obtained are apparently slightly more satisfactory. When the ease of handling petroleum ether, the relative toxicity and hazards, and the relative costs are compared, its superiority is even more evident. Therefore its adoption as an alternate fat solvent by the A.O.A.C. is recommended.

The screening method studied probably needs further work. Experience gained in actual use will prove valuable. The method is probably satisfactory for control officials in groceries, markets, and small meat plants where rapid visual evidence of fat content is desired. The method could be of value in controlling composition by the processors themselves. Its use as a laboratory screening method where contract average on fat content is not required should ease the work load at times when a large volume of samples are received.

COMMENTS OF COLLABORATORS

Collaborator 1.—Method 4 was altered to avoid excess sediment formation in Paley bottle necks by the use of 12 ml of sulfuric acid (A.C.S. sp. gr. 1.84) instead of 18 ml. This gave fat columns reasonably free of sediment. Drying losses (in reaching constant weight) were more erratic with diethyl ether than with petroleum ether. Suggest changes in procedure as follows: (1) Remove solvent from extracted fat at low temperature by use of a stream of dry air directed into the extraction flask; (2) Dry extracted fats at low temperature in a vacuum oven.

Collaborator 2.—Although both are considered equally effective as extracting solvents, petroleum ether consistently yielded a clearer, lighter colored solution and dried fat, while ether often gave darker or turbid solutions and left ether-insoluble residue in the dried fat. In some cases this residue amounted to 20–30 mg. Some lint from the paper thimbles was often found in the fats. There seemed to be no advantage in using 6 hour drying at 100–102°C. over 2 hour drying at 125°C.

Collaborator 3.—Method 4 was modified by digesting samples with the acid for 1 hour at room temperature. Petroleum ether is as efficient as anhydrous ethyl ether as a fat extraction solvent at a great saving in cost. Results on pork sausage, using Method 4, were not considered acceptable as they were excessively high.

Collaborator 4.—Method 4 was modified by (1) adding the acid in several small portions, (2) adding a five minute centrifuging, and (3) adding the hot water in two portions first to the base of the bottle neck after the 5 minute centrifuging and then to bring the fat into the graduations after the two minute centrifuging. All tests were tempered in a 60°C. water bath for five minutes before reading.

Collaborator 6.—Foreign material appeared most frequently in fats by method

TABLE 1.—*Comparison of fat methods*

COLLABORATOR	SAMPLE	METHOD 1		METHOD 2		METHOD 3		METHOD 4	
		AVERAGE	RANGE	AVERAGE	RANGE	AVERAGE	RANGE	AVERAGE	RANGE
1	Bologna and skim milk	25.4	0.3	24.9	0.2	25.3	0.4	25.5	0.6
	Pork sausage	41.8	0.7	41.6	0.4	41.9	0.5	43.1	1.0
	Franks and 32% mixed cereal	17.5	0.3	17.5	0.1	17.7	0.5	17.8	0.4
	Ham (trimmed)	6.1	0.1	6.0	0.0	6.1	0.6	5.5	0.4
2	Ground beef	19.5[a]	0.4	19.3[a]	0.3	19.6[a]	0.8	18.4	1.0
	Ground beef	24.3	0.8	23.6	0.7	23.7	1.3	23.4	0.6
	Ground beef	25.5	0.7	26.3	0.7	26.0	1.1	24.0	1.5
	Ground beef	21.3[a]	0.6	21.6	0.5	21.5[a]	0.7	20.0	1.5
	Ground beef	23.6	0.4	24.6	1.3	23.5[a]	0.3	22.5	1.0
	Ground beef	23.4[a]	0.2	23.6	0.3	23.8[a,c]	0.2	21.3	0.5
	Ground beef	25.2[a]	0.5	25.7[a]	0.5	25.4[a]	0.4	23.1	1.0
	Ground beef	24.4	0.6	24.6	0.4	24.8	0.3	23.8	0.5
	Pork sausage	42.5	0.4	42.9	0.6	42.3	0.6	43.8[b]	2.0
	Pork sausage	32.2[a]	0.6	34.6[a]	0.2	33.0	0.7	31.9[b]	1.5
4	Pork sausage	40.5	0.3	40.4	0.5	39.6	0.5	42.0	2.0
	Pork sausage	35.9	0.8	35.9	0.4	35.0	0.4	35.7	0.5
	Pork sausage	43.3	0.5	43.7	0.4	42.7	0.9	43.0	0.0
	Pork and gravy	10.6	0.1	10.6	0.1	10.2	0.3	10.0	0.0
	Beef sausage	18.2	0.5	18.0	0.4	18.0	0.4	18.0	0.0
	Franks	30.0	0.4	29.9	0.3	29.7	0.4	30.5	0.7
	Ground beef	24.0	0.6	24.2	0.2	24.1	0.3	24.1	0.5
	Liver sausage	30.6	1.0	30.9	0.9	30.8	0.5	31.4	0.5
	Pork sausage	38.5	0.5	38.2	0.7	38.3	0.7	38.5	1.0
	Bologna	31.3	0.6	31.1	0.4	30.9	0.7	32.3	0.5

4	Lunchmeat	22.2	1.0	22.5	1.0	21.8	1.6	21.7	1.0
	Pork and gravy	24.6	0.1	24.5	0.1	24.1	0.2	24.4	0.5
	Pork sausage	37.4[c]	1.0	36.9	1.5	37.0	1.0	38.7	2.0
	Franks	25.4	0.7	25.5	0.4	25.4	0.5	25.8	0.5
5	Ground beef	16.8	0.6	16.6	0.4	16.8	0.4	16.6	0.2
	Pork sausage	33.5	0.5	33.6	0.7	33.5	0.4	35.4	0.5
	Pork sausage	33.3	0.3	33.2	0.1	33.5	0.5	33.0	0.0
	Ground beef	6.4	0.4	6.5	0.0	6.6	0.7	6.6	0.4
6	Ground beef	16.6[a]	0.5	16.5	0.2	16.5[a]	0.5	15.5	0.0
	Ground beef	19.0	0.8	18.8	0.1	19.0[a]	0.8	18.2[b]	1.0
	Ground beef	20.0	0.9	19.7	0.7	19.7	1.0	20.0	0.0
	Ground beef	16.8	0.4	16.7	0.4	16.7	0.4	16.6	0.5
	Ground beef	20.1	0.3	20.4	0.6	20.1	0.6	20.2	0.5
	Franks and 10% starch	20.7	0.4	20.8	0.2	20.9[a,c]	0.1	21.5	0.0
	Bologna and 10% skim milk	18.9	0.2	19.0	0.1	18.8	0.2	18.4	0.5
	Beef and gravy	10.1[a]	0.4	9.9	0.1	10.2[a]	0.9	9.6	0.0
	Beef and gravy	19.0	0.1	18.8	0.5	18.7	0.5	20.0[b]	0.8
	Beef and gravy	11.3	0.2	11.1	0.2	11.2	0.1	11.7[b]	0.4
	Beef and gravy	13.1	0.1	13.1	0.2	12.9	0.6	12.0	0.0
	Beef and gravy	22.0	0.1	22.0	0.1	22.1	0.2	20.2	0.4
	Beef and gravy	9.5	0.0	9.5	0.1	9.8[a]	0.0	10.0	0.0
7	(Not reported)	24.1	0.1	23.9	0.2	24.1	0.3	24.7	0.3
		23.7	0.6	23.8	0.8	23.7	0.4	23.4	0.7
		34.6	0.9	34.8	0.5	34.5	0.4	34.6	0.3
		33.1	1.4	33.0	0.9	32.6	0.5	32.9	0.7

[a] Considerable extraneous material in one or more flasks.
[b] Considerable extraneous material in one or more fat columns.
[c] Three results only.

TABLE 1—(*continued*)

COLLABORATOR	SAMPLE	METHOD 1		METHOD 2		METHOD 3		METHOD 4	
		AVERAGE	RANGE	AVERAGE	RANGE	AVERAGE	RANGE	AVERAGE	RANGE
7		28.6	0.3	28.6	0.4	28.6	0.7	28.3	0.6
		19.7	0.4	19.6	0.4	19.3	0.7	20.2	1.0
		26.3	0.7	26.5	0.3	26.4	0.8	26.6	0.8
		22.7	0.3	22.8	0.5	22.8	0.4	22.8	0.6
		25.8	1.0	26.0	0.8	25.3	0.5	26.4	1.1
		23.2	0.7	23.1	0.3	22.9	0.5	23.1	0.5
		29.3	0.9	29.3	0.7	29.7	0.8	29.6	0.8
		22.6	0.5	22.5	0.9	22.6	0.5	22.5	0.4
		22.6	0.2	22.5	0.7	22.2	0.4	22.6	0.8
		21.9	0.2	22.2	0.2	21.8	0.4	22.4	0.8
		21.8	1.2	21.9	0.8	21.0	0.5	21.2	0.8
Average of 60 Samples		24.21	0.51	24.26	0.44	24.11	0.54	24.12	0.64
3	Pork sausage	37.3	1.2	37.1	0.6			37.9	0.7
	Pork sausage	32.4	0.5	32.3	0.2			33.7	0.5
	Pork sausage	39.4	0.8	39.2	0.5			40.9	2.0
	Pork sausage	42.8	0.6	42.4	1.1			44.0[c]	2.0
	Pork sausage	35.7	1.3	35.2	0.6			36.7	0.5
	Ground beef	26.6	0.1	26.7	0.1			27.4	0.5
	Ground beef	22.3	0.2	22.4	0.1			23.1	0.5
	Ground beef	24.0	0.6	23.9	0.7			25.0	0.0
	Ground beef	27.1	0.0	27.0	1.0			27.4	0.3
	Ground beef	22.2	0.4	22.0	0.5			22.0	1.5
	Pork sausage	37.1	0.4	37.4	1.0			37.3	2.0
	Ground beef	26.4	0.3	25.9	0.6			26.0	0.0

[c] Three results only.

3 and in some cases in method 1. Fats by method 2 were always clear and tended to a lighter color indicative of less oxidative change. Alundum thimbles were used and samples were ground in the thimble after drying. There was a definite tendency for starchy samples to mat together during ether extraction, but not during petroleum ether extraction. Samples dried at the higher temperature or overnight were hardened and much more difficult to grind properly.

No modification of method 4 gave as good results as the method submitted. Mixing with the water before addition of acid produced much more charring and darker columns with more foreign material. Less volume of stronger acid was less satisfactory for the same reasons.

Collaborator 7.—Collaborator 7 used two other screening methods, a modified Minnesota Babcock method and one using nitric and hydrochloric acids as the digesting agents. His results appeared as good as those with method 4.

LIST OF COLLABORATORS

J. M. McCoy, Meat Inspection Laboratory, Agricultural Research Service, U. S Department of Agriculture, Washington, D. C.

Veterinary Section, Second Army Area Medical Laboratory, Ft. Meade, Md.

Veterinary Section, Third Army Area Medical Laboratory, Ft. McPherson, Ga.

Veterinary Section, Fourth Army Area Medical Laboratory, Ft. Sam Houston, Texas

Veterinary Section, Fifth Army Area Medical Laboratory, St. Louis, Mo.

Veterinary Branch, Sixth Army Area Medical Laboratory, Oakland Army Base, Calif.

Veterinary Division, A.M.S.G.S., Walter Reed Army Medical Center, Washington, D. C.

RECOMMENDATIONS

It is recommended*—

(1) That the following changes be adopted:

(a) Add the following statement to **23.6,** "Petr. ether, **10.69,** may be used as the fat solvent."

(b) Insert in **23.6,** after " . . . 125°," the following: "If moisture content is not desired on same portion, less vigorous drying, 1.5 hrs at 125° or 6 hrs at 100°, is recommended."

(2) That the screening method (method 4) here reported be further studied with a view towards possible adoption as a procedure.

(3) That other aspects of fat determination in meat products be studied, such as:

(a) Suitability of various extraction apparatus. No particular apparatus is now specified.

(b) Minimum extraction time needed. A daytime 6–8 hour extraction may be sufficient.

(c) Type of thimble to recommend. Alundum thimbles can be used for drying, grinding, and extraction without transfer. Paper thimbles frequently add lint to the extracted fat.

(d) Influence and amount of fat oxidation in the various methods of drying the sample and extracting the fat.

* For report of Subcommittee C and action of the Association, see *This Journal,* **38,** 75, 76 (1955).

No reports were received on chemical tests for identification of meats, creatin in meat products, or serological tests for identification of meats.

REPORT ON MICROBIOLOGICAL METHODS

EXAMINATION OF EGGS AND EGG PRODUCTS

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

A year of further review of the first action method for eggs and egg products, **36.1–36.11,** has indicated the need for a few minor changes. These revisions are dictated in part by experience and in part by a desire to maintain uniformity with the companion method in *Standard Methods for the Examination of Dairy Products*, 10th ed., 1953, of the American Public Health Association. Changes which are merely editorial will not be set forth here.

The test for putrefactive anaerobic types of microorganisms, **36.7,** is being deleted in its entirety since there is no indication that this group is of special significance in egg products, and since it is not known that the test is being used by those examining egg products. The media employed in this test, **36.11(d)**, should also be deleted.

Preparation of stock buffer solution, **36.11(b)**, has been revised to provide for the adjustment of the *p*H to 7.2 prior to dilution to volume, rather than after the dilution.

RECOMMENDATIONS

It is recommended*—

(1) That with the foregoing noted changes, this topic be continued as first action.

(2) That collaborative work on the various methods be undertaken.

REPORT ON FEEDING STUFFS

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

RECOMMENDATIONS

It is recommended†—

(1) That the study of microscopic examination be discontinued.

---

* For report of Subcommittee C and action of the Association, see *This Journal*, 38, 76, 77 (1955).
† For report of Subcommittee A and action of the Association, see *This Journal*, 38, 62, 63 (1955).

(2) That work on the following be continued:

Crude fat or ether extract
Crude protein in feeding stuffs
Drugs in feeds
Gossypol
Molasses
Mineral constituents of mixed feeds
Sampling
Fat in fish meal

(3) That the method for moisture in highly acid milk by-products, *This Journal*, **37**, 99 (1954), be made official.

(4) That **22.10** be changed to read as follows:

Det. N as under **2.23**. Multiply results by 6.25, or in the case of wheat grains by 5.70.

(5) That the method for crude fat or ether extract (*Direct Method—Official*), **22.25**, be changed to read:

Large quantities of sol. carbohydrates may interfere with complete extn of the fat. In such cases ext. with H_2O before proceeding with detn. Ext. ca **2** g sample, dried as under **22.3** or **22.7**, with the anhyd. ether. Use a thimble having a porosity permitting rapid passage of the solvent. The period of extn may vary from 4 hrs at ether condensation rate of 5–6 drops per min. to 16 hrs at the ether condensation rate of 2–3 drops per min. Dry ext. at 100° for 60 min., cool, and weigh.

(6) That the method for crude fat or ether extract (*Indirect Method—Official*), **22.26**, be changed to read:

Det. moisture as under **22.3** or **22.7**; then ext. dried substance as under **22.25**, and dry again. Report loss in wt as ether ext.

(7) That the method for crude fat in baked dog food (*This Journal*, **37**, 98 (1954)) be made official.

(8) That the *Acid Titration Method*, **22.53**, and the *Alkaline Titration Method*, **22.54**, for hydrocyanic acid formed by hydrolysis of glucosides in beans be made official.

(9) That the *Knapheide-Lamb Method*, **22.58–22.60**, and the *Elmslie-Caldwell Method*, **22.61**, for iodine in mineral mixed feeds be made official.

(10) That the method for Enheptin (*This Journal*, **37**, 105 (1954)) be made official.

(11) That the method for *p*-arsanilic acid (*This Journal*, **37**, 106 (1954)) be made official. (Note: Add "Applicable in the presence of nitrophenide but not sulfonamides such as sulfaquinoxaline.")

(12) That sulfaquinoxaline in poultry feeds, **22.66**, be deleted and the new procedure outlined by the Associate Referee be adopted as first action.

(13) That the method for copper in mineral feeds, as submitted by the Associate Referee, be adopted as first action.

REPORT ON SAMPLING OF FEEDS

COMPARISON OF PROBE SAMPLING DEVICES WITH HAND SAMPLING OF PELLET-GROUND FEED MIXTURES

By V. C. Midkiff and Bruce Poundstone,* *Associate Referee*
(Kentucky Agricultural Experiment Station, Lexington, Ky.)

Sampling of dairy feeds made up of pellets and ground mixtures presents a problem to industry and feed officials. It has become apparent that the customary probe sampler could not be relied upon to give a representative sample of such products. In a study of four feed sampling devices, Stout Money, and Wineburg[1] showed that double tube slotted probes and a standard single tube trier included too high a proportion of finely divided particles.

The present study was designed to consider three sizes of probes and a hand sampling procedure for sampling pellet and ground feed mixtures. Three slotted brass tube samplers, each approximately 36 inches long and closed at one end with a solid cone tip, and varying in size as shown below, were used:

Sampler No. 1—$\frac{3}{4}''$ diameter with slot $\frac{1}{2}''\times 24''$
Sampler No. 2—$1\frac{1}{4}''$ diameter with slot $\frac{7}{8}''\times 26''$
Sampler No. 3—$1\frac{1}{2}''$ diameter with 4 openings $1\frac{1}{4}''\times 2''$ along one side

These three samplers were inserted into the bag of feed with the slot or slots down and then were rotated 180° so the material would fall into the sampler by gravity. Hand samples were also taken by inserting the hand as far into the material as possible. Each core sample or hand sample was treated as a separate sample.

One hundred pounds of a ground feed was re-mixed with an auger-type mixer. The analysis of the mixture was 14.35 per cent protein, verified by 16 analyses. Fifty pounds of this mixture was then mixed with 50 pounds of a re-mixed feed composed of pellets 5/16″ in diameter. The analysis of the pelleted feed was 33.77 per cent protein, based upon 16 analyses. (This wide difference in protein values was selected so that the final results would show the greatest possible variation due to particle size discrimination.)

The resulting 100 pounds of mixture (theoretical protein value 24.06 per cent) was bagged and sampled by each of the methods described above. Samples were first taken by inserting the probes through the bag

* Acknowledgment is gratefully made to Robert Mathews for making the No. 2 Sampler, J. A. Shrader for making the No. 3 Sampler, Robert Price for assisting in preparing materials for sampling and samples for analysis, Olin Spivey for the analytical work, and the Hales & Hunter Company for supplying the ground feed and pellets for this study.

[1] Stout, P. R., Money, J. W., and Wineburg, M. P., *Cereal Chemistry*, **30**, 48 (1953).

material. After the bag was opened another set of samples was secured with the probes and an additional set by hand. Results were as follows:

	Through Bag	*End of Bag Open*
Sampler No. 1	22.38% protein	21.72% protein
Sampler No. 2	23.00% protein	23.35% protein
Sampler No. 3	22.98% protein	23.13% protein
By Hand		23.54% protein

Another mixture was made of the pellets and mixed feed in the proportion of $\frac{1}{3}$ pellets and $\frac{2}{3}$ mash (theoretical value 20.82 per cent protein). This was sampled through the bag material and by hand through the open end with the following results:

Sampler No. 1	18.97% protein
Sampler No. 2	19.89% protein
Sampler No. 3	20.06% protein
By Hand	19.92% protein

CONCLUSIONS

1. The smallest probe showed more discrimination as to particle size and samples thus secured showed protein values of little more than 90 per cent of theoretical. As shown in this and other studies, this size probe cannot be depended upon as a sampler for this type of material.

2. The large sampler (No. 3) showed improvement over the other types. It is believed that with smaller pellets (most dairy feeds use $\frac{3}{16}''$ pellets instead of $\frac{5}{16}''$) this sampler might provide a means of sampling these feeds without requiring an opening in the end of the bag large enough to insert the hand or a small scoop.

3. Hand methods of sampling seem the most satisfactory of any method tried. Close to theoretical results were achieved with hand methods.

COMMENTS

1. In no case was the theoretical value for protein reached. This was not anticipated and the original material had been destroyed before this fact became known. Therefore further studies could not be made. Workers recalled that as the stream of the mixture poured from the mixer into the the bag (even though the stream was $6'' \times 9''$ in size, and the bag was filled quickly) the mixture seemed to form a cone in the bag with the finer material at the center and the pellets tending to work to the sides. Cores, though taken diagonally, probably picked up more of the "cone" proportion of the mixture. The same was probably true of hand sampling. This lack of uniformity in the mixture may, in part at least, explain why results failed to reach theoretical values and may also indicate the difficulty of getting a sample that can truly be said to represent the mixture.

2. Field conditions were not duplicated in this study, in that the bags

of feed were not trucked or otherwise handled. This study was limited to the effectiveness of sampling a known sample by different methods. The only resemblance to field conditions was that the product was bagged and that most of the cores were taken through the bagged material.

3. Because of the nature of this type of material it may always be desirable to get a fairly large sample, larger than is normally taken, in order to be representative of the total product. If only one bag is sampled, cores should be taken from different positions in the bag and a special effort should be made to take some cores next to the edge. If cores are taken from each of several bags the operator should make certain that cores are taken from different positions in the various bags, with some coming from near the edge.

It is recommended† that a collaborative study be undertaken on a commercially prepared dairy feed consisting of pellets, ground materials, and flakes, as well as on a feed made up of pellets and ground material only.

REPORT ON MINERAL CONSTITUENTS OF MIXED FEEDS

COPPER DETERMINATION IN MINERAL FEEDS

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

This report is a continuation of the work started last year[1] wherein tetraethylene pentamine is used to develop the copper color-complex. The sample distributed was a typical mineral feed and contained, in addition to copper, the approximate percentages of the following: Ca, 23.4; P, 9.3; Co, 0.013; Mn, 0.078; Fe, 0.88; and NaCl, 7.8.

Twenty-six collaborators reported average results (Table 1).

The method is simple and fast, and checks by individual analysts are good. The procedure will adapt to control work on a mass production basis, and results will come within an acceptable tolerance for accuracy.

Each analyst was asked to compare results obtained with fresh tetraethylene pentamine with results achieved by using the reagent purchased one year previously. Some found no change in the reagent. Others reported a wide difference in results (Table 1) when the old reagent was used. Conclusions are that the reagent changes after being opened, but if it is kept in dark glass bottles, tightly closed, and in the dark, no significant deterioration will occur within one year, and results will be accurate if the same reagent is used for the color development of standards and unknown.

It is recommended* that the method be made official.

---

† For report of Subcommittee A and action of the Association, see *This Journal*, 38, 62, 63 (1955).

[1] *This Journal*, 37, 246 (1954). The procedure is adapted from the method described by T. B. Crumpler *Anal. Chem.*, 19, 325 (1947).

* For report of Subcommittee A and action of the Association see *This Journal*, 38, 62, 63 (1955)

TABLE 1.—*Collaborative results for copper*

COLLABORATOR NO.	NEW AMINE	1 YEAR OLD AMINE	COLLABORATOR NO.	NEW AMINE	1 YEAR OLD AMINE
	per cent	*per cent*		*per cent*	*per cent*
1	0.226	0.232	14	0.244	0.243
2	.250	.260	15	.269	.268
3	.260	.321	16	.241	.244
4	.245		17	.231	.244
5	.237		18	.263	
6	.243		19	.241	.218
7	.245	.240	20	.231	.228
8	.235	.240	21	.249	
9	.238		22	.244	
10	.250		23	.245	
11	.241		24	.274	.370
12	.250		25	.244	
13	.217	.200	26	.214	

Average: 0.243% Cu
Mean Deviation: 0.0096%
Standard Deviation: 0.013%

COPPER DETERMINATION IN MINERAL FEEDS

REAGENTS

(a) *Tetraethylene pentamine.*

(b) *Copper sulfate.*—$CuSO_4.5H_2O$, reagent grade.

STANDARDS

Dissolve 1.9645 g $CuSO_4.5H_2O$ in H_2O and dil. to 500 ml (1 ml = 1 mg Cu). Use from 1 to 10 ml of this soln to prep. set of stds in 100 ml Pyrex glass-stoppered vol. flasks. Add 4 ml HCl, dil. to 50 ml, add 5 ml tetraethylene pentamine, dil. to vol. with H_2O, stopper, and mix thoroly. Prep. blank, using all reagents except Cu. Filter blank and stds before reading.

DETERMINATION

Ash 8 g sample 2 hrs at 600° and transfer to 200 ml vol. flask with 20 ml HCl and 50 ml H_2O. Boil 5 min., dil. to vol., and mix thoroly. Allow soln to settle. (Aliquots may be taken from this soln for detn of Ca, P, and Co.) Pipet 50 ml aliquot into 100 ml glass-stoppered vol. flask, add 5 ml tetraethylene pentamine, dil. to vol. with H_2O, and mix thoroly. Filter soln before comparing in colorimeter or spectrophotometer with stds. Read color within 30 min., using red (No. 66) filter, or if using spectrophotometer, a wavelength of 620 mμ. Report % Cu to third place to right of decimal (.000%).

COMMENTS OF COLLABORATORS

Collaborator 8.—"I found that standard curves prepared this year with both old and new reagents agreed within experimental error with the standard curve prepared last year. I think this method is worthy of consideration as an A.O.A.C. method for copper."

Collaborator 9.—"A new curve was determined for the old reagent. It was different from that of the new reagent but results were the same."

Collaborator 11.—"No difference was obtained with tetraethylene pentamine which had been opened about one year previously."

Collaborator 14.—"It would not appear that the reagent decomposes appreciably if it is kept in a stoppered bottle."

Collaborator 15.—"It appears that even though there is some decomposition, if the same tetraethylene pentamine is used for the standard solutions and samples the results will be correct. The method appears to be very satisfactory."

Collaborator 17.—"Two determinations were made with last year's tetraethylene pentamine stock, and the results were referred to a standard curve produced with the same reagent. The material was much more difficult to handle, and duplication of points on the curve was difficult. We find that addition of the tetraethylene pentamine is best accomplished by the use of a glass syringe (no needle) rather than the suggested graduated cylinder."

Collaborator 20.—"(1) A definite but small change in tetraethylene pentamine (TP) does occur after exposure to air and light as shown by comparison of the absorbance of the old stock reagent when read against a reagent blank containing fresh reagent. (2) This change occurs fairly rapidly after initial exposure but stability occurs at a value which apparently doesn't change further under ordinary working conditions. (3) Over the range of 10 to 100 micrograms of copper, standard curves prepared with the old and with the fresh reagent showed no variation in the linearity and absorbances. This holds true only when a large excess of TP (at least 5 ml) is present. When sufficient excess of TP is not present, the curve is lowered."

Collaborator 21.—"Results could not be duplicated by use of polarograph."

Collaborator 24.—"The old reagent gives higher results."

COLLABORATORS

(The order of listing of results has no bearing on the order of listing of collaborators.)

Loyd L. Nesbitt, Chemist & Assistant Director, Lime Crest Research Laboratory, Newton, N. J.

Ralph M. Morgan, Department of Agriculture and Immigration, Division of Chemistry, 1123 State Office Building, Richmond 19, Va.

J. C. Edwards, Department of Agriculture, Chemical Division, Tallahassee, Fla.

O. U. Walling, Mississippi State Chemical Laboratory, State College, Miss.

E. D. Schall, Department of Agricultural Chemistry, Purdue University, Agricultural Experiment Station, Lafayette, Ind.

Curtis L. Godfrey, Associate State Chemist, Texas Agricultural Experiment Station, Agricultural and Mechanical College of Texas, College Station, Texas

L. E. Bopst, State Chemist, Inspection and Regulatory Service, College Park, Md.

George F. Rickey, Division of Food Science and Technology, New York Agricultural Experiment Station, Cornell University, Geneva, N. Y.

R. T. Wetherbee, Chemist in Charge, Regulatory Service, University of Vermont and State Agricultural College, Burlington, Vt.

Graeme L. Baker, Department of Chemistry Research, Montana State College, Agricultural Experiment Station, Bozeman, Mont.

W. C. Geagley, Chief Chemist, Bureau of Chemical Laboratories, Department of Agriculture, Lansing, Mich.

Earl S. Packard, University of Maine, Agricultural Experiment Station, Orono, Me.

Harry J. Fisher, Chief Chemist, Connecticut Agricultural Experiment Station, New Haven, Conn.

Sherman Squires, Connecticut Agricultural Experiment Station, New Haven, Conn.

V. C. Midkiff, University of Kentucky, Agricultural Experiment Station, Lexington 29, Ky.

C. A. Metzger, Department of Agriculture and Inspection, Lincoln, Neb.

Charles V. Marshall, Superintendent of Laboratory, Plant Products Division, Department of Agriculture, 79 Sussex Street, Ottawa 2, Ontario

L. A. Koehler, State Laboratories Department, Box 900, Bismarck, N. D.

Van P. Entwistle, Supervising Feed Chemist, Feed Laboratory, Department of Agriculture, Sacramento 14, Calif.

James E. Launer, Department of Agriculture, Salem, Ore.

C. O. Gourley, Chief Chemist, The Beacon Milling Company, Inc., Cayuga, N. Y.

E. H. Probst, Cooperative G. L. F. Exchange, Inc., Mills Division, Buffalo, N. Y.

D. J. Mitchell, State Chemist, State Chemical Laboratory, Vermillion, S. D.

C. Tyson Smith, Regulatory Service, University of Massachusetts, Agricultural Experiment Station, Amherst, Mass.

John F. Wintermantel, Products Control Department, General Mills, Inc., 1081 Twenty First Avenue S. E., Minneapolis 14, Minn.

W. B. Griem and D. N. Willett, Feed and Fertilizer Section, Biochemistry Building, Department of Agriculture, Madison 6, Wis.

Leo J. Faneuf, New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick, N. J.

REPORT ON CRUDE FAT IN FEEDS

By HAROLD H. HOFFMAN (Florida Department of Agriculture, Tallahassee, Fla.), *Associate Referee*

CRUDE FAT OR ETHER EXTRACT

Thirty-nine feed control officials from Canada, Hawaii, and various states completed detailed questionnaires on crude fat in feeds in 1952. More than half of the reporting laboratories deviated from the 16 hour low-heat method on some or all of their analyses. Another questionnaire sent out this year brought responses from 28 laboratories and again it appeared that more than half made use of a short method. The 4 hour high heat extraction on the Goldfisch apparatus was the most popular modification.

Several state and federal control officials have asked that the shorter method be officially recognized. The General Referee on feeding stuffs has recommended that the difference between the official and the 4 hour high-heat methods be studied collaboratively with the thought that it might be possible to utilize the latter as an alternate method.

Previous collaborative comparisons of these methods were summarized in a previous report of the Associate Referee.[1]

COLLABORATIVE WORK

Feed control officials were invited to participate in this study by performing fat determinations by the two procedures on as many of their

[1] HOFFMAN, H. H., *This Journal*, **34**, 558 (1951).

official samples as possible. It was requested that the same equipment and quality of ether be used for comparing methods on any given sample and that solvent condensation rates would approach those obtained by low and by high heat on the Goldfisch extractor. All extractions were to be made on samples previously dried according to sections **22.3** or **22.7.**

Thirteen laboratories sent in comparative results on a total of 1,806 samples. Collaborators reported the average differences by the two methods for various types of feeds as shown in Table 1.

DISCUSSION

The fat averaged 0.072 per cent higher by the 16 hour low heat method. Collaborators 5 and 10 obtained slightly higher results by the 4 hour high heat extraction.

When grouped by type of feed, only ground hays and rabbit feeds averaged more fat by the shorter method.

Reporting only average analyses for groups of samples probably concealed wide differences on single samples. Collaborator 8 mentioned large differences in some cases.

Collaborator 9 recommended use of paper thimbles to accommodate rapid solvent passage as he found Alundum tubes unsatisfactory for high heat.

Because of the wide acceptance of the 4 hour high-heat extraction it appears that the slightly lower results obtained thereby should not serve as a deterrent to official recognition of this procedure. The solvent condensation rates required by the proposed changes will not limit the selection of apparatus to one make.

Although not covered by this collaborative study the intermittent 30 minute drying intervals have been replaced by a single 60 minute drying period. Answers on the 1952 questionnaire previously referred to indicated that only one laboratory out of 37 used the alternate weighing and drying of the extract or residue.

It is proposed that sections **22.25** and **22.26** be changed to read as follows:

22.25 DETERMINATION

Large quantities of sol. carbohydrates may interfere with complete extn of the fat. In such cases ext. with H_2O before proceeding with detn. Ext. ca 2 g sample, dried as under **22.3** or **22.7**, with the anhyd. ether. Use a thimble having a porosity permitting rapid passage of the solvent. The period of extn may vary from 4 hrs at the ether condensation rate of 5–6 drops per min. to 16 hrs at the ether condensation rate of 2–3 drops per min. Dry ext. at 100° for 60 min., cool, and weigh.

22.26 *Indirect Method—Official*

Det. moisture as under **22.3** or **22.7**; then ext. dried substance as under **22.25**, and dry again. Report loss in wt as ether ext.

TABLE 1.—*Average fat differences in feeds run by official and 4 hour high heat extraction methods*[a]

TYPE OF FEED	COLLABORATOR (average differences in per cent fat)													AVERAGE BY TYPE
	1	2	3	4	5	6	7	8	9	10	11	12	13	
Dairy cattle	.08 (37)	.11 (33)		.09 (5)	.02 (36)	.23 (38)	.46 (5)	.11 (81)	.03 (27)	.03 (90)	.01 (14)	.09 (60)	.37 (35)	.108 (461)
Dog food					.27 (5)	+.06 (1)			.01 (8)	+.33 (6)	.05 (5)	.31 (20)		.130 (45)
Horse Mule				+.16 (5)		.10 (1)		.13 (3)	.06 (4)	.02 (3)	.23 (4)		.08 (3)	.050 (23)
Poultry Turkey	.14 (92)	.15 (67)		+.19 (5)	+.13 (36)	.10 (63)	.23 (5)	+.04 (66)	.05 (17)	+.07 (128)	+.04 (13)	.06 (60)	.07 (12)	.032 (564)
Rabbit		.12 (7)		+.12 (5)		.11 (4)			+.03 (5)	+.01 (7)	+.06 (2)	+.04 (12)		+.003 (42)
Swine	.11 (10)			.02 (5)	+.03 (36)	.07 (15)	.13 (5)	+.17 (8)	.02 (4)	.00 (30)	.09 (6)		.18 (16)	.029 (135)
Animal products	.20 (15)		.09 (1)	.90 (3)	.04 (21)	.11 (3)	.39 (4)	.06 (1)	.04 (5)	.06 (12)		.33 (30)	.04 (2)	.201 (97)
Cereal Grains	+.15 (1)	.19 (17)		+.30 (5)					+.07 (5)	.11 (30)	+.05 (5)			.068 (63)
Ground hays	+.27 (19)		+.03 (3)	+.41 (5)		.39 (2)	+.29 (4)		+.03 (5)	.04 (14)	+.02 (1)	.09 (20)		+.075 (73)
Oil meals	.06 (82)		.03 (2)	.15 (5)	.25 (8)	.09 (14)	+.01 (5)	.29 (1)	.03 (6)	.03 (51)	+.18 (2)	.14 (36)	.27 (2)	.076 (214)
Wheat feeds	.14 (11)			.33 (5)		.09 (1)	.17 (4)		.04 (3)	.31 (2)		.24 (12)		.199 (38)
Misc.			.13 (2)	.91 (9)	.28 (1)	.09 (10)	.04 (4)	.01 (7)		+.04 (18)				.179 (51)
Average By Collaborator	.079 (267)	.143 (124)	.040 (8)	.139 (57)	+.004 (143)	.131 (152)	.147 (36)	.034 (167)	.021 (89)	+.006 (391)	.011 (52)	.137 (250)	.250 (70)	.072 (1806)

[a] Figures in parentheses indicate number of samples. Plus (+) values indicate higher fat by 4 hour high heat.

CRUDE FAT IN BAKED DOG FOOD

Collaborative study on this subject was not carried out in 1954. However, minor editorial changes have been made in the method, adopted as first action in 1953, as follows:

On page 98, Vol. **37** (1954), change line 10 of the method to read, " . . . 25 ml petr. ether (b.p. below 60°). Stopper and again . . . "

In line 15, substitute "digested charge" for "liquid."

In line 22, substitute "air" for "desiccator."

In line 23, after "weighed," add "against a counterpoise similarly treated."

In line 24, insert "at 100°" after "dry."

In line 24, substitute "air" for "desiccator" and after "weigh," add "against a counterpoise similarly treated . . . "

In line 25, change last sentence to read, "Correct this wt by blank detn on reagents used."

RECOMMENDATIONS

It is recommended* that Sections **22.25** and **22.26** be changed to read as above and be continued as official. It is further recommended that the method for crude fat in baked dog food (*This Journal*, **37**, 98 (1954)), be changed as above and be made official.

COLLABORATORS

Grateful acknowledgment is made to the following who participated in this study (the order does not agree with the listing in the table).

Richard S. Harding, Colorado Department of Agriculture, Denver, Colo.

Helen Kocaba and Harry J. Fisher, Connecticut Agricultural Experiment Station, New Haven, Conn.

Y. Kawano and G. Donald Sherman, University of Hawaii, Honolulu, T. H.

Ronald F. Larsen, Feed and Fertilizer Laboratory, Boise, Idaho.

E. D. Schall, Purdue University, Lafayette, Ind.

Marvin L. Schreiber, Kansas State Board of Agriculture, Topeka, Kan.

V. C. Midkiff, Kentucky Agricultural Experiment Station, Lexington, Ky.

Edwin R. Jackson and M. P. Etheredge, State College, Miss.

Leo J. Faneuf, New Jersey Agricultural Experiment Station, New Brunswick, N. J.

Alfred W. Avens, New York Agricultural Experiment Station, Geneva, N. Y.

Willis Richerson, Oklahoma State Department of Agriculture, Oklahoma City, Okla.

D. J. Mitchell, State Chemical Laboratory, Vermillion, S. D.

Curtis L. Godfrey, Texas Agricultural Experiment Station, College Station, Texas.

* For report of Subcommittee A and action of the Association, see *This Journal*, **38**, 62, 63 (1955).

REPORT ON DRUGS IN FEEDS

DETERMINATION OF SULFAQUINOXALINE

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

The official method for sulfaquinoxaline was compared collaboratively with a new method devised to recover more of the drug from old feeds. The study revealed that a gain of approximately 10 per cent in drug recovery from old feeds is possible with the new method. The new method also permits the co-determination of arsanilic acid when the arsenical is present with sulfaquinoxaline in the same feed.

Analysts have complained that the official method does not provide for a feed blank and that it does not always recover all the sulfaquinoxaline known to have been added. It has also been reported that as feeds age, less drug is recovered. Unground feeds assay slightly higher than the same feeds after grinding. And while unground feeds decline in recoverability of the drug at a slow rate, ground feeds sometimes show a rapid decline.

Another aspect of drug loss is known. In a communication accompanying his report Collaborator W. R. Flach observed loss of sulfaquinoxaline due to the heat caused by pelleting feeds. (His study of the two methods is more extensive than those of other collaborators and shows a noticeable gain in recoverability with the collaborative method.) Either the drug undergoes alteration through oxidation or some of it becomes "bound" to feed protein so that the official method fails to release all of it from old feeds.

The possibility that a proteolytic enzyme may free sulfaquinoxaline from a protein complex led Szalkowski and Mader[1] to study the effect of ficin in the treatment of feed solutions. Results on all feeds were higher than by the official method. Aside from use of the enzyme, two essentials of the ficin procedure are the use of stronger alkali to dissolve the drug and of HCl to precipitate protein.

Shortly after the ficin procedure was advanced the Associate Referee collaborated with these authors on several old composite feeds. In some cases, old samples run by the enzyme method yielded about 10 per cent more of the drug than when analyzed by the official method. However, in almost all cases the ficin procedure gave practically the same results when the enzyme was omitted.

Studies of tryptophane and its ability to couple with N-1-naphthylethylenediamine[2] have led to greater caution in selecting methods for diazotizable drugs. Extensive protein hydrolysis, whether by strong

[1] Szalkowski, C. R., and Mader, W. J., *This Journal*, **37**, 544 (1954).
[2] Merwin, R. T., *ibid.*, **37**, 257 (1954).

alkali or by a proteolytic enzyme, must be avoided in the determination of sulfaquinoxaline.

Since all feed solutions, when hydrolyzed, have varying quantities of diazotizable tryptophane, the conditions and extent of maximum interference were studied. Results of many experiments proved that several factors increased the possibility of interference. These were: (1) Extensive or prolonged feed hydrolysis, (2) prolonged or strong diazotization, and (3) mild acidity during diazotization. These conditions are avoided in the collaborative method and amino acid interference is greatly minimized.

As a result of extensive experiments the writer concluded that the enzyme was not essential, but that the ficin procedure itself had great merit. The essential difference between the official and the ficin methods is the means taken to remove protein from alkaline feed solutions. Experimental studies on old feeds indicated that the lower yield by the official method might be due to adsorption of the drug by the zinc hydroxide precipitate at the nearly neutral *p*H of filtration. The Associate Referee found that practically the same effect as that produced by a proteolytic enzyme could be obtained by a simple heating of the alkaline feed solution instead of the cold extraction process of the ficin procedure.

Last year it was reported[2] that tryptophane interference may be detected by its characteristic of exhibiting slowly increasing absorbance during spectrophotometric readings. Very slight increases on some feeds may occasionally be detected if alkaline hydrolysis is prolonged and if instrument readings are delayed 20 to 30 minutes after the ten minute period specified for color development. In studying the possibility of tryptophane interference, it has been noted many times that unmedicated feed blanks cannot validly be deducted as color blanks when the collaborative method is strictly followed. Tryptophane couples slowly in the absence of sulfaquinoxaline but even more slowly in its presence.

COLLABORATIVE METHOD FOR SULFAQUINOXALINE

REAGENTS

(a) *Sodium nitrite soln.*—0.10%. Prep. fresh.

(b) *Ammonium sulfamate soln.*—0.50%.

(c) *N-1-naphthylethylenediamine dihydrochloride soln.*—0.10%. Store in dark bottle.

DETERMINATION

To 5 g ground feed in a 250 ml vol. flask, add 150 ml H_2O and 5 ml 0.5 *N* NaOH, and place in a boiling H_2O bath for 15 min. Remove, cool, dil. to vol. with H_2O, mix, and let feed settle. Transfer 50 ml of the supernatant liquid to a 100 ml vol. flask, add 3 ml concd HCl, and dil. to vol. Mix and filter thru 18.5 cm Whatman No. 2 paper or equiv., discarding first 15 ml filtrate if turbid.

To 10 ml filtrate in two 50 ml beakers, add 2 ml sodium nitrite soln and let stand 3 min. Add 2 ml ammonium sulfamate soln and let stand 2 min. Finally add 1 ml of the coupling reagent to the first beaker and 1 ml H_2O to the second beaker. Mix solns thoroly after adding each reagent. Let stand 10 min.; then read absorbance in

spectrophotometer at 545 mμ. Deduct feed blank from sample absorbance and refer to std graph for quantity of drug. Divide by 1000 for percentage.

SULFAQUINOXALINE STANDARDS

Add 5 ml 0.5 *N* NaOH and 50 ml H_2O to 0.250 g pure sulfaquinoxaline in 500 ml vol. flask. Dissolve and dil. to vol. with H_2O. Place 5 ml in 100 ml vol. flask and dil. to vol. with H_2O.

To 100 ml vol. flasks add 2, 4, 6, 8, and 10 ml dild std, add 3 ml concd HCl, and dil. to vol. with H_2O.

Treat 10 ml aliquots of final dilns with same quantities and strengths of reagents as in method and take readings, corresponding to 5, 10, 15, 20, and 25 micrograms sulfaquinoxaline, against H_2O blank. Plot on graph paper.

NOTES.—Strict precautions should be observed in using the method. Time limits should not be exceeded, and the strengths of the various reagents should be those specified. Spectrophotometric readings should not be delayed much longer than the 10 minute period for color development. Of equal importance is the necessity of using clean cells and of discarding readings taken with nitrogen gas formation on cell walls. This condition leads to the greatest source of error in lack of reproducibility in absorbance measurements.

COLLABORATIVE STUDY

Copies of the method were sent to collaborators with the request that they choose a sulfaquinoxaline feed sample, preferably two to three months old, which they had found low in guarantee. They were also asked to submit four assays, run concurrently, by the official and the collaborative methods. By letting the collaborators select their own feeds, a greater variety of comparisons was thus obtained. Their results are shown in Table 1.

It is seen that all the collaborators except one obtained higher recoveries of sulfaquinoxaline by the collaborative method. The average increase for all is 9.80 per cent. Collaborator No. 10 reported a gain of 24 per cent, whereas No. 3 reported a slight loss. It is apparent that results vary according to the type of feed formulation, its age, the level of the guarantee, conditions of storage, and variations in technique. In two cases guarantees were exceeded, though only slightly. Guarantees were not reported for three feeds.

A year's experience with laboratory processed feeds showed that the method did not yield more than the amount of drug added.

COMMENTS OF COLLABORATORS

No. 2.—"Preliminary treatment with ficin solution before proceeding with the collaborative method yielded 0.0193 and 0.0181 per cent averages, or 6.6 per cent and 9 per cent more respectively than by the collaborative method."

No. 3.—"Filtration was very slow." (See comment of collaborator No. 9.)

No. 4.—One determination by the ficin procedure was included. The figure, 0.0061 per cent, is 9 per cent lower than his average for the collaborative method.

No. 5.—"You will note the pelleted feed is lower even though the guaranteed level is the same as the mash feed type. This would indicate that there is some loss of sulfaquinoxaline due to the heat of pelleting."

TABLE 1.—*Comparison of sulfaquinoxaline methods*

COLLABORATOR	GUARANTEE	A.O.A.C. 22.66	COLLABORATIVE METHOD	COLLABORATOR	GUARANTEE	A.O.A.C. 22.66	COLLABORATIVE METHOD
		(per cent)	*(per cent)*	5		.0128, .0130	.0138, .0143
		.0158, .0157	.0163, .0161	(cont'd)	.0175	.0130, .0130	.0143, .0143
1	.0175	.0157, .0160	.0157, .0160		(pellets)	av = .0130	av = .0142
		av = .0158	av = .0160				
						.0138, .0138	.0156, .0156
		.0147, .0164	.0174, .0183		.0175	.0140, .0138	.0162, .0163
		.0164, .0164	.0183, .0183		(pellets)	av = .0139	av = .0159
		.0154	.0182				
2		av = .0159	av = .0181			.0133, .0128	.0140, .0145
					.0175	.0130, .0128	.0138. .0143
		.0139, .0140	.0165, .0172		(pellets)	av = .0130	av = .0142
		.0140, .0160	.0171, .0159				
		.0130	.0161	6	.05	.0335, .0350	.0400, .0405
		av = .0142	av = .0166			.0360, .0360	.0405, .0400
						av = .0351	av = .0403
		.0154, .0153	.0153, .0149				
3	.0161	.0157, .0154	.0151, .0154	7	.015	.0090, .0092	.0100, .0098
		av = .0155	av = .0152			.0084, .0091	.0096, .0098
						av = .0089	av = .0098
		.0051, .0052	.0072, .0054				
4	.0125	.0054, .0054	.0070, .0071	8	.0125		.0108, .0108
		av = .0053	av = .0067				.0115, .0115
							av = .0112
		.0165, .0163	.0175, .0170				
5	.0175	.0160, .0165	.0170, .0173	9		.0174, .0179	.0197, .0197
	(mash)	av = .0163	av = .0172			.0186, .0181	.0198, .0200
						av = .0180	av = .0198
		.0175, .0180	.0180, .0180				
	.0175	.0178, .0178	.0185, .0182	10	.0175	.0095, .0100	.0118, .0122
	(mash)	av = .0178	av = .0182			.0095, .0100	.0121, .0126
						av = .0098	av = .0122
		.0173, .0173	.0185, .0185				
	.0175	.0173, .0170	.0183, .0180				
	(mash)	av = .0172	av = .0183				

Grand average (omitting No. 8)	.0153	.0168

Increase of collaborative method over AOAC method = 9.8%.

No. 8.—"Blanks on the same feed are variable." (A very slight variation in feed blanks is normal.) "We would suggest that emphasis be shifted to a method which will determine sulfaquinoxaline and arsanilic acid in the same sample." (The collaborative method can be extended to serve such a purpose.)

No. 9.—Better precision was experienced by the collaborative method. "We also found that Celite filter aid produced rapid filtering and very clear filtrates."

The one disadvantage of the method acknowledged by all who have tried it is the slow filtration after protein precipitation. The use of Celite filter aid suggested by Collaborator Turner is a valuable contribution in shortening filtration time and avoiding the possibility of turbidity.

ARSANILIC ACID DETERMINATION

The incorporation into feeds of arsanilic acid, anhydrous sodium arsanilate, or sodium arsanilate.$4H_2O$ mixed with sulfaquinoxaline

makes development of a method for the two drugs desirable. Richard O. Brooke and Collaborator John Reid, of the Wirthmore Research Laboratory, Malden, Mass., remove sulfaquinoxaline from a clarified solution of the two drugs with ether, leaving arsanilic acid for direct determination. Sulfaquinoxaline is determined by noting the total absorbance produced by the coupled drugs, subtracting the absorbance due to arsanilic acid, and applying the difference for evaluation of the first drug.

In the same way, the collaborative method can be used for dual drug assays. After noting the total absorbance in the usual way, 35 ml of the clear filtrate is placed in a separatory funnel and extracted three times with 50 ml portions of ether. The aqueous layer is transferred to an Erlenmeyer flask and a stream of air is passed through it for 10 minutes to remove dissolved ether. Two 10 ml portions of this solution are then treated as in the regular sulfaquinoxaline assay, one portion serving as a feed blank. Readings of arsanilic acid absorbance are taken at 545 mμ and referred to standards for arsanilic acid prepared in the same manner as the sulfaquinoxaline standards. Total absorbance minus arsanilic acid absorbance yields absorbance due to sulfaquinoxaline.

This procedure has been used successfully in the author's laboratory but the exact details for separation of the drugs were not worked out in time for collaborative study.

ACKNOWLEDGMENT

The Associate Referee is indebted to the following collaborators, whose names are in order of tabulation, for their very efficient help:

Harold H. Hoffman, Feed Laboratory, State Department of Agriculture, Tallahassee, Fla.

W. J. Mader, Analytical Research Dept., Chemical Control Div., Merck & Co., Rahway, N. J.

John Reid, Wirthmore Research Laboratory, Malden, Mass.

Charles Weber, New Jersey Agricultural Experiment Station, New Brunswick, N. J.

W. R. Flach, Eastern States Farmers' Exchange, Buffalo, N. Y.

C. A. Luhman, State Department of Agriculture, Sacramento, Calif.

Sigmund W. Senn, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

Charles V. Marshall, Department of Agriculture, Ottawa, Ontario, Canada

James N. Turner, The Park & Pollard Co., Buffalo, N. Y.

Earl S. Packard, University of Maine, Agricultural Experiment Station, Orono, Me.

RECOMMENDATIONS

It is recommended*—

(1) That the official method (**22.66**) for sulfaquinoxaline be deleted, first action.

(2) That the collaborative method for sulfaquinoxaline, herein described, be adopted as first action.

* For report of Subcommittee A and action of the Association, see *This Journal*, **38**, 62, 63 (1955).

(3) That the method for Enheptin ®, *This Journal*, **37**, 105 (1954), be adopted as official.

(4) That the method for *p*-arsanilic acid, *This Journal*, **37**, 106 (1954), be adopted as official with the heading: "Applicable in the presence of nitrophenide but not sulfonamides such as sulfaquinoxaline."

(5) That studies of methods for nitrophenide be continued.

(6) That a method for arsanilic acid or sodium arsanilate in sulfaquinoxaline feeds be studied.

(7) That studies of methods for nitrosal, nitrofurazone, and furazolidone be assigned to separate Associate Referees.

REPORT ON GOSSYPOL IN FEEDS

EFFECT OF VARIATION IN SAMPLE WEIGHTS AND ALIQUOTS ON THE DETERMINATION OF FREE GOSSYPOL

By CARROLL L. HOFFPAUIR, *Associate Referee*, and WALTER A. PONS, JR.
(Southern Utilization Research Branch, Agricultural Research Service, U. S. Department of Agriculture, New Orleans 19, La.)

Growing interest in the use of cottonseed meal in mixed feeds for swine and poultry has focused attention on the need for a reliable analytical method for estimating the free gossypol content of such feeds (1). Since the concentration of cottonseed meal in typical mixed feeds will vary from about 10 to 20 per cent by weight, it is apparent that fairly large sample weights, large sample aliquots, or both will be required.

Although satisfactory analytical methods are available for the determination of free gossypol in cottonseed meals (2, 3), several problems arise in connection with the application of these methods to mixed feeds. These involve the extraction of gossypol from the sample, the effect of larger sample aliquots, and the presence of interfering constituents in mixed feeds. Since it is necessary to establish suitable conditions for extraction before undertaking a study of possible interferences, the influence of variation in sample weight, aliquot volume, and temperature of color development on the determination of gossypol was studied.

Accepted methods (2, 3, 4) for the analysis of cottonseed meal, as well as a recently proposed procedure for mixed feeds (5), specify extraction of the free gossypol with 50 ml of solvent in a 250 ml flask by agitating for one hour on a mechanical shaker. This type of extraction offers several advantages. It is simple and reproducible and also minimizes hydrolysis of the "bound" gossypol which is present in cottonseed meals. Consequently this extraction procedure was used throughout this investigation, and aliquots of the filtered extracts were reacted with *p*-anisidine (2, 3) or with aniline (4) for the determination of free gossypol.

TABLE 1.—*Effect of sample weight and aliquot on the determination of free gossypol in cottonseed meal with p-anisidine at 60°C.*

ALIQUOT	FREE GOSSYPOL[a]		
	1 G SAMPLE	3 G SAMPLE	5 G SAMPLE
ml	*per cent*	*per cent*	*per cent*
2	0.0320	0.0285	0.0261
5	0.0310	0.0262	0.0235
10	0.0291	0.0233	——

[a] Cottonseed meal extracted with 70 per cent acetone.

Preliminary results using 70 per cent acetone for extraction and employing the *p*-anisidine reaction at 60°C. for color development (2) (modified by the use of thiourea in the reagent to prevent excessive reagent blanks) indicated that either increasing the sample weight above 1 gram or use of sample aliquots larger than 2 ml gave low results. Typical data are shown in Table 1. However, as shown in Table 2, when pure gossypol was dissolved in 70 per cent acetone, adequate recovery was obtained with aliquots varying from 2 to 10 ml in volume. This suggests that the reaction of the gossypol pigments in the meal extracts with *p*-anisidine at 60°C. was slower than that of pure gossypol under the same conditions. A modified color development procedure involving addition of thiourea to the *p*-anisidine reagent and the use of 80°C. for the color development reaction was tried. Details of this modified *p*-anisidine procedure are as follows:

METHOD

REAGENTS

(a) *p-Anisidine reagent.*—Dissolve 1 g recrystd *p*-anisidine and 0.2 g thiourea in 98% isopropyl alcohol, add 1 ml glacial acetic acid, and dil. to 100 ml with 98% isopropyl alcohol.

(b) *Acetic acid soln.*—Dil. 1 ml glacial acetic acid to 100 ml with 98% isopropyl alcohol.

TABLE 2.—*Recovery of gossypol from aliquots of solution of pure reagent in 70 per cent acetone by reaction with p-anisidine at 60°C.*

STANDARD SOLUTION	GOSSYPOL	
	PRESENT	FOUND
ml	*mg*	*mg*
2	0.010	0.010
2	0.025	0.024
5	0.025	0.024
5	0.060	0.060
5	0.125	0.124
8	0.200	0.196
10	0.050	0.049
10	0.125	0.124

DETERMINATION

Pipet duplicate aliquots of the aq. acetone ext. (aliquots can vary from 2 to 10 ml) into 25 ml vol. flasks. To one flask add 5 ml of the acetic acid soln and dil. to vol. with 80% isopropyl alcohol. To the other flask add 5 ml of the *p*-anisidine reagent and heat in H_2O bath at 80°C. for 45 min. Cool and dil. to vol. with 80% isopropyl alcohol. Prep. a reagent blank contg the same vol. of aq. acetone as used for the sample aliquot and process as for the sample aliquot.

The concentration of gossypol in the sample aliquot is determined as previously reported (2). Calibration curves for pure gossypol solutions are identical for the *p*-anisidine reaction whether conducted at 60°C. or at 80°C.

Values obtained by applying the modified procedure to aliquots of several meal extracts are reported in Table 3. It can be seen that essentially constant values were obtained for sample aliquots varying from 2

TABLE 3.—*Effect of aliquot volume in the determination of free gossypol in cottonseed meals*

SAMPLE	ALIQUOT OF EXTRACT	FREE GOSSYPOL	
		p-ANISIDINE REACTION (80°C.)	ANILINE REACTION (100°C.)
	ml	*per cent*	*per cent*
A	2	0.0356	0.0353
	5	0.0355	0.0350
	10	0.0348	0.0346
B	2	0.0295	0.0302
	5	0.0300	0.0299
	10	0.0278	0.0287
C	1	0.0310	——
	2	0.0312	0.0292
	3	0.0309	——
	5	0.0300	0.0277

to 10 ml. The use of aniline at 100°C. for color development, proposed recently by Miller (4), was also found to give constant values when applied to varying aliquots. Analyses of several types of cottonseed meals by both colorimetric procedures are shown in Table 4. These data indicate that comparable values are obtained by either colorimetric reaction.

When it was established that the modified *p*-anisidine or the aniline procedure permitted the use of larger aliquots of the extract, the effect of variation in weight of sample extracted was investigated. With the use of a commercial prepress-solvent extracted meal of low free gossypol content, samples ranging from 0.250 to 5.000 grams were extracted with both 70 per cent aqueous acetone and 90 per cent methyl ethyl ketone.

TABLE 4.—*Comparison of values for free gossypol in cottonseed meals by reaction with p-anisidine at 80°C. and with aniline at 100°C.*

TYPE OF MEAL	FREE GOSSYPOL[a]	
	p-ANISIDINE REACTION (80°C.)	ANILINE REACTION (100°C.)
	per cent	*per cent*
Prepress-solvent	0.0335	0.0350
Hydraulic-pressed	0.0937	0.0940
Screw pressed	0.0300	0.0314
Prepress-solvent	0.0328	0.0353
Prepress-solvent	0.0698	0.0700

[a] Samples extracted with 70 per cent acetone.

In each case 50 ml of solvent and a 1-hour extraction period were used. Free gossypol was determined by measuring the color produced when aliquots of the extracts were reacted with *p*-anisidine at 80°C. as well as with aniline at 100°C. The results (Table 5) show that sample weight has a definite effect on the extraction of the gossypol pigments. Similar results were obtained by using a 2-hour extraction period, indicating that equilibrium was attained in the shorter period. It is noted that as the sample weight increases the value obtained for gossypol decreases. It appears that for this type of extraction the use of samples larger than 1 gram introduces significant errors.

SUMMARY AND RECOMMENDATION

The application of accepted methods for the determination of free gossypol in cottonseed meal to the analysis of mixed feeds requires the

TABLE 5.—*Influence of sample weight on determination of free gossypol in cottonseed meals*

SAMPLE WEIGHT	FREE GOSSYPOL	
	EXTRACTED WITH 90% METHYL ETHYL KETONE[a]	EXTRACTED WITH 70% ACETONE[b]
grams	*per cent*	*per cent*
0.250	0.0400	0.0404
0.500	0.0388	0.0400
0.750	0.0388	0.0386
1.000	0.0372	0.0359
2.000	0.0326	0.0347
3.000	0.0306	0.0308
5.000	0.0285	0.0298

[a] Gossypol determined by reaction with aniline at 100°C. (4).
[b] Gossypol determined by reaction with *p*-anisidine at 80°C.

use of increased sample weights or increased aliquots, or both. Modification of the color development to use *p*-anisidine at 80°C. or aniline at 100°C. permits the use of larger aliquots, but the sample size should be limited to 1 gram or less to insure complete extraction of free gossypol.

It is recommended* that the study of the extraction of free gossypol from feeds be continued and that the effect of interfering constituents be investigated.

REFERENCES

(1) HOFFPAUIR, C. L., and PONS, W. A., JR., *This Journal*, **36**, 1108 (1953).
(2) PONS, W. A., JR., and GUTHRIE, J. D., *J. Am. Oil Chemists Soc.*, **26**, 671 (1949).
(3) American Oil Chemists' Society, *Official and Tentative Methods, Ed. 2*, rev. to 1953, Chicago, 1946–1953.
(4) MILLER, W. J., *J. Am. Oil Chemists Soc.*, **32**, 29 (1955).
(5) STORHERR, R. W., and HOLLEY, K. T., *J. Agr. Food Chem.*, **2**, 745 (1954).

No reports were received on crude protein in feeding stuffs, fat in fish meal, microscopic examination, or molasses.

REPORT ON SOILS AND LIMING MATERIALS

By W. H. MACINTIRE (The University of Tennessee Agricultural Experiment Station, Knoxville 16, Tenn.), *Referee*

RECOMMENDATIONS

In accordance with the request of the Chairman of the Editorial Board, the chapters on Soils and on Liming Materials were studied and a number of editorial changes and clarifications were recommended for the guidance of the Board in the forthcoming revision of *Official Methods of Analysis*. It is recommended†—

(1) That these editorial changes, which clarify the text without changing the procedures, be made.

(2) That the following material be deleted: **3.49,** the side heading "(**a**) *Electrometric*" and the colorimetric procedure headed "(**b**) *Colorimetric*"; **3.50,** the "Method of Statement."

The following recommendations, listed in the Referee's 1953 report, are repeated with the urgent request for collaboration from the responsible Associate Referees:

(3) That the exchangeable hydrogen values indicated by the calcium-acetate official procedure be checked further against liming experiments on different types of soil exposed to natural weather conditions and be analyzed for calcium sorptions and *p*H values.

---

* For report of Subcommittee A and action of the Association, see *This Journal*, **38**, 62, 63 (1955).
† For report of Subcommittee A and action of the Association, see *This Journal*, **38**, 67 (1955).

(4) That use of the flame photometer be further studied as a means for the determination of replaceable potassium.

(5) That studies on soil *p*H be continued.

(6) That the colorimetric determination of molybdenum be studied by the Associate Referee and that the collaborators be requested to make determinations by means of the methods used in their respective laboratories and by the procedure reported recently by Will and Yoe, *Anal. Chem.*, **25**, 1363 (1953).

(7) That a proposed "automatic" titration device, or devices, be made the objective of collaborative study of the determination of fluorine.

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

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

(10) That the utilization of carmin as an indicator in the determination of the boron content of soils be studied further, and that *p*-nitrobenzenazo-1,8-dihydroxynaphthalene-3,6-disulfonic acid, or "chromotrope-B," be studied as a suitable reagent for that determination.

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

(12) That the Associate Referee on exchangeable calcium and magnesium continue his work.

(13) That the potentiometric titration method for the determination of calcium carbonate equivalence of limestones and dolomites which carry undue amounts of interfering materials, described in the following report of the Associate Referee and the Referee, be added as first action to section **1.4.**

(14) That the following section, entitled "Proportions of Calcium and Magnesium in Magnesic Limestones," be added as first action to section **1.4:**

Acidify the titrd soln, **1.4**, slightly, transfer to 250 ml vol. flask, and make to vol. Withdraw 50 ml aliquot and det. Ca as in **22.48**, beginning, "Dil. to ca 100 ml." Subtract its $CaCO_3$ equivalence from total $CaCO_3$ equivalence, **1.4**, and assign difference as the $CaCO_3$ equivalence of the Mg content of the dolomite.

REPORT ON NEUTRALIZING VALUE OF LIMESTONES

DETERMINATION OF CALCIUM CARBONATE EQUIVALENCE OF LIMESTONE AND DOLOMITE THROUGH POTENTIOMETRIC TITRATION TO *p*H 7

By W. M. SHAW, *Associate Referee*, BROOKS ROBINSON, and W. H. MACINTIRE, *Referee* (The University of Tennessee Agricultural Experiment Station, Knoxville 16, Tenn.)

The official procedure for the determination of the neutralization value of agricultural limestone and dolomite (1) consists of dissolving a 1 gram charge in 50 ml of 0.5 *N* HCl, boiling 5 minutes to drive off the CO_2, and back-titrating with 0.25 *N* NaOH, using phenolphthalein indicator. Through many years, this procedure proved satisfactory. Recently, however, several control laboratories encountered a Missouri by-product, "high-grade" dolomite, the titration of which indicated a $CaCO_3$ value substantially below the joint value of the directly determined contents of calcium and magnesium. This dolomite has a high content of ferrous iron which obscured the color of the indicator until addition of a considerable excess of NaOH in the back-titration. Apparently, extra NaOH was also consumed when the precipitated Fe, Al, and Mn hydroxides adsorbed the Ca, Mg, and Na cations as the *p*H of the medium neared 7, and such adsorption was increased greatly when the back-titration was carried to *p*H 8. (Similar end point uncertainty, but of less degree, has been experienced in the titrimetric evaluation of processed dolomites that contained particles of coal dust.)

Because federal requirements and state laws prescribe that liming materials carry guarantees of minimal calcium carbonate equivalence, it is important that control officials be provided with an analytical procedure that will assure accurate determination of the $CaCO_3$ equivalence of a limestone or dolomite, regardless of its content of extraneous matter.

The problem of exactness in the back-titration was attacked by means of:

I. Potentiometric titration curves for solutions of C.P. precipitated $CaCO_3$, high-grade limestone, and dolomite characterized by an unusual content of iron and SiO_2.

II. Potentiometric titration to *p*H 7 and *p*H 8 of systems of known contents of $CaCl_2$, HCl, and $FeCl_2$.

III. Analyses for calcium and magnesium of representative limestone and dolomites to afford computations of total $CaCO_3$ equivalence.

I. Potentiometric Titration Curves.—The potentiometric back-titrations of the HCl solutions of 1 gram charges of the several carbonate materials are depicted by the curves in Fig. 1 which also serve to locate the titration equivalence point and to illustrate the effect of impurities of the

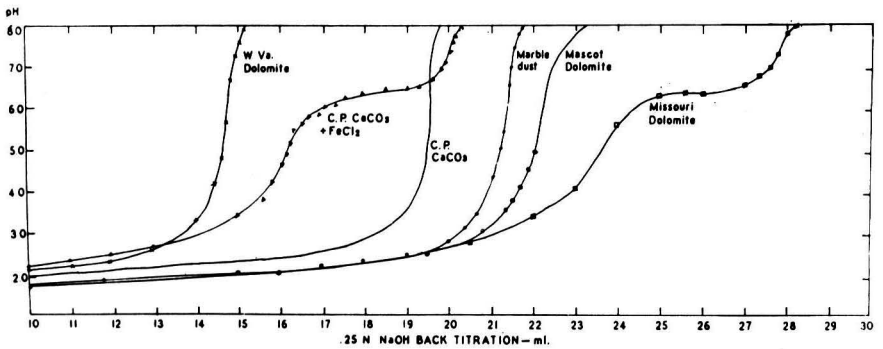


FIG. 1.—Potentiometric titration curves.

limestone and dolomites upon the course of the titrations. The equivalence point is recognized when the ratio of *p*H increase to successive drops of the titrant reaches a maximum (3, p. 14), and the accuracy of the titration is reflected by the magnitude of the ratio or the "jump" in *p*H. In the titration of a limestone or dolomite solution, in which iron hydroxides and suspensions of hydrated silica are engendered, the inflection point may not be at *p*H 7.

The development of the buffered zone of *p*H 6 to *p*H 7 in the back-titration of the Missouri dolomite is attributed to iron content. This phenomenon was simulated in the titration of the $FeCl_2$-fortified solution of the pure $CaCO_3$. In the NaOH titration of excess HCl, a portion of the acid was entrapped by the $Fe(OH)_2$ precipitate with resultant slowing of the reaction. Therefore, the final potentiometric reading should be taken only after allowance of sufficient time for equilibration after each drop of NaOH.

TABLE 1.—*Effect of iron on the titration of HCl-$CaCl_2$ solutions to pH 7 and pH 8*

ADDED TO NEUTRAL SOLUTION OF $CaCl_2$:		0.25 *N* NaOH USED IN TITRATING TO:		EXCESS 0.25 *N* NaOH AT:		PER CENT ERROR (ON BASIS OF $CaCO_3$)	
Fe_2O_3 (ON BASIS OF $CaCO_3$)	0.5 *N* HCl	*p*H 7	*p*H 8	*p*H 7	*p*H 8	*p*H 7	*p*H 8
per cent	*ml*	*ml*	*ml*	*ml*	*ml*		
none	none	0	0.05	0	0	0	0
1.03	2	3.97	4.15	−0.3	.10	0	− .12
2.58	5	10.10	10.38	.10	.33	− .12	− .41
5.15	10	20.10	20.70	.10	.65	− .12	− .81
7.72	15	30.28	30.92	.28	.87	− .35	−1.09
10.30	20	40.50	41.29	.50	1.24	− .62	−1.55

The Mascot dolomite carries a quartz content of from 8 to 10 per cent, but the buffering effect developed only between *p*H 7 and *p*H 8. However, the potentiometric titration would not be vitiated by a corresponding silica content derived from silicates.

The curve for the precipitated $CaCO_3$ showed an inflection point slightly below *p*H 7, whereas the inflection point for the marble, which contained a small amount of SiO_2 and iron, was midway between *p*H 6 and 7.

The inflection point of the solution of the high grade West Virginia dolomite was near *p*H 6, and for the Mascot dolomite, at *p*H 6.5. The solution of the Missouri dolomite of relatively high iron content registered

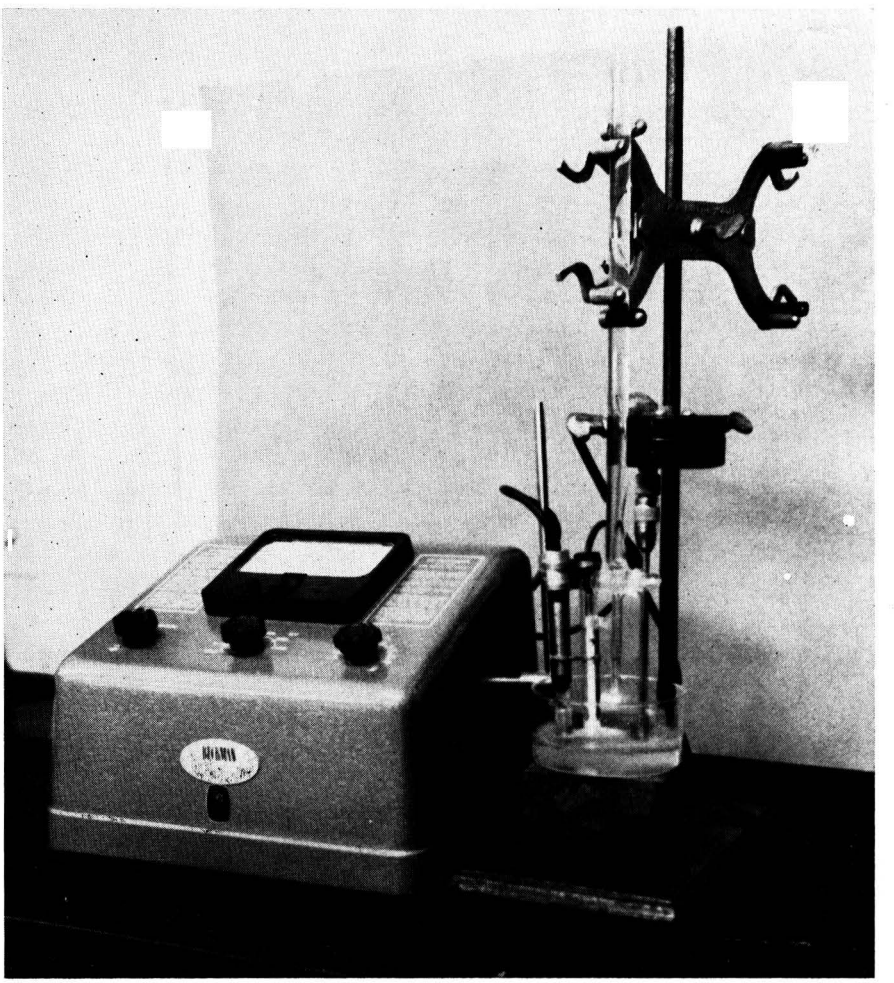


FIG. 2.—Titration assembly.

TABLE 2.—*Analyses of the limestone and three dolomites used in the potentiometric titrations*

(All results in per cent)

CONSTITUENTS	GRAY-KNOX MARBLE[a]			MASCOT DOLOMITE[b]			MISSOURI DOLOMITE[b]			W. VA. DOLOMITE[a]		
	1	2	AV.	1	2	AV.	1	2	AV.	1	2	AV.
Loss on ignition	42.44	42.41	42.42	42.61	42.74	42.67	42.74	42.86	42.80	46.49	46.45	46.47
Insoluble + SiO_2	1.66	1.68	1.67	8.79	8.66	8.72	7.77	7.84	7.80	1.56	1.55	1.56
R_2O_3	.98	.96	.97	1.14	1.10	1.12	5.86	5.86	5.86	1.05	1.46	1.26
CaO	53.65	53.65	53.65	29.57	29.50	29.54	27.46	27.63	27.54	30.28	30.28	30.28
MgO	.92	.87	.90	17.85	18.01	17.93	16.93	16.87	16.90	21.55	21.31	21.43
Sum	99.65	99.57	99.61	99.96	100.01	99.98	100.76	101.06	100.90	100.93	101.05	101.00
	Computed $CaCO_3$ equivalence of CaO + MgO											
Ca	95.74	95.74	95.74	52.77	52.64	52.71	49.00	49.31	49.16	54.04	54.04	54.04
Mg	2.78	2.16	2.47	44.30	44.70	44.50	42.02	41.87	41.95	53.49	52.89	53.19
Sum	98.02	97.90	97.96	97.07	97.34	97.20	91.02	91.18	91.10	107.53	106.93	107.20
Titrimetrically to pH 7.0, *Av.*			98.1			96.9			90.2			106.4
Computed value minus titration value			−.1			0.3			0.9			0.8

[a] 325-mesh separate.
[b] 60-mesh material.

inflection at *p*H 7.3. With the exception of the latter value, the evaluated materials registered inflections below *p*H 7.

The data of Fig. 1 also help in the selection of proper titration end points for the various materials. For limestone and dolomite of low iron content the variation between the *p*H 6 and *p*H 7 end points amounts to only 0.1 to 0.3 per cent in $CaCO_3$ evaluation. However, for limestones and dolomites of high iron content, such a change in end point choice induces $CaCO_3$-equivalence errors of from 4 to 5 per cent. But, when the solution of a limestone of high iron content is titrated to *p*H 7 instead of *p*H 7.3 (the indicated inflection point) the resultant error will be only 0.2 per cent. Such a variation from the absolute neutralization value of a commercial limestone or dolomite would be a small fraction of the tolerance allowed by the control chemist.

From these considerations it appears obvious that back-titration of the solution of 1 gram of limestone in 50 ml of 0.5 *N* HCl to *p*H 7 affords an accurate evaluation of $CaCO_3$ equivalence or neutralization value for limestones and dolomites, regardless of variance in character.

II. Potentiometric Titration of Systems of $CaCl_2$, HCl, and $FeCl_2$ to pH 7 and pH 8.—The interference of iron is illustrated by the data of Table 1. Portions of a $CaCl_2$ solution corresponding to hydrochloric acid solutions of 1 gram charges of $CaCO_3$ were prepared and neutralized to *p*H 7, and then modified by additions of various amounts of ferrous chloride and of HCl in slight excess. These mixtures were potentiometrically titrated with 0.25 *N* NaOH to *p*H 7, and then to *p*H 8. Excess NaOH titration over added HCl is expressed as ml of 0.25 *N* NaOH and as the per cent $CaCO_3$ negative error attributable to cation adsorption. The data of Table 1 show that even at titration to *p*H 7 some cation sorption occurred in the acidified system that had a high content of iron (above 5 per cent Fe_2O_3). The sorption in that system was much greater at *p*H 8, at which point the titrations of acidic solutions of limestones and dolomites of lower iron content are also affected to a measurable extent.

The findings of Table 1 support the previous conclusion from titration curves, *viz.*, that accurate evaluations of all grades of limestone and dolomite are obtained when the prescribed solutions are titrated potentiometrically with NaOH to *p*H 7.

III. Direct Analyses for Computation of $CaCO_3$ Equivalences.—The analyses reported in Table 2 for the limestone and three dolomites were made primarily for computation of the respective $CaCO_3$ equivalences of the $CaCO_3$ and $MgCO_3$ contents and to establish the degree of concordance between the values so determined and those established by potentiometric titrations, and also to point up the distinctive compositions of the four carbonate materials.

The summation of analytical values in Table 2 indicates a plus error of about 1 per cent in the Missouri and West Virginia dolomites. This is

0.5 per cent in excess of the tolerance allowed by Hillebrand and Lundell (2) and probably is due to high results for calcium. The computed $CaCO_3$ equivalences for the respective determinations of Ca plus Mg, as shown in the lower part of Table 2, show good agreement with potentiometric titration values obtained at *p*H 7 for the marble and Mascot dolomite, but are nearly 1 per cent higher for the Missouri and West Virginia dolomites. A difference of less than 1 per cent $CaCO_3$ equivalence between computed and titration results is a reasonable one especially as the nature and extent of the difference was foreshadowed in the summation of the analytical results, as noted earlier. This circumstance and the general agreement between the values by titration and the computed values prove the reliability of an evaluation of a limestone or dolomite by back-titration of its 1 gram solution in 50 ml of 0.5 *N* HCl to *p*H 7.

RECOMMENDATION

It is recommended* that the following method be adopted as an alternative procedure for determination of the neutralization value of limestones which bear undue proportions of interfering substances. The procedure is applicable to all carbonate, caustic, and hydrated liming materials.

POTENTIOMETRIC TITRATION PROCEDURE FOR LIMESTONES AND MAGNESIC LIMESTONES OF HIGH CONTENT OF IRON OR COLORING MATTER

Proceed as in **1.4** thru "Cool." Transfer to 100×50 mm crystg dish and place dish in position to accommodate glass and calomel electrodes of *p*H meter, buret contg 0.25 *N* NaOH, and mechanical stirrer (Fig. 2). Adjust stirrer to moderate speed and avoid splash. Deliver NaOH rapidly to attain *p*H 5, then dropwise until soln attains *p*H 7 and remains constant 1 min. while stirring. In case *p*H 7 should be over-titrd, add from 1 ml Mohr pipet just enough 0.5 *N* HCl to bring *p*H below 7 and back-titr. slowly to *p*H 7. Record and add the extra amount of 0.5 *N* HCl to initial 50 ml. % $CaCO_3$ equivalence of sample = 2.5 (ml HCl − ml NaOH/2).

REFERENCES

(1) *Official Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Washington, D. C., 1950.
(2) *Applied Inorganic Analysis*, HILLEBRAND, W. F., and LUNDELL, G. E. F., John Wiley & Sons, Inc., New York, 1929.
(3) *Potentiometric Titrations*, KOLTHOFF, I. M., and FURMAN, N. H., John Wiley & Sons, Inc., New York, 1926.

* For report of Subcommittee A and action of the Association, see *This Journal*, **38**, 67 (1955).

REPORT ON MOLYBDENUM IN SOILS AND METHODS FOR ITS DETERMINATION

By W. O. Robinson (Chemical Laboratories, Soil Conservation Service, U. S. Department of Agriculture, Beltsville, Md.), *Associate Referee*

This year three collaborators reported on molybdenum in three soils of quite different composition. It was recommended last year that the collaborators describe in detail the method in use in their laboratories. Methods of the three collaborators differed either in the preparation of the soil sample or in the colorimetric reagent used.

There was good agreement of duplicates and triplicates by the same analysts, and agreement between different analysts was better than might be expected. The average of molybdenum for the Norfolk sand, subsurface, was 0.7 p.p.m., range 0.4–1.0; for the lateritic Nipe surface soil, average 2.5, range 2.0–2.9; and for the DeKalb silt loam, composite sample, average 13.1, range 10.7–15.9. The results on the DeKalb silt loam by the dithiol method are consistently higher than by the thiocyanate method, but this generalization does not hold for the other soils.

As Sandell (4) states, there are definite advantages in using the sodium carbonate fusion and extraction method in the preparation of the sample. Iron, titanium, and manganese are eliminated, particularly if a little sodium peroxide is added to the solution of the sodium carbonate melt in the cold and heated a few minutes before filtering. The fusion is necessarily made in a platinum crucible. It is neither necessary nor expedient to use hydrochloric acid to wash out the crucible and cover if molybdenum only is wanted. The platinum error is negligible if this acid wash is not used. Another advantage of the sodium carbonate fusion and after-treatment with sodium peroxide is that the molybdenum should all be in the hexavalent state at the start of the color development. Then, when reduced by stannous chloride, it should all be in the quinquevalent state which gives the maximum molybdenum color by thiocyanate.

It has been objected that the precipitated silica carries down molybdenum but Sandell states that the quantity so carried down is negligible. There would seem to be an advantage in first decomposing the soil by HF plus H_2SO_4 and then fusing the residue with sodium carbonate for the determination of total molybdenum.

COLLABORATIVE METHODS AND RESULTS

The results of the collaborators are given in Tables 1 and 2 and the methods used by the collaborators are given below:

Collaborator No. 1, A. L. Willis, Ontario Experiment Station, Guelph, Canada, did much work comparing different methods of preparation of the test solution and also in comparing details of the colorimetric part of the procedure, although he used

TABLE 1.—*Molybdenum content of soil samples as reported by three collaborators*

COLLABORATOR	1	2	3
	p.p.m.	*p.p.m.*	*p.p.m.*
Norfolk sand, surface, Pontiac, S. C.		0.1	0.2, 0.2
Norfolk sand, subsurface, Pontiac, S. C.	1.0, 0.9, 0.9	0.4, 0.2	0.55, 0.40
Nipe soil, surface, Puerto Rico	2.4, 2.3, 2.8	2.4, 2.9	2.0, 2.4
DeKalb silt loam, composite, Bloom, Va.	11.2, 10.8, 10.7	15.7, 15.4, 15.9	12.4, 12.8

the thiocyanate color development and isopropyl ether extraction in all cases. Dr. Willis remarks that " . . . regardless of the method of soil treatment or color development we have arrived at about the same result for each soil."

Dr. Willis has used several combinations of methods of soil treatment and color development. He states " . . . the method we have used predominantly is as follows: 2 g air dried, 100 mesh soil (silk bolting cloth) is weighed into a Pt crucible and heated for three hours in a muffle at 500°C. to destroy organic matter. After cooling, the following reagents are added: 5 ml redistd H_2O, 10 ml HF (48–51%), and 0.5 ml concd H_2SO_4 (care must be taken in adding the HF if free carbonates are present). The crucible is then placed on a sand bath at low temp. and the contents evapd slowly to dryness to avoid spattering. Five ml H_2O and 10 ml HF are again added and evapd to dryness (two additions of HF are usually sufficient to volatilize all the silica present). The crucible is then placed in a muffle furnace; the temp. is raised to 350°C. and held there for ca 1 hr to drive off all SO_3 fumes.

"The residue is then taken up in 5 ml redistd H_2O plus 10 ml redistd 6 *N* HCl and

TABLE 2.—*Molybdenum content of three soils reported by A. L. Willis*

SAMPLE AND METHODS	MOLYBDENUM
	(*p.p.m.*)
1. Norfolk sand, subsurface	
(a) Willis' method throughout	1.0 0.9
(b) Sodium carbonate fusion, and Sandell's color development, but using isopropyl ether extraction	0.3 0.4
(c) Willis' method of soil decomposition, and Sandell's color development, but using isopropyl ether extraction	0.4 0.6
(d) Sodium carbonate fusion, and Willis' color development	0.5 0.3
2. Nipe soil, 0–5″, Puerto Rico	
Method (a) as above	2.4 2.3
Method (b) as above	2.3 2.1
Method (c) as above	1.6 1.9
Method (d) as above	2.3 1.8
3. DeKalb silt loam, composite	
Method (a) as above	11.2 10.8
Method (b) as above	10.4 10.9
Method (c) as above	10.8 10.6
Method (d) as above	10.7 10.9

warmed in the crucible on a hot plate until all residue is loosened. The entire contents are then transferred with the aid of a policeman and hot H_2O to a 150 ml beaker. Ten ml more of the 6 *N* HCl is added, the beaker is covered with a watch glass placed on a hot plate at low heat, and the contents are digested for ca 2 hrs. At the end of this time practically everything soluble is in soln. The contents of the beaker are then filtered into a 100 ml vol. flask and the filter paper is washed with hot H_2O until the flask is up to vol. (This generally requires at least 5 washings with hot H_2O).

"A 25 ml aliquot of the test soln representing 0.5 g soil is transferred to a 125 ml separatory funnel and the following reagents are added in the order given: 4 ml 10% NH_4CNS, 0.5 ml 5 *N* $NaNO_3$, 5 drops 5% $FeCl_3$ (if the soln is distinctly yellow the $FeCl_3$ is omitted), and 5 ml 10% $SnCl_2$ in normal HCl. After mixing to destroy the color of the ferric thiocyanate, 10 ml isopropyl ether is added. The separatory funnels are placed on a mechanical shaker at approximately 275 rpm and shaken for 30 sec. After the two layers separate, the aq. phase is discarded and the ether is delivered into a colorimeter tube which is immediately covered with parafilm to prevent evaporation. Ten minutes after separation, the transmittance is determined in an Evelyn colorimeter with a 470 filter."

Collaborator No. 2, Lewis J. Clark, Section of Fertilizer and Agricultural Lime, Soil and Water Conservation Research Branch, Agriculture Research Service, Beltsville, Maryland, used the dithiol reagent according to Piper and Beckwith (3), but with certain important modifications. (Dr. Clark is preparing the method for publication and at present it is available only in his thesis (1).)

Collaborator, No. 3, R. F. Dever of this laboratory, used the sodium carbonate method of soil preparation and the thiocyanate color development as described by Hillebrand, *et al.*, (2) and in more detail by Sandell (4), for silicate rocks. It is described in *Soil Sci.*, **77**, 237 (1954), and is reproduced below:

REAGENTS

(a) *$SnCl_2$.*—Dissolve 30 g $SnCl_2$ in dil. HCl (50 ml concd HCl with enough H_2O to make 300 ml).

(b) *KCNS.*—5% soln.

(c) *$FeCl_3$.*—0.01 *N* (approx.).

(d) *Isopropyl ether.*—Practical grade, as furnished by Eastman Kodak Co., is suitable.

(e) *Na_2CO_3.*—Anhyd., practically free from molybdenum. A blank should be run on this reagent by fusion in the same Pt crucibles used for the detn. The blank should not exceed 0.2 ppm molybdenum on the basis of 2 g soil. Cleaning the crucible with alternate fusions of Na_2CO_3 and $KHSO_4$ should reduce the blank to this amount or below.

PROCEDURE

Mix the soil well and grind it in an agate mortar to pass a 100-mesh silk bolting cloth. Ignite 2 g soil in a large Pt crucible, mix thoroly with 10 g Na_2CO_3, and fuse thoroly to a quiet melt. Cool, thoroly disintegrate in about 150 ml H_2O, and put on steam bath overnight. To make sure the fusion is thoroly disintegrated, transfer to a small porcelain mortar and grind to a slurry. Transfer back to the dish and keep hot for several hours, stirring and adding H_2O if necessary. Filter, and wash 5 or more times with hot H_2O. Acidify the filtrate with HCl and evap. to dryness to dehydrate the silica. Take up with 7.5 ml concd HCl and 35 ml hot H_2O. Filter into a beaker, and wash 6 times with hot H_2O. Add to the filtrate 2–3 drops $FeCl_3$ soln, make up to 140 ml, and transfer to a 250 ml separatory funnel. Add 5 ml KCNS soln, shake, add 5 ml $SnCl_2$ soln, and shake again. Add 10 ml isopropyl ether, shake

thoroly, and compare with standards, or read from a prepd curve in a suitable spectrophotometer.

Prep. the std by dissolving 10 g Na_2CO_3 in ca 100 ml H_2O. Neutralize with an equiv. quantity of HCl, add 7.5 ml excess, and dil. to 140 ml. Add the std molybdenum soln, making up several concns corresponding to 1, 2, 5, etc. ppm molybdenum. Add 2–3 drops $FeCl_3$ soln and 5 ml KCNS and shake; add 5 ml $SnCl_2$, and shake again. Add 10 ml isopropyl ether and shake thoroly.

RECOMMENDATION

It is recommended* that the work be continued.

REFERENCES

(1) *The Determination and Distribution of Manganese, Copper, Zinc and Molybdenum in Thirteen Maryland Soils and Associated Rocks.* CLARK, LEWIS J. Thesis submitted to the University of Maryland, June 1954.
(2) *Applied Inorganic Analysis.*, *2nd* Ed., by HILLEBRAND, W. E., LUNDELL, G. E. F., BRIGHT, H. A., and HOFFMAN, J. I., John Wiley and Sons, Inc., New York, 1953.
(3) PIPER, C. S., and BECKWITH, R. S., *J. Soc. Chem. Ind.*, **67**, 374 (1948).
(4) *Colorimetric Determination of Traces of Metals, 2nd* Ed., by SANDELL, E. B. Interscience Publishers, Inc., New York, 1950.

No reports were received on boron, exchangeable hydrogen, exchangeable potassium, fluorine, hydrogen-ion concentration of soils, phosphorus, or zinc and copper.

REPORT ON SOIL CONDITIONERS

By L. T. ALEXANDER (Soil Survey Laboratories, Plant Industry Station, U. S. Department of Agriculture, Beltsville, Md.), *Referee*

The Associate Referee on Chemical Analysis of Soil Conditioners and the Associate Referee on Performance of Soil Conditioning Chemicals have both submitted reports, and the Referee agrees with their conclusions that investigations of their respective subjects be continued. It is evident that there is not sufficient information at this time to adopt the performance and composition methods for official use. To a certain extent the problem is not as acute as it was in 1953, owing to the lack of pressure for the sale of these materials.

It is recommended† that the work of both Associate Referees be continued.

---

* For report of Subcommittee A and action of the Association, see *This Journal*, **38**, 67 (1955).
† For report of Subcommittee A and action of the Association, see *This Journal*, **38**, 67 (1955).

REPORT ON CHEMICAL ANALYSIS OF SOIL CONDITIONERS

By FRED J. ROTH (California Department of Agriculture, Sacramento, Calif.), *Associate Referee*

The first six months' investigation of the general problem of chemical analysis of soil conditioners has been spent largely in determining what type of materials are sold for this purpose. It is evident that the term includes a wide variety of materials greatly differing in chemical and physical nature and that appropriate methods of analysis will have to be devised for each of several types.

The complexity of the problem is indicated by listing some of the materials currently marketed under the general term "soil conditioners."

ORGANIC MATERIALS

charcoal	redwood bark fiber
cotton trash	rice hulls
earthworm castings	sawdust
fish extract	seaweed
grape pomace	spent coffee grounds
leafmold	spent cocoa beans
olive pomace	straw and hay
peat	tobacco waste
peat moss	

MINERAL MATERIALS

diatomite	perlite
expanded vermiculite	pumice
glass wool	sand
hadite	scoria
lignite	sintered shale
montmorillonite and other clays	talc

MISCELLANEOUS MATERIALS

bacterial sprays	synthetic polyelectrolytes
enzymes	cellulose derivatives
fermented cactus juice	lignin derivatives
plant extracts	polyacrylates
wetting agents	polyvinylites
silicates	

A few of these items, such as fish extract or seaweed, may be regarded as ordinary fertilizers when they are sold with a guarantee of certain percentages of plant foods, but they are sometimes sold simply for "conditioning" the soil and with no claim as plant foods. Many of the materials being marketed reflect a rather general belief that organic matter and extracts, ferments, enzymes, hormones, and other derivatives of organic matter significantly improve soil structure and thus benefit plant growth. The agricultural value of some of the products is questionable but that is not under consideration in this particular investigation. The

materials are being marketed, and adequate chemical analysis or other significant examination of them is needed to confirm guaranteed percentages when they are sold either alone or in mixtures.

The mineral materials appear to exert their effect solely by virtue of their physical nature. Being light, porous, and absorbent, they tend to improve the texture of heavy clay soils that otherwise compact and become impervious to water. Preliminary examination of these materials has been limited to determination of moisture, organic matter (loss on ignition), and ash. The *p*H has been determined for whatever value it might have in establishing uniformity of material sold under a brand name. The percentage of soluble solids was determined to detect the presence of "chemical" constituents that might be valuable or harmful as contrasted to the "physical" constituents that are claimed to affect the structure of the soil. The fineness of some of these materials was determined, but no determination has been made of their absorbency or water-holding capacity.

The organic materials were similarly examined by proximate analysis and by determination of *p*H and total soluble solids. Many of them, as well as samples of the mineral materials, were analyzed for total nitrogen, total phosphoric acid, and water-soluble potash, but few contained any significant amount of these plant foods. The percentage of soluble solids in the dry materials was determined by extraction with water, evaporation, and weighing the residue. The soluble solids in the liquid materials were determined simply by evaporating and drying a weighed portion. The specific gravity and the surface tension of some liquid products were determined.

The analytical findings are not presented in this report inasmuch as the data have been included in the annual publications on fertilizing materials issued by the Bureau of Chemistry, California State Department of Agriculture. Some mineral materials contained less than 1 per cent moisture and more than 95 per cent ash. Some organic materials such as peat moss ranged in moisture from 10 to 70 per cent and in ash from 1 to 15 per cent. Some liquid products, claimed to contain enzymes of benefit to soils, showed by proximate analysis 99.9 per cent water.

The recent interest in synthetic polyelectrolytic soil conditioners prompted this investigation, but the nature, the action, and the purpose of these materials were not sufficiently different from those of other soil conditioners to enable the investigation reasonably to be limited to the new materials alone. At least a half-dozen greatly different compounds are involved. The compounds are large molecular-weight polymers whose composition is not fully known. Few of them have constituents or groupings subject to direct determination by chemical methods. A representative sampling of the synthetic products on the market showed that the marketed mixtures could most conveniently be divided into wettable or

soluble powders, powders for dry application, and liquids. The wettable or soluble powders analyzed contained total nitrogen ranging from 4 to 6.5 per cent, less than 0.05 per cent total phosphoric acid, 0.1 to 0.4 per cent potash, 3 to 7 per cent moisture, and 24 to 39 per cent ash. The *p*H at a 1:100 dilution ranged from 5.8 to 9.0. The powders for dry application contained 0.1 to 4.3 per cent total nitrogen, from less than 0.05 per cent to 0.5 per cent total phosphoric acid, and from 0.2 to 0.4 per cent potash; 1 to 8 per cent moisture, and 61 to 75 per cent ash. The *p*H ranged from 3.9 to 11.0. The liquid preparations contained 0.7 to 2.7 per cent total nitrogen, less than 0.05 per cent total phosphoric acid, less than 0.4 per cent potash, 77 to 91 per cent water, and 2 to 9 per cent ash. The *p*H ranged from 6.2 to 10.3.

The large amount of money spent for many of these soil conditioners justifies further work on the subject, and although the most informative data on some of them will probably be derived from physical rather than chemical tests, it is recommended* that the investigation be continued to determine the scope and usefulness of chemical analysis. The Associate Referee would appreciate receiving comments and suggestions, particularly on analytical methods for polyelectrolyte type of conditioners or any other suggestions on chemical classification of these materials.

REPORT ON PERFORMANCE OF SOIL CONDITIONING CHEMICALS

By Stephen J. Toth (New Jersey Agricultural Experiment Station,† New Brunswick, N. J.), *Associate Referee*

Widespread interest of industry has resulted in the production of many types of polymeric resins which are designed for the stabilization of soil aggregates. Many types of procedures have been developed to evaluate these products in the laboratory, but essentially all of them are based upon the measurement of some change in the physical properties of soils. Because of the numerous types of evaluation procedures employed, various workers have tended to differ widely in their results.

The present program has as its main objective the development of a satisfactory evaluation procedure or procedures which, it is hoped, will be adopted by workers in this field. The objectives of the first phase of the program, reported this year, were: To determine the types of procedures that are in use for evaluating soil conditioner chemicals; to have

* For report of Subcommittee A and action of the Association, see *This Journal*, **38**, 67 (1955).

† Journal Series paper of the New Jersey Agricultural Experiment Station, Department of Soils, Rutgers University, The State University of New Jersey, New Brunswick, N. J.

collaborators rate six samples of these materials by their procedures; and to select an evaluation procedure for future study.

Six samples of conditioner chemicals, properties of which are listed in Table 1, were furnished each collaborator.

TABLE 1.—*Nature of soil conditioner chemicals used in collaborative study*

SAMPLE	PHYSICAL STATE	PERCENTAGE OF ACTIVE POLYMER	POLYMER TYPE
1	Liquid	12.5	Polyacrylate
2	Liquid	17.5	Polystyrene–copolymer
3	Gel	9.0	Copolymer–VAMA[a]
4	Powder	100.0	Copolymer–VAMA
5	Powder	100.0	Copolymer–VAMA
6	Powder	80.0	Polyacrylate

[a] VAMA: The calcium salt of polyvinyl acetate-malic acid copolymer.

INSTRUCTIONS TO COLLABORATORS

Samples of six conditioners are to be evaluated, using a procedure or procedures in use in your laboratory. Furthermore, the soil or soils to be used in the test are to be of your selection. The following information is to be submitted to the Associate Referee:

(1) The relative rating of the six samples listed in decreasing order of effectiveness.

(2) An outline of the evaluation procedure.

(3) The soil or soils used (series, texture, and nature of dominant clay mineral, if it is known).

(4) Additional comments concerning the samples or procedures employed.

RESULTS

A summary of the relative ratings of the six conditioner samples submitted to the collaborators is listed in Table 2.

Evaluation procedures that can be classified in the general group of "wet-sieving" were used by 9 of the 11 collaborators who submitted reports to the Associate Referee. Other types of physical measurements used were sedimentation, modulus of rupture, permeability to water and/or air, and intrinsic permeability. Some of the collaborators used more than one soil type; one collaborator varied the *p*H. There was a tendency to vary the concentration of the soil conditioning chemical over a range extending from 0.020 to 0.125 per cent.

COMMENTS OF COLLABORATORS

The collaborators made several suggestions concerning methods and properties of the 6 samples submitted for analysis. These comments are quoted as follows:

(1) "The differences between aggregation percentages for the two soils for each material are very large, indicating that soil type is an

TABLE 2.—*Relative rating of six soil conditioning chemicals*

COLLABORATOR NO.	TYPE OF SCREENING PROCEDURE	SAMPLE NUMBER					
		1	2	3	4	5	6
		RELATIVE RATING[a]					
1	Wet-sieving	3	1	5	2	6	4
		2	1	4	6	5	3
		1	2	3	5	6	4
2	Wet-sieving	1	4	5	2	6	3
	Hydrometer	1	4	5	2	6	3
	Modulus of rupture	3	5	6	2	4	1
	Permeability to water	3	5	4	2	4	1
	Air-water permeability	3	5	4	2	3	1
3	Wet-sieving	1	4	5	3	6	2
4	Wet-sieving	3	4	5	2	6	1
5	Wet-sieving[b]	3	1	6	2	5	4
	Wet-sieving[b]	4	6	5	1	3	2
	Wet-sieving[b]	5	2	4	1	6	3
	Wet-sieving[b]	2	4	6	1	5	3
6	Wet-sieving	2	4	6	1	5	3
		4	1	5	2	3	6
7	Pipet-sedimentation	2	6	4	1	5	3
8	Wet-sieving	5	1	6	2	3	4
9	Intrinsic permeability	1[c]	1[c]	2[c]	1[c]	2[c]	1[c]
10	Wet-sieving	5	1	6	2	4	3
		5	1	6	2	3	4
11	Wet-sieving	2	6	5	3	4	1
	Average Relative Rating:	3	4	6	1	5	2

[a] Decreasing order of effectiveness 1 to 6.
[b] Evaluation by different laboratories.
[c] Omitted from average.

important consideration in evaluating the actual effect of soil conditioners."

(2) "Conditioner No. 2, when applied at the 0.1 per cent rate, exhibited a noticeable tendency to render both soils slightly water repellent."

(5) "Sample No. 2 displayed considerable water proofing at 0.02 per cent. At 0.1 per cent the water proofing was so bad that approximately 90 per cent did not wet during the one-half hour wet-sieving period."

(6) "It is felt by this laboratory that polymers must be worked in accordance with the particular application for which they are intended, i.e., surface *vs.* depth treatment."

(9) "The data show no difference between conditioners 1, 2, 4, and 6. Numbers 3 and 5 were less effective."

(10) "Procedures were designed to give uniformly applicable conditions of listing. Sieving and puddling were used to destroy natural aggregation as completely as possible."

DISCUSSION

The data presented in Table 2 are typical of results that have been obtained in evaluating soil conditioning chemicals. Relative ratings of each conditioner varied widely and were dependent upon the method used for evaluation, the soil type, and the concentration of the polymer. No two collaborators obtained identical results on the six samples submitted to the test program.

It is obvious from the results obtained that the development of a standard method or methods for the laboratory evaluation of soil conditioner chemicals is dependent upon the use of the following: A standard or a synthetic soil, a selected procedure or procedures which must be carefully followed, and standardized concentration of soil conditioner chemicals for the test.

Because of the variations obtained in rating the six conditioner chemicals by methods now used by the collaborators, it is recommended† that studies be continued next year on laboratory evaluation tests selected by the Associate Referee.

LIST OF COLLABORATORS

(1) R. B. Alderfer, Pennsylvania Agricultural Experiment Station, State College, Pa.

(2) L. E. Allison, U. S. Salinity Laboratory, Riverside, Calif.

(3) N. T. Coleman, North Carolina Agricultural Experiment Station, Raleigh, N. C.

(4) J. D. Dalton, American Cyanamid Co., Stamford, Conn.

(5) J. C. Engibous, Monsanto Chemical Co., St. Louis, Mo.

(6) R. P. Hopkins, Rohm & Haas Co., Philadelphia, Pa.

(7) J. P. Martin, California Agricultural Experiment Station, Riverside, Calif.

(8) A. J. Martinelli, General Aniline & Film Corp., Easton, Pa.

(9) W. A. Raney, Mississippi Agricultural Experiment Station, State College, Miss.

(10) C. S. Slater, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Md.

(11) C. L. W. Swanson, Connecticut Agricultural Experiment Station, New Haven, Conn.

REPORT ON EXTRANEOUS MATERIALS IN FOODS AND DRUGS

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

The Referee has worked closely with all of the Associate Referees on this subject in an endeavor to incorporate all pertinent refinements and

† For report of Subcommittee A and action of the Association, see *This Journal*, 38, 67 (1955).

changes into the various methods in time for the publication of the Eighth Edition of *Official Methods of Analysis.* Their cooperation has been most helpful.

With the gradual development of new and improved methods for extraneous materials, methods **35.1–35.4,** including apparatus, reagents, and general information and precautions, have become outmoded, and it is recommended* that an amended draft be substituted for the present material. This new material should appear in the Eighth Edition of *Official Methods of Analysis.*

Last year O. L. Kurtz, Associate Referee on Identification of Insect Contaminants in Food and Drug Products, outlined a program for determining characteristics to differentiate and identify those insects which contribute fragments to cereal grains and stored products. Because of the complexity and numbers of the insects, the topic was designed to operate on a long range basis; however, the Associate Referee has received some progress reports from cooperating laboratories which indicate that certain sections of the project will be completed during the coming year. The Referee therefore concurs with the Associate Referee that the insect fragment work be continued and completed for report at the next meeting, and that a similar program for collaborative study be extended to include other major food commodities and their major insect contaminants.

The Referee agrees with the Associate Referee on Extraneous Matter in Beverage Materials that the method for Cocoa, Imitation Cocoa, Cocoa Substitutes, Coffee, and Coffee Substitutes, **35.8,** be deleted, including the title, and that it be replaced with two methods, one for Coffee and Coffee Substitutes, and the other for Cocoa, Chocolate, Chocolate Liquor, and Imitation and Substitute Chocolate Products, both described in the report of the Associate Referee.

The Referee concurs with the recommendation of C. R. Joiner, the Associate Referee on Sediment Tests in Milk and Cream, that the first action method for sediment test on milk, **35.9–35.12,** as revised, *This Journal,* **35,** 99 (1952); **36,** 87 (1953); **37,** 77, 117 (1954), be adopted as official. The Referee also suggests that the present off-bottom method for collection of sample, **35.11(b),** be revised to read as follows:

"Take pint sample not more than ¼" off bottom of unstirred can of milk by inserting the sampler, and during upstroke of the plunger, draw the head of the instrument across the diam. of the can bottom or around the circumference if the can has a high center. Expel milk with the gun in the can, and then with short stroke, remove excess fluid from the pad."

The Referee accepts the report of the Associate Referee on Extraneous Materials in Dairy Products, and concurs in the following recommendations:

* For report of Subcommittee D and action of the Association, see *This Journal,* **38,** 85, 86 (1955).

(1) That the following procedures be deleted and replaced by the methods described in the report of the Associate Referee:

Quantitative separation of filth in cheese products, **35.13**
Qualitative separation of filth in cheese products, **35.14**
Filtration on paper of filth in dried milk, **35.15**
Filtration on sediment pad of filth in dried milk, **35.16**
Filth in cream, **35.17**

(2) That these methods, with the exception of the optional procedure (**e**) for hard skim-milk cheese, etc., be adopted as first action.

(3) That the first action method for filth in butter, **35.21,** be deleted and be replaced by the method described by the Associate Referee.

(4) That the new method for hard cheeses be studied collaboratively.

(5) That the methods for extraneous materials in cheese that contains mold, plant tissues, and spices be studied.

(6) That the study for methods for the identification of manure fragments in dairy products be continued.

The Referee accepts the report of the Associate Referee on Extraneous Materials in Nut Products. This past year, M. G. Yakowitz and her collaborators have made outstanding progress in standardizing these procedures. It is recommended that the method for shelled nuts, **35.22,** be revised in accordance with the suggestions of the Associate Referee. It is also recommended that work on nuts and nut products be continued, and that further work on methods for extraneous material in peanut butter be discontinued.

The Referee concurs in the report of the Associate Referee on Extraneous Materials in Cereal Grains, Cereal Products, and Confectionery, and recommends:

(1) That the method for insects, insect parts, and rodent hairs in rye meal, **35.36**(**b**), be revised as described by the Associate Referee and be adopted as first action.

(2) That the Tween 80–Versene procedure for examination of bakery products be studied during the coming year.

(3) That the method for internal insects in grains, *This Journal,* **36,** 309 (1953), be adopted as first action.

(4) That the method for pancreatic digestion of insect fragments and rodent hairs in white wheat flour, **35.29**(**a**), as revised, *This Journal,* **36,** 309 (1953), be adopted as first action.

(5) That the method for direct trapping of insect fragments and rodent hairs in baked products, prepared cereals, and alimentary pastes, **35.28**(**b**), be subjected to further collaborative study.

(6) That the method for filth in candy, **35.60,** be revised in accordance with the suggestions of the Associate Referee.

The Referee concurs in the recommendation of the Associate Referee on Extraneous Materials in Egg Products that the subject be given further

collaborative study. It is hoped that some of the suggested methods will be adopted as first action at the next meeting.

The Referee concurs with the following recommendations of W. G. Helsel, Associate Referee on Extraneous Materials in Fruit Products:

(1) That the procedures for extracting insects from berries, **35.52** and **35.53,** be deleted. These procedures should be revised to include use of the newer wetting agents and should be adapted to the recovery of thrips and other small insects.

(2) That the method for maggots in blueberries and cherries, **35.54,** be changed in status from a first action method to a recommended procedure. It is essentially a macroscopical procedure and is not suitable for collaborative study.

(3) That the method for rot in blackberries, raspberries, and other drupelet berries, *This Journal,* **35,** 337 (1952), be revised by changing the title from "frozen" to read "frozen with or without sugar" and the word "canned" to read "frozen in sirup, canned in sirup or water"

The Referee agrees with the suggestions of the Associate Referee on Extraneous Materials in Vegetable Products, and recommends that the method for molds in tomato products, **35.64,** and for fly eggs and maggots in tomato products, **35.67,** be revised in accordance with the recommendations of the Associate Referee. It is also recommended that the methods for filth in potato chips and for filth in sauerkraut, as described in the report of the Associate Referee, be adopted as first action. The Referee recommends that work be continued on the various aspects of the methods for extraneous materials in vegetable products as opportunity arises and material becomes available. This work should include further data on the method for fly eggs and maggots in tomato products with a view to obtaining more conclusive results.

The Referee concurs in the recommendation of the Associate Referee on Drugs and Spices that work be continued on this subject.

REPORT ON EXTRANEOUS MATTER IN BEVERAGE MATERIALS

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

It is recommended* that method **35.8,** "Cocoa, Imitation Cocoa, Cocoa Substitutes, Coffee, and Coffee Substitutes," be deleted and the following new method, **35.8,** entitled "Coffee and Coffee Substitutes" be adopted as first action:

* For report of Subcommittee D and action of the Association, see *This Journal,* **38,** 85, 86 (1955).

Weigh 100 g sample in 600 ml beaker, add 350 ml $CHCl_3$, and boil 15 min., stirring occasionally. Wash down sides of beaker with $CHCl_3$. Allow mixt. to cool and settle 15 min., with occasional stirring of top layer. Decant $CHCl_3$ and floating tissue onto ca 15 cm smooth filter paper in büchner, taking care not to disturb heavy residue in bottom of beaker. Repeat decantings with small quantities of $CHCl_3$[1] from wash bottle until practically no plant tissue remains with residue on bottom of beaker. Transfer residue from beaker to ashless filter paper and examine for filth. If residue is appreciable, ignite filter and det. weight of sand, soil, etc.

Air dry overnight or for an hour in an oven at ca 80°, transfer dried material to a 2 l Wildman trap flask, and add 400 ml hot H_2O. Boil 15 min., and if necessary, add small amounts of cold H_2O intermittently to prevent foaming. Cool mixt. below 20°. Trap off twice, using 35 ml and 25 ml gasoline for respective trappings. In the first trapping allow to stand 5 min. after stirring in the gasoline before filling the flask. Filter and examine.

It is recommended that the following new method be adopted as first action: "Cocoa, Chocolate, Chocolate Liquor, Imitation and Substitute Chocolate Products. Examine by **35.60(b)**."

REPORT ON EXTRANEOUS MATERIALS IN DAIRY PRODUCTS

By DOROTHY B. SCOTT (Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.),
Associate Referee

No work was done by the Associate Referee during the year on methods for the identification of dung fragments in dairy products. It is recommended* that work on this subject be done during the coming year.

Work on egg products is being reported elsewhere in *This Journal* (p. 268).

A new method was developed for extracting extraneous materials from cheese which is difficult to examine. This method has been incorporated into a complete reorganization of the dairy products methods. These changes in the methods are based upon current practices in the examination of dairy products for filth.

Dairy products as a group are highly variable in their composition and even in the physical character of similar chemical components. The amount and character of the casein or calcium caseinate, the fineness of its dispersion, the amount of water in the casein, to cite just a few examples, all contribute to the difficulties encountered in the routine examination of dairy products for insect parts, hairs or pieces of hairs, pieces of manure, or nondescript soil and debris.

[1] Specific gravity may be increased by addition of CCl_4 if necessary to float plant tissue. Do not add CCl_4 beyond equal parts CCl_4 to $CHCl_3$.

* For report of Subcommittee D and action of the Association, see *This Journal*, **38**, 85, 86 (1955).

The numerous varieties of cheese may be grouped into four divisions for the purpose of extracting extraneous materials.

Group 1 includes soft cheeses with high fat and moisture content such as cream, neufchatel, and creamed cottage cheese. It also includes semi-hard cheeses which are made from whole milk and partly skimmed milk. Usually they are aged for more than 60 days. Cheddar, colby, brick, swiss, gruyere, edam, and limburger belong in this group.

Group 2 includes hard cheeses, made from skimmed milk, which have been aged for long periods of time. Parmesan, romano, sardo, and other hard grating cheeses made from cow, sheep, or goat's milk or mixtures of these milks are in this group.

Group 3 includes cheeses made from skimmed milk and whey or from whey alone. They may contain some fat. The protein has been heated to high temperatures. Ricotta, ziger, and mysost are in this group.

Group 4 includes cheeses cured with mold and those containing plant tissues or spices for flavor. Blue, roquefort, gorgonzola, and kuminast are examples.

The hard cheeses are difficult to disintegrate in water and changes in the casein complex make it difficult to form colloidal solutions of the cheese solids.

A method in which the cheese is digested with enzymes, and in which some of the proteins are partially solubilized and then a colloidal solution in hot acid-alcohol solution is obtained, has been found satisfactory by the Associate Referee for the examination of cheese in groups 2 and 3. This method is (e) in the description of methods that appears below.

The cheeses in group 4 should be studied in the future for new methods of examination.

RECOMMENDATIONS

It is recommended*—

(1) That Methods **35.13, 35.14, 35.15, 35.16, 35.17,** and **35.21** be deleted and replaced by the following:

Filth in Butter, Cheese, Cheese Products, Dried Milk Products, and Dairy Products in General

Used independently or in various combinations the following procedures are those recommended for the products as specified. In all cases weigh out 225 g into a suitable container and use S&S ruled No. 410 filter paper for filtration. Cut hard cheese into small pieces approx. 5–7 mm wide and 2 mm thick.

(a) *Butter.*—Place container in H_2O bath or oven at ca 80°C. When fat has sepd, pour directly thru paper, using suction in a suction funnel and retaining most of the curd and H_2O in the container. Filter remaining material after the fat has passed thru and examine. To facilitate filtration of curd, wash filter paper with boiling H_2O during filtration. (For butter not filterable by this process, see (c)). Examine filter microscopically.

(b) *Evaporated milk; condensed milk; sweet cream, or milk; spray-dried whole,*

* For report of Subcommittee D and action of the Association, see *This Journal*, 38, 85, 86 (1955).

or skim milk.—Reconstitute dried or concd products. Dil. the product with an equal vol. of hot H_2O, or hot 3% $Na_2C_2O_4$ soln, or hot 2% Na_2CO_3 soln and filter, using suction. During filtration, continually wash the filter with a stream of nearly boiling H_2O to prevent the accumulation of a layer of particles which clog the paper. Examine filter microscopically.

(c) *Basic method for soft and semi-hard cheese, sour cream, some difficult dried whole and skim milks, and butter that cannot be filtered by* (a).—Heat sample in 1.5–2 l beaker with 800–1000 ml 2% H_3PO_4 soln with continuous stirring until the mixt. is boiling, or add cheese to boiling H_3PO_4 soln. Use slow speed mechanical stirrer. In order to disperse, continue boiling up to 20 min. Filter. Do not allow mixt. to accumulate on paper. During filtration, continually wash the filter with a stream of nearly boiling H_2O to prevent the clogging of the filter. When filtration is impeded, add H_2O, dil. (1–5%) alkali, dil. H_3PO_4 soln, and/or hot alcohol until paper is cleared; then resume addns of material and H_2O. Examine filter microscopically.

(d) *Basic procedure for hard skim milk cheese, Romano, Ricotta, Feta, Pecorino, Sardo, goat's milk cheese, Sbrinz, Goya, and those made of whey.*—After dispersing by (c) above, cool to 35–40°C. and adjust to ca *p*H 7.5, using NaOH soln, and then to *p*H 8, using Na_3PO_4 soln. Add the filtered aq. ext. from 10 g pancreatin. Readjust *p*H to 8 after ca 5, 20, and 60 min. Allow to digest at least 3 hrs, preferably overnight. Bring to *p*H 2 with H_3PO_4, bring to boil, boil for ca 15 min., filter, and examine.

(If cheese cannot be handled by method given above, separate into 2 ca equal portions. To each half add ca 250 ml or up to an equal portion alcohol, boil for addnl 15 min., filter, and examine.)

(e) *Optional procedure for hard skim-milk cheese, etc., as above.*—Add 400 ml H_2O to sample. Adjust to *p*H 8 with Calgon[1] (sodium hexametaphosphate) soln, add filtered soln from 10 g pancreatin, and then add 10 g Na sulfite. Warm to 40°C., stir until thoroly mixed, and adjust to *p*H 8 with Calgon soln at intervals of 15 min. until stabilized. Digest overnight. Add an equal vol. ethyl alcohol, stir, and adjust to *p*H 2 with HCl. Bring to boiling on a hot plate with constant gentle stirring. Boil for 15 min. and filter, keeping the paper clean with near boiling H_2O. In some cases, a constant stream of hot H_2O played on the paper will facilitate filtering. If some lumps of cheese remain in the bottom of the beaker, add acid-alcohol-H_2O soln, and repeat boiling and filtering operations.

(f) *Cheese containing mold, plant tissues, and spices.*—Use methods (c), (d), or (e) for dispersal of cheese. Pour thru a No. 140 sieve, washing thoroly with a forcible stream of H_2O. Transfer material retained on the sieve to beaker. Add 200 ml 2% H_3PO_4. Boil until lumpy residue is dissolved. Filter again on No. 140 sieve, washing thoroly with a forcible stream of hot H_2O. With ca 200 ml 60% alcohol, transfer the material on the sieve to a trap flask. Cool, trap off, using gasoline and H_2O, filter, and examine.

Sediment in Cream, Butter, Cheese, Cheese Products, Dried Milk Products, and Dairy Products in General

(a) *Rapid method for sweet cream and cream in which the curd remains relatively soft and easy to disperse and there is no mold.*—Place 1 pint cream in a beaker or pan of convenient size, ca 2 l, and add ca 1 pint hot H_2O (70–90°C.). More or less H_2O may be added so that the mixt. when ready for filtration will be 45–60°C. Remove whole flies or other large filth particles which float to the surface and which would be broken up by the stirrer. Place these on the sediment pad when completed. Place the pan under a malted milk stirrer and add, while stirring, sufficient 40% Calgon soln to make the mixt. slightly alk. to red litmus. Use not less than 25 ml; an excess will not interfere with filtration. Stir 30–60 sec. or until curd is broken up. Filter

[1] Calgon, Adjusted. Manufactured by Calgon Inc., Pittsburgh, Pa.

with vacuum thru a sediment pad (if the pad clogs, filter the remaining portion thru a fresh disk). Rinse pan and funnel onto a std size sediment disk (see below) with hot H_2O.

(b) Use procedures (c), (d), (e), or (f) and filter thru a std size sediment disk in holder which confines filtration area to a circle 1″ in diam. In prepn of sediment pad, violent mechanical agitation such as provided by a malted milk stirrer may be used with any of these methods to facilitate dispersion of the product.

(2) That the new method for hard cheeses be studied collaboratively.

(3) That the methods for extraneous materials in cheese that contains mold, plant tissues, and spices be studied.

(4) That the study of methods for the identification of manure fragments in dairy products be continued.

(5) That the methods described above be adopted as first action.

REPORT ON EXTRANEOUS MATERIALS IN NUT PRODUCTS

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

During the year, the methods for filth in shelled nuts (**35.22**) were restudied with a view toward standardizing those general procedures common to various filth methods. Based on experience obtained in these studies, it is recommended* that the entire section be revised as follows:

SHELLED NUTS

REAGENTS

(a) *Tween 80–60% alcohol solution.*—To 40 ml Tween 80 add 210 ml 60% alcohol, mix, and filter. Proportionate stock reagent quantities may be prepd.

(b) *Na_4EDTA–60% alcohol solution.*—Dissolve 5 g Na_4EDTA in 100 ml H_2O, add 150 ml 95% alcohol, mix, and filter. Proportionate stock reagent quantities may be prepd.

For Na_4EDTA (tetra sodium salt of ethylenediamine tetraacetic acid) use either (1) Versene, Regular Powder: Bersworth Chemical Co., Framingham, Mass.; or (2) Sequestrene Na_4: Alrose Chemical Co., Providence 1, R. I.

Whole Nut Meats and Large Pieces

(a) *Light filth (except peanuts with adhering testa.)*—Place 100 g sample in a 1 l trap flask. Add 10 ml 10% Na oleate soln and ca 600 ml 60% alcohol. Heat to boiling; then cool below 20°. Add 35 ml gasoline and ext. In the first trapping allow mixt. to stand 5 min. before filling the flask with 60% alcohol. Use 20 ml gasoline for the second extn. Filter and examine. Pour the residue in the trap flask onto a No. 40 sieve. Rinse both flask and residue with H_2O. Transfer the material on the sieve to a white enamel pan and examine for gross filth.

* For report of Subcommittee D and action of the Association, see *This Journal*, 38, 85, 86 (1955).

(b) *Light filth (peanuts with adhering testa.)*—Place 100 g peanuts in a 2 l trap flask and add 250 ml Tween 80–60% alcohol soln. Mix well and add 60% alcohol to make a total vol. of ca 800 ml. Trap off twice from 60% alcohol in the usual manner, using 75 and 40 ml portions of gasoline, resp. Stir occasionally during the first 20 min. after the flask is filled. Allow the flask to stand undisturbed 1 addnl hr for each of the 2 extns. Trap off and filter, using 60% alcohol as a rinse. (Acetone may be used to decolorize the material on the filter.) Examine filters.

Granulated Nut Meats or Mixtures Containing Substantial Amounts of Fine Granular Material

(a) *Heavy filth.*—Weigh 100 g sample into a 600 ml beaker. Add ca 350 ml petr. ether and boil gently 30 min., adding petr. ether to maintain the original vol. Decant the ether, taking care not to lose any coarse nut tissue, and discard the decantation. Add ca 300 ml $CHCl_3$ to the beaker and allow to settle 10–15 min. Pour off the floating nut meats and ca 2/3 of the $CHCl_3$ thru a 15 cm (or larger) filter paper in a büchner, using care not to disturb the residue in the bottom of the beaker. Repeat this sepn with smaller quantities of a mixt. of $CHCl_3$ and CCl_4 (1+1) until the residue becomes relatively free of particles of nut meats. Transfer the residue to ashless filter paper and examine for heavy filth. If an appreciable amount of sand and soil is present, ignite the filter paper in a tared crucible at ca 500° and weigh.

(b) *Light filth.*—Invert the filter paper contg the decanted nut tissue from (a) over a smooth sheet of paper. Break up any caked material and dry overnight at room temp. or in an oven at ca 80° for 1 hr. Proceed as follows:

(1) *Granulated pecans.*—Transfer dried nut meats to a heavy-wall 2 l trap flask. Add ca 300 ml 60% alcohol. Rinse down sides of trap flask and apply vacuum ca 10 min.[1] Occasionally swirl the contents of the flask to facilitate the removal of the entrapped air. Release the vacuum and trap off, using 50 and 25 ml portions of gasoline for the two trappings mentioned in **35.4**(a). In the first trapping, after stirring in the gasoline, allow to stand 5 min. before filling the flask with 60% alcohol. To the trappings add an amount of HCl equal to 1% of the vol., bring to boil, filter, and examine.

(2) *Granulated black walnuts.*—Transfer dried nut meats to a 2 l trap flask, assisting the transfer and rinsing the 15 cm filter paper with ca 300 ml alcohol. Stir slightly and allow the mixt. to soak 10 min. Add 250 ml Tween 80–60% alcohol soln and mix.[2] Quickly add 250 ml Na_4EDTA–60% alcohol soln and ca 70 ml gasoline. Stir immediately in the usual manner. Fill the flask with 60% alcohol. After the flask is filled, stir occasionally during the first 20 min. Allow the flask to stand undisturbed 1 addnl hr. Trap off, taking care not to disturb the interface. Rinse the rod and the neck of the flask with 60% alcohol and filter. If debris is present in trappings, add an amount of HCl equal to 1% of the vol. of the trappings, bring to a boil, and filter. Repeat the above extn, using 40 ml gasoline and 1.5 hrs standing. Examine the filters.

(3) *Granulated nut meats (except pecans and black walnuts).*—Follow the methods as given for (b)(2) thru addn of the 60% alcohol. Instead of allowing the mixt. to soak 10 min., heat the mixt. to an incipient boil. (Do not boil or the filth recovery will be lowered.) Cool to below 20° and follow the black walnut method as above, beginning with "Add 250 ml Tween 80–60% alcohol . . . ."

NOTE: Curculio larvae cannot be extd by gasoline flotation.

---

[1] Vacuum flask: Fit the mouth of the heavy-wall flask with a large 1-hole rubber stopper provided with a glass tube thru its center, of sufficient diam. to fit over the protruding rod of the trap flask. Control the vacuum with a 2-way glass stopcock in the vacuum line. If the stopcock tube is of sufficient diam. and length, it may be used as the glass tube thru the center of the stopper.

[2] Add reagents, mix gasoline, and fill flask with 60% alcohol without time interruption. Operate only 1 flask at a time for these 3 steps.

The essential differences between the present methods in section **35.22(a)** and those in the recommended section are as follows:

The present method for heavy filth, **35.22(a)**, has been deleted, since ordinary visual examination is suitable for detecting heavy filth in whole nut meats and large pieces.

A method for light filth in finely granulated pecans, consisting of a simple gasoline–60 per cent alcohol flotation after deaëration, has been incorporated in the granulated nut meat section **35.22(c)**. Other methods have been found to be unsatisfactory for handling this product.

The light filth procedure for granulated nut meats other than pecans and black walnuts has been modified to include a preliminary heating of the 60 per cent alcohol mixture before extraction. Various granulated nut meats, particularly those that have been roasted, float with the gasoline layer unless the defatted nut meat–60 per cent alcohol mixture is first heated to incipient boiling and cooled.

RECOMMENDATIONS

It is recommended*—

(1) That **35.22(a)**, **(b)**, and **(c)** be replaced by the modified methods described in this report.

(2) That further work on methods for extraneous material in peanut butter be discontinued.

REPORT ON EXTRANEOUS MATERIALS IN CEREAL GRAINS, CEREAL PRODUCTS, AND CONFECTIONERY

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

Present methods have been considered unsatisfactory for the determination of filth in rye flour. A method reported last year[1] by M. G. Yakowitz for determining filth in granulated black walnuts, in which Na_4EDTA and Tween 80 are used as reagents,[2] has now been adapted by Mrs. Yakowitz to rye flour and subjected to collaborative testing.

METHOD

Insects, insect fragments, and rodent hairs in rye flour.—Weigh 50 g flour into a 2 l trap flask. Add 300 ml 60% alcohol, mix, and let stand 10 min. Add 250 ml Tween 80–60% alcohol soln and mix.[3] Quickly add 250 ml Na_4EDTA–60% alcohol soln and ca 70 ml gasoline. Immediately stir for 1 min. in the usual manner. Fill the

* For report of Subcommittee D and action of the Association, see *This Journal*, **38**, 85, 86 (1955).

[1] *This Journal*, **37**, 119 (1954).

[2] For reagents, see preceding report, p. 262.

[3] Add reagents, mix gasoline, and fill flask with 60% alcohol without time interruption. Operate only one flask at a time for these three steps.

flask with 60% alcohol. Stir occasionally during the first 20 min. after the flask is filled. At the end of the 20 min. period, rotate plunger to remove flour which has settled on its top surface. Raise rod so that plunger is above mass of flour at bottom of flask and rinse exposed portion of rod with small quantity of 60% alcohol. Clamp rod in place (a clothes pin may be used) so that plunger is held above flour mass to minimize flour settling on it. Allow the flask to stand strictly undisturbed 1 addnl hr. Trap off, taking care not to disturb the interface layer, rinse neck of flask with 60% alcohol, and filter. Repeat extn, using 40 ml gasoline and 1.5 hrs standing. Examine filters microscopically.

Two 50 gram samples were sent to each of seven collaborators. Each sample contained 45 grams rye flour, 5 grams white flour highly contaminated by insect fragments, and 26 added rodent hair fragments. One sample was to be analyzed by the method given above, the other by any method the analyst thought best suited to the product. The results obtained by the analysts are shown in Table 1.

TABLE 1.—*Recovery of filth from check rye flour*

COLLABORATOR NO.	BY PROPOSED TWEEN 80-Na_4EDTA PROCEDURE			BY ALTERNATE PROCEDURES			
	WHOLE INSECTS OR EQUIVALENT	INSECT FRAGMENTS	RODENT HAIR FRAGMENTS	METHOD NO.	WHOLE INSECTS OR EQUIVALENT	INSECT FRAGMENTS	RODENT HAIR FRAGMENTS
1	2	134	23	**35.33**	2	56	16
2	5	155	18	**35.29**	2	15	8
3	5	161	22	**35.33**	2	95	14
4	2	127	18	**35.33**	1	107	20
5	2	164	17	**35.40**[a]	0	123	9
6	3	139	19	**35.22, 35.33**	0	48	15
7	0	175	18	Pancreatin digestion, 60% alcohol flotation	2	61	14

[a] Gasoline used instead of kerosene.

It is recommended* that this method be adopted as first action.

It has been suggested by N. Aubrey Carson, of the St. Louis District, U.S. Food and Drug Administration, that the Tween 80-Versene procedure be adapted to the methods for the examination of bakery products. Mr. Carson submitted some analytical data which shows that the suggested procedure has promise as a bakery products method. The method will be studied during the coming year.

Methods for the detection of internal insects in cereal grains are still being developed. While new and better methods may be developed later for the detection of internal insects, it has been found that the method

* For report of Subcommittee D and action of the Association, see *This Journal*, **38**, 85, 86 (1955).

given in the Associate Referee's report for 1952[4] has been used successfully by experienced analysts. It is recommended that this method be adopted as first action.

It is recommended that method **35.29(a)** as revised[4] be adopted as first action. This method has been found to give satisfactory results in the hands of experienced analysts.

It is recommended that the collaborative study of the revised method **35.28(b)** be continued, since the results obtained to date have not been conclusive.

It is recommended that the following be inserted in method **35.60(a)**:

Sieving for hard candies which contain materials which makes them difficult to filter by (**a**), *e.g., licorice candy.*—Follow (**b**), except substitute 0.5% HCl (1+70) soln for the 5–10% $Na_2B_4O_7 \cdot 10H_2O$ soln.

It is recommended that in method **35.60(b)**, line 5, "give a final alcohol rinse" be changed to read "give an alcohol and then a hot H_2O rinse." In line 9, before the word "alcohol" add "60%." The same addition should be made in the 10th line before the word "alcohol."

ACKNOWLEDGMENT

This Associate Referee and the Associate Referee on Extraneous Materials in Nut Products wish to thank Garland L. Reed of Cincinnati District; Mary C. Harrigan, Boston District; Helen T. Hyde, formerly of Cincinnati District; J. E. Roe, Denver District; William H. Munday, Minneapolis District; David Firestone, New York District; N. Aubrey Carson, St. Louis District, all of the Food and Drug Administration, for their cooperation in the study of these methods.

REPORT ON EXTRANEOUS MATERIALS IN VEGETABLE PRODUCTS

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

Two new methods and a few changes in existing methods in the section on Extraneous Materials in Vegetable Products are recommended this year. The changes are all of a minor nature, consisting of either rewording, clarifications, or small changes in procedure which have been shown to give better results than those previously used.

It is recommended* that in method **35.64**, second paragraph, second line, the phrase "and place small drop" be deleted, substituting "and with knife blade or scalpel place portion." In the third line of the same

[4] *This Journal*, **36**, 309 (1953).

* For report of Subcommittee D and action of the Association, see *This Journal*, **38**, 85, 86 (1955).

paragraph, delete "drop." In the fifth line of the paragraph, delete "drop" and insert "portion."

In this method, the statement regarding the magnification to be used for mold count of tomato products should be moved from the section on apparatus, **35.11(c)**, to section **35.64,** since it should be in the method itself.

Some confusion exists as to whether higher magnification can be used in checking the identity of filaments previously observed in the standard field of view. It is standard microanalytical procedure to use higher magnification when it is necessary to confirm the identity of objects. Many analysts have followed Mr. Howard's example and used higher magnification in the identification of occasional questionable filaments. It will promote greater uniformity and accuracy if this practice is specifically incorporated into the method.

It is recommended that the following changes be made:

In method **35.64,** at the end of the next to last paragraph, add: "Use magnification of 90–125×. In those instances where the identifying characters of the mold filaments are not clearly discernible in the standard field, use magnification of ca 200× (8 mm objective) to confirm the identity of mold filaments previously observed in the standard field." In the last paragraph of the same method, delete the last sentence.

In method **35.67** L. G. Ensminger[1] has shown that fifteen-minute trappings give better results than one-hour trappings. It is therefore recommended that the method be changed as follows: In the fourth line of (**a**), after "to settle," delete the remainder of that sentence and the next sentence, and insert "at 15 min. intervals trap off 15–20 ml bottom liquid and then gently shake contents of separator with a rotary motion to facilitate settling out of fly eggs and maggots." In the next to the last line of the first paragraph of the same method, after "are found" insert "during first hour" and continue with rest of sentence.

In the ninth line of **35.67(b)**, remove the sentence beginning "At end of 1 hr . . . " and insert "Draw off 25–30 ml bottom liquid before each 15 min. shake. These portions may be examined at once or successive portions may be combined for examination at end of hour. Pass drawn off portion through No. 10 sieve and rinse seeds and sieve thoroly, recovering both liquid portion and rinse H_2O in beaker." In the last paragraph of this method, after "If fly eggs or maggots are found" insert "during first hour."

During the last year, a series of samples of canned tomatoes were prepared and sent out for collaborative examination to determine the effect on fly egg and maggot recovery of (1) pulping with the Waring blendor and pulping by forcing the tomatoes through a No. 6 or No. 8 sieve, and (2) extracting with the 2 liter and 6 liter separatory funnels. Since it was found that the controls were contaminated, the results were inconclusive and unreliable, but there is some indication that both the Waring blendor and the 2 liter funnel are satisfactory. It is therefore recommended that when possible this work be repeated to obtain more conclusive results.

[1] *This Journal,* **37,** 730 (1954).

A method for detecting extraneous matter in potato chips has been studied. The method was apparently developed some time ago by R. T. Elliott, formerly of Seattle District, U. S. Food and Drug Administration. It has been tried and found satisfactory. It is recommended that the method, as follows, be adopted as first action.

FILTH IN POTATO CHIPS

Weigh 100 g into 1500 ml beaker. Crush chips into small pieces and cover with petr. ether. Let stand ca 5 min. and decant through filter. Again add ether and decant thru filter. Dry out all petr. ether from chips, transfer to 2 l trap flask, and add 500 ml 60% alcohol. Boil ca 30 min., replacing alcohol lost by evapn. Cool, add 35 ml gasoline, mix, let stand ca 5 min., and fill with 60% alcohol. Let stand, trap off twice, and filter in the usual manner. Examine filter papers microscopically.

During the past year a method for the detection of filth in canned sauerkraut was developed by analysts in the Buffalo District, U. S. Food and Drug Administration. The method has been tried by analysts from several other districts and found satisfactory. It is recommended that the method, as follows, be adopted as first action.

FILTH IN SAUERKRAUT—PROCEDURE

Use entire contents of can for containers of less than 2 lbs. Use 24 oz. well-mixed sample from larger containers. Wash thoroly, small portion at a time (ca 4 oz.), on an 8″ No. 8 screen nesting in an 8″ No. 20 screen with the washings finally passing thru an 8″ No. 140 screen. Wash the material remaining on the No. 20 screen with the washings passing thru the No. 140 screen. Transfer the material on the No. 20 screen to paper and examine at 10× for whole insects or large body parts. Transfer the material remaining on the No. 140 screen to paper and examine at 25× to 30×.

It is also recommended that work be continued on the various aspects of the methods for Extraneous Materials in Vegetable Products as opportunity and material become available.

REPORT ON EXTRANEOUS MATERIALS IN EGG PRODUCTS

By Joel J. Thrasher (Food and Drug Administration, Department of Health, Education, and Welfare, St. Louis 1, Mo.),
Associate Referee

The present method for extraneous matter in eggs (1) contains no procedure for the separation of extraneous materials from liquid or frozen eggs. An attempt was made to digest with phosphoric acid the material decanted from the sodium chloride specific gravity separation for chicken excrement. Almost none of the egg material was finely enough dispersed to filter through paper. Also, dried whole eggs treated by the present phosphoric acid procedure could not be filtered. This phosphoric acid procedure as written is limited to dried whole eggs. The present sodium hydroxide procedure does not recover hairs or feathers.

Work was undertaken to find a procedure by which both chicken excrement and extraneous matter could be determined successively on the same samples of egg products. An attempt was also made to increase the sensitivity of the uric acid test.

Technical ortho phosphoric acid, 75 per cent (1+11.5), has about the same specific gravity as the 5 per cent sodium chloride recommended for use in the current procedure for chicken excrement. The use of this concentration of phosphoric acid gave at least 90 per cent recoveries of added excrement ground to pass a No. 16 sieve. For other egg products, tests were conducted to find the minimum concentration of acid that would adequately separate the bulk of the egg material from the chicken excrement. In the proposed methods such concentrations are specified for each individual product. Work showed that the decanted material could be filtered successfully through paper by adjustment of the acid concentration and by proper heat treatment.

No general phosphoric acid digestion procedure was developed that would work for products containing albumen. For these products it was found that pepsin digestion is necessary and will work readily after proper coagulation of the albumen.

The lower limit of sensitivity of the color test for uric acid was determined to be about 0.2 micrograms when the proposed concentrations of reagents are used. The resultant color approximates that of E-2 or F-3, Plate 34, *Dictionary of Color*, by Maerz and Paul. No reagent blanks have shown color when the proposed reagents were used in the amounts and manner specified.

Collaborative samples were not submitted; the procedure worked out by the Associate Referee was sent to ten collaborators with instructions that each collaborator obtain eggs locally for testing. Reports were received from 6 collaborators.

CHICKEN EXCREMENT AND HEAVY FILTH

I. Frozen Eggs

(Thawed at not above room temperature)

(a) *Whole or yolks.*—Weigh 100 g directly into 250 ml centrifuge bottle. Add ca 75 ml (1+11.5) H_3PO_4 (75%, tech. grade), stopper, and shake well. Fill bottle with (1+11.5) H_3PO_4 and mix by swirling. Centrifuge at ca 1500 rpm for ca 5 min. Without disturbing the sediment decant the upper layer and bulk of liquid into a 1500 ml beaker and save for detn of extraneous matter and light filth. Repeat shaking and centrifuging until most of the egg material is decanted into 1500 ml beaker. Transfer centrifuged sediment to filter paper with H_2O.

Examine paper microscopically and remove white chalky or amorphous material to spot plate. Add 1 drop 1% NaOH to transferred material and macerate with glass stirring rod. With continuous stirring, add successively 2 drops 15%NaCN and 1 drop arsenophosphotungstic acid soln (**35.2**(e)). Prompt development of blue color demonstrates presence of uric acid or its salts.

(b) *Whites.*—Weigh 150 g into 250 ml centrifuge bottle and add (1+2) H_3PO_4

to fill bottle while stirring steadily. Centrifuge 2 min. at 1500 rpm. Let centrifuge stop without braking. Decant bulk of liquid and add an equal vol. of (1+1) H_3PO_4 to liquid in centrifuge tube. Shake well and centrifuge at 1000 rpm for 2 min., decanting closely. Shake once more with ca 50 ml (1+1) H_3PO_4 and allow to settle before decantation. Rinse sediment onto paper with cold H_2O and continue as in (a) above, beginning "Examine paper microscopically . . . ."

II. Dried Eggs

(a) *Whole eggs or yolks.*—(1) Weigh 25 g into 400 ml beaker, make to smooth paste with (1+9) H_3PO_4, and with continued stirring add H_3PO_4 (1+9) until beaker is almost full. After 10 min. stir top layer without disturbing sediment. Allow to settle 10 min. and repeat stirring. Allow to stand 10 min. more; then decant upper layer into 1500 ml beaker and save for detn of extraneous matter and light filth. Repeat flotation step to remove most of remaining egg material from sediment. Rinse sediment onto paper with H_2O. Continue as for frozen eggs, (a) above, beginning with "Examine paper microscopically . . . ."

Or, (2) weigh 25 g into 250 ml beaker and make to smooth paste with H_3PO_4 (1+9) by adding successive small vols while stirring thoroly. Transfer smooth paste to 250 ml centrifuge bottle, and fill bottle with (1+9) H_3PO_4. Centrifuge at 700–800 rpm for 2.5–3 min. Decant upper layer into 1500 ml beaker and save for detn of extraneous matter and light filth. Add (1+9) H_3PO_4 to centrifuge bottle, shake, centrifuge, and decant to complete sepn of egg material from sediment. Transfer sediment to paper with H_2O. Continue as for frozen eggs, (a) above, beginning with "Examine paper microscopically . . . ."

(b) *Whites.*—Proceed as for extraneous matter and light filth in dried eggs, (c).

EXTRANEOUS MATTER AND LIGHT FILTH

I. Frozen Eggs

(Thawed at not above room temperature)

(a) *Whole eggs or yolks.*—Weigh 100 g into 1500 ml beaker and add 600 ml (1+4) H_3PO_4 (75% tech. grade), or use the decanted material from the detn of chicken excrement in frozen eggs, (a) above, adding H_3PO_4 to make to (1+5) H_3PO_4 in final vol. of not less than 700 ml. Heat 5–7 min. in steam bath with stirring and transfer to hot plate. Boil vigorously for ca 5 min., guarding against violent frothing. (Up to 8 drops Dow-Corning Antifoam "A" emulsion may be used to control frothing.) Filter boiling soln immediately with full suction, pouring slowly onto center of paper. Wash paper with hot water.

(b) *Whites.*—Weigh 150 g into 800 ml beaker and heat on top of steam bath for 3 min. with thoro stirring. Do not allow any material to stick to bottom or to the sides. Lower beaker into steam bath and heat for 3 min. with stirring. Add ca 10 ml (1+3) HCl and cool to 35–40°. Adjust to *p*H 1.5–2.0, using (1+3) HCl or (1+3) NH_4OH. Add 100 ml 2% citric acid and filtered ext. of 1 g pepsin in 50 ml H_2O. Transfer to 38–40° oven for overnight digestion. Readjust frequently to *p*H of 1.5–2.0 during first 1.5 hrs. Filter with full suction, pouring slowly onto center of paper. Wash paper with warm H_2O.

II. Dried Eggs

(a) *Whole eggs.*—Weigh 25 g into 600 ml beaker and make to smooth paste with H_2O by adding successive small vols while stirring. Use a total of ca 150 ml. Heat on top of steam bath for 3–5 min. with thoro stirring. Lower into bath and heat 5–7 min. more, stirring well. Add 10 ml (1+3) HCl; then cool to 38–40°C Adjust to *p*H 1.5–2.0, using (1+3) HCl or (1+3) NH_4OH. Add 100 ml 2% citric acid and filtered ext. of 1 g pepsin in 50 ml H_2O. Transfer to 38–40° oven for overnight digestion. Readjust frequently to *p*H of 1.5–2.0 during first 1.5 hrs.

Transfer to 1500 ml or 2 l beaker, preferably stainless steel, adding sufficient H_3PO_4 to make to (1+5) H_3PO_4 in final vol. of not less than 700 ml. Use full heat for 5–7 min. in steam bath and then boil vigorously for ca 7 min. Control violent frothing by use of up to 8 drops Dow-Corning Antifoam "A." Filter boiling soln immediately under full suction, pouring slowly onto center of paper.

(b) *Yolks.*—(1) Weigh 25 g into 400 ml beaker and make to smooth paste with (1+5) H_3PO_4. Transfer to 2 l beaker with (1+5) H_3PO_4, using total of 700 ml. Or (2), use the decanted material from detn of chicken excrement in dried eggs, (a), adding sufficient H_3PO_4 to make to (1+5) H_3PO_4 in final vol. of not less than 700 ml. Continue as for detn of extraneous matter in frozen eggs, (a), beginning "Heat for 5–7 min in steam bath . . . ."

(c) *Whites.*—(1) *Powdered.*—Weigh 25 g into 600 ml beaker and stir to smooth paste with H_2O by adding successive small vols with thoro stirring. Use ca 150 ml total. Continue as for extraneous matter in frozen eggs, (b), beginning with "and heat on top of steam bath . . . ."

(2) *Flakes.*—Weigh 25 g into 600 ml beaker and add 200 ml warm H_2O (40°–50°). Soak with occasional stirring for at least 4 hrs. When egg material is completely dispersed (resembles a turbid soln), place on steam bath and coagulate with low heat, stirring steadily. Lower into bath for 2 or 3 min. at full heat, stirring steadily. Continue as for extraneous matter in frozen eggs, (b), beginning with "Add ca 10 ml (1+3) HCl."

As will be seen, the above procedure is a modification and extension of methods **35.43** and **35.44** in *Official Methods of Analysis* of the A.O.A.C.

COLLABORATIVE STUDY

Since samples were to be obtained locally, the types of samples used were not uniform. Collaborator 1 used frozen and dried yolks and whites; Collaborator 2, frozen whole eggs; Collaborator 3, dried and frozen whole eggs, frozen whites, and frozen yolks with added sugar and salt; Collaborator 4, fresh whole eggs, yolks, and whites, dried yolks, powdered whole eggs and whites, and flaked whites; Collaborator 5, frozen yolks and whites, dried yolks, and powdered and flaked whites; and Collaborator 6, frozen whole eggs.

The results obtained by the collaborators may be summarized essentially as follows:

Collaborators 1, 2, 3, 5, and 6 found the procedures for determination of chicken excrement and heavy filth and of extraneous matter and light filth to be satisfactory for frozen eggs (whole or yolks). Collaborators 1 and 3 considered the method for chicken excrement and heavy filth satisfactory for frozen whites; Collaborators 3 and 5 found it satisfactory for dried whole eggs and yolks, respectively. Collaborators 4 and 5 obtained satisfactory results in the determination of extraneous matter and light filth in dried egg yolks; Collaborator 3 had good results with this method for frozen whites, but Collaborator 1 felt that the procedure was unsatisfactory in this instance.

Collaborators 1 and 4 obtained unsatisfactory results in determining chicken excrement and heavy filth in dried whole eggs and yolks and also in dried whites; Collaborator 1 found the method for extraneous matter and light filth unsatisfactory for dried yolks, but satisfactory for

dried whites. Collaborator 5 considered that the procedure for extraneous matter and light filth was satisfactory for powdered whites and flaked whites; Collaborator 4 found it unsatisfactory for flaked whites, but obtained satisfactory results for powdered whole eggs and powdered whites when he used 2 grams of pepsin and 24 hour digestion. Collaborator 4 also applied both methods to fresh whole eggs and to fresh yolks and whites separately, and found the methods satisfactory in all instances.

DISCUSSION

The quality of pepsin seems to be rather important; although three collaborators reported no difficulty with one gram of pepsin, one needed two grams and an extended digestion time. It is difficult to be sure of the amount of pepsin needed and its quality, since its activity is so closely related to the optimum *p*H—2 ± 0.2 (2, 3). Since this narrow range of *p*H is difficult to check accurately and maintain for any length of time, the difficulty with whites of eggs may thus be accounted for in part. Proper coagulation of albumen prior to pepsin digestion is important since pepsin attacks raw egg albumen only very slowly (3). This fact could account for one collaborator's success with dried egg whites although he had trouble with frozen whites.

It is not easy to prepare a smooth paste of dried egg yolks, and several collaborators reported trouble with this step. Too much importance may have been attached to producing a paste free from lumps. The lumps will disperse in the follow-up step for extraneous materials, and since uric acid is stable to overnight pepsin digestion or to boiling for 10 minutes in (1+4) phosphoric acid, it would be recovered. A suggestion regarding the use of 35 ml of 95 per cent ethyl alcohol has not yet been tried.

It is recommended* that this subject be given further collaborative study in line with the comments of the collaborators and the above discussion.

ACKNOWLEDGMENT

The Associate Referee takes this opportunity to thank the following collaborators, all of the Food and Drug Administration:

E. Coulter, Chicago; R. Edge, Denver; M. McEniry, St. Louis; J. Thoms, New Orleans; and D. Tilden, San Francisco.

REFERENCES

(1) *Official Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Box 540, Benjamin Franklin Station, Wash. 4, D. C., 1950.

(2) WAKSMAN, S. A., and DAVISON, W. C., *Enzymes*, Williams and Wilkins Co., Baltimore, 1926.

(3) TAUBER, H., *Chemistry and Technology of Enzymes*, John Wiley & Sons, Inc., New York, 1949.

No report was given on extraneous matter in miscellaneous materials.

* For report of Subcommittee D and action of the Association, see *This Journal*, 38, 85, 86 (1955).

MONDAY—AFTERNOON SESSION

REPORT ON DISINFECTANTS

By L. S. STUART (Plant Pest Control Branch, Agricultural Research Service, U. S. Department of Agriculture, Washington 25, D. C.), *Referee*

Only one change is proposed for the official phenol coefficient method as it now appears in Chapter 5 of the 7th Ed. of *Official Methods of Analysis*. Under *Calculation*, **5.164,** the concluding note should be revised to read:

NOTE: The commonly accepted criterion that disinfectants for general use be at a diln equiv. in germicidal efficiency to 5% phenol against *S. typhosa*, thru use of the calculation $20 \times S.$ *typhosa* coefficient to det. the number of parts of H_2O in which 1 part of germicide should be incorporated, is subject to confirmation by use-dilution methods. Where this criterion is found invalid, the highest diln that will kill in the use-dilution method should be used as the index to the highest diln for use in practical disinfection.

No change is proposed for the first action use-dilution confirmation test. Following acceptance of this method in 1952 (*This Journal*, **36,** 67 (1953)), additional collaborative studies were made in cooperation with the Disinfectant Scientific Committee of the Chemical Specialties Manufacturers Association. The results of these studies showed that the method was quite precise with certain types of commercial products. However, with some commercial products the degree of agreement was only fair. It is therefore recommended* that the method remain first action.

Based on the information furnished by the Associate Referee, it is recommended that the fungicidal test as amended in 1953 (*This Journal*, **37,** 82 (1954)) be made official.

To avoid confusion in the interpretation of results obtained by the method, the following note should be added after *Operating Technic*, **5.170:**

NOTE: The highest diln which kills the spores within 10 min. is commonly considered as the highest diln which could be expected to disinfect inanimate surfaces contaminated with pathogenic fungi.

While it is universally recognized that adequate provisions must be made in all methods of testing disinfectants to avoid false reading due to bacteriostasis and fungistasis, the exact procedure specified in this method has been reported by the Associate Referee and other workers to be inadequate with certain types of products. Thus it is recommended that

* For report of Subcommittee A and action of the Association, see *This Journal* **38,** 66 (1955).

the present text of this method be clarified to emphasize the necessity of using adequate procedures to overcome these difficulties. This can probably be best accomplished by revising the sixth sentence under *Operating Technic*, **5.170,** to read:

To eliminate risk of faulty results owing to possibility of fungistatic action, make subtransfers from the initial dextrose broth subculture tubes to fresh tubes of dextrose-neopeptone broth, using the 4 mm loop prior to incubation; or make initial subcultures in dextrose-neopeptone broth contg either 0.05% sodium thioglycollate, 1.5% iso-octylphenoxypolyethoxyethanol, or a mixt. of 0.07% lecithin (Azolectin) and 0.5% sorbitan monoöleate (Tween 80), whichever gives the lowest results.

It is further recommended that the sporicidal test published by Ortenzio, Friedl, and Stuart (*This Journal*, **36,** 480 (1953)) be adopted as a first action method in accordance with the suggestion of the Associate Referee on Sporicides, and that the available chlorine germicidal equivalent concentration test, studied by the Associate Referee on Fungicides and Subculture Media, be adopted on the same basis.

REPORT ON FUNGICIDE AND GERMICIDAL RINSE TESTING METHODS

By L. F. Ortenzio (Plant Pest Control Branch, Agricultural Research Service, U. S. Department of Agriculture, Washington 25, D. C.), *Associate Referee*

In the recent book *Antiseptics, Disinfectants, Fungicides and Sterilization*, Stuart (1) outlined a procedure for determining the available chlorine germicidal equivalent concentrations of disinfecting solutions. This method, originally described by Cantor and Shelanski (2), was studied extensively in the laboratory of the Pesticide Regulation Section and some minor modifications were made to improve its precision and accuracy. Collaborative studies were then initiated. The results of these studies were sufficiently encouraging to justify a recommendation that this method be accepted as first action for determining the available chlorine germicidal equivalent concentrations of germicides used as disinfecting rinse solutions for previously cleaned non-porous surfaces.

In the collaborative studies, two samples were forwarded to each collaborator; one was a commercial 5.3 per cent aqueous solution of sodium hypochlorite and the other was a commercial iodine preparation containing 12.77 per cent of titratable iodine. Each laboratory was sent a copy of the procedure as published by Stuart (1) except that the *p*H 8.0 phosphate buffer was supplanted by a *p*H 8.5 phosphate buffer and the test culture increment volume of 0.2 ml was changed to a volume of

0.05 ml. Each laboratory was instructed to test the sodium hypochlorite preparation in solutions providing concentrations of 50, 100, and 200 p.p.m. of available chlorine and the iodine preparation in solutions providing concentrations of 6.25, 12.5, 25.0, 50.0, and 100.0 p.p.m. of titratable iodine.

Results were submitted by seven laboratories. The average number of negative increments for all solutions tested by the seven laboratories has been used to fill in a typical work sheet report reproduced as Table 1.

TABLE 1.—*Method of recording results*[a]

GERMICIDE	CONCN PPM	SUBCULTURE SERIES									
		1	2	3	4	5	6	7	8	9	10
NaOCl (Av. Cl)	200	−	−	−	−	−	+	+	+	+	+
	100	−	−	−	+	+	+	+	+	+	+
	50	−	−	+	+	+	+	+	+	+	+
I Prep. (Titratable I)	100	−	−	−	−	−	−	−	−	−	−
	50	−	−	−	−	−	−	−	−	+	+
	25	−	−	−	−	−	+	+	+	+	+
	12.5	−	−	+	+	+	+	+	+	+	+
	6.25	−	+	+	+	+	+	+	+	+	+

[a] − =No growth; + =growth.

The method of recording the results shown in Table 1 makes it possible to tell at a glance the concentration of the unknown germicide, in this case an iodine preparation, which gives the number of negative increments equivalent to solutions containing 200, 100, and 50 p.p.m. of available chlorine.

The average results shown in Table 1 are especially interesting because numerous investigators who have employed a variety of other methods have reported that the iodine preparation used has sufficient germicidal activity so that a solution containing 25 p.p.m. of titratable iodine is equivalent in disinfecting action to a solution containing 200 p.p.m. of available chlorine. It should be noted that in these tests both solutions provided 5 negative increments; this would seem to bear out conclusively the manufacturer's claim on this point. However, the results with solutions containing 12.5 and 6.25 p.p.m. of titratable iodine were not quite as good as results obtained with solutions containing 100 and 50 p.p.m. of available chlorine, respectively. One more negative increment was obtained in the available chlorine controls than in the iodine solutions. It will be shown later that these differences are probably within the range of allowable variation in the method, but they do suggest a slight inferiority in the 12.5 p.p.m. titratable iodine solution compared

to a solution of 100 p.p.m. of available chlorine, and in the 6.25 p.p.m. titratable iodine solution compared to a solution of 50 p.p.m. available chlorine.

The results submitted by all collaborators are summarized in Table 2. In only 2 instances was the standard deviation greater than a ±1 increment. This is rather remarkable for a biological test.

One laboratory reported difficulty in obtaining the phenol resistance of the test culture prescribed in the phenol coefficient test. One reported some difficulty in standardizing the control solutions of NaOCl. However, no laboratory made any specific objection to the procedure.

TABLE 2.—*Summary of results submitted by collaborating laboratories*

GERMICIDES AND CONCNS IN PPM	NO. OF NEGATIVE SUBCULTURE TUBES IN EACH SERIES REPORTED BY INDEPENDENT LABORATORIES							AV.[a]	STD. DEV.
	1	2	3	4	5	6	7		
NaOCl Control (Av. Cl)									
200	4	5	5	4	8	4	5	5	±0.8
100	2	4	3	3	4	2	2	3	±0.7
50	1	3	1	2	2	1	1	2	±0.7
I Prep. (Titratable I)									
100	10	10	10	10	10	10	10	10	±0
50	9	7	10	6	9	7	8	8	±1.1
25	5	3	9	3	7	4	4	5	±1.7
12.5	2	1	4	2	3	1	2	2	±0.7
6.25	1	1	1	1	1	1	0	0	±0.3

[a] Av. expressed as the nearest whole number.

It might be emphasized at this point that the adoption of this particular method which employs buffered standardized solutions of NaOCl as reference standards would set up a minimum standard of performance for available chlorine preparations as well as for chemicals of other types. Public health officials have repeatedly pointed out that certain chlorine-type germicides lacked the capacity and speed of kill necessary to disinfect previously cleaned surfaces in restaurants, taverns, and dairies. Thus, minimum standards based on 50, 100, and 200 p.p.m. of available chlorine derived from NaOCl in the presence of an $M/15$ phosphate buffer at pH 8.5 and a series of 1-minute exposure intervals would seem to be both reasonable and desirable.

To supplement the collaborative studies on the first action fungicidal test reported in 1953 (3) a series of studies were conducted on the relation of the phenol resistance of the spores of *T. interdigitale* to the end points obtained with commercial germicides of different types and on the efficacy of these products in actual use. These studies have shown that spores of lower resistance to phenol give a lower end point in the method with synthetic phenolic-type germicides than do spores of high resistance to phenol. This was not found to be true with cresylic acid, chlorine-type, and quaternary ammonium-type germicides.

In themselves these observations have only limited value in the absence of specific information on the efficacy of the various products in destroying pathogenic fungi on surfaces where they are commonly encountered. Extensive studies of the floors of locker rooms and shower stalls failed to provide conclusive information on this point. The distribution of the fungi residues in such places was so irregular that significant control data were not obtained. To overcome this difficulty and the objection raised by Oster and Golden (4, 5) and by Burlingame and Reddish (6) that results obtained with suspensions of conidiospores might not hold true for residues of other fungus forms such as dried mycelia and chlamydospores, a laboratory procedure was devised in which *T. interdigitale* was grown at room temperature on surfaces of previously sterilized ceramic tile squares, 3/4×3/4×3/16″, placed on top of sterile filter paper saturated with 30 ml of neopeptone broth in a sterile 150×15 mm Petri dish, and seeded with 1.0 ml of suspension containing 5,000,000 spores per ml. After 20 days' incubation, the tile squares were completely overgrown with a dense mycelial mat. Following a 7-day drying period at room temperature, it was found that these mats were so dense and water resistant that they were impermeable to the test solutions and the fungus would withstand 20 minutes' exposure to all concentrations of all of the germicidal solutions to which they were exposed. However, following a 3–5 minute scarification with sterile glass beads in a glass bottle shaken by hand, tile squares were obtained which carried dried fragments of all recognized fungus cell forms and which could also be readily immersed in aqueous solutions to provide contact between the organism and the fungicidal chemicals. It is reasonable to suppose that 10.0 ml volumes of dilutions of fungicides which would kill all of the fungus forms on this surface at 20°C. within 10 minutes, followed by a 5 minute wash in 30 per cent acetone, could be effective in actual use.

Table 3 presents results obtained with one commercial synthetic phenol preparation and one commercial cresylic acid preparation in the fungicidal test in which both freshly harvested spores and refrigerated spore suspensions were used. These results are compared with those of the simulated use test described above.

TABLE 3.—*Data obtained in fungicidal tests in which phenolic type germicides are used*[a]

GERMICIDE	DILUTION TESTED	A.O.A.C. FUNGICIDAL PROCEDURE		CERAMIC TILE CARRIER-USE DILN PROCEDURE FOR 10 MIN.
		FRESHLY HARVESTED SPORES RESISTING 1:65 DILN PHENOL FOR 10 MIN.	REFRIGERATED SPORES RESISTING 1:55 DILN PHENOL FOR 10 MIN.	
A	1–50	−	−	−
Synthetic	1–100	−	−	−
Phenol	1–150	−	+	+
	1–175	+	+	+
	1–200	+	+	+
	1–250	+	+	+
B	1–80	−	−	−
Cresylic	1–100	−	−	−
	1–125	−	−	−
	1–150	+	+	+
	1–200	+	+	+
	1–250	+	+	+

[a] + =Growth, − =no growth.

The data in Table 3 clearly show that the end point in the method with the synthetic phenol type preparation varied with the resistance of the spore suspension to the chemical control phenol. However, it also shows that there is a direct correlation between the maximum dilution which will kill in the simulated use test and the maximum dilution which will kill in the A.O.A.C. fungicide test in which the spores used have the resistance to phenol specified in the present method. While it might simplify the task of the laboratory technician to reduce the resistance requirement to the mean resistance found in the collaborative studies reported in 1953, the evidence developed on the relation of the end point in the method to the safe use-dilution would rule against any such change, at this time.

Similar studies employing quaternary ammonium germicides and mercurial type germicides produced erratic results which were traced directly to fungistatic effects of small amounts of these materials carried over to the dextrose-neopeptone subculture broth by the transfer needle in the A.O.A.C. fungicide test method. In many instances, these effects could not be avoided or overcome by the subtransfer method stipulated in the present test. On the other hand, they could be readily overcome by adding suitable neutralizing or reversing substances to the dextrose-neopeptone broth employed in subculturing. It is essential, therefore, that the method be amended to provide for the use of such materials in subculturing. By way of example, results obtained in the study with two commercial quaternary ammonium germicides are presented in Table 4.

TABLE 4.—*The effect of neutralizers in testing quaternary ammonium compounds against T. interdigitale*[a]

QUATERNARY GERMICIDE	DILUTIONS TESTED	MEDIA														
		NEOPEPTONE DEXTROSE ONE TRANSFER			NEOPEPTONE DEXTROSE SUBCULTURE						NEOPEPTONE DEXTROSE LETHEEN			NEOPEPTONE DEXTROSE 2% TAMOL N		
		EXPOSURE INTERVALS IN MINUTES														
		5	10	15	5	10	15	5	10	15	5	10	15	5	10	15
A	1–10	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+
	1–20	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+
	1–30	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+
	1–50	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+
	1–75	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
	1–100	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+
	1–150	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B	1–10	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+
	1–20	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+
	1–30	−	−	−	−	−	−	+	−	−	+	+	+	+	+	+
	1–50	−	−	−	−	−	−	+	+	−	+	+	+	+	+	+
	1–100	−	−	−	−	−	−	+	+	−	+	+	+	+	+	+
	1–150	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+
	1–200	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+

[a] − =No growth; + =growth.

It can be seen from Table 4 that the subculture procedure presently specified in the test only partially overcomes the fungistatic effect of the quaternary ammonium compounds. Growth in the initial subculture in the neopeptone broth containing either Letheen or Tamol N occurred with both products at a lower dilution than in the subculture procedure.

SUMMARY AND RECOMMENDATIONS

(1) A procedure for determining the available chlorine equivalent germicidal concentration of rinses recommended for disinfecting non-porous surfaces after cleaning was studied collaboratively. The results of these studies showed the method to be very precise. It is recommended* that this method be accepted as first action.

(2) Studies initiated in 1953 on the phenol resistance of the spores of *T. interdigitale* were continued. These studies have shown that there is good agreement between the end point in the method and the effective dilution in disinfecting inanimate surfaces when spores of the resistance specified in the present method are employed. If spores of resistance lower than that specified in the present method are employed, the dilutions of synthetic phenolic disinfectants active in relatively high dilutions which would apparently have to be employed to disinfect surfaces would

* For report of Subcommittee A and action of the Association, see *This Journal*, 38, 66 (1955).

be lower than would kill in the method. These observations rule against any change in the resistance requirement in the present method, at this time.

(3) Data have been presented to show that the subtransfer method for eliminating the risk of faulty results due to fungistatic action in the fungicidal test is not adequate for all types of germicides. It is recommended that the method be amended to provide for the use of subculture media containing recognized neutralizers or reversing substances.

ACKNOWLEDGMENT

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

(1) Dr. Cecil W. Chambers, Robert A. Taft Sanitary Engineering Center, Cincinnati, Ohio.

(2) Dr. Thaddeus Lewandowski, Pennsylvania Salt Manufacturing Company, Philadelphia, Pa.

(3) Mr. Alfred L. Sotier, Wyandotte Chemicals Corporation, Wyandotte, Mich.

(4) Dr. M. J. Pelczar, Bacteriology Department, University of Maryland, College Park, Md.

(5) Dr. A. Cantor, Industrial Toxicology Laboratory, Philadelphia, Pa.

(6) Miss Rebecca Shapiro, Hudson Laboratories, New York City, N. Y.

REFERENCES

(1) STUART, L. S., in *Antiseptics, Disinfectants, Fungicides and Sterilization*, 1st Ed., edited by G. F. Reddish, Lee & Febiger, Philadelphia, 1954, p. 125.

(2) CANTOR, A., and SHELANSKI, H. A., *Soap Sanit. Chemicals*, **27**, 133 (1951).

(3) ORTENZIO, L. F., *This Journal*, **37**, 616 (1954).

(4) OSTER, K. A., and GOLDEN, M. J., *Am. J. Pharm.*, **121**, 375 (1949).

(5) ———, and ———, *J. Am. Pharm. Assoc., Sci. Ed.*, **36**, 283 (1947).

(6) BURLINGAME, E. M., and REDDISH, G. F., *J. Lab. Clin. Med.*, **24**, 765 (19?9).

REPORT ON SPORICIDAL TESTS FOR DISINFECTANTS

By J. L. FRIEDL (Plant Pest Control Branch, Agricultural Research Service, U. S. Department of Agriculture, Washington 25, D. C.), *Associate Referee*

Since the beginning of regulatory activities under the Insecticide Act of 1910, officials have employed methods for testing sterilizers and sporicides based on the use of dried bacterial spores resistant to constant boiling hydrochloric acid. A preferred procedure for obtaining spores of the desired resistance and exposing them to germicides was developed and published in 1953 by Ortenzio, Stuart, and Friedl.[1] This preferred procedure has now been evaluated in collaborative studies.

[1] *This Journal*, 36, 480 (1953).

Two test organisms, *Bacillus subtilis* and *Clostridium sporogenes*, a supply of constant boiling hydrochloric acid, and samples of two unknown commercial germicides were forwarded to all collaborators, together with a detailed description of the method. Sample No. 1 was a commercial cresylic acid germicide recommended as a disinfectant at a dilution of 1:64. The manufacturer made no claims for its value as a sporicide. However, the collaborators were requested to test it as a sporicide at this one dilution. Sample No. 2 was a formaldehyde-type germicide for which the manufacturer made sporicide claims. It was recommended for use undiluted, and the collaborators were requested to test it undiluted.

The method used is as follows:

SPORICIDAL TEST

(Applicable for use with germicides to determine presence or absence of sporicidal activity and potential effectiveness in disinfecting against specified spore-forming bacteria)

REAGENTS

(a) *Culture media.*—(1) *Soil extract nutrient broth.*—Ext. 1 pound garden soil in 1 l distd H_2O, filter several times through S&S No. 588 paper (or equiv.), and bring to vol. The *p*H of this ext. should be 5.2 or higher. Add 5 g beef extract (Difco), 5 g NaCl, and 10 g Armour's peptone; boil for 20 min., adjust to *p*H 6.9, filter thru paper, and bring to vol. Dispense in 20×150 mm test tubes, plug with cotton, and sterilize at 15 lbs steam pressure for 20 min. Use this broth for propagation of test culture of *Bacilli.* (2) *Nutrient agar.*—Same as described in the official phenol coefficient method, **5.161**(a)(2). Use slants of this medium for maintaining stock culture of *Bacilli.* (3) *Fluid thioglycollate medium (U.S.P. XIV).*—Same as described in **5.161**(a)(3)(b), except to add 20 ml normal NaOH to each liter before dispensing for sterilization. Use this medium for subculturing spores exposed to constant boiling HCl. For spores exposed to unknown germicides use fluid thioglycollate medium, U.S.P. XIV. (4) *Soil extract meat egg medium.*—Add 1.5 g meat egg medium to individual 20×150 mm test tubes; then add 10 ml garden soil ext. as described above, plug with cotton, and sterilize at 15 lbs steam pressure for 20 min. Use this medium for propagating test cultures of *Clostridia* and maintaining stock cultures of species of this genus.

(b) *Test organisms.*—Any species of *Clostridia* or *Bacilli:* Strains of *Bacillus subtilis* and *Clostridium sporogenes* may be employed in routine evaluations, but the method is applicable for use with strains of *B. anthracis, Cl. tetani,* or other species in which the investigator may have interest.

(c) *Constant boiling HCl.*—Dil. analytical reagent grade HCl (33–37% HCl) with equal quantity of H_2O. Adjust sp. gr. if necessary to 1.10 (spindle). Place 1500 ml in 2 l flask connected to straight inner-tube condenser, using rubber or ground-glass stoppers. (Distg system should be of resistant glass constructed to completely condense all distillate and at the same time maintain exact atmospheric pressure thruout). To prevent superheating of any part of the system, place ten 1″ lengths of small diam. resistant glass tubing in boiling flask and so encase flask in sheet asbestos so that hot gases from flame strike flask only at bottom. Distil continuously at 5–10 ml/min. When 1125 ml has been distd, change receivers and catch next 225 ml (which is constant boiling HCl) in erlenmeyer, with end of condenser inserted into flask but not below surface of liquid. Use this for detg resistance of the dried spores. (The exact HCl concn may vary slightly, depending on the atmospheric

pressure. At 780 mm pressure, the concn will be 20.173% and at 730 mm pressure, it will be 20.293% or a mean molarity of approximately 5.5.

APPARATUS

(a) *Glassware.*—Pyrex lipped test tubes, 25×150 mm; 100 ml stoppered cylinders graduated in 1 ml divisions; supply of 15×110 ml Petri dishes matted with 2 sheets of filter paper. Sterilize all glassware 2 hrs in hot air oven at 180°C.

(b) *Water bath.*—Same as specified in **5.178**(b).

(c) *Racks.*—Same as specified in **5.178**(c).

(d) *Transfer loop and hook.*—Same as specified in the first action use-dilution confirmation test.

(e) *Suture loop carrier.*—From a spool of size 3 surgical silk suture, prep. std loops by wrapping the silk around an ordinary pencil 3 times, slipping the coil so formed off the end of the pencil, and holding it firmly with the thumb and index finger while passing another piece of suture through the coil, knotting and tying it securely. Then shear the end of the coil and knot suture off to within 1/16 in. This should provide an over-all length of ca 2.5 in. of suture in a 2 loop coil that can be conveniently handled in ordinary aseptic transfer procedures.

PROCEDURE

Grow all *Bacilli* in soil ext. nutrient broth and all *Clostridia* in soil ext. meat egg medium. Inoculate tubes, using 1 loop of the stock culture, and incubate at 37°C. for 72 hrs. Place supply of suture loop carriers in Petri dishes matted with filter paper and sterilize at 15 lbs for 20 min. Use new loops for each test. Place 5 sterile loops in each 72 hr culture; then agitate the tube vigorously, and allow to stand for 15 min. Withdraw the loops and place in sterile Petri dishes matted with 2 sheets of filter paper, and allow to dry for 22–26 hrs at room temp. Make all suture loop transfers with a 2–3″ nichrome wire hook, flamed in the conventional manner between transfers.

Transfer 10 ml constant boiling HCl into a sterile cotton-plugged 25×150 mm lipped test tube, place the tube in a constant temp. H_2O bath at 20°C., and allow it to come to temp. Transfer 4 dried contaminated loop suture carriers to the tube of acid. (Theoretically these transfers should be made simultaneously but in practice a few seconds elapse in making 4 successive transfers.) Transfer the remaining dried contaminated suture loop to a tube of the thioglycollate subculture medium as a viability control. After 5, 10, 20, and 30 min., withdraw individual loops from the acid and transfer to individual tubes of thioglycollate subculture medium. Rotate each tube vigorously for 20 sec. and incubate for 1 week at 37°C. Reliable readings can usually be made after 48 hrs incubation, but this will not always be true. If it appears that the *p*H of the subculture medium has been reduced by the acid carried over to a level below that which will permit growth of the test organism, transfer the loops to fresh tubes of media and reincubate for a second period of 7 days. The test spores should resist the HCl for at least 5 min. (Many will resist the HCl for the full 30 min. period and longer.) If this test shows that resistant dried spores are present (vegetative cells will not show a measurable resistance against constant boiling HCl), use replicate lots of dried contaminated suture loops drained and dried at the same time, and held at room temp. 7 days, to test the sporicidal activity of the germicide. (It has been established that spores dried and held under these conditions will retain their resistance for this period of time or longer.) Place 10 ml of the disinfectant, at the diln recommended for use or under investigation, in a 25×150 mm lipped test tube, place the tube in the H_2O bath at 20°C., and allow to come to temp. Select a set of 5 dried contaminated suture loops shown to be carrying resistant spores of the culture under study and use for tests on each diln of disinfectant. Transfer 1 suture loop immediately to a tube of thioglycollate

subculture medium as a viability control. Then transfer the remaining 4 loops to the diln of disinfectant in the H_2O bath. Remove individual loops at 4 selected intervals of time, e.g., 10 min., 30 min., 1 hr, and 2 hrs, transferring them to individual tubes of thioglycollate subculture medium. Shake all tubes thoroly and incubate for 1 week at 37°C. If no growth occurs, and if there is reason to suspect that lack of growth may be due to bacteriostasis, transfer each loop to a fresh tube of medium and incubate for a second period of 7 days at 37°C. Report the results as + (growth) or − (no growth) values.

NOTE: Dilutions of unknown germicides found to be effective against specific spores in this test might be expected to be effective in disinfecting against the same spores in actual use, providing an adequate contact period can be provided.

Complete data were submitted by four laboratories. The results which these four laboratories obtained with the control constant boiling hydrochloric acid are shown in Table 1. All four collaborators found that the spores of both test organisms resisted the constant boiling hydrochloric acid for 5 minutes. Growths were obtained in all instances in the first subculture after a 5-minute exposure. Three of the four collaborators found that the spores of *B. subtilis* resisted the acid for 10 minutes, two found the spores surviving after 20 minutes, but all found that the spores were killed after 30 minutes' exposure. Three of the four collaborators found that the spores of *Cl. sporogenes* survived after 30 minutes' exposure. Two of the collaborators found growths after subtransfer of the exposed spores which were not obtained in the primary culture. There were 8 instances where this occurred, representing 25 per cent of the total exposure periods in the study. This illustrates the importance of the subtransfer procedure in the method.

The results obtained with germicide No. 1 are shown in Table 2. None of the collaborating laboratories found germicide No. 1 capable of killing the spores of *B. subtilis* or *Cl. sporogenes* at the dilution tested. The primary value of the data on this product is the demonstration of complete agreement on its lack of sporicidal activity.

The results obtained with germicide No. 2 are shown in Table 3. All four collaborators found that the spores of *B. subtilis* were killed by germicide No. 2 within 30 minutes; two of the four laboratories found that they were killed within 10 minutes. Thus it would appear that this product is sporicidal for some spores. However, all four laboratories were in agreement that the product did not kill the spores of *Cl. sporogenes* in an exposure period of 120 minutes (2 hours). This illustrates the danger of drawing conclusions relative to sterilizing activity from the results obtained with resistant spores of a single species. The importance of the subtransfer procedure in this method is again clearly illustrated in the results shown in Table 3 for *Cl. sporogenes*. Three of the four laboratories would have found this product to be effective against the spores of this organism on the basis of the results obtained in the primary subcultures incubated for 7 days. Viable spores were not evident until the second incubation period of 7 days after subtransfer.

TABLE 1.—*Resistance of the test spores to constant boiling hydrochloric acid at 20°C.*[a]

LABORATORY	TEST CULTURE	EXPOSURE TIME IN MINUTES									
		0		5		10		20		30	
		FIRST CULTURE	SUB-TRANSFER	FIRST CULTURE	SUB-TRANSFER	FIRST CULTURE	SUB-TRANSFER	FIRST CULTURE	SUB-TRANSFER	FIRST CULTURE	SUB-TRANSFER
1	B. subtilis	+	+	+	+	+	+	−	−	−	−
	Cl. sporogenes	+	+	+	+	−	+	−	+	−	+
2	B. subtilis	+	+	+	+	+	+	+	+	−	−
	Cl. sporogenes	+	+	+	+	+	+	+	+	−	−
3	B. subtilis	+	+	+	+	−	+	−	−	−	−
	Cl. sporogenes	+	+	+	+	−	+	+	+	+	+
4	B. subtilis	+	+	+	+	−	−	−	+	−	−
	Cl. sporogenes	+	+	+	+	−	+	−	+	−	+

[a] + =growth; − =no growth.

TABLE 2.—*Results found with cresylic acid germicide at a dilution of 1:64*[a]

LABORATORY	TEST CULTURE	EXPOSURE TIME IN MINUTES									
		0		10		30		60		120	
		FIRST CULTURE	SUB-TRANSFER	FIRST CULTURE	SUB-TRANSFER	FIRST CULTURE	SUB-TRANSFER	FIRST CULTURE	SUB-TRANSFER	FIRST CULTURE	SUB-TRANSFER
1	B. subtilis	+	+	+	+	+	+	+	+	+	+
	Cl. sporogenes	+	+	+	+	+	+	+	+	+	+
2	B. subtilis	+	+	+	+	+	+	+	+	+	+
	Cl. sporogenes	+	+	+	+	+	+	+	+	+	+
3	B. subtilis	+	+	+	+	+	+	+	+	+	+
	Cl. sporogenes	+	+	+	+	+	+	+	+	+	+
4	B. subtilis	+	+	+	+	+	+	+	+	+	+
	Cl. sporogenes	+	+	+	+	+	+	+	+	+	+

[a] + =growth.

TABLE 3.—*Results found with formaldehyde-type germicide when tested undiluted*[a]

LABORATORY	TEST CULTURE	EXPOSURE TIME IN MINUTES									
		0		10		30		60		120	
		FIRST CULTURE	SUB-TRANSFER	FIRST CULTURE	SUB-TRANSFER	FIRST CULTURE	SUB-TRANSFER	FIRST CULTURE	SUB-TRANSFER	FIRST CULTURE	SUB-TRANSFER
1	B. subtilis	+	+	+	+	−	−	−	−	−	−
	Cl. sporogenes	+	+	+	+	−	+	−	+	−	+
2	B. subtilis	+	+	−	−	−	−	−	−	−	−
	Cl. sporogenes	+	+	−	+	−	+	−	+	−	+
3	B. subtilis	+	+	−	−	−	−	−	−	−	−
	Cl. sporogenes	+	+	+	+	+	+	+	+	+	+
4	B. subtilis	+	+	−	+	−	−	−	−	−	−
	Cl. sporogenes	+	+	−	+	−	+	−	+	−	+

[a] + =growth; − =no growth.

One collaborator reported that trypticase-soy broth gave a more efficient recovery of small numbers of surviving spores of *B. subtilis* than did the thioglycollate medium specified. This point should be given consideration in future studies. A fifth collaborator submitted complete data after the foregoing report was compiled. The only variation from the results reported above was that germicide No. 1 was found to kill the spores of *B. subtilis* at the longest exposure intervals. This result may be a reflection of some deficiency in the thioglycollate medium for this particular organism.

It is evident that the method has sufficient precision for referee use, and it is therefore recommended* that it be adopted as first action.

Acknowledgment should be given to the following individuals who acted as collaborators in these studies:

Dr. Samuel Molinas, Food and Drug Administration, Washington, D. C.
Mr. John F. Gain, Winthrop-Stearns, Inc., Rensselaer, N. Y.
Dr. Albert F. Guiteras, Hudson Laboratories, New York, N. Y.
Dr. A. Haldane Gee, Foster D. Snell, Inc., New York, N. Y.

No report was given on media for disinfectant testing.

REPORT ON PESTICIDES

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

Reports were received from six Associate Referees, four of which contained results of collaborative studies, and the Referee will summarize and comment briefly on the subject matter in these reports.

Benzene Hexachloride.—The Associate Referee continued as chairman of the Industry-Government Committee which was organized to study means of improving the method for gamma benzene hexachloride, **5.149–5.153.** Specifically, this method was compared in a collaborative study with a proposed modification involving polarography and partition chromatography. The results that were obtained by both methods were essentially the same, although some of the collaborators felt that the proposed modification merited further collaborative evaluation.

A new method for the determination of gamma benzene hexachloride, known as the radioactive isotope dilution method (1), was investigated by some of the collaborators and the results appeared to be highly precise and accurate. The Referee believes that it should receive further collaborative study.

---

* For report of Subcommittee A and action of the Association, see *This Journal*, 38, 66 (1955).

Parathion.—The Associate Referee concluded his work on parathion in the present report and requested that he be relieved of this assignment so that he could devote his full attention to the study of systemic insecticides during the coming year. A new Associate Referee on parathion was appointed. On the basis of collaborative results obtained during the past year, it is now possible to recommend adoption of a first action method for parathion. The results were highly satisfactory.

Pyrethrins.—The Associate Referee in a collaborative study compared two new procedures for the determination of total pyrethrins with the mercury reduction method, **5.113–5.114.** These procedures are known as the ethylenediamine method (EDA) (3) and the Levy-Estrada colorimetric method (2). The colorimetric method gave results that were in very good agreement with the mercury reduction method, but results by the EDA method were higher and less precise.

Rotenone.—The Associate Referee is continuing his work on rotenone.

Dieldrin.— The Associate Referee has presented some very interesting results on the determination of dieldrin by partition chromatography. For approximately a year, the Beltsville Laboratory of the Pesticide Regulation Section has been making use of a similar procedure for the analysis of dieldrin formulations. This appears to be another example of the usefulness of partition chromatography when applied in the analysis of insecticidal formulations.

Rodenticides.—The determination of warfarin in so-called concentrates by a spectrophotometric method does not appear to offer any serious problem. The determination of warfarin in baits and particularly in pelletized bait is not entirely satisfactory, however. The same situation appears to exist in the case of Pival baits and pelletized Pival baits.

Quaternary Ammonium Compounds.—Ultraviolet absorption is proving useful in the qualitative and quantitative determination of quaternary ammonium compounds in disinfectants. The determination of quaternary ammonium nitrogen by adaptation of the ferricyanide method, **27.30,** has also proved very useful, and the Referee believes that this method should be subjected to a collaborative study on disinfectants.

Several Associate Referees, although not giving reports for this year, have reported progress on their assignments.

At the 1953 meeting of the Association of Economic Poisons Control Officials, it was suggested that the uniform sampling procedure adopted by that Association be submitted for adoption by the Association of Official Agricultural Chemists. There is some question in the mind of the Referee regarding the advisability of placing sampling procedures in *Official Methods of Analysis* of the A.O.A.C. However, there is a precedent for such action, inasmuch as sampling procedures are found in several chapters of the book, and the Referee agrees that it would be desirable if official status could be given to a sampling procedure for

pesticides. After consultation with officials of this Association, the Referee favors adoption of a condensed version of the uniform sampling procedure which appears in the 1954 report of the Association of Economic Poisons Control Officials.

Recommendations 9 and 10 are made in connection with a revision of methods.

RECOMMENDATIONS

It is recommended*—

(1) That the Associate Referee on benzene hexachloride initiate a collaborative study of the radioactive isotope dilution method for the determination of gamma benzene hexachloride and continue the evaluation of the proposed modification of the partition chromatographic method, **5.149–5.153.**

(2) That the O'Keefe and Averell titration method as modified in reports of the Associate Referee be adopted as first action for the determination of parathion in technical parathion, dusts, wettable powders, and emulsifiable concentrates.

(3) That the investigation of analytical methods for the determination of pyrethrins be continued.

(4) That the study of methods for rotenone be continued, and that the ultraviolet procedures (*This Journal*, **37**, 630 (1954)) be studied collaboratively.

(5) That collaborative work be initiated on the determination of dieldrin by partition chromatography.

(6) That the method for the determination of warfarin in concentrates (*This Journal*, **37**, 634 (1954)) be adopted as first action; that a collaborative study be made on the determination of warfarin in finished baits; and that further investigational work be done on the determination of Pival in baits.

(7) That collaborative work be done on the ferricyanide method, **27.30,** for the determination of quaternary ammonium compounds in disinfectants and that further study be made of spectrophotometric methods.

(8) That potassium bromate be substituted for sodium bromate in official methods for the determination of arsenic which at the present time specify the use of sodium bromate.

(9) That the note added to the method for the determination of 2,4-dichlorophenoxy acetic acid (2,4-D) (*This Journal*, **34**, 674 (1951)) be deleted.

(10) That the dead-stop end point procedure for titration in the method for the determination of parathion (*This Journal*, **35**, 384 (1953)) be included in the method.

* For report of Subcommittee A and action of the Association, see *This Journal*, **38**, 65, 66 (1955.)

(11) That a condensation of the uniform sampling procedure which appears in the 1954 report of the Association of Economic Poisons Control Officials be adopted by the Association as a procedure for the sampling of pesticides.

REFERENCES

(1) CRAIG, J. T., and TRYAN, P. F., *Anal. Chem.*, **25**, 1661 (1953).
(2) LEVY, L. W., and ESTRADA, R. E., *J. Agr. Food Chem.*, **2**, 629 (1954).
(3) HOGSETT, J. N., *et al.*, *Anal. Chem.*, **25**, 1207 (1953).

REPORT ON BENZENE HEXACHLORIDE

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

Modified Partition Chromatographic Procedure.—The modified partition chromatographic procedure for benzene hexachloride that the joint industry-government committee found accurate to ±3 relative per cent (1) was submitted for collaborative study and was compared by the cooperating laboratories with the present A.O.A.C. chromatographic procedure.

To recapitulate briefly, the method is based on a procedure suggested by Rosin and Radan (2). There are three essential steps: (a) A chromatographic separation on silicic acid which yields a fraction containing the relatively pure gamma isomer; (b) a cryoscopic correction applied to this fraction to correct for impurities that may be present; (c) a polarographic assay of combined fore-run and tail-run to assay any of the gamma isomer that may not have been collected in the gamma fraction.

Twenty-four laboratories originally signified their desire to participate in the study. However, only 9 of the submitted reports gave most of the data requested. These results are summarized in Table 1.

Since so few results had been obtained and since the results of the A.O.A.C. and modified procedure were similar, no major changes in the present A.O.A.C. procedure are recommended.* However, the committee agreed that the following improvements could be made in this procedure:

(*a*) A melting-point determination should be made on the main gamma cut.

(*b*) A modification of the extraction procedure should be introduced.

(*c*) In general, the various changes made in the modified partition chromatographic procedure pertinent to the chromatographic separation step itself should be adopted.

* For report of Subcommittee A and action of the Association, see *This Journal*, **38**, 66 (1955).

TABLE 1.—*Summary of results obtained by the modified partition chromatographic procedure for gamma isomer in benzene hexachloride; gamma content of the unknown found by infrared analysis to be 15.9%*

LABORATORY	NO. OF RUNS	PER CENT GAMMA BY A.O.A.C. PROCEDURE			NO. OF RUNS	PER CENT GAMMA BY MODIFIED PROCEDURE		
		MEAN	MEAN DEVIATION	RANGE		MEAN	MEAN DEVIATION	RANGE
1	2	15.22	±0.28	0.56	3	15.86	±0.07	0.20
2	—	—	—	—	2	14.9	±0.05	0.10
3	—	—	—	—	4	15.10	±0.11	0.47
4	3	14.5	±0.3	0.8	2	15.2	±0.1	0.2
5	3	11.11	±0.36	0.87	3	12.28	±0.34	0.83
6	3	14.87	±0.02	0.05	3	14.82	±0.07	0.19
7[b]	2	14.46	±0.03	0.06	5	14.03	±0.70	1.47
8	3	15.61	±0.09	0.09	3	15.13	±0.07	0.18
9	3	15.15	±0.05	0.05	3	15.11	±0.26	0.65
Average of means[c]		15.07				15.16		
Average deviation of means[c]		±0.31				±0.21		
Range of means[c]		1.11				1.04		

[a] These analyses were made on a sample containing 12.9% of gamma.
[b] Trouble had been encountered in getting a good separation of the gamma. With the silicic acid used even 0.1 g gamma overloaded the column.
[c] Results obtained by laboratories 5 and 7 were omitted in calculating average values.

(*d*) Some of the difficulties that might be encountered by an analyst in using the procedure should be mentioned.

There was general agreement that the modified procedure was superior to the present A.O.A.C. method, and that the advantages of one method over the other would be more apparent if additional laboratories not associated with the development of the method had submitted data. The committee will therefore enlist the aid of other laboratories, and the collaborative study will be repeated.

Radioactive-Isotope Dilution Procedure for Gamma Benzene Hexachloride.—The joint industry-government committee, headed by J. T. Craig, has compiled an interim report on their evaluation of the Craig and Tryan radioactive isotope dilution method (3) for determining the gamma isomer in benzene hexachloride. Typical results are shown in Table 2. In this procedure, pure gamma isomer made radioactive by the incorporation of chlorine^{36} is added to the sample being analyzed, and pure gamma fraction is then isolated from the mixture. The decrease in radioactivity from the original to the diluted level is a measure of the gamma

TABLE 2.—*Summary of results obtained by the radioactive isotope dilution method for gamma-isomer determination in benzene hexachloride; gamma content based on infrared analysis, sample 1, 13.1%; sample 2, 15.9%*

LABORATORY	NO. OF RUNS	SAMPLE 1			NO. OF RUNS	SAMPLE 2[a]		
		AVERAGE	AVERAGE DEVIATION	RANGE		AVERAGE	AVERAGE DEVIATION	RANGE
		per cent gamma				*per cent gamma*		
1	6	12.9	±0.25	0.2	6	15.5	±0.17	0.5
2	6	12.8	±0.05	0.2	6	15.95	±0.08	0.3
3	2	12.98	0	0	—	—	—	—
4	5	13.0	±0.20	0.5	—	—	—	—

[a] The same sample tested by the modified partition chromatographic method (Table 1).

benzene hexachloride in the sample. This method which is inherently an absolute one—that is, not subject to interferences—combines precision and accuracy with simplicity and requires only that a weighable sample, not necessarily quantitative, be isolated from the mixture.

At least one large chemical company is now using the radioactive-isotope dilution method for all their gamma-isomer determinations in technical benzene hexachloride. It has given excellent results wherever tried. Collaborators in the group studying the modified partition chromatographic procedure have been asked to use this method, if equipment is available, along with the chromatographic procedure in analyzing the standard samples.

REFERENCES

(1) HORNSTEIN, I., *This Journal*, **37**, 623 (1954).
(2) ROSIN, J., and RADAN, G. B., *Anal. Chem.*, **25**, 817 (1953).
(3) CRAIG, J. T., and TRYAN, P. F., *ibid.*, **25**, 1661 (1953).

REPORT ON PARATHION

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

Because of the wide disagreements in the results shown in the report presented at the annual meeting of this Association last year, it was recommended that the modified O'Keefe and Averell titration method for parathion be given further collaborative study, and that the study should include also the modified procedure for the extraction of *p*-nitrophenol from parathion emulsifiable concentrate (*This Journal*, **37**, 625 (1954)).

During the past year P. A. Averell and the Associate Referee made some studies on the suggested modifications. We concluded that the difficulties have been ironed out. All the procedures, including all the

TABLE 1.—*Results of the collaborative study on parathion samples of 1954*

ANALYST	TECHNICAL PARATHION		EMULSIFIABLE CONCENTRATE		WETTABLE POWDER	
	PARATHION	*p*-NITROPHENOL	PARATHION	*p*-NITROPHENOL	PARATHION	*p*-NITROPHENOL
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	99.74	0.13	26.75	0.05	23.60	0.83
	99.68	0.14	26.79	0.05	23.60	0.83
	99.80	0.13	26.77	0.05	23.52	0.83
2	99.2	0.12	26.53	0.08	23.4	0.74
	98.6	0.14	26.53	0.07	23.5	0.75
	97.6	0.14	26.58	0.06	23.6	0.74
	99.8					
	99.8					
	100.5					
3	100.05	0.13	26.89	0.05	22.94	0.89
	99.41	0.12	26.64	0.05	22.73	0.87
4	99.2	0.15	26.9	0.046	23.5	0.89
	99.3	0.15	27.0	0.048	23.5	0.86
	99.2	—	—	—	23.5	—
5	99.28	0.23	26.54	0.12	23.44	0.86
	99.00	0.15	26.46	0.10	23.54	0.87
	98.80	0.20	26.53	0.10	23.40	0.94
6	99.58	0.13	26.71	0.08	23.70	0.72
	99.45	0.15	26.56	0.07	23.48	0.68
	99.70	0.15	26.60	0.09	23.56	0.64
7	98.79	—	26.71	—	23.18	—
	98.50	—	26.58	—	23.42	—
	99.08	—	26.54	—	23.11	—
	—	—	26.59	—	23.33	—
	—	—	26.54	—		
	—	—	27.12			
	—	—	26.91			
8	99.11	0.14	26.95	0.56	23.15	0.93
	99.22	0.15	26.90	0.56	23.32	0.95
	99.24	0.14	26.90	0.58	22.90	0.92
	100.08	0.15	26.90	0.53	22.75	0.93
	99.76	0.15	26.91	0.56	23.35	0.94
	99.77	0.15	27.28	0.59	23.32	0.95
	99.77	0.15	27.29	0.55	23.34	0.95
	99.02	0.17	27.27	0.57	23.04	0.95
	99.33	0.17	—	—	23.10	0.95
	99.43	0.14	—	—	23.20	0.97
	99.62	0.14	—	—	23.32	0.96
	99.49	0.14				
	99.62	0.14				
	99.49	0.14				
	99.50	0.14				
	99.47	0.14				
	99.65	0.14				
9	99.5	—	24.0[a]	—	23.9	—
	99.7	—	23.2[a]	—	24.0	—
	99.7	—	23.5[a]	—	24.1	—
	99.2	—				
	99.4					
Average	99.41 ±0.79		26.78 ±0.30		23.38 ±0.46	

[a] Values omitted from the average.

modifications in the titration method, have been checked independently by us, and our results showed very good agreement.

Therefore, for a wider confirmation of the workability of the method, three samples, including a sample of technical parathion, a 25 per cent emulsifiable concentrate, and a 25 per cent wettable powder, were sent out last June to 12 laboratories for collaborative study. Eight of the laboratories have already reported their results, which are shown in Table 1.

In general, all the collaborators were satisfied with the modified O'Keefe and Averell titration method. The results reported by the various laboratories are surprisingly close and uniform.

Three minor improvements, however, have been suggested by two of the collaborators:

(1) The extraction time for parathion dust and wettable powder should be increased from 1 hour, as specified in the method, to 2 or 3 hours in order to be sure of complete extraction.

(2) At the end of the step in which the ether solution of parathion is reduced, a total of 30 ml of hydrochloric acid, rather than 10 ml, should be used.

(3) The final volume of solution at the time of titration, especially when the dead-stop end point titration procedure is used, should be close to 125 ml instead of 250 ml.

Since some collaborators used the indicated volumes in their studies, these are incorporated into the revised method.

It is recommended* that the O'Keefe and Averell titration method as modified in this and the previous reports for the quantitative analysis of technical parathion, parathion dust, parathion wettable powders, and parathion emulsifiable concentrates be adopted as first action.

COLLABORATORS

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

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

P. A. Averell, American Cyanamid Company, Stamford, Conn.

H. A. Thomson, U. S. Rubber Co., Naugatuck Chemical Division, Elmira, Ontario, Canada

Charles V. Marshall, Ontario Department of Agriculture, Ottawa, Ontario, Canada

R. L. Caswell, Production and Marketing Administration, U. S. Department of Agriculture, Beltsville, Md.

Kenneth Helrich, New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick, N. J.

R. C. Scott, Research and Development Department, Pittsburgh Coke and Chemical Company, Pittsburgh, Pa.

* For report of Subcommittee A and action of the Association, see *This Journal*, 38, 66 (1955).

REPORT ON PYRETHRINS

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

It was recommended at the 1953 meeting of the Association (1) that the investigation of analytical methods for the determination of pyrethrins, including the hydrochloric acid modification of the official mercury reduction method, should be continued. The joint collaborative program for this purpose, which was established with the Insecticide Chemical Analysis Committee of the Chemical Specialties Manufacturers Association, was aimed primarily toward the development of an entirely new method of analysis of the pyrethrins. Samples of commercial 2000 mg concentrates of Kenya and Congo flowers were forwarded to 17 American and foreign collaborators; analytical results were received from 12, although not all the analytical procedures followed the program requested by the Associate Referee.

The analytical methods selected for this work were the spectrophotometric method of Shukis and Wachs (2) and a modification of the ethylenediamine (EDA) method as used in the determination of allethrin (3). As a control, each collaborator was requested to analyze his samples by the official mercury reduction method and to report both Pyrethrin I and Pyrethrin II.

It appeared early in the program that further work with the spectrophotometric method was not merited. While the method works satisfactorily for the analysis of pyrethrum flowers, where a volatile solvent which can be completely evaporated is used, it was shown in practice to be virtually impossible to remove the high-boiling solvents (usually deodorized kerosenes) normally found in commercial extracts. The results obtained by using this method were uniformly absurd; few of the collaborators had the necessary equipment, and those that did found the method to be tedious and time consuming.

Ten collaborators analyzed the test solutions by the EDA method and the official mercury reduction method. The results are shown in Table 1. Although results were received from 10 collaborators, only 9 are included because the results which the tenth laboratory obtained by using the EDA method did not agree statistically with the results obtained by the other collaborators, and it was indicated that there had been some gross error in procedure.

The average deviation of the results obtained with the EDA method in each case is approximately twice that obtained by using the official mercury reduction method. Furthermore, there was a general feeling on the part of the collaborators that the use of the EDA method for the pyrethrins had a number of serious drawbacks. The large quantities and

TABLE 1.—*Comparative percentages of pyrethrin content of 2000 mg extracts of Kenya and Congo flowers*

COLLABORATOR	TOTAL PYRETHRINS, AVERAGE PERCENTAGE BY WEIGHT			
	KENYA FLOWERS		CONGO FLOWERS	
	A.O.A.C. METHOD	EDA METHOD	A.O.A.C. METHOD	EDA METHOD
	per cent	*per cent*	*per cent*	*per cent*
A	2.45	2.75	2.54	2.63
B	2.46	2.79	2.58	2.71
C	2.39	2.62	2.64	2.67
D	2.44	2.71	2.57	2.56
E	Not reported	2.40	Not reported	2.58
F	2.33	2.67	2.46	2.69
G	2.34	2.89	2.39	2.79
H	2.56	Not reported	2.59	Not reported
I	2.33	2.89	2.61	3.06
Average of all collaborators reporting	2.41	2.71	2.55	2.71
Average deviation	0.06	0.12	0.06	0.11

the cost of the pyridine and ethylenediamine necessary make the method expensive for routine work unless a method for their recovery can be established. The end point is not sharp, and while the technique was easily mastered and the method was found to be very rapid, the variation in the results obtained by the different collaborators indicates that more modification is necessary before it can be considered for further collaborative work.

The Levy-Estrada colorimetric method (4) was used in a preliminary exploration of the possibility of a colorimetric determination for total pyrethrins. Such a method would be in line with the general desire of the laboratories participating in this program for the development of methods of analysis for the pyrethrins to be directed towards those procedures which require relatively simple technique and instrumentation. The results of this preliminary work are shown in Table 2.

It should be noted that where previous methods under consideration have generally given higher results for total pyrethrins than the official mercury reduction method, the Levy-Estrada method gives results almost identical with those obtained by the mercury reduction method in the case of Kenya flowers, and somewhat lower in the case of Congo flowers.

The principal drawback of colorimetric methods announced in recent years (5, 6) has been their dependence upon a standard curve which was established with values obtained by the mercury reduction method. They

TABLE 2.—*Colorimetric determination of pyrethrins by Levy-Estrada method*[a]

	TOTAL PYRETHRINS, PERCENTAGE BY WEIGHT	
	A.O.A.C.[b] METHOD	COLORIMETRIC[c] METHOD
	per cent	*per cent*
Kenya 2000 mg extract	2.33	2.31
Congo 2000 mg extract	2.61	2.48

[a] Using the OTI as standard for 1 mg/ml. [b] Average of 2 determinations. [c] Average of 3 determinations.

were therefore not suitable for primary work. However, recent developments in the field of partition chromatography (7–9) have indicated that it may be possible to obtain pure Pyrethrin I and II which could then be used as standards in the establishment of reference curves suitable for use in colorimetry.

No work was done during 1954 on the previously proposed and rescinded modification of the official mercury reduction method which called for the use of hydrochloric acid instead of sulfuric acid to neutralize the saponifying alkali. Although considerable work has been done on establishing the nature of the pyrethrin-like acidic material (10, 11) which can be recovered from the barium sulfate residues resulting from the use of sulfuric acid (which are normally discarded), the insecticidal activity of the esters from which this acidic material is obtained has not been demonstrated or disproved. It has not been possible, therefore, to state positively that the higher values for Pyrethrin I, obtained when hydrochloric acid is used in the official mercury reduction method, are due to insecticidally-active esters.

It is recommended* that the investigation of analytical methods for the determination of pyrethrins be continued.

COLLABORATORS

The Associate Referee wishes to thank the following collaborators for their cooperation in this work:

Stephen S. Voris, Seil, Putt & Rusbey, Inc., New York, N. Y.
G. W. Flint, Standard Oil Co. (Indiana), Whiting, Ind.
Mark L. Hill, Gulf Oil Corp., Philadelphia, Pa.
Howard A. Jones, U. S. Industrial Chemicals, Inc., Baltimore, Md.
Ralph L. Tracy, John Powell Laboratories, Inc., Port Jefferson, Long Island, N. Y.
J. B. Johnson, Carbide & Carbon Chemicals Co., South Charleston, W. Va.
A. A. Schreiber, McLaughlin-Gormley-King Co., Minneapolis, Minn.
Charles V. Marshall, Department of Agriculture, Ottawa, Ontario, Canada
Cipriano Cueto, formerly with Bureau of Entomology & Plant Quarantine, Savannah, Ga.

* For report of Subcommittee A and action of the Association, see *This Journal*, 38, 66 (1955).

John Versocki, Boyle-Midway, Inc., Cranford, N. J.

A. J. Feuell, Colonial Products Advisory Bureau (Plant & Animal), South Kensington, London, England

Luis W. Levy, Centro Quimico, Quito, Ecuador

REFERENCES

(1) KELSEY, D., *This Journal*, **37**, 628 (1954).
(2) SHUKIS, A. J., *et al.*, *Soap Sanit. Chemicals*, **27**, 124 (1951).
(3) HOGSETT, J. N., *et al.*, *Anal. Chem.*, **25**, 1207 (1953).
(4) LEVY, L. W., and ESTRADA, R. E., *J. Agr. Food Chem.*, **2**, 629 (1954).
(5) CUETO, C., and DALE, W. E., *Anal. Chem.*, **25**, 1367 (1953).
(6) EDWARDS, F. I., *ibid.*, **24**, 1357 (1952).
(7) LORD, K. A., *et al.*, *J. Sci. Food Agric.* **3**, 419 (1952).
(8) WARD, J., *Chemistry & Industry*, 586 (1953).
(9) BROWN, N. C., *et al.*, *Pyrethrum Post*, **3**, 3 (1954).
(10) BECKLEY, V. A., and HOPKINS, J., *Soap Sanit. Chemicals*, **30**, 141 (1954).
(11) MITCHELL, W., *J. Sci. Food Agric.* **5**, 246 (1953).

REPORT ON DIELDRIN

By J. B. McDEVITT, JR. (Louisiana Agricultural Experiment Station, Baton Rouge, La.,) *Associate Referee*

The Associate Referee's report on this topic is based upon a study of partition chromatography as a means of determining dieldrin alone or in mixtures.

The method used was that described by H. F. Beckman (*Anal. Chem.*, **26**, 922 (1954)), except that the column was prepared from 75 g H_2SiO_3,

TABLE 1.—*Analysis of laboratory prepared samples*

GRAMS ADDED	GRAMS RECOVERED
0.0300	0.0309
0.0300	0.0300
0.0300	0.0307

TABLE 2.—*Analysis of commercial samples*

PER CENT GUARANTEE	PER CENT FOUND
2.0	2.07, 2.07
1.5	1.52, 1.42
1.5	1.39, 1.55
15.5	17.4 , 17.00
15.8	17.3 , 16.5 , 17.2

60–70 ml nitromethane, and 270 cc mobile solvent. After the mixture was poured in the column, a long glass rod was used to stir out all bubbles and quickly make a smooth column.

The samples analyzed consisted of recrystallized dieldrin, commercial dusts, and liquid concentrates. Results obtained are shown in Tables 1 and 2.

It is recommended* that further investigational work be done before samples are sent out to collaborators.

REPORT ON RODENTICIDES

THE DETERMINATION OF WARFARIN AND PIVAL

By J. B. LaClair (State Department of Agriculture, Bureau of Chemistry, State Office Building No. 1, Sacramento 14, Calif.), *Associate Referee*

The results obtained in the 1953 collaborative study[1] on warfarin in warfarin-starch mixtures showed that the method could give average results within a 5 per cent error, but some of the individual errors ran as high as 13 per cent. To check the procedure and to discount the effect of spectrophotometer variance a collaborative study was undertaken on the analysis of a warfarin-starch mixture of known composition wherein all collaborators used the same spectrophotometer. The results are given in Table 1.

With the acceptance of "Pival" (2-pivalyl-1,3-indandione) as a worthwhile anticoagulant rodenticide, it has become necessary to evaluate proposed methods for the analysis of this compound, both in materials containing 0.50 per cent, and in prepared baits containing 0.025 per cent.

Some of the chemical and physical properties of 2-pivalyl-1,3-indandione (mol. wt. 230.25), as submitted by Ringwood Chemical Corporation, are as follows: The material is a yellow crystalline solid with an uncorrected melting point of 108–109.5°C. The absorption spectrum shows two major and two minor absorption peaks in the ultraviolet region at 250, 283, 313, and 325 millimicrons. The point of maximum absorption is at 283 millimicrons.

The compound contains both an enolic hydroxyl and a ketone group which exhibit typical reactions. Thus, salts of sodium, potassium, calcium, aluminum, iron, and magnesium are readily formed. Of these, only the sodium and potassium salts are water soluble. 2-Pivalyl-1,3-indandione is only slightly soluble in water (18 p.p.m. at 25°C.), but it is readily

* For report of Subcommittee A and action of the Association, see *This Journal*, **38**, 66 (1955).
[1] *This Journal*, **37**, 634 (1954).

TABLE 1.—*Collaborative analysis of a sample containing 0.600% warfarin in cornstarch*

COLLABORATOR[a]	WARFARIN FOUND	RECOVERY OF WARFARIN
	per cent	*per cent*
1	0.599	
	0.605	
	0.603	
Collaborator's average	0.602	100.33
2	0.603	
	0.602	
Collaborator's average	0.603	100.50
3	0.599	
	0.589	
Collaborator's average	0.594	99.00
4	0.599	
	0.602	
Collaborator's average	0.601	100.17
Average of all collaborators	0.600	100.00

[a] All collaborators used the same spectrophotometer.

soluble in common organic solvents such as ethyl and methyl alcohols, ether, benzene, petroleum ether, toluene, mineral oil, and corn oil.

The Ringwood Chemical Corporation supplied the following procedure for the determination of 2-pivalyl-1,3-indandione in starch mixtures and aqueous sodium salt solutions.

ANALYSIS OF PIVAL AND PIVALYN BY ULTRAVIOLET SPECTROPHOTOMETRY

ANALYSIS OF PIVAL-STARCH MIXTURES (0.5% PIVAL)

Weigh 0.5 g sample into a 250 ml beaker or erlenmeyer and add 50 ml 3A or C.P. ethyl alcohol which has been treated with alkali.[2] Heat on a steam bath for 10 min. with constant stirring or swirling. Decant the alcohol thru filter paper (Whatman No. 44 or equiv.) into a 250 ml vol. flask. Repeat extn 4 times, using 40 ml portions of 3A or ethyl alcohol, and combine filtrates. Wash filter paper thoroly with 30 ml warm alcohol and adjust vol. to 250 ml at 25°C.

To prep. blank, weigh 0.1 g starch (same type used in concentrate) in a beaker or erlenmeyer and add 50 ml 3A or ethyl alcohol. Heat on a steam bath for 10 min. with constant stirring or swirling. Filter thru filter paper (Whatman No. 44 or equiv.) into a 50 ml vol. flask, and adjust the vol. to 50 ml with alcohol.

[2] To each liter of alcohol used in the analysis add 1.0 ml of a solution prepd by dissolving one pellet (0.13 g) of NaOH in 50 ml alcohol. (3A denatured alcohol contains 100 parts of 95% ethyl alcohol and five parts of absolute methanol.)

Det. the absorbance (A) of the Pival solution at 283 mμ in 1 cm quartz cells against the above prepd blank. A Beckman Model DU spectrophotometer with slit width of 0.70 mm was used in this work. The expected accuracy is ±2%.

CALCULATIONS

$$\text{Per cent Pival} = \frac{A_{283} \times 230.3 \times 100}{2.46 \times 10^4 \times 4 \times \text{wt sample in g}} = \frac{A_{283} \times 0.2340}{\text{wt sample in g}}$$

ANALYSIS OF AQUEOUS SOLUTIONS OF PIVALYN (SODIUM SALT OF PIVAL)

Dil. 10 ml of the soln (usually 4.23×10^{-2} moles per liter; 1.066% Pivalyn or 0.97% Pival) to 1 l with distd H_2O. Dil. 10 ml of the dild soln to 100 ml after the pH is adjusted to a point between 10 and 11.

Det. absorbance of this soln at 283 mμ with 1 cm quartz cells, using distd H_2O as a blank.

CALCULATION

$$\text{Per cent Pivalyn} = A_{283} \times 1.015$$

Some interest has been shown in rodenticide baits containing both warfarin and Pival, and an excellent method for the assay of baits containing both warfarin and Pival has been developed by Francis Coon of Wisconsin Alumni Research Foundation. Through the courtesy of Mr. Coon and the Wisconsin Alumni Research Foundation the procedure is given as follows:

METHOD FOR DETERMINING WARFARIN AND PIVAL IN A BAIT

OUTLINE

1. Construction of a standard curve for warfarin at 283 mμ.
2. Extraction of the bait with ether to remove both warfarin and Pival.
3. Extraction of the ether with 1% sodium pyrophosphate.
4. Determination of the absorbance of the pyrophosphate solution at 283 mμ.
5. Passage of a portion of the original ether solution through a column of Attaclay (Pival is adsorbed on the column and warfarin passes through).
6. Collection of about 30 ml of ether from the column and extraction by pyrophosphate.
7. Determination of the absorbance of the pyrophosphate at 308 mμ.

PROCEDURE

(A) Prep. a std curve for warfarin at 283 mμ by dissolving a weighed quantity of pure warfarin in 1% aq. sodium pyrophosphate and dilg to the following concns: 0.5, 1.0, 1.5, and 2.0 mg per 100 ml of the pyrophosphate soln. Det. the absorbance of each at 283 mμ, using 1 cm quartz cells and a 1% sodium pyrophosphate soln as a blank. A straight line is obtained when concn is plotted against absorbance.

(B) Ext. a 12–15 g sample of the bait with diethyl ether by (a) shaking with 100 ml ether in a glass-stoppered bottle for 1 hr, or (b) extg in a Soxhlet for 1 hr, transferring the ether ext. quantitatively to a 100 ml vol. flask, and dilg to vol. with ether. Transfer a 5 ml aliquot of either ext. to a 100 ml flask contg 50 ml 1% aq. sodium pyrophosphate. Stopper and shake for 2 min. Transfer entire contents to a 50 ml centrifuge cone and if an emulsion has formed, centrifuge for 1 min. Draw off the ether layer and any emulsion. Ext. the pyrophosphate soln successively with two 10 ml portions of ether and two 10 ml portions of Skellysolve B (either redistd or shaken with pyrophosphate solution). Centrifuge and draw off the upper layer each time. Transfer about 4 ml of the pyrophosphate soln to a 1 cm quartz cell and det. the

absorbance at 283 mμ, using a 1% sodium pyrophosphate soln, treated as above, to set the instrument at zero.

(*C*) For the warfarin detn, prep. a chromatographic column (chromatographic tube 11 mm I. D.×200 mm long, fitted with a capillary stopcock) by inserting a glass wool plug above the stopcock and pouring onto it a slurry of 2 to 3 g Attaclay and ether. Force the excess ether to the top of the clay by means of air pressure applied to the top of the column. Carefully deliver a 5 ml aliquot of ext., (*a*) or (*b*) above, onto the column. Place a 50 ml centrifuge cone, contg 10 ml of the pyrophosphate soln, under the column, force the 5 ml aliquot thru the column, and fill the tube with ether. Force an addnl 20–25 ml ether into the cone, stopper it, shake 2 min., and then centrifuge to break any emulsion. Draw off the ether layer and ext. the pyrophosphate soln with ether and Skellysolve B as previously described. Transfer a portion of the pyrophosphate soln to a 1 cm quartz cell and det. its absorbance at 308 mμ, using a treated pyrophosphate soln to zero the instrument.

CALCULATIONS

$$\text{Per cent Warfarin} = \frac{A_{308} \times 0.2}{0.461 \times \text{wt sample in g}}$$

From the warfarin value obtained, calc. the amount of warfarin in the aliquot taken for the reading at 283 mμ. Convert this amount to the same terms used in constructing the warfarin std curve at 283 mμ (mg/100 ml). From the curve, read off the absorbance due to warfarin. Subtract the absorbance due to warfarin from the total absorbance found at 283 mμ and use this value to calc. Pival as follows:

$$\text{Per cent Pival} = \frac{A_{283}\ (corr.)}{1.082 \times \text{wt sample in g}}$$

DISCUSSION

So far the above method has been used on simple baits only.

The importance of using neutral or slightly alkaline alcohol as a solvent for Pival cannot be overemphasized. It is not uncommon for commercial ethyl alcohol, including U.S.P. quality, to contain appreciable amounts of acid. When an acidic alcohol is used as a solvent for Pival the ultraviolet absorption spectrum is changed radically, giving absorption peaks at 238, 276, and 286 (max.) mμ compared with 224, 249, 283 (max.), 312, and 324 mμ when neutral or slightly alkaline alcohol is used. These changes are believed by Ringwood Chemical Corporation to be due to the fact that in Pival there is a tautomeric system, with an equilibrium mixture of keto and enol forms. This equilibrium is shifted by *p*H changes.

The absorption maximum at 283 mμ appears to be quite constant, while the location of all other peaks may vary 1 to 2 mμ from sample to sample of Pival. There appears to be an appreciable deviation from Beer's law at 283 mμ when concentrations of Pival are less than 0.4 mg per 100 ml.

Section (*B*) of the Coon procedure for Pival-warfarin baits is also applicable to baits containing only Pival. It will be noted that this section is an adaptation of the warfarin procedure[3] except that greater dilutions

[3] *This Journal*, 35, 372 (1952), Procedure III.

are necessary. A procedure submitted by Ringwood Chemical Corporation differs only in aliquots and volumes, and final calculation is the same.

Apparently some difficulty has been experienced by others in determining Pival in pelleted baits, and they have obtained usually low results. It might be necessary to use a modification of the Eble method,[4] as is sometimes necessary in the assay of pelleted warfarin baits.

There seems to be some discrepancy in the value reported for the absorptivity value (a) (extinction coefficient) for Pival. The value for (a) of 1.082 (where $a = A/bc$; $b = 1$ cm, $c =$ mg/100 ml) seems to be the accepted value.

SUMMARY AND RECOMMENDATIONS

Collaborative analysis of a warfarin-cornstarch mixture containing a known percentage of warfarin, in which all collaborators used the same spectrophotometer, gave excellent results.

Some of the chemical and physical properties of "Pival" (2-pivalyl-1,3-indandione), one of the new anticoagulant rodenticides, are listed and procedures for the analysis of Pival preparations and mixed Pival-warfarin baits are discussed.

It is recommended*—

(1) That the procedure for warfarin in preparations containing about 0.5 per cent-0.6 per cent warfarin (*This Journal*, **37**, 634 (1954)) be adopted as first action.

(2) It is recommended that a collaborative study be undertaken on the determination of warfarin in prepared baits.

(3) It is recommended that further work be undertaken to test and develop methods for the analysis of Pival.

ACKNOWLEDGMENT

The author wishes to thank Motomco Inc., 10 Murray Street, New York, N. Y., for the Pival samples; Ringwood Chemical Corporation, Ringwood, Illinois, and Wisconsin Alumni Research Foundation, Madison, Wisconsin, for procedures; and C. L. Gordon, H. K. Specht, and H. S. Stocks, of the California Department of Agriculture, Bureau of Chemistry, who acted as collaborators.

---

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

* For report of Subcommittee A and action of the Association, see *This Journal*, **38**, 66 (1955).

REPORT ON QUATERNARY AMMONIUM COMPOUNDS

By R. L. CASWELL (Pesticide Regulation Section, Plant Pest Control Branch, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Md.), *Associate Referee*

Identification by Ultraviolet Spectroscopy.—Several quantitative methods are available for the determination of quaternary ammonium compounds in commercial germicides and sanitizers (1–3). It is sometimes necessary to identify the specific quaternary present in the product. A method involving the preparation and the determination of the optical crystallographic properties of the reineckate of the unknown quaternary has been published by Tillson, *et al.* (4).

The three compounds most frequently encountered are:

(1) *p*-Diisobutylphenoxyethoxyethyldimethylbenzyl ammonium chloride.

(2) Alkyldimethylbenzyl ammonium chloride (alkyl C_8H_{17} to $C_{18}H_{37}$).

(3) Alkyltolylmethyltrimethyl ammonium chloride (alkyl C_9H_{19} to $C_{15}H_{31}$). (This was formerly called methyldodecylbenzyltrimethyl ammonium chloride.)

These compounds may be distinguished by means of ultraviolet spectrophotometry. A solution containing about 20 mg of quaternary ammonium compound in 100 ml of water is prepared. If a turbid solution is obtained, a water-ethyl alcohol mixture may be used as solvent. The absorbance of the solution is determined from 290 mμ to 245 mμ with water (or water-ethyl alcohol) as the blank. A Beckman Model DU spectrophotometer, with 1 cm silica cells, may be used for the measurement.

The following characteristic maxima, calculated for a concentration of 20 mg in 100 ml for comparison, were obtained for the compounds mentioned above.

Wavelength, mμ	*Absorbance*
***p*-Diisobutylphenoxyethoxyethyldimethylbenzyl ammonium chloride**	
274	0.603
269	0.617
264	0.497
Alkyldimethylbenzyl ammonium chloride	
269	0.206
263	0.283
257	0.254
Alkyltolylmethyltrimethyl ammonium chloride	
279	0.549
273	0.531

The results are often roughly quantitative as well as qualitative. If interfering substances are present, the absorbances may be corrected by means of a base line technique, if it is assumed that the absorption is linear with the wavelength, as used by Clements and Newburger in the calculations for the determination of dichlorophene (5).

For some products it may be necessary to remove the interfering substances by acidifying the aqueous solution with hydrochloric acid and extracting with ether. This treatment has removed enough of the interfering material to permit identification of the quaternary.

This identification is not specific; the compounds cetyldimethylbenzyl ammonium chloride and dodecylacetamidyldimethylbenzyl ammonium chloride each show absorption maxima at 269, 263, and 257 mμ as alkyl dimethylbenzyl ammonium chloride does. The absorption appears to be characteristic of the benzyl group. Also, para-diisobutylcresoxyethoxyethyldimethylbenzyl ammonium chloride shows the same maxima as para-diisobutylphenoxyethoxyethyldimethylbenzyl ammonium chloride. A compound such as cetyltrimethyl ammonium bromide that has no strongly absorbing groups cannot be identified by this method.

Ferricyanide Method (1).—The ferricyanide method has been used successfully in this laboratory for many commercial products. It appears to be applicable to all the preparations encountered except some formulations of alkyltolylmethyltrimethyl ammonium chloride.

RECOMMENDATIONS

It is recommended*—

(1) That the ferricyanide method for quaternary ammonium compounds be studied collaboratively.

(2) That the spectrophotometric procedure be further investigated and that it be subjected to collaborative study.

REFERENCES

(1) *Official Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Box 540, Benjamin Franklin Station, Washington 4, D. C., 1950, p. 463.
(2) CASWELL, R. L., *This Journal*, **34**, 675 (1951).
(3) WILSON, J. B., *ibid.*, **37**, 379 (1954).
(4) TILLSON, A. H., EISENBERG, W. V., and WILSON, J. B., *ibid.*, **35**, 459 (1952).
(5) CLEMENTS, J. E., and NEWBURGER, S. H., *ibid.*, **37**, 192 (1954).

No reports were given on aldrin, allethrin, DDT and related compounds, dithiocarbamates, herbicides, phenolic disinfectants, physical properties of economic poisons, piperonyl butoxide, systemic insecticides, or volatility of ester forms of hormone type herbicides.

* For report of subcommittee A and action of the Association, see *This Journal*, **38**, 66 (1954).

REPORT ON DAIRY PRODUCTS

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

The two main subjects discussed at length in last year's report, the detection of adulteration of butterfat with foreign fats and the freezing point of milk, continue to hold the attention of analytical chemists in the dairy field. In spite of diligent work, methods are still in the development or survey stage and have not reached the point required for adoption by the Association.

Foreign Fats.—The only recommendation to be acted upon by the Association at this meeting with regard to methods for the detection of foreign fats in butterfat comes as the result of an extensive study of the Reichert-Meissl determination. Certain changes are recommended for convenience in carrying out the determination, such as the substitution of an all-glass distillation apparatus for the apparatus constructed with rubber stoppers. This change has no effect on the Reichert-Meissl value; it is important, however, in the Polenske determination. Carborundum is recommended as a substitute for pumice, since satisfactory pumice cannot always be obtained and unsatisfactory pumice often results in the loss of determinations through bumping. This change in antibumping agent will result in slightly higher Reichert-Meissl and Polenske values; on the average, approximately one quarter of a Reichert-Meissl unit and one half a Polenske unit. This increase is hardly significant in the interpretation of results by the Reichert-Meissl determination. The recommended changes have the advantage of significantly increasing the Polenske value of such oils as palm kernel oil; this may be of assistance in the interpretation of the results of analyses of mixtures containing this type of oil, particularly in view of the fact that the Polenske determination is not as precise as the Reichert-Meissl value.

This report is very important when considered in the light of several current surveys of the Reichert-Meissl and Polenske values for normal market samples of butterfat. In one of the most comprehensive surveys ever attempted, Professor Jackson, of the University of Wisconsin, has collected hundreds of samples of butterfat from all over the country, over a period of a year, for examination for Reichert-Meissl value and refractive index. His results have been supplemented by those of Dr. Keeney of the University of Maryland, who determined the molar per cent butyric acid on these samples by his chromatographic procedure. Canadian scientists have performed a similar study and the combined data will be a much firmer foundation for the interpretation of Reichert-Meissl values than the older literature on this subject.

Studies of other methods of analysis are also continuing. Dr. Mahon,

of the Canadian Food and Drug Division, is attempting to adapt chromatographic procedures to regulatory operations, and his studies may develop a method which can be subjected to collaborative study during the coming year. The tocopherol determination, which is an index of vegetable oil adulteration, is being studied by Mr. Windom, of the Army Medical Service Graduate School, who was appointed late in the year.

The most specific test for the presence of vegetable oils, the sterol acetate test, was studied collaboratively during the past year. Although many collaborators were able to use the procedure with success, even with small amounts of coconut oil, the Associate Referee has concluded that the method requires additional refinement. His observation that the addition of cholesterol intensifies rather than diminishes the melting point differences in the presence of phytosterol acetate is most unexpected, but it may prove to be the key to satisfactory collaborative results.

The Seidenberg test, discussed in the previous report, has also been assigned to an Associate Referee. It is hoped that work can be done on this procedure during the coming year.

The American Dairy Association is making arrangements for the establishment of a "butter bank" that will include many seasonally spaced market samples of butter or butter oil from widely scattered locations throughout the country. Chemists may use these samples in establishing the normal range of constants for present methods or for new methods that may be developed. Full details of this project have not yet been announced. The advantage of this type of sample is that it reflects commercial samples. These samples should not be confused with authentic samples which are collected and prepared under the immediate supervision of a responsible individual throughout their entire history.

Cryoscopy of Milk.—Shortly after the beginning of the year (1954), a questionnaire was distributed to State officials having regulatory control over milk and to some city officials, requesting their cooperation in the determination of the freezing points of authentic milks. It was intended to obtain information on the many variables which may contribute to the freezing-point variation, and instructions for the survey were issued by the Associate Referee, Dr. Dubin, and by the Chairman of Subcommittee C, Dr. Robertson. A brief report to this meeting by Dr. Robertson indicates the progress that is being made on this survey but recommendations on this subject must obviously be postponed until the survey has been completed. The cooperation of the participants in this survey is greatly appreciated by the Association.

Reconstituted Milk.—The Associate Referee appointed during the year has gathered a considerable amount of authentic data on the protein-reducing value of raw milk, pasteurized milk, and homogenized pasteurized milk, which will assist in the interpretation of results obtained by this method. The samples submitted to ascertain the reliability of the

method, however, as so often happens with fresh dairy products, arrived in an unsatisfactory state, and therefore the collaborative studies will be repeated during the next year, using samples of nonfat dry milk solids which have been subjected to various heat treatments.

Method Revisions.—In the course of numerous studies by the Associate Referee on the Babcock test, detailed directions for conducting the Babcock test have been submitted to the Association.[1] Action on these revisions has been deferred pending studies on a detergent-type test. Certain features in the preparation of sample and in the determination itself, as outlined in the recommendations, reflect better technique in conducting the test, correct possible misunderstandings, and more nearly reflect actual practice. Accordingly, several recommendations have been given for revision of sections **15.1–15.2,** Collection and Preparation of Sample, which deal with preserving and mixing the sample, and in section **15.27,** changing the volume of added sulfuric acid from 17.5 ml to "approximately 17.5 ml," since the quantity of acid in actual practice is varied according to the amount required to obtain a clear fat column.

Similarly, method **15.123,** Selection and Preparation of Sample of Cheese, is being revised to separate the collection of sample from the preparation. The sentence in the method, "For inspection purposes reject rind, but for investigations requiring absolute quantity of fat in cheese include rind in sample," has always been obscure and ambiguous. It does not reflect present day regulatory or commercial practice which always replaces the rind portion of a sample in the hole of a plug. Separation of collection and preparation of sample is also necessary because of the adoption of specific procedures for cottage cheese.

The method for the determination of citric acid in milk is based on the corresponding procedure under fruit acids, as are many of the other citric acid methods in *Official Methods of Analysis.* An exception is the method for citric acid in cheese which was developed and studied collaboratively prior to the adoption of the present fruit methods. Since the appearance of the 7th Edition the method for citric acid in fruits has been thoroughly studied and revised; therefore, the present method for milk, which is cross-referenced to the fruit method at the point where the lead salts are isolated, must also be revised. The isolation procedure for milk remains the same as at present.

The figure "0.04" to indicate properly pasteurized milk in the phosphatase method, **15.46,** should have been "0.047" and should be corrected. In the preparation of cream sample, **15.52,** heating at 50° is poor technique, since application of such a high temperature is apt to cause further oiling off. Melting of the fat can be accomplished satisfactorily at the specified 38° if the sample is left at this temperature a sufficient length of time.

[1] *This Journal,* 37, 235 (1954); 36, 183 (1953); 35, 202 (1952).

RECOMMENDATIONS

It is recommended*—

(1) That the method for the determination of soluble and insoluble volatile acids (Reichert-Meissl and Polenske values), **26.26–26.27**, be revised as recommended by the Associate Referee and be retained as official, and that the work be continued.

(2) That the procedure for the collection of sample of milk, **15.1**, be revised as follows: In the third paragraph substitute for lines 3 and 4 the following:

"... completely fill containers, stopper tightly, and identify. Tablets contg $HgCl_2$, $K_2Cr_2O_7$, or other suitable preservative, weighing not more than 0.5 g for 8 fl. oz. milk, or 36% soln of HCHO, 0.1 ml (2 drops) per fl. oz., may be used unless presence. ..."

(3) That the procedure for the preparation of sample of milk, **15.2**, be revised as follows:

"Bring sample to ca 20°, mix until homogeneous by pouring into clean receptacle and back repeatedly, and promptly weigh or measure test portion. If lumps of cream remain undispersed, warm sample in H_2O bath to ca 38° and continue mixing until homogeneous, using policeman, if necessary, to reincorporate any cream adhering to container or stopper. Where practical, and fat remains dispersed, cool warmed samples to ca 20° before transferring test portion.

"When Babcock method, **15.27**, is used, adjust both fresh and composite samples to ca 38°, mix until homogeneous as above, and immediately pipet portions into test bottles."

(4) That the procedure for preparation of samples of cream, **15.52**, be revised to delete heating to 50°.

(5) That the official Babcock method, **15.27**, for the determination of fat in milk be revised as follows and the revised method be adopted as official:

DETERMINATION

"With pipet (b), transfer 18 g prepd sample, **15.2**, to milk test bottle. Blow out milk in pipet tip ca 10 sec. after free outflow has ceased. Add portionwise ca 17.5 ml H_2SO_4 (not less than 14 ml) (sp. gr. 1.82–1.83 at 20°) tempered at 15–20°, washing all traces of milk into bulb. Shake until all traces of curd have disappeared, place bottle in heated centrifuge, counterbalance, and after. ..."

Insert at end of line 9: "(not less than 3 min.)."

Insert at end of last line of the second paragraph: "and adjust quantity of H_2SO_4 added accordingly."

(6) That the citric acid methods, **15.5–15.7** and **15.101**, be revised to refer to the revised procedure in fruits[2] and be adopted as first action.

(7) That the first action method for the determination of the critical temperature of dissolution of butter and oleomargarine (*This Journal*, **37**, 93 (1954)) be adopted as official.

(8) That the procedure for selection and preparation of sample of cheese, **15.123**, be revised as follows:

* For report of Subcommittee C and action of the Association, see *This Journal*, **38**, 73, 74 (1955).
[2] *This Journal*, **34**, 74 (1951); **35**, 79 (1952); **36**, 78 (1953).

COLLECTION OF SAMPLE

"When cheese can be cut, take narrow, wedge-shape segment reaching from outer edge to center. When not permissible to cut cheese, take sample with cheese trier. If only one plug can be obtained, take it perpendicularly to surface of cheese at point ⅓ distance from edge to center and extending either entirely or half way thru. When possible draw 3 plugs, 1 from center, 1 from point near outer edge, and 1 from point midway between other 2. Use ca ¾" of rind portion to plug hole."

PREPARATION OF SAMPLE

"Cut wedge sample into strips and pass 3 times thru sausage-type machine. Grind plugs in sausage-type machine (preferable method), or cut or shred very finely and mix thoroly."

(9) That the figure "0.04" in the first line of second paragraph of **15.46** be changed to "0.047."

(10) That work of the Associate Referees on the detection and estimation of foreign fats in dairy products be continued, including methods of extraction of fat from dairy products preparatory to the determination of fat constants.

(11) That methods for the determination of acidity and sucrose in ice cream and frozen desserts be subjected to collaborative study.

(12) That further collaborative work be done with the mechanical shaking method for the preparation of butter samples.

(13) That work on the Babcock method for the determination of fat in homogenized milk be continued.

(14) That studies be made of substitutes for sulfuric acid in the Babcock method for fat in milk and cream, to include comparison with the results by the Roese-Gottlieb method.

(15) That studies on the detection of reconstituted milk be continued.

(16) That the procedure for sampling cheese be re-studied.

(17) That studies on the freezing point of milk be continued.

(18) That studies of the procedure for the preparation of sample of pressurized cream be continued.

REPORT ON DETECTION OF RECONSTITUTED MILK IN FLUID MARKET MILK

By CASSIUS L. CLAY, *Associate Referee*, MARY B. BURKE, and GLADYS JUNKER (Division of Food and Drugs, Chemical-Toxicological Laboratories, Louisiana Department of Health, New Orleans, La.)

For some years there has been need for a reliable chemical method for the detection of reconstituted milk in fluid market milk. Improvements in manufacturing methods for non-fat dried milk solids in recent years have resulted in a much higher quality of product with minimum physical

and chemical alterations in the resulting milk powder. This has rendered the Evenson (1) and related methods obsolete. The method presented here has been developed by R. P. Choi (2) and Associates of the Research Laboratories of the American Dry Milk Institute, in cooperation with the Chemical-Toxicological Laboratories of the Louisiana State Department of Health. This method is based on an increase in the protein-reducing substances of fluid milk when reconstituted milk is added thereto. The collaborative study for the past year has been centered on determining the variations in protein-reducing substances of authentic raw milks due to location, breed of cows, pasteurization, and homogenization.

The following is the method submitted to the collaborators:

DETECTION OF NON-FAT DRY MILK SOLIDS IN FLUID MILK

REAGENTS

(Use A.R. grade reagents and distilled H_2O throughout)

(a) *Acetic acid 5%.*—Dil. 50 ml glacial acid to 1 l with H_2O.

(b) *Saturated urea soln.*—Saturate ca 200 ml H_2O with urea.

(c) *Buffer soln.*—Dissolve 2.0 g NaOH in H_2O and dil. to 250 ml. Dissolve 10.2 g potassium acid phthalate in H_2O and dil. to 250 ml. Mix 158.8 ml NaOH soln with 200 ml of the phthalate soln and dil. to 800 ml in a graduate. Adjust final soln to *p*H 5.6 by addn of NaOH or phthalate soln.

(d) *Potassium ferricyanide soln, 1%.*—Dissolve 10 g $K_3Fe(CN)_6$ in H_2O and dil. to 1 l.

(e) *Trichloroacetic acid, 10%.*—Dissolve 100 g trichloroacetic acid in H_2O and dil. to 1 l.

(f) *Ferric chloride, 0.1%.*—Dissolve 0.1 g (or 0.1 ml of liquefied portion of $FeCl_3$) in 100 ml H_2O. Make fresh daily.

SPECIAL EQUIPMENT

(a) *Centrifuge tubes, 50 ml graduated, Pyrex, conical red line.*—(Like Curtin No. 3922 or Fisher No. 5-525R.)

(b) *Constant temperature water bath.*—Adjusted to 70°C.

(c) *Spectrophotometer.*—Beckman Model B with matched set of 10 mm Corex cells, or similar equipment.

REFERENCE CURVE

To prep. std soln weigh exactly 0.1147 g potassium ferrocyanide ($K_4Fe(CN)_6 \cdot 3H_2O$) and dil to 1 l with H_2O in vol. flask. Dil. 50 ml of this soln to 100 ml in vol. flask. This is working std soln. Each ml = 0.05 mg anhyd. $K_4Fe(CN)_6$. Because of air oxidation, use at once.

Pipet 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 ml of working std soln into a series of clean, dry test tubes. Add H_2O by pipet to give a total vol. of 5 ml. To a control (used to set spectrophotomter for 100% transmittance) add 5 ml H_2O. Then to all tubes add 5 ml "blank soln" prepd as follows:

Dil. 3 ml urea soln to 15 ml with H_2O in centrifuge tube, add 5 ml buffer soln, 5 ml ferricyanide soln, and 5 ml trichloroacetic acid soln. Mix with clean stirring rod. (Blank soln need not be heated or filtered for reference curve. However, when running samples, heat and filter blank to duplicate test conditions.)

Develop color with 1 ml of the 0.1% $FeCl_3$ soln, stir, and allow to stand exactly

10 min. Set spectrophotometer to read 100% transmittance at 610 mμ with control soln. Read stds in % transmission and plot % transmittance against mg potassium ferrocyanide on semi-log graph paper.

DETERMINATION

Mix sample thoroly by pouring into clean receptacle and back until homogeneous mixt. is assured. With a pipet, measure 15 ml prepd sample into a 50 ml graduated centrifuge tube contg 15 ml H_2O. Add 3 ml of the 5% acetic acid soln, mix thoroly with clean glass stirring rod, and centrifuge for 5 min. at 1000 to 1500 rpm. Decant the supernatant liquid[1] and wash ppt twice with 15 ml H_2O, mixing each time with clean glass rod, centrifuging for 5 min., and decanting each time.

To the ppt (and also to clean graduated centrifuge tube for blank) add 3 ml of the urea soln and make up to 15 ml with H_2O.[2] Mix thoroly with clean glass rod. Add 5 ml buffer soln and 5 ml 1% $K_3Fe(CN)_6$ soln; mix with clean glass rod. Heat in H_2O bath at 70° for exactly 20 min. and cool in ice water.

When cool, add 5 ml 10% trichloroacetic acid, mix with stirring rod, and filter.[3] Use first few ml of filtrate to wash sides and bottom of receiver, and discard. Pour rest of soln on filter and allow to filter completely.[4]

Add 5 ml H_2O to clean, dry test tubes; then add 5 ml clear filtrate. Add 1 ml 0.1% $FeCl_3$ to develop color. Mix with glass stirring rod and allow to stand exactly 10 min.[5] Set spectrophotometer to read 100% transmittance at 610 mμ against blank and read samples.

From reference curve, det. the amount of reducing substance in terms of mg of potassium ferrocyanide per 100 ml of milk by multiplying the value obtained from the curve by 40.[6]

NOTES

[1] A very small amount of curd (possibly cream) may be floating on top of tube after centrifuging. If major part of curd is well packed in bottom of tube, this suspended material may be poured off with the liquid portion. However, if milk contains excessive cream, curd forms layer at top of tube and liquid cannot be poured off without losing some of curd. If this occurs, samples are not representative and should not be run.

[2] Samples become gummy if mixed before addition of water and results may be inaccurate.

[3] Use 11 cm No. 40 Whatman filter paper.

[4] If filtrate is cloudy, refilter until clear. A second filtration is usually sufficient.

[5] This 10 min. waiting period is critical as maximum color is reached at this time. Read sample within 2 to 3 min. after the 10 min. waiting period. If a series of samples are run, stagger addition of $FeCl_3$ so that each sample can be read after standing exactly 10 min.

[6] Derivation of factor of 40: Total volume before filtration (30 ml) represents 15 ml fluid milk, and 5 ml filtrate, equivalent to 2.5 ml milk, is used to develop the color.

ADDITIONAL NOTES ON METHOD

If possible, samples should be run when fresh. Freezing samples for preservation causes erratic results probably due to coagulation of protein. This makes sampling difficult. Refrigeration of sample at 37°F. appears to be the best method for preserving samples.

Duplicate samples should not show more than 1% difference in transmittance.

---

[1] (See notes at end of method.)

If the potassium ferricyanide solution appears green, or contains a blue precipitate, a new solution should be made. A new reference curve should then be run.

Analyses should be completed the same day they are begun. It is particularly important that samples do not stand too long after cooling or after filtration.

It is most important that no oxidizing or reducing fumes (H_2S, Cl, HNO_3, etc.) be present in the laboratory when samples are run.

COLLABORATIVE STUDY

Table 1 gives the range of protein-reducing substances (P.R.S.) in 411 samples of authentic raw milks from six states. These show a variation in P.R.S. values of 0.90 minimum to 4.18 maximum, with an average

TABLE 1.—*Protein-reducing substance values of authentic raw milks, July–August 1954*

COLLABORATING LABORATORY	NO. OF SAMPLES	P.R.S. VALUES[a]			NO. OF COWS
		MAXIMUM	MINIMUM	AVERAGE	
Alabama Dept. of Agriculture	21	2.48	1.64	2.17	275
Florida Dept. of Agriculture	39	3.48	1.48	2.19	1,083
Kansas Dept. of Health	9	3.36	2.34	2.90	202
Louisiana Dept. of Health	236	3.72	0.90	2.30	5,688
Oklahoma Dept. of Health	50	2.92	1.34	1.90	928
Tennessee Dept. of Agriculture	37	4.18	1.38	2.62	435
Washington D. C. Dept. of Health	19	2.88	1.86	2.41	1,744
Total	411	4.18	0.90	2.28	10,355

[a] Protein reducing substances expressed as mg $K_4Fe(CN)_6$ per 100 ml of milk.

value of 2.28. This value is in close agreement with the average value of 2.44 obtained by Choi (2) on a number of raw milk samples.

Table 2 shows the effect of standard pasteurization and combined homogenization-pasteurization processes on the P.R.S. values of raw and pasteurized market milks. Except in two instances these values are slightly higher in the pasteurized milks than in the raw milks. The homogenized milks test slightly higher than the standard pasteurized milks since in normal commercial practice homogenized milks are subjected to slightly higher temperatures for longer periods of time in order to control bacterial counts.

Table 3 gives the results of tests on the effects of freezing on the P.R.S. values of milk. These tests show that frozen milks give variable results

TABLE 2.—*Protein-reducing substance values in raw and pasteurized market milks*

COLLABORATING LABORATORY	NO. OF SAMPLES	AVERAGE P.R.S. VALUES			TYPE OF PASTEURIZATION
		RAW	STAND. PAST.	PAST. HOMOG.	
Alabama	11	2.03	1.91	2.84	1[a]
Dept. of Agriculture	3	2.32	—	3.25	2[b]
Kansas	3	2.54	2.97	3.98	1
Dept. of Health	2	2.96	4.03	4.23	2
Louisiana	15	2.03	1.76	2.35	1
Dept. of Health	6	2.00	2.61	2.97	2
Tennessee	2	2.59	—	3.84	1
Dept. of Agriculture	2	2.99	3.54	3.76	2
Washington D. C.	3	2.05	2.10	2.97	1
Dept. of Health	—	—	—	—	—
Total	47				

[a] 1: Short time, high temperature.
[b] 2: Holding.

above and below those of fresh fluid milk. This is probably explained by the inability to secure uniform samples after thawing the frozen milks. Other methods of preserving milk samples for the reconstitution test have shown that all common chemical preservatives interfere with the tests, namely, formaldehyde, mercuric chloride, boric acid, hypochlorites, chloramines, and sodium benzoate. Storage of samples under refrigeration at 37°F. gives consistent results as long as the milk does not clabber. Storage of pasteurized milk samples has been successfully maintained for two weeks at this temperature without appreciable variation in the P.R.S. values.

Table 4 shows a series of tests on the comparative P.R.S. values of whole milk and the skim milk portions of the same fluid milk. It is noted

TABLE 3.—*Effect of freezing upon P.R.S. values*

LABORATORY	TOTAL NO. OF SAMPLES	AV. P.R.S. VALUES OF FRESH MILK	AV. P.R.S. VALUES OF FROZEN MILK
Louisiana Dept. of Health	42	2.36	2.38[a]

[a] Twenty-three of the frozen samples showed higher results (max. dev. 1.16, min. dev. 0.08; av. dev. 0.46) and 19 showed lower results (max. dev. 1.24, min. dev. 0.02; av. dev. 0.51) than the same samples when fresh. Samples frozen approximately 6 wks. to 2 months.

TABLE 4.—*Comparative P.R.S. values of raw and pasteurized whole milk and skim milk (July–August 1954)*

SAMPLE NO.	TYPE	P.R.S. VALUE OF WHOLE MILK	P.R.S. VALUE OF SKIM MILK PORTION
1	Market, past.	2.68	1.54
2	Authentic, raw	1.96	0.80
3	Authentic, raw	1.96	0.80
4	Market, past.	3.00	2.26
5	Market, past.	3.08	2.60
6	Market, past.	3.20	2.86
7	Market, past.	2.82	2.56
8	Market, past.	3.20	2.58
9	Market, past.	2.84	2.34
10	Market, past.	2.58	2.22

that the skim milk portions show a consistently lower P.R.S. value than the whole milk portions of the same milk.

Table 5 shows the increase in P.R.S. values in 45 samples of fluid milk to which known amounts of reconstituted milk were added. The reconstituted milk was made up by making 10 grams of the dried skim milk powder to a volume of 100 ml with distilled water. From this table it is noted that a definite increase in P.R.S. values occurs with the increase in percentage of added reconstituted milk. The non-fat dried milk solids used in this series of tests (tests were made by R. P. Choi and Associates) represents the milk of 17 different manufacturers having more or less wide national distribution. The results of these tests show that the products of the various manufacturers differ considerably in protein-reducing substances. The low-heat spray process powders, which normally are the only milk powders used to reconstitute fluid milk commercially, are lower in P.R.S. values and vary less than the intermediate- and high-heat spray process powders. The intermediate-heat powders are frequently used in ice cream products, while the high-heat powders are used principally in bakery and confectionery products.

Table 6, which has been prepared by Howard Edelson of the Statistical Branch of the U. S. Food and Drug Administration, shows the effects of breed of cattle and number of cows in the herd on the P.R.S. values and indicates that neither of these factors significantly affects the protein-reducing values.

Unfortunately, in tests of identical samples containing from 0 to 10 per cent of reconstituted milk submitted by special airmail to 12 collaborating laboratories, the results were so variable they are not reported here. In all cases the milk samples had soured, clabbered, or separated on receipt and this undoubtedly accounts for the great variation in results obtained.

TABLE 5.—*Increase in P.R.S. values at different levels of addition of reliquefied spray process nonfat dry milk solids to pasteurized milk*

NONFAT SAMPLE USED[a]	P.R.S. VALUES AT DIFFERENT LEVELS OF ADDED RELIQUEFIED MILK				INCREASES IN P.R.S. VALUES AT DIFFERENT LEVELS OF ADDED RELIQUEFIED MILK		
	per cent				*per cent*		
	0	3	5	10	3	5	10
1-B	2.36	2.72	3.08	3.56	0.36	0.72	1.20
2-A	2.00	2.56	2.92	3.28	0.20	0.56	0.92
3-C	2.36	2.64	2.71	3.28	0.28	0.35	0.92
4-C	2.36	2.72	3.00	3.36	0.36	0.64	1.00
5-A	1.92	2.56	2.84	3.28	0.64	0.92	1.36
6-A	1.92	2.64	2.71	3.20	0.72	2.79	1.28
7-A	1.92	2.56	2.84	3.28	0.64	0.92	1.26
8-A	1.92	2.68	2.76	3.20	0.76	0.84	1.28
9-A	2.12	2.48	2.74	3.64	0.36	0.62	1.52
10-A	2.12	2.56	2.96	3.76	0.44	0.84	1.64
11-A	2.12	2.92	3.12	3.64	0.80	1.00	1.52
12-A	2.12	2.68	2.24	3.04	0.56	0.72	0.92
13-A	2.20	2.56	2.96	3.48	0.36	0.76	1.28
14-A	2.20	2.56	3.12	3.60	0.36	0.92	1.40
15-A	2.12	3.04	3.20	4.60	0.92	0.08	2.48
16-D	2.12	3.40	3.76	5.76	1.28	1.64	3.64
17-A	2.12	2.96	3.28	4.12	0.84	1.16	2.00
18-A	1.80	2.68	2.84	4.12	0.88	1.04	2.32
19-E	2.12	2.68	2.84	3.64	0.56	0.72	1.52
20-F	1.80	2.08	2.36	2.56	0.28	0.56	0.76
21-F	1.80	2.00	2.20	2.44	0.20	0.40	0.64
22-F	1.80	2.36	2.56	3.04	0.56	0.76	1.24
23-G	1.80	2.44	2.68	3.20	0.64	0.88	1.40
24-G	1.80	2.28	2.44	3.04	0.48	0.64	1.24
25-G	1.80	2.56	2.76	3.48	0.76	0.96	1.68
26-H	1.80	2.44	2.68	2.84	0.64	0.88	1.04
27-H	1.80	2.56	2.76	3.04	0.76	0.96	1.24
28-H	1.80	2.56	2.76	3.04	0.76	0.96	1.24
29-I	2.00	3.28	3.64	4.52	1.28	1.64	2.52
30-J	2.00	3.00	3.64	4.96	1.00	1.64	2.96
31-C	2.28	2.92	3.20	4.08	0.64	0.92	1.80
32-C	2.28	3.00	3.28	4.28	0.72	1.00	2.00
33-A	2.00	4.28	5.00	7.67	2.28	3.00	5.67
34-K	2.00	2.44	3.00	4.08	0.44	1.00	2.08
35-L	1.80	2.40	2.71	3.84	0.60	0.91	2.04
36-M	1.80	2.40	2.44	3.00	0.60	0.64	1.20
37-J	2.00	4.20	5.16	7.67	2.20	3.16	5.67
38-N	2.00	2.92	3.36	4.60	0.92	1.36	2.60
39-C	2.28	3.00	3.36	4.00	0.72	1.08	1.72
40-O	2.00	2.92	3.08	4.36	0.92	1.08	2.36
41-P	2.00	3.72	4.92	7.80	1.72	2.92	5.80
42-Q	1.92	2.84	3.00	4.28	0.92	1.08	2.36
43-Q	2.00	2.60	3.20	3.80	0.60	1.20	1.80
44-B	2.36	4.28	5.60	8.40	1.92	3.24	6.04
45-B	2.12	2.96	3.16	4.80	0.84	1.04	2.68

[a] The reliquefied milks added to samples No. 1 to 28 were made with "Low-Heat Spray Process" powder, those from No. 30 to 45 were made with "High-Heat Spray Process" powder, and No. 20 was made with "Intermediate-Heat Spray Process" powder. Normally, for fluid milk only "Low-Heat Spray Process" powders are used. Each letter represents products of one factory.

TABLE 6.—*Effect of size of herd and breed upon average P.R.S. values*

NO. IN HERD	BREED[a]													
	J		H		G		JH		JG		MIXED		TOTALS BY SIZE	
	No.[b]	*P.R.S.*	*No.*	*P.R.S.*	*No.*	*P.R.S.*	*No.*	*P.R.S.*	*No.*	*P.R.S.*	*No.*	*P.R.S.*	*No.*	*P.R.S.*
0–9	9	2.62	2	2.33	0	—	0	—	1	2.12	2	2.02	14	2.46
10–19	70	2.26	9	2.33	4	2.23	2	3.12	1	1.74	11	1.85	97	2.23
20–29	44	2.11	9	2.44	3	2.20	1	2.58	1	2.88	12	2.70	70	2.28
30–39	12	2.45	9	2.63	2	2.11	0	—	0	—	4	2.61	27	2.51
40–49	8	2.20	4	2.39	1	3.20	0	—	0	—	3	2.39	16	2.34
50 & Over	6	2.11	5	2.56	0	—	0	—	0	—	1	1.96	12	2.29
Totals by Breed	149	2.25	38	2.47	10	2.29	3	2.94	3	2.25	33	2.32	236	2.30

Standard Deviation between Duplicates = .059
Average Difference between Duplicates = .057

[a] J = Jersey, H = Holstein, G = Guernsey.
[b] No. of samples.

SUMMARY AND RECOMMENDATION

This method seems to offer definite possibilities for the detection of reliquefied or reconstituted dried milk solids in fluid market milk. Even without information as to the original raw milk which is processed, it seems definite that the addition of reconstituted milk in excess of 20 per cent can be positively detected. Information as to the P.R.S. value of the original raw milk will allow the detection of as little as 5 per cent of reconstituted milk. After chemists have become familiar with the technique of this method, no difficulty is experienced in securing good checks of duplicate samples.

It is recommended* that this method be given further study during the next year. This study should include investigation of possible variations due to seasonal conditions and feeding stuffs. The study should also include the effects of any unusual methods of processing milks, including a thorough study of the influences of excessive time and temperatures. The study should include a sufficient number of tests by collaborating laboratories on samples of milk containing known amounts of reconstituted milk prepared in these laboratories from identical samples of various types of non-fat dried milk solids. Replies from nearly all of the regulatory laboratories in the United States have indicated a willingness on the part of about two-thirds of these laboratories to participate in the collaborative study during the coming year.

ACKNOWLEDGMENT

Thanks are expressed to the eight collaborating laboratories who participated in the study this year as indicated in the tables of this report.

REFERENCES

(1) Evenson, O. L., *J. Dairy Sci.*, **5**, 97 (1922).
(2) Choi, R. P., Koncus, A. F., Cherrey, G., and Remaley, R. J., *J. Milk Food Technol.*, **16**, 241 (1953).

* For report of Subcommittee C and action of the Association, see *This Journal*, **38**, 73, 74 (1955).

REPORT ON FOREIGN FATS IN DAIRY PRODUCTS

A CRITICAL REVIEW OF THE REICHERT-MEISSL AND POLENSKE DETERMINATIONS

By SAM D. FINE (Food and Drug Administration, Department of Health, Education, and Welfare, Denver, Colorado),
Associate Referee

INTRODUCTION

In April 1953, Dr. A. H. Robertson, Director of the New York State Food Laboratory, suggested to the Referee on Dairy Products the desirability of using apparatus more modern than that depicted in section **26.27** of the 7th Edition of *Official Methods of Analysis* for the Reichert-Meissl and Polenske determinations on fat from dairy products. He proposed use of commercial apparatus with all ground-glass, standard taper joints, use of an electrically heated mantle in place of a gas burner, use of powdered Alundum in place of pumice, and the use of a fritted glass filter in place of filter paper. Mr. Walter C. Bartsch, Chief Chemist of the Pioneer Ice Cream Division of The Borden Company, had proposed some of these modifications to Dr. Robertson.

The Referee agreed that provision for an alternate apparatus would be desirable, but because of the long-established nature of the determination and the rigid specification of apparatus and technique, he felt that before changes of the nature suggested could be adopted, it would be necessary to demonstrate that those changes would give the same values as the present official methods.

The Referee arranged for a series of determinations on butterfat samples by methods **26.26–26.27** and by the suggested revisions of those methods. Chemists of the New York State Food Laboratory, the Pioneer Ice Cream Division of the Borden Company, Brooklyn, New York, and the Denver and Minneapolis Districts of the Food and Drug Administration participated in this work in the spring and summer of 1953.

The Denver laboratory procured the apparatus[1] depicted in section **26.27** of *Official Methods of Analysis*, and a glass joint apparatus[2] was also obtained. A Glas-Col 470 watt heating mantle, designed to fit a 300 ml flask and controlled by a variable transformer, was used with the glass joint apparatus. Six-mesh carborundum chips were used as the antibumping agent in the ground glass joint apparatus. Corning medium porosity fritted Gooch-type crucibles were used with this apparatus for the filtration step. One suggestion for modernizing the methods was that the saponification be carried out by using the electric mantle with the flask

[1] Arthur H. Thomas Co., p. 511, 1950 catalog.
[2] Scientific Glass Apparatus Co., p. 133, catalog J-52.

connected to a reflux condenser. At Denver it was found that three to four times as long was required for saponification by this procedure, compared to saponification over a flame. In addition, the point of complete saponification was more difficult to determine when the mantle was used, and care had to be taken to prevent bumping and loss of the sample through the top of the condenser. Accordingly, all saponifications were carried out at both Denver and Minneapolis by means of a gas flame.

The Minneapolis laboratory obtained a Glas-Col 245 watt heating mantle which did not have the hemispherical metal shell used on the 470 watt mantle. They found that the 245 watt mantle caused overheating of the distillation flask at the liquid level. Thus, toward the end of the distillation, decomposition of the floating acids occurred and erroneous Polenske values resulted. The mantle used at the Denver laboratory was sent to the Minneapolis laboratory, and no further difficulties were encountered. It was found that the heating element in the 245 watt mantle extended to the top of the unit, while in the 470 watt mantle it was at least three fourths of an inch from the top.

Carborundum proved to be a superior anti-bumping agent, and the glass joint apparatus, heated with an electric mantle, was more convenient than the rubber-stoppered apparatus heated with a gas flame. At Denver it was noted that with the fritted glass filters, small amounts of insoluble liquids passed through; this would cause erroneous Polenske values. Also, filtration time with the glass filters was much longer than when filter paper was employed. It was the conclusion of this laboratory, later borne out by work on fats with high Polenske values, that the use of fritted glass filters was not an improvement in the procedure.

In the four laboratories participating in this work in the spring and summer of 1953, 98 determinations were made on butterfat samples by methods **26.26–26.27.** Then, utilizing the modern apparatus and the other suggested changes, a total of 96 determinations were made by the same chemists on the same samples. The average Reichert-Meissl value for the 98 determinations was 27.1 and the average for the 96 determinations with modern apparatus was 28.1. The Referee concluded that the matter warranted further study by an Associate Referee, and in the fall of 1953, the problem was assigned to the author of this report.

HISTORY OF THE METHODS

The Associate Referee felt that a review of the development of the Reichert-Meissl and Polenske methods was in order, since the procedures are empirical, and since many individuals believed there was a great mass of authentic data associated with one particular, rigidly specified apparatus and procedure. The opinion had been expressed that changes in apparatus and procedure, which admittedly resulted in changes in

Reichert-Meissl and Polenske values, would invalidate all of the preceding authentic data. The Associate Referee found that the methods have undergone many changes during their development, and that it is only since 1930 that *Official Methods of Analysis* has contained the present procedures.

Hehner and Angell (1) in 1874, after showing that butter contained more butyric acid than was then generally supposed, attempted to estimate the butyric acid by distillation. They finally abandoned the method because of discordant results which were due primarily to the bumping of the liquid and the use of too strong an acid.

Lechartier (2), tried to separate the volatile acids of butterfat from the non-volatile acids by saponifying with sodium hydroxide, adding tartaric acid, and distilling off the volatile acids. The distillate was neutralized with barium hydroxide, and the barium salts were weighed. Practical application of this procedure met with difficulties due to the impossibility of distilling off the total amount of volatile acids without some decomposition.

In 1879, Reichert (3) showed that by isolating a definite proportion of the volatile soluble acids under certain conditions, a useful analysis in the examination of butterfat could be made. He originally proposed to determine the number of cubic centimeters of 0.1 *N* alkali required for neutralization of the soluble volatile fatty acids from 2.5 grams of sample. A slow stream of air was used in the distilling flask to prevent bumping. Of interest is the fact that Reichert called the procedure "Hehner's method." Time of distillation was not specified.

Meissl (4), in 1879, suggested 5 grams as a more convenient sample. He used a "few small pieces of pumice" to prevent bumping. Distillation was "within about one hour."

In 1887, Wollny (5) studied the Reichert-Meissl method thoroughly, and pointed out various possible sources of error. He was the first investigator to specify an apparatus in detail. His apparatus differed from present day versions in that the condenser was inclined at an angle of 50° instead of being vertical. This method was adopted in 1900 by a joint committtee of the British Government Laboratory and the Society of Public Analysts as the standard method for the determination of the soluble volatile fatty acids in margarine and butter (6). Pumice ("three or four fragments of pumice or broken pipestems") was specified as the anti-bumping agent, and distillation time was listed as 28 to 32 minutes.

Bulletin 13 of the Division of Chemistry, Department of Agriculture, 1887, refers to Hehner and Angell's publication of 1874, and the Reichert and Meissl publications of 1879. With various modifications the methods were then in use in the United States. Caldwell, New York State Board of Health, substituted short spirals of platinum wire and pieces of pumice stone for the current of air used by Reichert. Crampton of the U. S.

Department of Agriculture used "rough pumice loaded with stout platinum wire."

Bulletin 24 of the Division of Chemistry, 1890, Proceedings of the 6th Annual Convention of the Association, contains a report by H. W. Wiley on the analysis of butter. Wollny's method was used. Following is an excerpt from the procedure in Bulletin 24: "A few pieces of pumice stone thrown white-hot into distilled water and kept therein are placed in the flask, which is connected by a bulb bent tube with an appropriate condensing apparatus. . . . in as nearly as possible 30 minutes." Bulletin 28, also dated 1890, contains the official methods of the Association, and Wollny's apparatus is depicted, together with the procedure specified in Bulletin 24.

Leffman and Beam (7), in 1891, proposed a time-saving modification by providing for saponification with sodium hydroxide in glycerin. Proceedings of the 9th Annual Convention of the Association, Bulletin 35 of the Division of Chemistry, 1892, reports a study of the Leffman and Beam method, but it was not adopted. Glass and block-tin condensers were also studied; glass condensers only were considered suitable. The distilling apparatus was not described, but the same method of pumice preparation and a distillation time of 30 minutes were specified.

Proceedings of the 15th Annual Convention of the Association, 1898, Bulletin 56 of the Division of Chemistry, 1899, reports a new study of the Leffman and Beam saponification procedure as compared with the then official Reichert-Wollny method. The Referee, C. L. Penney, recommended the adoption of the Leffman-Beam procedure, and it was included in the official methods in 1899, published as Bulletin 40, Revised Edition, Division of Chemistry. Wollny's apparatus, without dimensions, was depicted. The method of pumice preparation and distillation time of about 30 minutes remained unchanged.

Polenske (8), in 1904, determined the water-insoluble volatile fatty acids in fats, and specified in detail the apparatus as it is given in the 7th Edition of *Official Methods of Analysis*. Pumice for anti-bumping was specified as "0.5 gram of granulated pumice stone with grains 1 mm in diameter." Distillation time was 19–20 minutes.

Bulletin 107, Bureau of Chemistry, 1907, contained both the Leffman and Beam saponification procedure, and the earlier procedures which used alkali, alcohol, and pressure. Wollny's apparatus was depicted, and the pumice preparation and distillation time remained the same.

In 1911, Revis and Bolton (9) used the Polenske apparatus and the Leffman and Beam saponification. For an anti-bumping agent they specified "0.1 gram powdered pumice sieved through butter-muslin." Distillation time was specified as 19–21 minutes.

The Polenske method, which does not appear in Bulletin 107, was included in Association of Official Agricultural Chemists, *Methods*, 1916,

which antedates the First Edition of *Official Methods of Analysis*. No reference to its inclusion is found in the proceedings of the various conventions, and it was not subjected to collaborative study at that time. The method was continued in the First and Second Editions of *Official Methods of Analysis* and the apparatus including dimensions of the principal components was depicted. Distillation time for the Polenske determination was specified at 20 minutes, while the specified distillation time for the Reichert-Meissl and Leffman and Beam methods was 30 minutes. The Reichert-Meissl method, as included in the first two editions of *Official Methods of Analysis*, did not specify any particular type of distilling apparatus. On the other hand, in both editions the Leffman and Beam method specified the Polenske apparatus.

In the 1929 report (10) of G. S. Jamieson, Referee on Fats and Oils, it was recommended that a study be made of "the combined procedure for the determination of the Reichert-Meissl and Polenske values, which is now quite commonly used in place of the separate procedures described in *Methods of Analysis*." (The combined procedure used the Leffman and Beam method of saponification.) Jamieson's 1930 report (11) gave collaborative results on five fat samples and recommended the adoption of the combined procedure as official and its substitution for the separate methods then listed under soluble acids and insoluble acids. The recommendation was approved, and the combined procedure entered the Third Edition of *Official Methods of Analysis* and has remained unchanged down to and including the Seventh Edition. Polenske's apparatus was designated in the combined procedure, and the names of Leffman and Beam were dropped, although the saponification procedure used is the method they devised.

EFFECTS OF VARIABLES

The Associate Referee has tried to find which of the proposed modifications in apparatus and procedure were responsible for the changes in Reichert-Meissl values on butterfat noted in the 1953 study. Since the methods appear in the chapter on fats and oils, it was also considered necessary to consider the effect of the proposed modifications on fats with high Polenske and low Reichert-Meissl values, in contrast with the low Polenske and high Reichert-Meissl values of butterfat.

Dr. A. H. Robertson, in a private communication to the Associate Referee, had called attention to the variation in the dimensions of the glass joint apparatus purchased for his laboratory for these determinations. The Associate Referee noted the same variations in apparatus supplied to his laboratory by a second firm. To devise practical specifications it is, of course, necessary to know the variations to be expected in fabricating apparatus of this type to stipulated dimensions.

The Society of Public Analysts in 1936 (12) described the apparatus

pictured in the 7th Edition of *Official Methods of Analysis* in minute detail, and listed maximum and minimum figures for each dimension. The Associate Referee prepared a drawing of a glass joint apparatus, changing the dimensions as required by the standard taper joints, and submitted it to the Corning Glass Works with the request that maximum and minimum values to be expected in good commercial practice be indicated for each dimension. Figure 1 depicts the glass joint apparatus,

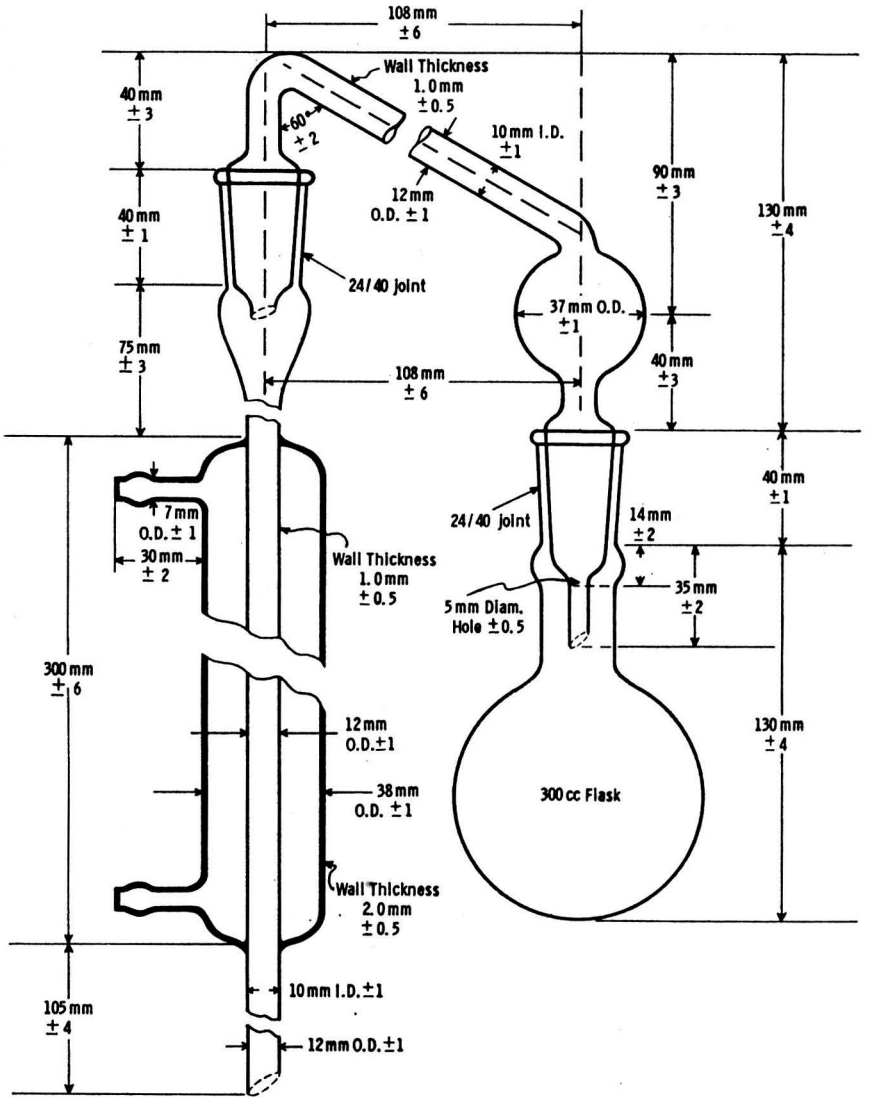


FIG. 1.—"Modern" all-glass distilling apparatus (dimension of tube at flask end of distilling head below joint is 10 ±1 mm I.D.)

with tolerances for each dimension, as supplied by Corning Glass Works. In all of the ensuing work, "modern apparatus" refers to the glass joint apparatus meeting the dimensions of Figure 1. "Old apparatus" refers to the apparatus meeting the approximate dimensions of that depicted in the 7th Edition.

A preliminary study on butterfat, using the modern apparatus with carborundum and electric heating, and comparing results with the old apparatus, pumice, and gas heating, indicated that the carborundum was causing most of the increase in Reichert-Meissl values. It has been known for many years that the type of anti-bumping agent has a marked effect upon Polenske values. In 1938, R. S. McKinney (13) reported on Reichert-Meissl and Polenske values of five fat samples, where for one series of determinations, pieces of pumice were used and for a second series, fine pumice was used. The fine pumice gave higher Polenske values than the pieces of pumice, but was not nearly so efficient an anti-bumping agent. McKinney recommended further study of the Polenske determination with powdered pumice. Subcommittee C recommended continuation of the study in 1938, and an Associate Referee on the subject was appointed. No reports were made and the topic was not included for further study by Subcommittee C in 1943. However, the Referee on Dairy Products furnished the author of this report with some unpublished work done by J. A. Mathews, using powdered pumice, lump pumice, glass beads, and 20 mesh zinc as anti-bumping agents. Mathews found that lump pumice gave an average Polenske value on a palm kernel oil of 17.3; on the same oil, glass beads gave an average Polenske value of 22.35, while powdered pumice and 20 mesh zinc each gave a value of 23.3.

William Weiss, Statistician, Division of Food, Food and Drug Administration, was requested by the Associate Referee to design an experiment to allow statistical evaluation of the effect of the variables of anti-bumping agent, apparatus, and method of heating. Table 1 shows the design of this experiment. Three determinations were specified for each

TABLE 1.—*Design of experiment*[a]

ANTI-BUMPING AGENT	MODERN APPARATUS		OLD APPARATUS	
	ELECTRICITY	GAS	ELECTRICITY	GAS
Carborundum	(2)	(6)	(7)	(3)
	(15)	(11)	(13)	(12)
	(18)	(22)	(24)	(20)
Pumice	(5)	(8)	(1)	(4)
	(10)	(14)	(9)	(16)
	(21)	(17)	(19)	(23)

[a] Figures indicate order in which the 24 determinations were run.

TABLE 2.—*Comparison of modern and old apparatus*

Butterfat

ANTI-BUMPING AGENT	MODERN APPARATUS				OLD APPARATUS			
	ELECTRICITY		GAS		ELECTRICITY		GAS	
	R-M	*P*	*R-M*	*P*	*R-M*	*P*	*R-M*	*P*
Carborundum	27.77	1.70	27.34	1.89	27.66	1.75	27.53	1.83
	27.90	1.77	27.38	1.96	27.72	1.74	27.50	1.94
	27.81	1.64	27.47	1.93	27.63	1.81	27.37	1.85
Pumice	27.23	1.46	26.87	1.74	27.22	1.45	27.03	1.51
	27.10	1.41	26.95	1.45	27.18	1.35	26.89	1.52
	27.10	1.42	26.89	1.46	27.17	1.45	26.96	1.56

TABLE 3.—*Comparison of modern and old apparatus*

Palm Kernel Oil

ANTI-BUMPING AGENT	MODERN APPARATUS				OLD APPARATUS			
	ELECTRICITY		GAS		ELECTRICITY		GAS	
	R-M	*P*	*R-M*	*P*	*R-M*	*P*	*R-M*	*P*
Carborundum	5.68	9.03	5.68	9.21	5.78	8.65	5.93	8.78
	5.93	9.31	5.74	9.37	5.90	8.83	6.09	8.89
	5.79	9.37	5.83	9.82	5.78	9.18	5.77	9.41
Pumice	5.20	6.65	5.58	8.53	5.16	6.68	5.69	8.09
	5.11	6.61	5.80	8.78	5.32	6.55	5.53	7.92
	5.28	7.26	5.56	8.41	5.31	6.68	5.62	8.02

TABLE 4.—*Scheme for evaluating effect of time*

Either Apparatus

	CARBORUNDUM	PUMICE
Electricity	25 min.	25 min.
	30 min.	30 min.
	35 min.	35 min.
Gas	25 min.	25 min.
	30 min.	30 min.
	35 min.	35 min.

TABLE 5.—*Effect of time, method of heating, and anti-bumping agent*
Butterfat—Modern Apparatus

	CARBORUNDUM			PUMICE		
		R-M	*P*		*R-M*	*P*
Electricity	25 min.	27.57	1.70	25 min.	26.84	1.31
	30 min.	27.86	1.75	30 min.	27.03	1.47
	35 min.	28.03	1.79	35 min.	27.24	1.46
Gas	25 min.	27.16	1.85	25 min.	26.94	1.65
	30 min.	27.31	1.81	30 min.	26.97	1.59
	35 min.	27.58	1.95	35 min.	27.41	1.83

combination, with the order of the determinations (numbers in parenthesis) determined by reference to a table of random numbers.

T. J. Klayder of the Denver laboratory made all of the determinations to demonstrate the effect of these variables, as well as those reported on the effects of time of distillation, amount of glycerol-soda reagent, and pressure. Since the work in 1953 had demonstrated that insoluble, liquid volatile acids were in part passing through the fritted glass filters, filter paper (S&S No. 589, White Ribbon) was used for all of the determinations. All saponifications were carried out over a gas flame. The same butterfat and reagents were used in all 24 distillations reported in Table 2, and the same palm kernel oil and reagents were used in all 24 distillations reported in Table 3.

Richmond and Hall (14) reported in 1920 that time of distillation does not make any material difference in the Reichert-Meissl and Polenske values of butterfat, but does affect the same values for coconut oil. William Weiss designed the experiment shown in Table 4 to allow statistical evaluation of the effect of varying times of distillation. The same butterfat and reagents were used in the 12 distillations reported in Table 5 as for the 24 distillations reported in Table 2. The same palm kernel oil

TABLE 6.—*Effect of time, method of heating, and anti-bumping agent*
Palm Kernel Oil—Modern Apparatus

	CARBORUNDUM			PUMICE		
		R-M	*P*		*R-M*	*P*
Electricity	25 min.	5.74	8.92	25 min.	5.10	6.53
	30 min.	5.80	8.96	30 min.	5.09	7.01
	35 min.	5.75	9.71	35 min.	5.17	6.45
Gas	25 min.	5.73	9.42	25 min.	5.46	8.56
	30 min.	5.97	9.25	30 min.	5.55	8.54
	35 min.	5.71	9.20	35 min.	5.53	8.06

TABLE 7.—*Effect of amount of alkali, method of heating, and anti-bumping agent*

Butterfat—Modern Apparatus

	CARBORUNDUM			PUMICE		
		R-M	*P*		*R-M*	*P*
Electricity	18 ml	27.26	1.83	18 ml	27.34	1.79
	20 ml	27.54	1.67	20 ml	27.06	1.45
	22 ml	27.62	1.86	22 ml	27.22	1.53
Gas	18 ml	27.41	1.88	18 ml	27.26	1.83
	20 ml	27.32	1.76	20 ml	27.36	1.81
	22 ml	27.41	2.05	22 ml	27.53	2.13

was used in the 12 distillations reported in Table 6 as for the 24 reported in Table 3. While the effect of time was being studied, it was discovered that the instructions in **26.27** " . . . collect 110 ml of distillate in as near 30 min. . . . " are susceptible to different interpretations. The majority of the analysts queried on this point believed that the 30 minutes was measured from the time the first drop left the tip of the condenser until the 110 ml flask was filled. However, some interpreted the instruction to mean that the 30 minute period should be measured from the time the preset burner was placed under the distillation flask.

In order to evaluate the effect of varying amounts of glycerol-soda reagent, the same design as in Table 4 was used. Volumes of 18, 20, and 22 ml were substituted for the 25, 30, and 35 minute time variables. Since the reagent has a high viscosity, it was added to the flasks by weights equivalent to the above volumes. It was determined that with 22 ml of the reagent there was present an excess of 0.38 ml of H_2SO_4 (1+4). The same butterfat and reagents were used for the 12 distillations reported in Table 7 as for the 24 reported in Table 2 and the 12 reported in Table 5.

TABLE 8.—*Effect of amount of alkali, method of heating, and anti-bumping agent*

Palm Kernel Oil—Modern Apparatus

	CARBORUNDUM			PUMICE		
		R-M	*P*		*R-M*	*P*
Electricity	18 ml	4.67	8.27	18 ml	4.23	6.11
	20 ml	4.65	8.15	20 ml	4.17	6.60
	22 ml	4.67	8.41	22 ml	4.41	7.23
Gas	18 ml	4.70	8.72	18 ml	4.43	7.88
	20 ml	4.93	8.71	20 ml	4.58	7.87
	22 ml	4.70	8.82	22 ml	4.67	8.07

A different palm kernel oil was used for the 12 distillations reported in Table 8 than for the 24 reported in Table 3 and the 12 reported in Table 6.

William Weiss evaluated the data in Tables 2, 3, 5, 6, 7, and 8. His conclusions with respect to the effects of the variables follow:

Apparatus.—A comparison of modern apparatus and old apparatus was made in the experiments reported in Tables 2 and 3. There was no apparatus effect on R-M values in either experiment, nor on Polenske values of butterfat. However, a strong apparatus effect is shown in the Polenske values of palm kernel oil. In this instance the modern apparatus averaged 0.39 higher than the old, and this difference was consistent for each combination of heat source *vs.* anti-bumping agent. Restrictions on type of apparatus need be placed only on the Polenske determinations of palm kernel and similar types of oils. Neither apparatus offers greater precision than the other.

Heat.—An examination of the data in Tables 2, 3, 5, 6, 7, and 8 with respect to electric *vs.* gas heating leads to several definite conclusions.

There is a real difference due to the type of heat employed. The following tabulation lists the average excess of values for gas over those for electricity when carborundum and pumice are used as the anti-bumping agents:

Table No.	*Reichert-Meissl*		*Polenske*	
	Carborundum	*Pumice*	*Carborundum*	*Pumice*
2	−0.32	−0.24	0.16	0.08
3	0.03	0.40	0.19	1.55
5	−0.47	0.07	0.12	0.28
6	−0.06	0.39	0.09	1.73
8	0.12	0.29	0.47	0.29
7	−0.26	0.19	0.11	0.33
Average	−0.16	0.18	0.19	0.88

In each experiment the Polenske determinations by gas heat were greater than those by electricity; the average excess of gas over electricity ranged from 0.08 to 1.73. For Reichert-Meissl determinations, significant differences exist between the two forms of heat, but in some cases electricity gives a significantly greater response, and in others, gas gives the greater response. This is meaningful only if an assumption, which cannot be tested statistically, is made. This assumption is that electric heat gave higher values (as shown by the negative values above) in the experiments represented by the data of Tables 2, 5, and 7, by reason of the combination of butterfat, Reichert-Meissl determination, and carborundum. With carborundum the Reichert-Meissl determinations on palm kernel oil did not show significant differences between the two types of heating. With pumice as the anti-bumping agent, gas heat gave higher Reichert-Meissl values in all cases but one.

Since there is a significant difference between the heating methods, one or the other, but not both, should be recommended.

There is no significant difference in precision whether electricity or gas is the heating agent; the combined average standard deviation for replicates for gas is 0.14, and for electricity, 0.13. (One set of replicates for electricity was not included in the calculation of the standard deviation since one of the three observations was apparently an aberrant value.)

Anti-bumping agent.—In each experiment, the average of the determinations with carborundum as the anti-bumping agent exceeded those with pumice. The average differences varied widely from experiment to experiment. The following tabulation lists the average excess of the carborundum values over those for pumice:

Table No.	Reichert-Meissl		Polenske	
	Electricity	Gas	Electricity	Gas
2	0.58	0.50	0.32	0.40
3	0.58	0.21	2.32	0.96
5	0.78	0.24	0.34	0.18
6	0.64	0.19	2.54	0.90
8	0.39	0.22	1.63	0.81
7	0.43	0	0.20	−0.02
Average	0.57	0.23	1.23	0.54

In every case but one, the difference between the two anti-bumping agents was less with gas heat than with electricity. The greatest difference occurred when palm kernel oil was used with electricity as the heating agent.

Carborundum offers no greater precision than pumice. For the combined experiments the standard deviation for Reichert-Meissl replicates is 0.09 for carborundum and 0.09 for pumice; on the Polenske determinations, 0.21 for carborundum and 0.23 for pumice. The following tabulation lists the standard deviations for replicates for the data of Tables 2, 3, 6, 7, and 8. (The time experiment on palm kernel oil, Table 6, was included since there was no time effect:)

Table No.	Reichert-Meissl		Polenske	
	Carborundum	Pumice	Carborundum	Pumice
2	0.07	0.08	0.07	0.04
	0.07	0.04	0.04	0.01
	0.05	0.03	0.04	0.06
	0.09	0.07	0.06	0.03
3 & 6	0.13	0.08	0.18	0.36
	0.08	0.13	0.32	0.19
	0.07	0.09	0.27	0.08
	0.16	0.08	0.34	0.09
	0.03	0.04	0.45	0.30
	0.03	0.05	0.12	0.28
7 & 8	0.01	0.12	0.13	0.56
	0.13	0.12	0.06	0.11
	0.11	0.14	0.10	0.18
	0.05	0.14	0.15	0.18
Combined S.D.	0.09	0.09	0.21	0.23

The Polenske determinations show less stability in precision from experiment to experiment; the range of standard deviations on Reichert-Meissl determinations is 0.01 to 0.16, while for Polenske determinations, the range is 0.01 to 0.56.

Distillation time.—The data of Table 5 (butterfat) show a very significant effect of distillation time upon Reichert-Meissl values. The average increase from 25 to 35 minutes was 0.44. On the other hand, the average increase of Polenske values was 0.13. The data of Table 6 (palm kernel oil) definitely show no effect of distillation time upon either Reichert-Meissl or Polenske values. This aspect is inconclusive, but it may be inferred that under carefully controlled test conditions a change of distillation time of plus or minus 5 minutes is permissible in the determination of the Polenske value.

Amount of reagent.—There is no significant effect of changing the amount of reagent from 18 to 22 ml.

Summary.—Based on the data of Tables 2, 3, 5, 6, 7, and 8, an analyst, at one laboratory, making one determination, should obtain a value within ±0.18 on Reichert-Meissl, and within ±0.44 on Polenske, of the true value for that laboratory.

V. H. Kirkham (15) reports that the Polenske value is a function of the distillation pressure, and that if values are not corrected to normal pressure, serious errors are liable to be introduced. He reported a Polenske value (on the same butterfat) of 2.68 at 760 mm and 2.06 at 627 mm. The Reichert-Meissl value was stated to be a logarithmic function of the pressure, and the errors introduced by ordinary variations in atmospheric pressure were said to be quite small. A butterfat yielding an R-M value of 27.99 at 760 mm gave a result of 27.60 at 627 mm. Kirkham made his observations at Nairobi, British East Africa, which has an elevation of about 5,500 feet. Variations in pressure were artificially achieved by attaching a closed chamber to the receiver end of the condenser, and attaching a manometer and pump to this chamber.

The Denver laboratory is also at an elevation of about 5,500 feet above sea level. With the cooperation of the Minneapolis District laboratory, located at an elevation of about 840 feet above sea level, experiments to evaluate the effect of pressure were undertaken. A sample of butterfat, and a sample of palm kernel oil were analyzed with the same apparatus and reagents at each laboratory, with maximum precautions to insure identical techniques. Results are summarized in Table 9.

Kirkham's conclusions that the Polenske and Reichert-Meissl values increase with increasing pressure are confirmed. Correction of the average of four determinations on butterfat at each of the two laboratories to standard pressure of 760 mm by the use of Kirkham's formulas yields the results shown in Table 10. Kirkham worked only with butterfat, and his formulas apply only to that fat. (Application of the Reichert-Meissl correction formula to the Reichert-Meissl results on palm kernel oil yields lower values at 760 mm than those at lower pressures. This is contrary to the experimentally observed results.)

RANGE OF REICHERT-MEISSL VALUES ON AUTHENTIC BUTTERFATS

Values reported in the literature for authentic butterfat are confusing to the regulatory chemist. The following ranges in Reichert-Meissl values have been reported for authentic butterfat:

Jacobs (16)—22 to 32
Winton (17)—24 to 34
Leach-Winton (18)—22.4 to 36.4
Woodman (19)—24 to 34
Handbook of Chemistry & Physics (20)—17.0 to 34.5

Woodman (19) reports that values as low as 12 and as high as 40 have been found in butterfat. It is well known that the Reichert-Meissl values of butterfat are influenced by such factors as feed, season, and characteristics of individual cows. Cranfield and Taylor (21), in 1915,

TABLE 9.—*Effect of barometric pressure*

	DENVER					MINNEAPOLIS					
	Butterfat										
Elevation	5500 ft.					840 ft.					
					Av.					*Av.*	
Barometric reading (mm)	635.2	635.0	634.4	633.5	*634.5*	739.4	739.4	739.4	741.7	*740.0*	739.1
R-M value	27.63	27.68	27.85	27.73	*27.72*	28.43	28.74	28.70	28.28	*28.54*	28.48[a]
P value	1.86	1.74	1.80	1.72	*1.78*	2.41	2.42	2.21	2.55	*2.40*	2.45[a]
	Palm Kernel Oil										
Barometric reading (mm)	635.7	635.5	635.5	635.3	*635.5*	739.1	739.1	739.1	739.1	*739.1*	739.1
R-M value	4.95	4.79	4.91	4.87	*4.88*	5.44	5.31	5.54	5.42	*5.43*	5.66[a]
P value	8.46	8.62	8.58	8.48	*8.54*	10.01	9.75	9.97	9.65	*9.85*	9.73[a]

[a] Single determinations by a second chemist.

TABLE 10.—*Correction of R-M and P values of butterfat to 760 mm (15)*

	DENVER		MINNEAPOLIS	
	R-M	*P*	*R-M*	*P*
Observed atm. pressure	634.5	634.5	740	740
Observed value[a]	27.72	1.78	28.54	2.40
Corrected value	28.22	2.16	28.60	2.47
Difference from obs. value	+0.50	+0.38	+0.06	+0.07

[a] Average of 4 determinations.

reported that butterfat from cows on poor pastures had lower Reichert-Meissl values than did the butterfat from the same cows when the diet was changed to include cottonseed cake, linseed cake, etc. Addition of vegetable fats and oils to cows' diets tends in general to lower both the Reichert-Meissl and Polenske numbers (22, 23, 24).

Hilditch and Slightholme (25) reported on changes in the Reichert-Meissl values due to changes in season and feeding conditions. They found that dry summer and autumn pastures lower the Reichert-Meissl values. Arup (26) reported on the seasonal variations of Reichert-Meissl and Polenske values of Irish butter.

Various investigators (27–29) have also noted that there is some variation in the Reichert-Meissl values from cows fed normal rations but Overman and Garrett (27) concluded that the variations were not large enough to be of other than scientific interest. Stout and Wilster (29) studied butterfat from three sections of Oregon where different types of feeding were used, and found values ranging from 26.03 to 31.5. Hawley (30) pointed out that published figures purporting to be the Reichert-Meissl values of the milks of single animals should be accepted with reserve in the absence of a statement of the stage of lactation of the animals, or of the daily yield of milk at the time of sampling. Balavoine (31) reports that a survey of the literature shows a relationship between the annual variations in the vitamin content of cows' milk and butter and the variations in Reichert-Meissl number and refractive index. He noted that cows producing milk with a lower Reichert-Meissl number are healthier.

During a period of four to five years, Spitzer and Epple (28) examined 500 samples of creamery butter. The lowest Reichert-Meissl number obtained was 26.45, the highest was 32.10, and the average was 29.91.

Some agencies have set limits for Reichert-Meissl values, and butterfats having values below a prescribed limit are considered to be adulterated. For example, the Food and Drug Division of the Canadian Department of National Health and Welfare, in Trade Information Letter No. 92, (August 10, 1953) states that butterfat shall have a Reichert-Meissl number of not less than 24, and a Polenske value of not more than 3.5; where the Polenske value exceeds 10 per cent of the Reichert-Meissl value, there shall be deemed to have been an addition to the milk fat of fat other than that of cows' milk.

Lewkowitsch (32) states that the minimum value generally recognized among analysts (not officially) is 24 in England and France, 25 in Germany, and 23 in Sweden. This text also states that in 1904 Belgium defined butter as abnormal in composition if the Reichert-Meissl value fell below 28; that an Italian law of 1894 defined butter as genuine only when the Reichert-Wollny number is not below 26; and that an Act of the United States Congress of July 1, 1902, gives as one of the chemical

standards for butterfat a Reichert-Meissl number of not less than 24. (A check was made with the Internal Revenue Service which is charged with the enforcement of this Act, and it was learned that Lewkowitsch is in error, since neither the Act, the regulations promulgated under it, or any public correspondence lists a limiting Reichert-Meissl value.)

Various groups, including the Food and Drug Administration and the Universities of Maryland and Wisconsin, are presently gathering new data on butterfat. Precautions are being taken to make certain that the butterfats represent mixed milk from large herds. This will obviate the possibility of abnormalities, due to individual animals, which may significantly affect the Reichert-Meissl values. Samples are being collected throughout the year in order to allow evaluation of seasonal changes. Perhaps this new data, taken in conjunction with the results on the 500 samples reported by Spitzer and Epple (28), will serve to narrow the wide range presently reported in the literature.

METHOD OF EXTRACTION OF FAT FROM DAIRY PRODUCTS

The method of extraction of fat from dairy products can significantly affect both the Reichert-Meissl and Polenske values. Martin, Rutz, and Whitnah (33) reported the changes in these constants on butterfats extracted from ice creams by three methods. The procedure they designated as the Minnesota reagent churning method was shown to have insignificant effects upon the butterfat during extraction.

It was suggested to the Associate Referee that, if at all possible, the Sager-Sanders detergent reagent (34) should be used to separate the fat from ice cream. An authentic ice cream was prepared. The Reichert-Meissl value was determined on the butterfat used in its manufacture, separating the fat from the butter by method **15.115.** The fat was also separated from the ice cream with the Sager-Sanders detergent reagent and by a modification of the Minnesota reagent churning method. Reichert-Meissl values on the fat separated by the Sager-Sanders detergent reagent were significantly lower than those on the fat obtained by the modified Minnesota reagent churning method. It was learned by discussion between the Referee and Mr. Sager that approximately 3 per cent of the reagent remains in the fat. Attempts to wash out the reagent were not successful and additional work is planned on separation procedures.

SUMMARY

Reichert-Meissl and Polenske determinations have been made on butterfat and palm kernel oil by **26.26** and **26.27,** and by modifications of those methods, utilizing glass joint apparatus, an electric heating mantle, and 6 mesh carborundum as the anti-bumping agent. The modifications result in changes in both the Reichert-Meissl and Polenske values.

The effects of apparatus, heating method, and anti-bumping agent have been studied experimentally and statistically evaluated. In addition, the effects of varying the times of distillation and varying the amounts of glycerol-soda reagent have been similarly studied and evaluated.

Based on the data obtained at Denver, Colorado, it can be stated:

(1) For butterfat, the type of apparatus had no effect on either the Reichert-Meissl or Polenske values. For palm kernel oil, the type of apparatus had no effect on the Reichert-Meissl value, but had a very significant effect on the Polenske value. Thus, either apparatus may be used *except* in the determination of Polenske values on high Polenske value fats. Neither apparatus offers greater precision than the other.

(2) The method of heating has a significant effect, and only one method should be used. On the basis of precision, there is no preference between electric and gas heating, since both were equally precise.

(3) The anti-bumping agent affects both the Reichert-Meissl and Polenske values of both butterfat and palm kernel oil, with carborundum increasing the values of both constants significantly. Only one should be used, but there is no preference on the basis of precision.

(4) Time of distillation affected both the Reichert-Meissl and Polenske values of butterfat, but did not affect the values of these constants for palm kernel oil.

(5) Varying the amounts of glycerol-soda reagent (from 18 to 22 ml) did not affect the Reichert-Meissl and Polenske values of butterfat and palm kernel oil.

Based on the data obtained at Denver, Colorado, and Minneapolis, Minnesota, it can be stated:

The pressure effect reported by Kirkham (15) on the Reichert-Meissl and Polenske values of butterfat was confirmed. It was also found that the Reichert-Meissl and Polenske values of palm kernel oil are significantly affected by varying pressure.

A review of the history of the methods reveals that many changes and improvements have been incorporated since the determinations were originally devised. There is no great mass of authentic data associated with any one particular apparatus or procedure. It is doubtful however, if the various modifications have changed the Reichert-Meissl values of butterfats more than 2–3 units.

Ranges in Reichert-Meissl values for butterfat taken from the literature are so wide as to cause suspicion of their authenticity in the practical sense of that term. It may well be that the extremely low and high values reported by some authorities represent abnormalities which are not encountered in butterfat from truly authentic, mixed herd milk.

Methods of extraction of butterfat from ice cream can significantly affect the Reichert-Meissl value of the butterfat.

CONCLUSIONS AND RECOMMENDATIONS

On the basis of the results reported, it can be concluded:

1. When dealing only with Reichert-Meissl values, either the apparatus depicted in section **26.27** of the 7th Edition, *Official Methods of Analysis*, or that depicted in Figure 1 of this report, may be used. With Polenske values, however, the apparatus must be specified.

2. Since the statistical analysis indicates there is no advantage to the use of electrical heating from the standpoint of precision, it is believed that gas heating should be continued as the only method. Aside from the fact that it is much cheaper, there is the possibility that other electric mantles (besides the one supplied to the Minneapolis laboratory) would be so constructed as to cause charring of the floating acids in the distillation flask and consequent erroneous values.

3. As carborundum is a superior anti-bumping agent, it should replace pumice. Six-mesh carborundum was used in the course of this work.

It is recommended*—

(1) That the methods embodied in **26.26** and **26.27**, 7th Edition, *Official Methods of Analysis*, be changed as follows:

(a) Replace the reagent, pumice stone, with 6-mesh carborundum.

(b) Include a diagram of a distillation apparatus as specified in Figure 1 of this report in addition to the present Figure 46.

(c) Change the last sentence in the first paragraph of **26.27** to read: "Rest flask on piece of asbestos board having hole 5 cm in diam. in center, and so regulate flame as to collect 110 ml of distillate in as near 30 min. as possible (measure time from passage of first drop of distillate from condenser to receiving flask). Allow distillate to drip into receiving flask at temperature not higher than 18–20°."

(2) That the effect of pressure on Reichert-Meissl and Polenske values of fats be further evaluated.

(3) That data be collected on authentic butterfats under current production methods from mixed herd milks, in an effort to discover the range limits for Reichert-Meissl values of pure butterfat.

(4) That methods of extraction of butterfat from dairy products preparatory to determination of fat constants be studied.

ACKNOWLEDGMENT

Grateful appreciation is extended to the following individuals who have participated in the work reported in this paper:

T. J. Klayder of the Denver District, Food and Drug Administration, who performed the analytical work to demonstrate the effects of the variables and studied the methods of extraction of butterfat from ice cream.

William Weiss of the Division of Food, Food and Drug Administration,

* For report of Subcommittee C and action of the Association, see *This Journal*, **38**, 73, 74 (1955).

who designed the experiments to evaluate the effects of variables and analyzed the data obtained from those experiments.

Dr. William Horwitz, Division of Food, Food and Drug Administration, Dr. A. H. Robertson, New York State Department of Agriculture and Markets, and Mr. Walter C. Bartsch, The Borden Company, all of whom made valuable suggestions.

Jesse E. Roe, Thos. C. Dunn, and William A. Bosin of the Food and Drug Administration, C. L. Losacco of the New York State Department of Agriculture and Markets, and Walter C. Bartsch and Marion M. Glasser of The Borden Company, all of whom carried on analytical work in 1953 with the old and the modern apparatus.

William Munday and Richard Heuerman of the Food and Drug Administration, who, with T. J. Klayder, made the analytical determinations to evaluate the effect of pressure.

REFERENCES

(1) HEHNER, O., and ANGELL, *Butter: Its Analysis and Adulteration*, London, 1874.
(2) LECHARTIER, *Ann. agron.*, 456 (1875).
(3) REICHERT, *Z. anal. Chem.*, **18**, 68 (1879).
(4) MEISSL, *Dinglers Polytech. J.*, **233**, 229 (1879).
(5) WOLLNY, R., *J. Soc. Chem. Ind.*, **6**, 831 (1887).
(6) ANON., *Analyst*, **25**, 309 (1900).
(7) LEFFMAN, H., and BEAM, W., *ibid.*, **16**, 153 (1891).
(8) POLENSKE, E., *Z. Nahr. Genussm.*, **7**, 274 (1904).
(9) REVIS, C., and BOLTON, E. R., *Analyst*, **36**, 333 (1911).
(10) JAMIESON, G. S., *This Journal*, **12**, 203 (1929).
(11) JAMIESON, G. S., *ibid.*, **13**, 255 (1930).
(12) ANON., *Analyst*, **61**, 404 (1936).
(13) MCKINNEY, R. S., *This Journal*, **22**, 618 (1939).
(14) RICHMOND, H. D., and HALL, G. F., *J. Soc. Chem. Ind.*, **39**, 80 (1920).
(15) KIRKHAM, V. H., *Analyst*, **45**, 293 (1920).
(16) JACOBS, M. B., *Chemistry and Technology of Food and Food Products*, Vol. I, D. Van Nostrand Co., Inc., New York, 1944, p. 583.
(17) WINTON, A. L., and WINTON, K. B., *Structure and Composition of Foods*, Vol. III, John Wiley & Sons, Inc., New York, 1937, p. 87.
(18) LEACH, A. E., and WINTON, A. L., *Food Inspection and Analysis*, Fourth Edition, John Wiley & Sons, Inc., New York, 1920, p. 571.
(19) WOODMAN, A. G., *Food Analysis*, Third Edition, McGraw-Hill Book Co., Inc., New York, 1931, p. 216.
(20) *Handbook of Chemistry and Physics*, Twenty-First Edition, Chemical Rubber Publishing Co., Cleveland, Ohio, 1936–1937, p. 796.
(21) CRANFIELD, H. T., and TAYLOR, M. G. D., *Analyst*, **40**, 433 (1915).
(22) HILL, O. J., and PALMER, L. S., *J. Dairy Sci.*, **21**, 529 (1938).
(23) BROWN, J. B., and SUTTON, T. S., *ibid.*, **14**, 125 (1931).
(24) SUTTON, T. S., BROWN, J. B., and JOHNSTON, E. W., *ibid.*, **15**, 209 (1932).
(25) HILDITCH, T. P., and SLIGHTHOLME, J. L., *Biochem. J.*, **24**, 1098 (1930).
(26) ARUP, P. S., *Analyst*, **57**, 610 (1932).
(27) OVERMAN, O. R., and GARRETT, O. F., *J. Agr. Research*, **45**, 51 (1932).
(28) SPITZER, G., and EPPLE, W. F., *Purdue Agr. Expt. Sta. Bull.* **254** (1921).

(29) STOUT, R. E., and WILSTER, G. H., *J. Dairy Sci.*, **22**, 424 (1939).
(30) HAWLEY, H., *Current Sci. (India)*, **12**, 153 (1943).
(31) BALAVOINE, P., *Mitt. Lebensm. Hyg.*, **32**, 203 (1941).
(32) LEWKOWITSCH, J., *Chemical Technology and Analysis of Oils, Fats, and Waxes*, Vol. II, Fourth Edition, Macmillan & Co., Ltd., London, 1909, p. 678.
(33) MARTIN, W. H., RUTZ, W. D., and WHITNAH, C. H., *Ice Cream Trade J.*, **45**, No. 11, 48; No. 12, 40 (1949).
(34) SAGER, O. S., and SANDERS, G. P., *Milk Industry Foundation, Convention Proceedings*, Laboratory Section, 1, September (1952).

REPORT ON FOREIGN FATS IN DAIRY PRODUCTS

STEROL ACETATE TEST

By J. H. CANNON (Food and Drug Administration, Department of Health, Education, and Welfare, St. Louis, Missouri), *Associate Referee*

Last year no collaborative samples were submitted for the reasons given in the Associate Referee's report (1). Certain further revisions of the test are explained under Discussion of Method in the present report. This year, six fat samples were sent to each of twelve collaborators. The composition of the samples was as follows:

No. 1.—Butterfat 90%—hydrogenated coconut fat[1] 10%.
No. 2.—Butterfat, 100%.
No. 3.—Butterfat 90%—hydrogenated cottonseed oil[2] 10%.
No. 4.—Identical with No. 1.
No. 5.—Identical with No. 3.
No. 6.—Identical with No. 2.

The collaborators also received a copy of the following method. In addition, each collaborator was furnished a micro filter and photo copies (Fig. 1) showing the crystalline forms of sterol acetates.

STEROL ACETATE TEST FOR VEGETABLE OILS IN BUTTER

REAGENTS

(a) *Digitonin soln.*—1.0% w/v in 95% EtOH.
(b) *Acetic anhydride.*—Reagent grade.
(c) *Ethyl ether.*—U.S.P. grade.

APPARATUS

(a) *Micro filter.*—Specially designed (Fig. 2).
(b) *Hypodermic syringe.*—2 ml or 5 ml, with 1 inch, 26 gage needle.

PROCEDURE

To 15 g filtered fat in a lipless 100 ml beaker, add 4 g KOH pellets dissolved in 4 ml H_2O. Then add 20 ml 95% alcohol and heat 30 min. on steam bath, using 100

[1] A stiff, white, semisolid fat, m.p. 110°F., obtained from the Blanton Co., St. Louis, Mo.
[2] A fairly soft, white, semisolid fat obtained from the Blanton Co., St. Louis, Mo., exact degree of hydrogenation not known.

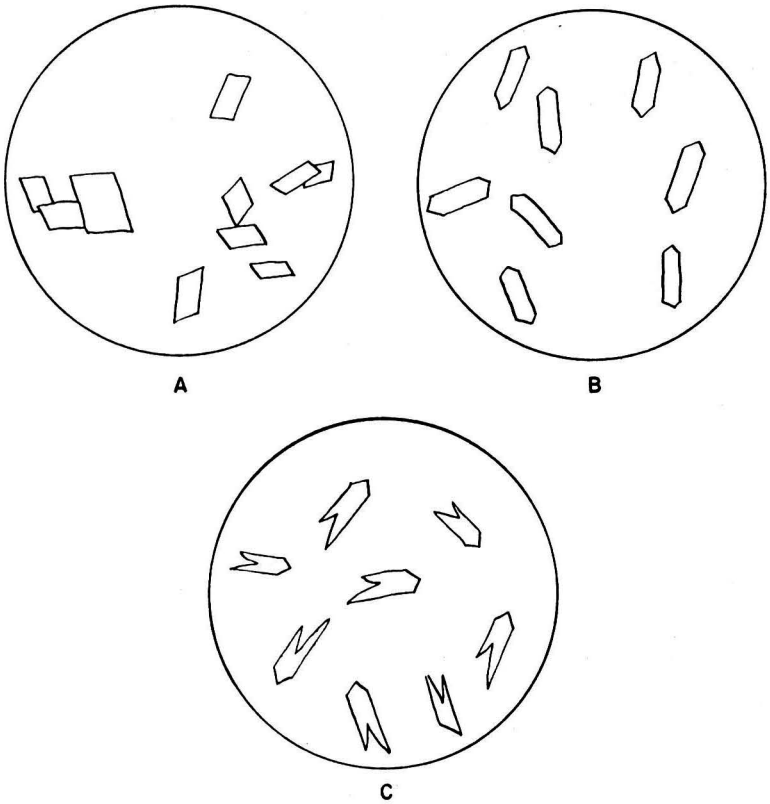


FIG. 1.—Crystalline forms of sterol acetates. (A) Pure cholesterol. (B) Pure phytosterol. (C) Mixed cholesterol-phytosterol.

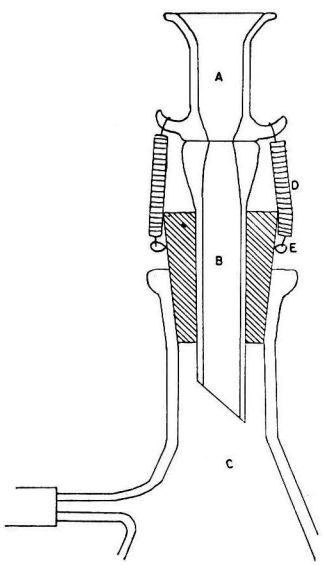


FIG. 2.—Glass micro filter for sterol acetate precipitates. (A), Top portion of filter, capacity 1 ml. (B), Lower portion of filter. Ground surfaces between (A) and (B) hold filter pad. "A" and "B" held together by springs, (D). (C), filter flask. (E), wire twisted around stopper to hold lower end of springs.

ml short-neck, round-bottom flask of cold water sitting in the mouth of the beaker as a condenser. Shake mixt. from time to time.

Add 60 ml H_2O, mix, and pour into 180 ml 95% alcohol in a 400 ml beaker. Warm to ca 40°C. and add 40 ml 1% digitonin in alcohol. Cool to about 6°–10°C. for 3 hours (or overnight) and filter on an 11 cm Büchner funnel. Wash with H_2O to remove soap, then with alcohol to remove H_2O. Finally, wash with several portions of ether.

Dry the paper and ppt for 10–15 min. at 100°C. The ppt can then be easily removed from the paper. Weigh the ppt and multiply the weight by 0.24 to obtain the equivalent weight of sterol.

Transfer the ppt to a test tube and add 2 ml acetic anhydride. Boil gently until the ppt is completely dissolved. Allow to cool somewhat, add 4 ml of 95% alcohol, and mix.

Filter the hot soln into a 20 ml beaker. Place beaker on a small hot plate and bring soln just to the boiling point. Add H_2O drop by drop, until the sterol acetate is just about to ppt, but still remains in soln at the boiling point.

Allow to cool, and filter the resulting ppt on a micro filter. Rinse beaker and filter with three 1 ml portions of 80% alcohol. (This washing is easily managed if a 2 ml hypodermic syringe fitted with a one inch 26 gage needle is used as a wash

TABLE 1.—*Sterol recovery as mg sterol per 15 g sample*

COLLABORATOR	SAMPLE NO.	FOUND	SAMPLE NO.	FOUND	SAMPLE NO.	FOUND
1	2	37.8	1	34.9	3	37.2
	6	38.1	4	34.4	5	39.7
2	2	35.2	1	34.2	3	32.8
	6	26.8	4	34.2	5	39.4
3	2	22.2	1	36.8	3	35.1
	6	28.0	4	21.6	5	35.2
5	2	36.2	1	31.1	3	37.7
	6	42.1	4	44.1	5	41.0
6	2	36.2	1	35.7	3	38.9
	6	37.8	4	35.6	5	38.9
7	2	34.4	1	31.2	3	34.8
	6	32.1	4	31.2	5	33.8
8	2	37.1	1	40.0	3	37.7
	6	38.0	4	34.9	5	39.8
9	2	35.4	1	37.1	3	37.5
	6	39.7	4	34.2	5	37.5
Average		34.8		34.5		37.3
Std. Deviation		4.5		4.5		2.29

bottle.) Transfer the ppt to a 5 ml beaker and add 1 ml 95% alcohol. Heat to dissolve ppt. Cool to recrystallize the acetate and again filter. Repeat the last recrystallization twice. Finally, determine the melting point. If m. p. is 117°C. or higher, vegetable fat is indicated.

Dissolve the remaining sterol acetate in 2 ml alcohol in a 20 ml beaker and add 3 drops of 40% aqueous KOH soln. Heat on steam bath for 5 min. Add 10 ml H_2O and transfer the liquid to a 125 ml separatory funnel. Add 50 ml ether and shake. Allow layers to separate and draw off the aqueous layer. Wash ether with three 5 ml portions of water and evaporate ether to dryness in a 50 ml beaker.

Add 10 ml of 70% alcohol to the residue and heat to dissolve. Cool, place a drop of the clear alcohol on a slide, and place on microscope stage. Examine the drop at 100× or 200× for typical crystals of phytosterol. (See diagrams, Fig. 1.)

COLLABORATIVE RESULTS

The collaborative results are presented in three tables, each representing one phase of the method.

TABLE 2.—*Melting points of sterol acetates* (°C.)

COLLABORATOR	SAMPLE NO.	M.P.	SAMPLE NO.	M.P.	SAMPLE NO.	M.P.
1	2	115	1	117	3	121
	6	115	4	117	5	120
2	2	115.5	1	118	3	119
	6	116	4	118	5	120
3	2	112	1	115	3	120
	6	114	4	113	5	120
4	2	115.1	1	117.1	3	120.1
	6	115.5	4	116.9	5	120.2
5	2	116	1	118	3	120
	6	116	4	110	5	117
6	2	115	1	117	3	121
	6	115.5	4	117	5	122
7	2	111	1	—	3	122
	6	115	4	117.8	5	122
8	2	111.3	1	110.2	3	118.5
	6	111	4	111.9	5	118.3
9	2	114	1	114	3	119
	6	114	4	114	5	119
Average		114.3°C.		115.4°C.		120.0°C.
Std. Deviation		1.8°C.		3.1°C.		1.2°C.

Table 1 shows somewhat higher and less variable sterol values for the cottonseed oil mixture (Nos. 3 and 5) than for butterfat or for the coconut oil mixture. Values for the coconut oil mixture (Nos. 1 and 4) are about the same as for butter.

Table 2 shows that 10 per cent of cottonseed oil can be detected with a high degree of certainty by the method. The results on the coconut oil mixture are slightly higher than for butter, but are much more variable. The collaborators' comments help explain this variability.

TABLE 3.—*Microcrystalline forms of the sterol ppts (see Fig. 1)*

COLLABORATOR	SAMPLE NO.	FORM	SAMPLE NO.	FORM	SAMPLE NO.	FORM
1	2	A	1	C	3	C
	6	A	4	C	5	C
2	2	A	1	C	3	C
	6	A	4	C	5	C
3	2	A	1	C	3	C
	6	C	4	C	5	C
4	2	A	1	C	3	C
	6	A	4	C	5	C
5	2	A	1	A	3	C
	6	A	4	A	5	—
6	2	A	1	C	3	C
	6	A	4	C	5	C
7	2	A	1	C	3	C
	6	A	4	C	5	C
8	2	A	1	A	3	C
	6	A	4	A	5	C
9	2	—	1	—	3	C
	6	—	4	—	5	C

Table 3 shows that microscopic examination of the crystalline form of the purified sterols is a valuable test for use in conjunction with the melting point test. The presence of 10 per cent of cottonseed oil was indicated with a high degree of certainty by this test. The presence of 10 per cent of coconut oil was indicated with a somewhat lesser degree of certainty. Collaborator's comments help explain the uncertainty associated with the coconut oil mixture.

The results of another collaborator were received too late for inclusion in the above tabulations. This collaborator experienced difficulty in the

determination of melting points and in the crystal identification. Time for repeat runs was not available. This analyst will be asked to collaborate next year in the hope that the experience gained in the present study will help him obtain more consistent results.

COMMENTS OF COLLABORATORS

Collaborator No. 1.—"The weight of sterols was affected by the type of digitonin used."

Collaborator No. 2.—"Digitonin must be heated in alcohol to effect solution. Three crystallizations may not be enough. It might be suggested that if a sharp melting point is not obtained, again recrystallize the acetate and take another melting point."

Collaborator No. 3.—"A fast filter paper was used for the filtration of the digitonin steroid precipitates as it took considerable time to filter with a fine porosity paper. In the filtrations of the sterol acetates, a fine porosity filter paper (Blue Ribbon S & S No. 589) was employed, yet so much of the acetates went through the paper in your micro filter that I was able to crystallize them only twice before taking melting points. Thus, inadequate purification may have affected the reported melting points. M.P.'s were determined by an electric block type apparatus, which does not have an accuracy greater than 1°C. Apparatus was calibrated with acetanilid.

"I allowed crystallization of the acetates to proceed for ½ to 1 hour prior to filtration. Possibly, 4 or more hours should be allowed for each crystallization so that formation of larger crystals would cut down on filtration loss.

"Since so little precipitate was left, I encountered difficulty in determining types of sterols present by examining crystals the same day crystals were formed. By leaving crystals in 70% alcohol overnight, I obtained very satisfactory, definite crystal formations. Hence, I feel it would be advisable to include an overnight crystallization period for the crystal identification test."

Collaborator No. 4.—"No particular difficulty was encountered in applying the method except that in the final recrystallizations it was necessary to use less than 1 ml of alcohol to obtain sufficient material for the subsequent steps."

Collaborator No. 5.—"Melting points were determined on a Fisher melting point apparatus.

"In the microscopic test, Sample No. 5 exhibited a large number of very small, rod-like crystals, the long axis about 5 times or more the short axis. In general, I have little confidence in results by this test. There is a large amount of suspended matter in and on the drop of solution on the slide and this makes observation very difficult. The typical crystals apparently are few in number so that even with repeated observations the writer was uncertain as to which of the illustrated types they might belong. With the exception of Samples 1 and 3, which are believed to have contained crystals definitely identified as illustrated for A, and C, respectively, the reported identifications are essentially guess work. Also, for the crystals of Sample 3, one end did not terminate in a uniform taper as illustrated, but had some notching at both ends.

"The second paragraph directs filtering on a Büchner funnel but does not designate paper porosity. Perhaps this is not important but it would be well to know the maximum porosity that may be used.

"My only adverse comments concern the last paragraph as discussed above, and the many manipulations which make the method rather time-consuming."

Collaborator No. 6.—"Since we had no lipless 100 ml beakers or 100 ml round-botton flasks at hand, the fats were saponified in 125 ml standard taper Erlenmeyer flasks equipped with air condensers.

"Some trouble was experienced in getting suitable crystals for microscopic examination with Samples 2 and 6. Possibly the alcohol evaporated too rapidly to allow good crystals to develop. Typical cholesterol crystals were more readily obtained when the crystallization was allowed to take place on a slide under a cover glass.

"With Sample 4, crystals resembling cholesterol, as well as those indicating a mixture, were obtained. No crystals strictly typical of phytosterol were observed, although Samples 3 and 5 exhibited large numbers which appeared intermediate between B and C (on drawing), that is, more elongated and having less pronounced notches at one end."

Collaborator No. 7.—"Many crystals from samples 3 and 5 were indented at both ends but there were some exactly like your drawing for type C."

Collaborator No. 8.—"It appeared advisable to weigh the digitonin-sterol precipitate directly on the 11 cm filter paper which had been previously heated and tared. Attempts to remove the precipitate before weighing it create a loss of 35–50 mg.

"It was noticed that the recrystallized sterol acetate from Samples 3 and 5 was in the form of a silvery, lustrous, fibrous mat. This may be another indication of the presence of phytosterol.

"The amount of sterol acetate recovered from 2 samples after re-crystallization was much smaller than from the others. In one case crystal formation was slow and the crystals were few and small in size. However, they were identifiable. No crystals formed in the other case (Sample 1). To be more certain, I dried the solution completely in both cases, dissolved the residue in 5 ml of hot 70% alcohol (instead of 10 ml), and continued in the same manner. The crystals appeared more rapidly and were slightly larger, making for easier identification. Sample 1 again showed no identifiable crystals with this slight modification. The same procedure was used on the other samples, whose crystals were previously identified with no difficulty. Again, the crystals appeared to be a little easier to identify.

"Too much sterol acetate may be lost upon recrystallization. It is assumed that the purpose of this is to remove interferences and obtain more characteristic crystals of phytosterol, if present. In the writer's experience failure of crystals to appear in the 2nd or 3rd recrystallization may occur in some cases, and evaporation of the 1 ml of solution to a point where crystals do appear may be necessary."

Collaborator No. 9.—"Melting points were determined with the Fisher melting point apparatus. Only Samples 3 and 5 gave distinct tests for phytosterol. Crystalline structure was indistinct in the other samples and no definite conclusions could be drawn as to the presence or absence of phytosterol."

DISCUSSION OF METHOD

Last year the Associate Referee's report mentioned a carbon column purification procedure which, at that time, appeared promising. However, continued studies have shown that the carbon column procedure could not be relied on in all cases and it has been concluded that the digitonin method of purifying sterols is generally more dependable. Therefore, the method submitted this year omitted the carbon purification.

After sending out this year's samples another interesting observation was made. It was observed that addition of pure cholesterol to coconut oil samples just before the digitonin precipitation resulted in higher-melting acetates than those obtained without such addition. Results are shown in Table 4. The cholesterol was added because unexpectedly small

TABLE 4.—*Effect of adding cholesterol[a] to coconut oil*

DETERMINATION	SAMPLE DESCRIPTION	CHOLESTEROL ADDED	M.P.
			(°C.)
1	Coconut oil, crude (Inv. 32-937 K, Sub A)	None	112
2	Same as above	30 mg	127
3	Coconut oil, crude (Inv. 87-290 K)	None	112
4	Same as above	30 mg	125

[a] M.p. of the acetate prepared from this lot of cholesterol was 115°C.

residues were obtained when the method was applied to certain coconut oils as shown in Table 5. It was felt that the low melting points were somehow related to the low residue weights. In short, the Associate Referee suspected that some phytosterol from the coconut oil was being lost because of the solubility of its digitonide in the soap solution. He felt that the addition of cholesterol to increase the bulk of precipitate might bring down more of the phytosterol digitonide, either by salting out, by co-precipitation, or by adsorption. The striking effects of the addition of cholesterol to coconut oil encouraged the Associate Referee to run similar determinations on 10% coconut oil–90% butterfat mixtures. Results are given in Table 6.

The results in Table 6 would seem to show little advantage in adding cholesterol to mixtures containing a large proportion of butterfat, which already contains considerable cholesterol. However, it was found that the addition of cholesterol produced a dry, flaky digitonide precipitate which was distinctly easier to remove from the filter paper.

TABLE 5.—*Examination of pure coconut oils by the collaborative method*

DETERMINATION NO.	SAMPLE DESCRIPTION	WT. OF DRIED DIGITONIN PPT	STEROL ACETATE M.P.
		(*mg*)	(°C.)
1	Refined	21	127
2	Refined	24	128
3	Unrefined	31	115
4	Unrefined	21	115
5	Refined	30	—
6	Refined	23	119
7	Unrefined	12	112
8	Unrefined	18	112
9	Unrefined	6	112
10	Refined	21	120

TABLE 6.—*Effect of adding cholesterol to a 10% coconut oil-90% butterfat mixture*

DETERMINATION	CHOLESTEROL ADDED	M.P.
	(*mg*)	(°C.)
1	None	115.5
2	None	117
3	None	117
4	30	117
5	30	117.5
6	30	117

The filtration of the minute sterol acetate precipitates which result from the last recrystallizations presents a problem in technique. The Associate Referee has tried a number of microfilters and found the type shown in Figure 2 to be the best yet devised. So far as the Associate Referee knows it is not commercially available. Those sent with the collaborative samples this year were made by the Associate Referee and some of them may not have been perfectly fabricated. If made as shown in the diagram, they should be satisfactory. The capacity of "A," Figure 2, should be close to 1 ml. Filter pad No. 740-E, Schleicher & Schuell, has been found suitable for the filter. These pads are supplied for the assay of penicillin and other antibacterial substances, and come in boxes of 1000 disks. The disks are 12.7 mm in diameter and the funnel should have ground surfaces (between "A" and "B") of about this diameter. The diameter of the openings at the constricted ends of "A" and "B" should be about 3.5 to 4 mm.

According to Lewkowitsch (2), rapid crystallization of a mixture of cholesterol and phytosterol yields crystals which simulate phytosterol. He reproduces drawings (made by Zetsche in 1898) of crystals resulting from evaporation of a solution containing 7 per cent phytosterol and 93 per cent cholesterol. These crystals have a distinctive notch on one end and the typical phytosterol point on the other end as shown at C, in Figure 1. Good crystals may sometimes be difficult to obtain but, if found, seem to be a reliable indication of adulteration of butter with vegetable fat.

RECOMMENDATIONS

The Associate Referee believes that the method can be depended upon to detect as little as 10 per cent of added vegetable fat if employed with sufficient "know-how." It involves micromanipulations which, like other micro techniques, require considerable practice. Possibly, more detailed instruction at critical points in the method is desirable. Accordingly, it is recommended*—

* For report of Subcommittee C and action of the Association, see *This Journal*, 38, 73, 74 (1955).

(1) That the method be modified to include the addition of 30 mg of pure cholesterol to the saponified solution just before the addition of the digitonin solution.

(2) That more detailed instruction regarding micro technique be included in the method.

(3) That the modified method be submitted to further collaborative study.

ACKNOWLEDGMENT

The thanks of the Associate Referee are extended to C. I. Kruisheer, Director of the Government Dairy Station of Leyden, Holland, for valuable suggestions, and for making available to the Associate Referee the work of P. C. den Herder (3) on the detection of vegetable fats in butter. Thanks are also extended to the collaborators for their cooperation in this study.

REFERENCES

(1) CANNON, JAMES H., *This Journal*, **37**, 239 (1954).

(2) LEWKOWITSCH, J., *Chemical Technology of Oils, Fats, and Waxes*, p. 372, MacMillan & Co., New York (1904).

(3) P. C. den HERDER, *Systeme De Recherches Pour Deceler La Falsification Du Buerre Pas La Margarine*, p. 9; Imprimerie S.A.D. 123, Boulevard Barthelemy, 23 Bruxelles.

No reports were given on cryoscopy of milk, fat in dairy products, fat in homogenized milk, frozen desserts, preparation of butter samples, pressurized cream, sampling and preparation of sample of soft cheeses, and sampling, fat, and moisture in hard cheeses.

REPORT OF THE REFEREE ON COSMETICS

By G. ROBERT CLARK (Division of Cosmetics, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.), *Referee*

The Referee concurs with the recommendations* of the Associate Referee on Deodorants and Anti-Perspirants that the proposed methods for boric acid and phenolsulfonic acid be adopted as first action.

The Referee recommends that the proposed methods for analysis of mascaras, eyebrow pencils, and eye shadow be submitted to collaborative study.

The Referee recommends that the following methods, now first action, be adopted as official. References are to the Seventh Edition of *Official*

* For report of Subcommittee B and action of the Association, see *This Journal*, **38**, 70, 71 (1955).

Methods of Analysis of the Association of Official Agricultural Chemists.

Deodorants and Anti-Perspirants.

Aluminum and zinc, **33.1–33.6.**

Face Powders.

Fats and fatty acids as stearic acid, **33.9**
Boric acid, **33.10**
Total zinc, **33.11, 33.12**
Acid-soluble calcium, **33.13**
Acid-soluble magnesium, **33.14**
Barium sulfate, **33.15, 33.16**
Total titanium and iron, **33.17, 33.18**
Total iron, **33.19–33.20**
Total titanium, **33.21**
Total oxides of iron, titanium, and aluminum, **33.22**
Total aluminum, **33.23**
Acid-insoluble calcium, **33.24**
Acid-insoluble magnesium, **33.25**
Silica, **33.26**
Starch, **33.27**

Vanishing Cream.

Test for type of emulsion, **33.43**
Water, **33.44**
Ash, **33.45**
Chloroform-soluble material, **33.46**
Glycerol, **33.47–33.49**

The Referee recommends that the following topics be continued:

Cold permanent wave preparations
Hair dyes and rinses
Deodorants and anti-perspirants
Mascaras, eyebrow pencils, and eye shadows

REPORT ON MASCARAS, EYEBROW PENCILS, AND EYE SHADOWS

By PAUL W. JEWEL (Max Factor & Co., Hollywood 28, Calif.),
Associate Referee

A thorough examination has been made of the methods suggested during the course of this project, and all conceivable means of improving them have been investigated. This year's work has shown that no further progress is likely to be made in developing a more comprehensive method for the analysis of mascara.

The introduction of the non-ionic emulsifiers has not affected the usefulness of this method since these materials can be extracted with chloroform in the same manner as the waxes. The increase in popularity of the cream type of mascaras poses no new problem, since they become typical cake type products when the water is evaporated. The still newer solvent type fits into the analytical scheme well enough so that the experienced analyst will have no difficulty.

The method submitted here contains all the best features of those reported earlier.

ANALYSIS OF MASCARAS

PRELIMINARY TEST

Place in a beaker a small piece of mascara or portion of cream mascara which has been evapd to dryness, add 10 ml $CHCl_3$, and boil for few min. If the mass appears to dissolve completely except for the pigments, proceed with Method II; if not, proceed with Method I.

METHOD I

(a) *Fatty acids, oils, and waxes.*—Place weighed sample, 3–4 g, in a 250 ml beaker. Add 100 ml 10% HCl and heat to 80–90°C. As soon as mascara melts, stir rapidly with a glass mechanical agitator, continuing heating for 30 min. Place the beaker, with the agitator, in refrigerator until temp. reaches 3–5°C. Break up lumps with agitator and filter thru CS&S No. 589 paper. Wash residue once with distd H_2O, transfer it from the paper to the original beaker with stream of H_2O from a wash bottle, and again ext. with 10% HCl, heating, stirring, and chilling as before. Following final extn with distd H_2O, place filter paper with residue in the beaker, and dry at 100°C. for 2 hrs. After drying, ext. residue with three 50 ml portions of boiling $CHCl_3$, filter thru CS&S No. 589 paper, and collect the exts in a tared dish. Place the filter paper in the beaker and again ext. with 50 ml boiling $CHCl_3$, filter thru fresh paper, and evap. the combined filtrates to dryness on the steam bath. Add 5 ml abs. alcohol, again evap. to dryness, dry in oven at 100°C. for 2 hrs, cool, and weigh. This weight represents total fatty acids, oils, and waxes in sample.

(b) *Acid number of oils, fatty acids, and waxes.*—Weigh ca 2 g sample of ext. from (a), dissolve in boiling abs. alcohol, and titrate with 0.50 *N* alc. KOH, using phenolphthalein as indicator. Calc. acid number in usual manner.

(c) *Arsenic.*—Digest 1 g sample of mascara according to the directions given in *Official Methods of Analysis*, 7th Ed., 1950, page 370, under *Preparation of Sample*. Det. according to the directions given on page 371 with the modification described by Cahill and Walters.[1]

(This procedure employs No. 24 threads impregnated with mercuric bromide held in 2 mm capillary tubes, instead of the paper strips specified by the official method. Accurate and reproducible results are given by the modified method if the amount of arsenic present is between 1 and 3 mmg. This modification makes it possible to determine arsenic in very small samples of mascara, and difficulties of digestion, with consequent loss of arsenic, are obviated. It is imperative that a suitable blank be run with each digestion. Exactly the same amounts of reagents must be used and they must be boiled down in a Kjeldahl flask in exactly the same manner as in the digestion. The reagents used in any set of determinations should come from the same bottle, since the amount of arsenic in C.P. reagent chemicals varies considerably from lot to lot.)

[1] Sister Emily Cahill and Sister Louisella Walters, *Ind. Eng. Chem., Anal. Ed.*, **14**, 90 (1942).

METHOD II

Total base.—Weigh ca 3 g sample of mascara, wrap in filter paper (Munktell 00), turning ends in so as to make seal, and tie with thread. Placed wrapped sample in double thickness extn thimble, plug opening with cotton, and ext. with $CHCl_3$ in Soxhlet extractor at such rate that siphon dumps about once every 10 min. Continue extn for 8 hrs; then evap. solvent on steam bath, dry in oven for 30 min., cool, and weigh. Repeat oven drying until all $CHCl_3$ odor is gone and wt remains constant. Report result as % total base. (When most of $CHCl_3$ has been removed by evapn, addn of few ml of abs. alcohol will facilitate removal of last traces.)

Triethanolamine.—To total base contained in extn flask, add 10 ml 95% alcohol, and heat until base is dissolved or dispersed. Add 10.00 ml 0.50 *N* H_2SO_4, heat to boiling, add 50 ml distd H_2O, heat until fats melt, and chill thoroly in refrigerator. When mixt. is thoroly chilled, filter thru paper into titration flask. Wash waxes several times with H_2O and titrate filtrate with 0.50 *N* aq. NaOH, using Me red as indicator. Net titration in ml *N* acid divided by 7.0 = g triethanolamine. Express results in terms of % triethanolamine in original sample.

Total acid as stearic acid.—Return filter paper from triethanolamine detn to extn flask, add 50 ml abs. alcohol, heat to dissolve, and titrate with 0.50 *N* alc. KOH, using phenolphthalein as indicator. Calc. total acid found as stearic acid, using 208 as acid number for stearic acid, and report result as % stearic acid.

ANALYSIS OF EYEBROW PENCILS AND EYE SHADOW

Eyebrow Pencils.—Eyebrow pencils are usually made by manufacturers of ordinary pencils and are similar in structure except for the pigment. There is very little base present and it is of little importance. Arsenic may be determined by the same method described in the mascara analysis (Method I). Coal-tar dyes will appear in the chloroform extract together with such small amounts of excipient and lubricant as may be present. No further analysis of these pencils need be made.

Eye Shadow.—Eye shadow is a simple milled mixture of pigments with a soft oily base. It presents little difficulty in analysis. A weighed sample of the material is dissolved in petroleum ether and filtered. The pigments may then be investigated for traces of toxic elements or for coal-tar colors. The base is mainly mineral oil together with small amounts of waxes to give body.

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

* For report of Subcommittee B and action of the Association, see *This Journal*, **38**, 70, 71 (1955).

REPORT ON DEODORANTS AND ANTI-PERSPIRANTS

DETERMINATION OF BORIC ACID AND PHENOLSULFONIC ACID

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

BORIC ACID

A method for the determination of boric acid in deodorants and anti-perspirants was presented in a previous report (1). During the past year the proposed method has been submitted to collaborative study.

Portions of the following solution, containing a number of ingredients likely to be found in deodorants and anti-perspirants, were sent to the collaborators:

Boric acid	15.11 g
Aluminum sulfate	40.0 g
Zinc chloride	40.0 g
Magnesium chloride	40.0 g
Urea	40.0 g
Glycerol	40.0 g
Water q.s.	1 l

The collaborators were directed to use 10 ml aliquots of the solution, and to make duplicate determinations. Their results are given in Table 1.

TABLE 1.—*Analyses for boric acid by collaborators*

COLLABORATOR	WEIGHT OF BORIC ACID IN SAMPLE[a]		RECOVERY[b]
	ADDED	REPORTED	
	mg	*mg*	*per cent*
1	151.1	152.3	100.8
		150.4	99.5
2	151.1	151.2	100.1
		150.3	99.5
3	151.1	153.0	101.3
		151.9	100.5
4	151.1	147.4	97.6
		141.9	93.9
			Av. 99.1

[a] A 10 ml aliquot of the solution supplied.
[b] Calculated by the Associate Referee.

Collaborators who submitted results were:

H. E. Gakenheimer, Food and Drug Administration, Baltimore, Md.
A. C. Thomson, Food and Drug Administration, Cincinnati, Ohio
G. G. McKeown, Department of National Health and Welfare, Ottawa, Canada
M. Dolinsky, Food and Drug Administration, Washington, D. C.

The composition of the solution was not known by the collaborators. Their results range from 93.9 per cent to 101.3 per cent of the amount added. The average recovery of boric acid was 99.1 per cent.

G. G. McKeown reported some difficulty in the extraction of the boric acid from the ash, as the ash tended to fuse to the crucible and resisted solution even with strong acid. Consistent results were obtained when the ash was scraped free and pulverized during the washing process.

PHENOLSULFONIC ACID

An alternate method for the determination of phenolsulfonic acid in deodorants and anti-perspirants was proposed at the 1953 A.O.A.C. meeting (2). During the past year the method has been submitted to collaborative study.

TABLE 2.—*Analyses for phenol sulfonic acid by collaborators*

COLLABORATOR	PHENOLSULFONIC ACID PRESENT IN SAMPLE[a]		RECOVERY[b]
	ADDED	REPORTED	
	per cent (w/v)	*per cent (w/v)*	*per cent*
1	3.14	3.10	98.7
		3.12	99.4
2	3.14	3.18	101.3
		3.18	101.3
3	3.14	3.13	99.7
		3.15	100.3
4	3.14	3.11	99.0
		3.14	100.0
			Av. 99.9

[a] A 10 ml aliquot of the solution supplied.
[b] Calculated by Associate Referee.

Portions of a solution containing the following ingredients were sent to the collaborators:

Zinc phenolsulfonate	25.00 g
Glycerol	5.0 g
Urea	5.0 g

Calcium nitrate	5.0	g
Aluminum chloride	5.0	g
Zinc chloride	5.0	g
Magnesium chloride	5.0	g
Sodium borate	5.0	g
Hydrochloric acid (concd)	25	ml
Water	q.s. 500	ml

The collaborators were directed to make duplicate determinations on 10 ml aliquots of the solution. Their results are shown in Table 2.

Collaborators who submitted results were:

G. G. McKeown, Department of National Health and Welfare, Ottawa, Canada
A. Kleinman, Food and Drug Administration, Chicago, Ill.
S. H. Newburger, Food and Drug Administration, Washington, D. C.
M. Dolinsky, Food and Drug Administration, Washington, D. C.

Again, the composition of the solution was not known by the collaborators, and in this case, results reported vary from 98.7 per cent to 101.3 per cent of the amount added. The average recovery was 99.9 per cent.

RECOMMENDATIONS

It is recommended*—

(1) That the proposed method for boric acid be adopted as first action.

(2) That the proposed method for phenolsulfonic acid be adopted as first action.

(3) That the study of deodorants and anti-perspirants be continued.

REFERENCES

(1) CLEMENTS, J. E., *This Journal*, **36**, 791 (1953).
(2) ———, *ibid.*, **37**, 798 (1954).

No reports were received on cold permanent waves, cosmetic creams, or hair dyes and rinses.

REPORT OF THE REFEREE ON COAL-TAR COLORS

By KENNETH A. FREEMAN (Division of Cosmetics, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.), *Referee*

The Referee concurs in the recommendation† of the Associate Referee that the spectrophotometric procedure for the determination of 4-toluene-azo-2-naphthol in D&C Red No. 35 be adopted as first action, that the

* For report of Subcommittee B and action of the Association, see *This Journal*, **38**, 70, 71 (1955).
† For report of Subcommittee B and action of the Association, see *This Journal*, **38**, 71, 72 (1955).

chemical procedure for the determination of 4-toluene-azo-2-naphthol in D&C Red No. 35 be submitted to further study, and that the topic, Subsidiary Dyes in D&C Colors, be continued.

The Referee concurs in the recommendation of the Associate Referee that the method for the determination of higher sulfonated dye in FD&C Yellow No. 6, with specific directions for the preparation of standards, be changed from first action to official. He also concurs in the recommendation that the topic Subsidiary Dyes in FD&C Colors be continued.

The Referee recommends that the following topics be continued:

Paper chromatography of coal-tar colors
Heavy metals in coal-tar colors
Sulfonated amine intermediates in coal-tar colors
Volatile amine intermediates in coal-tar colors
Non-volatile unsulfonated amine intermediates in coal-tar colors
Inorganic salts in coal-tar colors
Sulfonated phenolic intermediates in coal-tar colors
Unsulfonated phenolic intermediates in coal-tar colors
Intermediates in triphenylmethane colors
Arsenic and antimony in coal-tar colors
Lakes and pigments

Method **34.2** for the separation of coloring matters in foods, drugs, and cosmetics involves separation by wool dyeing followed by stripping and re-dyeing on fresh wool. The stripping and re-dyeing may, in some instances, be repeated several times. The dyeing is accomplished in acid solution and the stripping in alkaline solution in the case of acid colors, and in the case of basic colors, dyeing is done in alkaline solution and the stripping in acid solution. All dyeings and strippings are done from boiling solutions.

Method **34.11** for the identification of coal-tar colors directs the analyst to test the behavior of the dyed wool, obtained in the procedure outlined above, with various chemical reagents. The resulting color of each piece of cloth is then noted and compared with the data contained in Table 1, Chapter 34. The unknown is tentatively identified as identical to that color listed in the table which has the same color reactions as the unknown. It is recommended that in the procedure the color reactions of the unknown dye be compared with dyeings of known colors of about the same dye concentration.

Experience has shown that separations by wool dyeing and identifications by color changes produced on dyed fibers with acids and alkalies are unreliable. Many coal-tar colors decompose rapidly under the conditions employed in the dyeing and stripping process and may be lost completely. The decomposition products may change the hue of the spot tests in such a manner that erroneous identifications are made.

If two or more colors of the same type are present, complete separation

can seldom be accomplished by the wool dyeing procedure. If separation is not complete, misleading results will be obtained from the spot tests. The conditions of the dyeing and stripping may cause decomposition of the cloth and yield products which sometimes alter the appearance of the dye in the spot tests. The appearance of the dye in the spot tests may also be altered by the presence of various components of the original product which have not been completely separated from the dye. A further source of error is the variation of shade produced in the spot tests by differences in concentration of color on the cloth.

It is therefore recommended that the following changes in Chapter 34 be made:

(1) Delete the method for separation by wool dyeing, **34.2.**

(2) Change method **34.5**(**a**) to read "*Water-soluble colors.*—Obtain aq. soln as free as practicable from suspended matter, alcohol, acids, alkalies, and salts. Liquids require no prepn except removal of any alcohol that may be present."

(3) In method **34.8**(**b**) delete the following: "Proceed as under **34.2,** omitting fixation of color on wool and. . . . "

(4) Delete methods **34.10,** I. *General;* **34.11,** II. *By Color Changes Produced with Acids and Alkalies;* and **34.12,** III. *By Special Tests.*

(5) Delete TABLE 1.—*Color reactions produced on dyed fibers by various reagents.*

Many years of experience have shown that the most reliable method for identifying a given color is to compare its spectrophotometric characteristics with those of a known color. It is therefore recommended that the following be adopted as first action:

I. Oil-Soluble Dyes

Prep. a soln of the isolated dye of suitable concn in $CHCl_3$. Prep. the spectrophotometric curve of this soln. Compare this curve with those of known dyes in $CHCl_3$ solns detd on same instrument under same conditions.

II. Water-Soluble Dyes

Prep. practically neutral aq. soln of the dye having a concn suitable for spectrophotometric analysis with cells and instrument available. Divide into 3 portions and to one portion add few crystals of NH_4 acetate. To second portion add HCl to make ca 0.1 *N.* To third portion add NaOH to make ca 0.1 *N.* Prep. the spectrophotometric curves of the three solns. Compare the 3 curves of the unknown dye with curves of known dyes detd under same conditions and with same spectrophotometer.

If the spectrophotometric data of the unknown color cannot be correlated with the data of a known color, the unknown color may be a mixt. The unknown color may then be subjected to chromatography. For oil-soluble colors the procedure of Weiss (1) is recommended. For water-soluble colors either Tilden's paper chromatographic procedure (2, 3) or the following column chromatographic procedure may be used.

COLUMN CHROMATOGRAPHY

(**a**) *Preparation of the column.*—Insert glass wool plug into chromatograph tube and tamp it lightly into constricted end. Prep. thin aq. slurry of powd. cellulose, such as Solka-Floc BW 40, and pour into column. Allow liquid to drain off as cellulose settles and add more slurry until column is of sufficient height. When liquid

level has dropped almost to top of adsorbent bed, add 20% NaCl wash soln. Just before last portion of soln enters adsorbent bed, close constricted end of column. The column may be used immediately or may be stored for several weeks before use.

(b) *Procedure.*—To the neutral aq. soln of the color, add solid NaCl in amount sufficient to make 20% NaCl soln. Pour this soln into chromatograph column so that adsorbent bed is not disturbed; then open constricted end of tube. When last portion of this solution is ready to pass into adsorbent bed, add few ml of 20% NaCl soln. If any color moves down column at moderate rate, continue washing with 20% NaCl soln. If all color remains at or near the top of column, change to 10% NaCl soln. If this solution fails to move any color down column, change to 5% NaCl soln. Continue cutting concn in half until color moves down column at moderate rate. Continue addn of appropriate concn of NaCl soln until color is eluted and collected. If color seps into two or more bands on column, collect each band separately. (In some cases it may be necessary to change to still more dil. NaCl soln to elute upper bands of color.) If two or more bands of color are found, examine each spectrophotometrically as described previously. If this procedure gives no indication that there is more than one color present, assume that color is not a mixt.

It is further recommended that the methods entitled "Extraction, Separation, and Identification of Coloring Matter in Cereal Foods," **13.114** and **13.115,** be revised in the following manner:

(1) In method **13.114,** delete the last portion of the first paragraph beginning with: "Confirm with wet and spot reactions . . . " and insert "Proceed with spectrophotometric identification as in Chapter 34."

(2) In method **13.114**(1), delete the portion of the method beginning: "Test ca 5 ml portion of the petr. ether ext. . . . " and insert "Proceed with spectrophotometric identification as in Chapter 34."

(3) In method **13.114**(3), delete the portion of the paragraph beginning: "after addn of NaCl and acetic acid is re-extd with amyl alcohol . . . " and insert "is examined spectrophotometrically as in Chapter 34."

(4) In method **13.115,** delete second and third paragraphs and insert: "Centrifuge and decant clear soln into clean vessel. Examine this soln spectrophotometrically as in Chapter 34."

The Referee on Cereal Foods concurs in these recommendations.

It is further recommended that the method for coloring matter in dairy products, **15.38,** be revised by deleting the term "under **34.11**" where it appears and substituting the term "in Chapter 34." The Referee on Dairy Products concurs in this recommendation.

It is further recommended that the official method for coal-tar colors in oils, fats, and waxes (*This Journal,* **34,** 76 (1951); **35,** 84 (1952)) be revised in the following manner:

(1) Delete in the second paragraph, line 8, beginning "Dissolve residue in 10 ml 70% alcohol," to end of paragraph and insert "Identify color spectrophotometrically as in Chapter 34."

(2) Delete sentence of line 17 of third paragraph: "Dye the color on silk as described above and identify by spot tests." and insert: "Identify color spectrophotometrically as in Chapter 34."

The Referee on Oils, Fats, and Waxes concurs in these recommendations.

REFERENCES

(1) WEISS, LOUIS C., *This Journal*, **34**, 453 (1951).
(2) TILDEN, DORIS H., *ibid.*, **35**, 423 (1952).
(3) ———, *ibid.*, **36**, 802 (1953).

REPORT ON SUBSIDIARY DYES IN D&C COLORS

4-TOLUENE-AZO-2-NAPHTHOL IN D&C RED NO. 35

By LOUIS KOCH (H. Kohnstamm & Co., Inc., Brooklyn, N. Y.),
Associate Referee

Collaborative studies on the estimation of 4-toluene-azo-2-naphthol in D&C Red No. 35, both chemically[1] and spectrophotometrically[2] were undertaken with the assistance of the following individuals whose cooperation is greatly appreciated:

A. B. Fuhr, National Aniline Division, Allied Chemical & Dye Corporation, Buffalo, N. Y.
T. G. Webber, E. I. du Pont de Nemours and Company, Wilmington, Del.
H. Holtzman, Ansbacher-Siegle Corporation, Staten Island, N. Y.
F. H. Hedger, Chas. Pfizer & Co., Inc., Brooklyn, N. Y.
I. Hanig, H. Kohnstamm & Co., Inc., Brooklyn, N. Y.

Samples of D&C Red No. 35, with and without subsidiary pigment adulterant, gave the results outlined in Tables 1–4.

TABLE 1.—*Total subsidiary dye (per cent) found by the spectrophotometric method*

ANALYST	SAMPLE			
	A	B	C	D
1	0.25	0.68	1.22	2.17
2	0.17	0.59	1.09	2.12
3	0.34[a]	0.68	1.16	2.06
4	0.26	0.76	1.12	1.70
5	0.23	0.76	1.20	2.54
Associate Referee	0.26	0.84	1.21	2.10

[a] Used a 0.24% value for calculating results in Table 2.

[1] KOCH, L., *This Journal*, **36**, 796 (1953).
[2] ———, *ibid.*, **37**, 803 (1954).

TABLE 2.—*Added subsidiary dye (per cent) found by the spectrophotometric method*

ANALYST	SAMPLE		
	B	C	D
1	0.43	0.97	1.92
2	0.42	0.92	1.95
3	0.44	0.92	1.82
4	0.50	0.86	1.44
5	0.53	0.97	2.31
Associate Referee	0.58	0.95	1.84
Subsidiary Dye Added	0.50	1.00	2.00

TABLE 3.—*Total subsidiary dye (per cent) found by the chemical method*

ANALYST	SAMPLE			
	A	B	C	D
1	0.63	1.09	1.31	1.47
3	0.24	0.64	1.10	2.11
4	0.16	0.60	1.03	1.85
5	1.55	0.73	1.68	2.01
Associate Referee	0.22	0.76	1.37	2.22

TABLE 4.—*Added subsidiary dye (per cent) found by the chemical method*

ANALYST	SAMPLE		
	B	C	D
1[a]	——	——	——
3	0.40	0.86	1.87
4	0.44	0.87	1.69
5[a]	——	——	——
Associate Referee	0.54	1.15	2.00
Subsidiary Dye Added	0.50	1.00	2.00

[a] Sample "A" value too high to permit calculation.

COMMENTS OF COLLABORATORS

A. B. Fuhr: In the chemical procedure, close attention was needed during distillation to avoid foaming and bumping. The spectrophotometric procedure is preferable for convenience and speed. However, agreement between blank runs was not good. Better reproducibility of blank might be obtained by precise definition of time, temperature, and volume in the crystallization.

H. Holtzman: The spectrophotometric method is rather lengthy and time consuming, but works smoothly and with good reproducibility.

F. H. Hedger: The procedures appeared to work satisfactorily in the hands of two separate technicians.

DISCUSSION

Of the two proposed methods, the spectrophotometric procedure offered the least difficulty to all collaborators and gave results whose accuracy would warrant its use in regulatory work. Erratic findings with the chemical procedure by at least two analysts indicate that its acceptance as an official procedure must be held in abeyance until further study reveals the cause of this seeming unreliability.

RECOMMENDATIONS

It is recommended*—

(1) That the spectrophotometric procedure be adopted as first action.

(2) That the chemical method be submitted to further study.

REPORT ON SUBSIDIARY DYES IN FD&C COLORS

LOWER SULFONATED DYE IN FD&C BLUE NO. 1

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

FD&C Blue No. 1 is a trisulfonated dye obtained by condensing one mole of *o*-sulfobenzaldehyde with two moles of ethylbenzylaniline sulfonic acid (EBASA). At the 1952 A.O.A.C. meeting, a preliminary report on lower sulfonated dyes in FD&C Blue No. 1 was presented in which it was shown that commercial samples of this color contained up to 3 per cent of lower sulfonated dye of unknown composition.[1] The lower sulfonated dye can be isolated by extraction.[2]

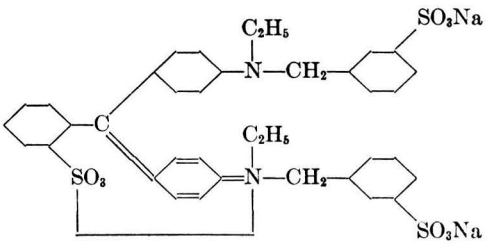
During the past year, work has been continued on this topic in an attempt to determine the nature and source of this subsidiary color. In order to accomplish this purpose, it became necessary to prepare a number of disulfonated subsidiaries of FD&C Blue No. 1, in which one mole of EBASA was replaced by an unsulfonated amine. Theoretically, compounds of this type could be prepared directly *via* several routes; however, it was found more expedient to condense *o*-sulfobenzaldehyde with a mixture containing both EBASA and the appropriate unsulfonated amine, and then to separate the resulting mixture into its individual

* For report of Subcommittee B and action of the Association, see *This Journal*, **38**, 71, 72 (1955).

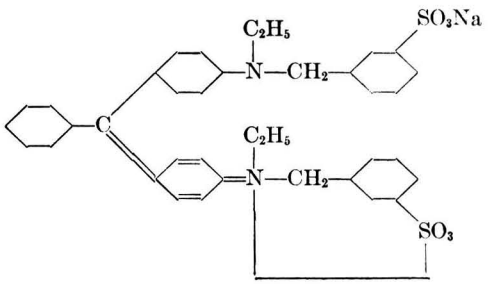
[1] DOLINSKY, M., *This Journal*, **36**, 798 (1953).

[2] *Official Methods of Analysis*, 6th Ed., A.O.A.C., p. 293 (1945).

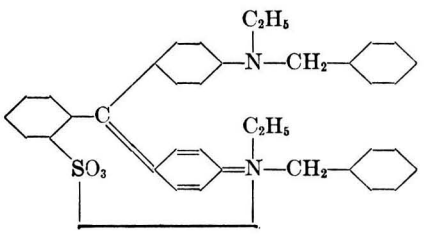
components by taking advantage of their marked differences in solubility. These differences were established through preliminary work with the following dyes of known composition:



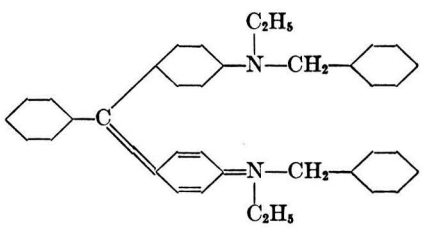
FD&C Blue No. 1
(Trisulfonated)



FD&C Green No. 1
(Disulfonated)



(Monosulfonated)



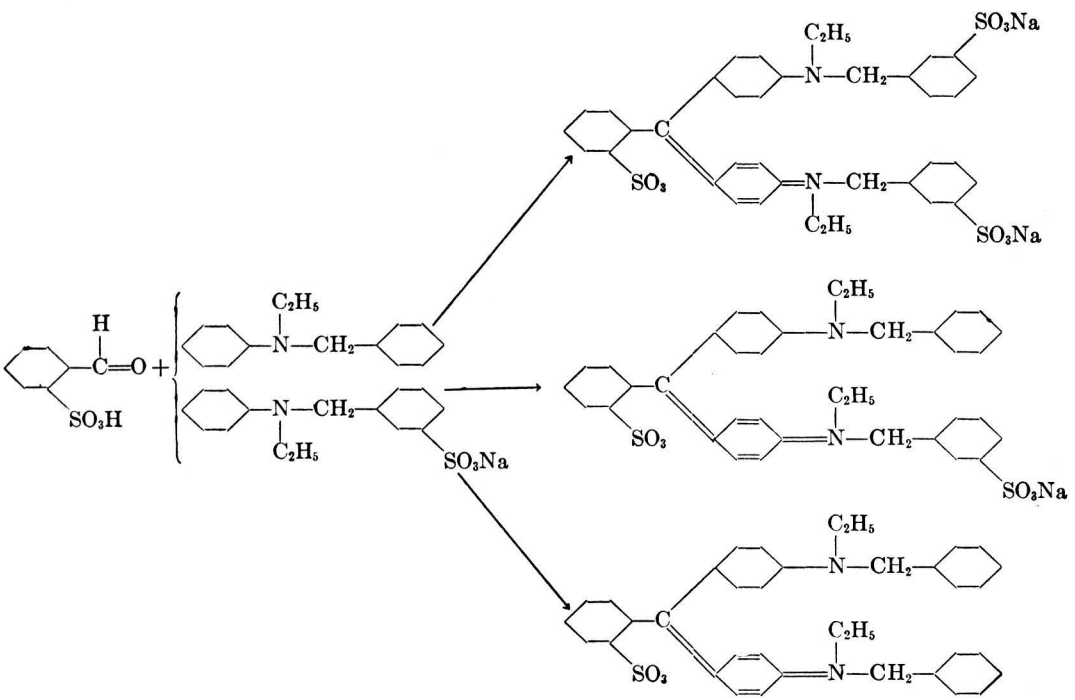
(Unsulfonated)

The FD&C Blue No. 1 and FD&C Green No. 1 were commercial samples. The monosulfonated and unsulfonated dyes were prepared by the Associate Referee. Solubility and extraction data for the four dyes are summarized in Table 1.

TABLE 1.—*Solubility and extraction data*

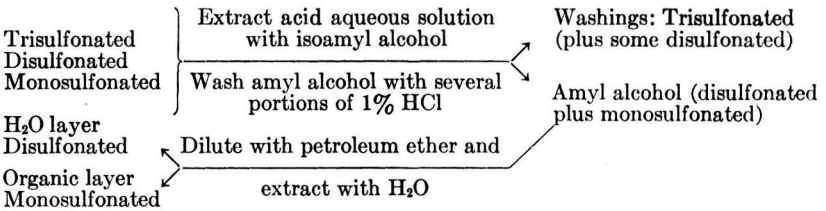
DYE	SOLUBILITY		EXTRACTED		
	H_2O	BENZENE	FROM DIL. ACID INTO ISOAMYL ALCOHOL	FROM ISOAMYL ALCOHOL INTO 1% HCl	FROM (1+1) ISOAMYL ALCOHOL–PETR. ETHER INTO H_2O
FD&C Blue No. 1	sol.	insol.	slightly	yes	yes
FD&C Green No. 1	sol.	insol.	yes	slightly	yes
Monosulfonated	very sl. sol.	insol.	yes	no	no
Unsulfonated	insol.	sol.	yes	no	no

The condensation of *o*-sulfobenzaldehyde with a mixture of EBASA plus an unsulfonated amine will result in a mixture of trisulfonated, disulfonated, and monosulfonated products:



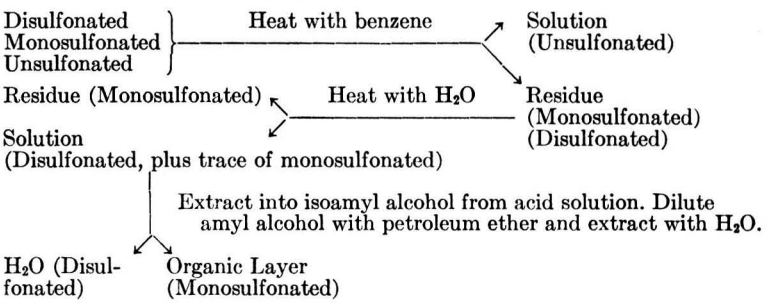
The extraction data of Table 1 indicate that this mixture should be readily separated in the following manner:

SEPARATION SCHEME I



Likewise, the condensation of benzaldehyde with a mixture of EBASA plus an unsulfonated amine will yield a mixture of disulfonated, monosulfonated, and unsulfonated products which can be separated as follows:

SEPARATION SCHEME II



These extraction schemes were applied to a number of three-component mixtures which, in each case, were found to separate as postulated into three distinct components, so that the monosulfonated and disulfonated products could be obtained free of other dyes and their absorption spectra measured. The method used for the preparation of the mixed dyes is as follows:

A mixture of 0.1 mole of *o*-sulfobenzaldehyde, 0.15 mole of EBASA, and 0.15 mole of unsulfonated amine was refluxed with 250 ml of (1+4) concentrated HCl-water for 16 hours. The mixed leuco compounds were dehydrated with absolute alcohol and ether, and the last traces of moisture were removed by boiling with benzene. Two hundred mg of the product, 200 mg of chloranil, and 25 ml of dimethylformamide were heated on the steam bath with occasional stirring for 1 hour. The mixture was then diluted to 200 ml with water and filtered through a fritted glass funnel, and the filtrate was extracted by separation scheme I. (The A.O.A.C. extraction procedure[2] may also be used.)

Table 2 shows the absorption maxima of a number of disulfonated dyes separated in this manner. That the subsidiary color in commercial samples of FD&C Blue No. 1 must be one or more of these dyes was indicated by the following preliminary data:

(1) The subsidiary color found in commercial samples of FD&C Blue No. 1 is extracted almost quantitatively from petroleum ether-isoamyl alcohol by water, which indicates that the material is disulfonated.

TABLE 2.—*Absorption maxima of some disulfonated subsidiaries of FD&C Blue No. 1 (neutral aqueous solution)*

DYE	MAJOR PEAK	MINOR PEAK
R = phenyl RX = *o*-hydroxyphenyl RY = *o*-sulfophenyl	mμ ±2	mμ ±2
R-C-EBASA | EBASA	621	428
RX-C-EBASA | EBASA	622	440
RY-C-EBASA | EBA	626	407
RY-C-EBASA | R-N-C_2H_5 | H	612	398
RY-C-EBASA | R-N(CH_3)$_2$	623	408
RY-C-EBASA | R-N(C_2H_5)$_2$	629	409
RY-C-EBASA | R-NCH_3 | C_2H_5	627	408
Subsidiary color from commercial FD&C Blue No. 1	617–620	402

(2) The subsidiary shows a typical triphenylmethane type absorption spectrum, with a sharp peak near 617 mμ. The minor peak near 402 mμ, characteristic of dyes derived from *o*-sulfobenzaldehyde, is also present. Dyes derived from benzaldehyde, such as FD&C Green No. 1, show minor peaks at significantly longer wavelengths.

(3) The color changes to green in acid and fades rapidly in alkali—reactions typical of triphenylmethane dyes.

(4) The crude extracted color had a nitrogen content (semimicro Kjeldahl) of 4.2 per cent. The probable presence of two nitrogen atoms is thus indicated.

It can be seen from Table 2 that the absorption spectrum of the material extracted from the commercial FD&C Blue No. 1 is not identical with the absorption spectrum of any of the postulated subsidiary colors.

Only one of the colors, that made from N-ethylaniline, shows an absorption maximum below 620 mμ. The spectrophotometric data thus indicate that the extracted material is a mixture, one component of which must be the derivative from N-ethylaniline.

ORIGIN OF THE SUBSIDIARY COLOR

Any unsulfonated amine present in commercial EBASA may give rise to a lower sulfonated subsidiary. When alkaline solutions of several commercial EBASA samples were extracted with ether, only 0.1 to 0.2 per cent of unsulfonated material was found. The unsulfonated amine in the EBASA obviously cannot account for the presence of several per cent of subsidiary in the finished dye.

No increase in ether extract was obtained by heating an alkaline solution of EBASA on the steam bath for two hours, or upon refluxing EBASA in acid solution under conditions which would be used in the preparation of FD&C Blue No. 1.

It appears that the lower sulfonated dye is not derived from unsulfonated amines present in the intermediates, but is formed from FD&C Blue No. 1 through decomposition of the dye. Exposure of FD&C Blue No. 1 solutions to strong alkali for a very short time resulted in a marked increase in subsidiary dye content. The following experiment is typical:

Commercial FD&C Blue No. 1 (10 mg) was extracted by the A.O.A.C. procedure.[2] The residual color was then re-extracted and found to be completely free of lower sulfonated dye. Five ml of 30 per cent sodium hydroxide was then added to the subsidiary-free solution (5.8 mg of FD&C Blue No. 1 in 50 ml of salt-acetate solution), and after about 10 seconds, the solution was diluted with a large volume of salt-acetate solution and the extraction was repeated. The solution then had a lower sulfonated dye content of 0.42 mg (7.3 per cent). The extracted color was spectrophotometrically identical with the subsidiary ordinarily obtained, showing a major peak at 617 mμ and a minor peak at 402 mμ.

Triphenylmethane dyes are known to decompose rapidly and completely under the influence of strong alkali. The N-ethylaniline subsidiary could be formed from FD&C Blue No. 1 as an intermediate step in this decomposition by the elimination of a sulfonated benzyl group. Several other intermediate products may be postulated, particularly the subsidiaries from N-ethyl-N-methylaniline and from N-ethylbenzylaniline. A combination of this type could readily account for all of the spectrophotometric properties of the extracted subsidiary color.

SUMMARY

(1) A method for obtaining a number of lower sulfonated subsidiaries of FD&C Blue No. 1 is presented, and absorption maxima of the dyes are shown.

(2) The subsidiary dyes found in commercial samples are disulfonated products and appear to be a mixture, one component of which is the subsidiary obtained by substituting one molecule of N-ethylaniline for one molecule of EBASA.

(3) The subsidiary color appears to be a decomposition product, possibly caused by the action of alkali during the manufacturing process.

RECOMMENDATIONS

It is recommended* that the topic be continued.

It is further recommended that the method for higher sulfonated dye in FD&C Yellow No. 6 be changed from first action to official with specific directions for the preparation of standards. This method has been in routine use for approximately one year and has given satisfactory results.

No reports were given on arsenic and antimony in coal-tar colors; heavy metals in coal-tar colors; identification of coal-tar colors; inorganic salts in coal-tar colors; intermediates derived from phthalic acid; intermediates in triphenylmethane dyes; lakes and pigments; nonvolatile unsulfonated amine intermediates in coal-tar colors; paper chromatography of coal-tar colors; spectrophotometric testing of coal-tar colors; sulfonated amine intermediates in coal-tar colors; sulfonated phenolic intermediates in coal-tar colors; unsulfonated phenolic intermediates in coal-tar colors; and volatile amine intermediates in coal-tar colors.

REPORT ON STANDARDIZATION OF MICROCHEMICAL METHODS

By C. L. Ogg (Eastern Regional Research Laboratory,† Philadelphia 18, Pa.), *Referee*

Studies on two methods, for sulfur and for alkoxyl groups, were conducted during the past year. These investigations included two series of tests on alkoxyl methods, the first to compare the Clark apparatus with other commonly used apparatus for the analysis of methyl and ethyl esters, and the second to test a tentative method using the Clark apparatus. These studies have been made despite the existence of the present

* For report of Subcommittee B and action of the Association, see *This Journal*, **38**, 71, 72 (1955).

† A laboratory of the Eastern Utilization Research Branch, Agricultural Research Service, U. S. Department of Agriculture.

first action procedure because this procedure has never been subjected to a good collaborative study to test the interlaboratory precision obtainable. Earlier work showed the Clark method to be better than the other commonly used procedure, but the accuracy and precision of the data by the tentative Clark method tested this year were not satisfactory and more work will need to be done before the method can be recommended for first action adoption.

However, this method is required in the determination of methoxy groups in lignin and guiacol in other sections of *Official Methods of Analysis*. Since the present method is not unsatisfactory for this purpose, it should be retained for these analyses.

The other study during the past year concerned the gravimetric determination of sulfur. The present first action procedure specifies a volumetric determination of the sulfate formed either by catalytic or Carius combustion. However, the volumetric method cannot be used if the material contains phosphorus, and consequently it is necessary to establish a gravimetric procedure for such materials. A previously tested procedure did not give satisfactory interlaboratory precision. This procedure was revised and retested with satisfactory results.

The first action methods for Kjeldahl nitrogen, carbon and hydrogen, sulfur, and chlorine and bromine produced precise and accurate results in the collaborative studies leading to their initial adoption, and they have been proved further by two to five years of use as first action methods. Consequently, it is recommended that they be made official.

Some minor editorial modifications in apparatus will be inserted into Chapter 37 to conform with the latest recommendations of the Committee on Microchemical Apparatus of the American Chemical Society.

It is recommended†—

(1) That the first action Kjeldahl method for nitrogen be made official.

(2) That the first action procedure for carbon and hydrogen be adopted as official.

(3) That the first action Carius and catalytic combustion procedures for the sulfur and the volumetric procedure for determining the sulfate formed by the combustions be made official.

(4) That the single ignition procedure tested this year for determining gravimetrically the sulfate from the Carius and catalytic combustions be amended as proposed and adopted as first action.

(5) That the first action procedure for chlorine and bromine be made official.

(6) That the first action method for methoxyl and ethoxyl groups be restricted to the determination of methoxy groups in lignin and guiacol.

(7) That the method for determining methoxyl and ethoxyl groups be studied further.

† For report of Subcommittee C and action of the Association, see *This Journal*, 38, 77 (1955).

(8) That the study of the determination of nitrogen by the Dumas or similar procedure be continued.

(9) That studies of micromethods for determining acetyl groups be continued.

(10) That studies of micromethods for the determination of phosphorus be initiated as soon as practicable.

REPORT ON MICROANALYTICAL DETERMINATION OF ALKOXYL GROUPS. PART II

By AL STEYERMARK (Hoffmann-La Roche Inc., Nutley, N. J.),
Associate Referee

In a previous collaborative study (15) and an interim study reported below, the results indicated that the Clark method for alkoxyl groups (1, 2, 3, 6) was the best of those most widely used, and therefore a procedure based on it was submitted for collaborative study. This report presents the results obtained with this tentative procedure, as well as results of the first study conducted during the year.

TENTATIVE PROCEDURE

REAGENTS AND APPARATUS

(a) *Acetic acid–potassium acetate–bromine solution.*—Dissolve 10 g potassium acetate in sufficient acetic acid to make 100 ml, and add 3 ml bromine.

(b) *Sodium acetate solution.*—Dissolve 25 g sodium acetate, $NaC_2H_3O_2 \cdot 3H_2O$, in enough H_2O to make 100 ml.

(c) *Starch indicator.*—(1) Prep. 1% soln of H_2O-sol. starch by triturating 1 g with a few ml cold distd H_2O and adding this mixt. to ca 95 ml boiling 20% aq. NaCl soln. Boil resulting mixt. 5 min., cool, and filter. Keep soln in refrigerator. Or, (2) Mix ca 2 g finely powd. potato starch with cold H_2O to thin paste; add ca 200 ml boiling H_2O, stirring constantly, and immediately discontinue heating. Add ca 1 ml Hg, shake, and allow starch to stand over the Hg.

(d) *Sodium thiosulfate solution.*—0.01 *N*. Bring to boil 1 l distd H_2O to remove CO_2, and cool while loosely covered. Dissolve 2.48 g sodium thiosulfate, $Na_2S_2O_3 \cdot 5H_2O$, in the H_2O and dil. to 1000 ml. Transfer to brown bottle equipped with rubber stopper. Add 1 ml $CHCl_3$ and shake bottle for few min. ($CHCl_3$ acts as preservative. Use of rubber stopper is preferred to ground glass, as former absorbs $CHCl_3$, increasing efficiency of preservative. This soln is quite stable as long as a pool of $CHCl_3$ is present, but it should be stdzed at ca 1 month intervals. In absence of $CHCl_3$, stdzn should be done every few days.) *Standardization:* Accurately weigh 2–3 mg solid potassium biiodate (using porcelain boat or charging tube), transfer to 125 ml glass-stoppered erlenmeyer, and dissolve in 5 ml distd H_2O. To this soln add 1.5 ml concd HCl and 1 ml *freshly prepd* 4% KI soln, and stopper flask. After 2 min., dil. soln with H_2O to 20 ml and titr. liberated I with the $Na_2S_2O_3$ soln. When I color has almost disappeared (yellow), add several drops starch indicator. Add thiosulfate until blue color has been converted to faint pink at most (end point). *Calculation:* $N_{Na_2S_2O_3} = (\text{mg } KH(IO_3)_2 \times 0.03077)/\text{ml } Na_2S_2O_3$.

(e) *Hydriodic acid.*—Place 250 ml ca constant boiling (57%) HI (sp. gr. 1.7) in 500 ml round-bottom flask connected by ground joint to air condenser, and reflux 2 hrs while stream of CO_2 or N is bubbled thru by means of glass tube extending to bottom. (At no time should acid vapors be allowed to come in contact with organic material.) As soon as refluxing stops, discontinue flow of gas. Cool, and store in glass-stoppered bottle.

(f) *Scrubber solutions.*—(1) 5% soln of $CdSO_4$ in distd H_2O; and (2) 5% soln of $Na_2S_2O_3$ in distd H_2O.

(g) *Apparatus.*—Similar to modified Clark app. (6).

DETERMINATION

Fill trap of app. half way with equal parts of the 2 scrubber solns, and fill receiver ⅔ full of the freshly prepd acetic acid–K acetate–Br soln. Weigh ca 10 mg sample in Pt boat and place in bottom of boiling flask. Add 2.5 ml melted phenol from wide-tip pipet and 5 ml of the HI, and connect boiling flask. Pass CO_2 thru app. from capillary side arm of flask at uniform rate of 15 ml/min. Allow reaction mixt. to remain at room temp. 30 min. With manteled micro burner, boil liquid at such rate that vapors of boiling liquid rise into condenser, but not more than half way, and continue boiling 60 min. Disconnect flask, remove the receiver, and rinse delivery tube and contents of receiver into 125 ml erlenmeyer contg 5 ml of the Na acetate soln.

Adjust vol. to ca 50 ml and add formic acid dropwise until excess bromine is destroyed.

Remove any bromine vapors by blowing air over liquid; then add 0.5 g KI and 5 ml 10% H_2SO_4. Swirl soln to dissolve KI and mix contents; then titr. liberated I with the $Na_2S_2O_3$ soln to the end point, using starch indicator (see stdzn of thiosulfate, above).

Det. blank on all reagents by making detn without sample, and subtract blank from vol. $Na_2S_2O_3$ soln used when sample was present. Calc. % alkoxyl group by the following: % alkoxyl group = (ml in detn − ml in blank) × normality × equiv. wt. × 100/sample wt in mg. Equiv. wts are as follows: methoxyl, 5.17; ethoxyl, 7.51.

A. RESULTS OBTAINED FOR ESTERS BY VARIOUS METHODS

In the first study (15) on the determination of alkoxyl groups, the ethoxyl and methoxyl were present in the form of ethers (*p*-ethoxybenzoic acid and vanillin, respectively). It has been suggested that esters might present more difficulty in analysis than ethers, due to the ease with which ethyl and methyl alcohols are liberated on hydrolysis and to their possible loss by distillation before reaction with hydrogen iodide (11, 12). Therefore, it was decided that the study of alkoxyl groups must include this type of compound. Consequently, two additional samples, namely, benzocaine (ethyl *p*-aminobenzoate)[1] and methyl *p*-aminobenzoate[1] were sent to the collaborators with the request to analyze them by the *same* procedures which they had used on the ethers (15), to analyze each sample at least four times, and to include all results obtained unless there was some definite reason for not doing so. Treatment of the results was identical to that of previous studies (7–10, 13–17). In the following tables, *n* is

[1] The samples submitted were benzocaine (ethyl *p*-aminobenzoate), U.S.P., J. T. Baker Chemical Co.; and methyl *p*-aminobenzoate, Eastman Kodak Co., recrystallized three times from dilute methanol. Before being sent to the various collaborators, these samples were analyzed with the following results: Calcd for benzocaine, $C_9H_{11}O_2N$: C = 65.43; H = 6.71. Found: C = 65.47, 65.27, 65.59; H = 6.55, 6.56, 6.48. Calcd for methyl *p*-aminobenzoate, $C_8H_9O_2N$: C = 63.56; H = 6.00. Found: C = 63.50, 63.36, 63.48, 63.47, 63.71, 63.55, 63.67; H = 6.16, 5.70, 5.66, 6.00, 5.74, 6.14, 5.87, 5.70.

the number of values reported by each collaborator; $\bar{x}$ is the mean of his data; and s is the standard deviation of his data, calculated by the formula:

$$s = \sqrt{\frac{\Sigma(x - \bar{x})^2}{n - 1}}$$

where x is equal to the individual values. Also given in the tables are the values for $\bar{x}$—theory, $\bar{\bar{x}}$, which is the mean of the $\bar{x}$'s; $\bar{\bar{x}}$—theory, $\bar{s}$, which is the mean of the s's; and $s_{\bar{x}}$, which is the standard deviation of the $\bar{x}$'s, calculated by the formula:

$$s_{\bar{x}} = \sqrt{\frac{\Sigma(\bar{x} - \bar{\bar{x}})^2}{n - 1}},$$

The total number of collaborators and the total number of determinations performed are also shown in the tables.

The variance ratio, F, was used to compare the precision of the results obtained by the different methods. It was calculated by the equation: $F = (s_{\bar{x}})^2_a/(s_{\bar{x}})^2_b$, where $(s_{\bar{x}})^2_a$ is always the larger value. If the calculated F value was greater than the critical value ($F_{0.05}$ or $F_{0.01}$) obtained from the table of F values (10, 17), the difference in precision between the two groups of data was significant at the 10 or 2 per cent level, respectively, and the procedure with the lower $s_{\bar{x}}$ was the more precise. The data were significant at the 10 or 2 per cent level rather than at the 5 or 1 per cent level, respectively, due to the fact that the numerator had been chosen.

In addition, t values were calculated to determine whether or not the means for the two groups which were being compared were significantly different. The formula used was:

$$t = (\bar{x}_a - \bar{x}_b)\sqrt{\frac{n_a n_b(n_a + n_b - 2)}{(n_a + n_b)[\Sigma(x_a - \bar{x}_a)^2 + \Sigma(x_b - \bar{x}_b)^2]}},$$

where n_a and n_b are the number of values in groups a and b respectively, x_a and x_b are individual values for the two groups, and $\bar{x}_a$ and $\bar{x}_b$ are the means of the values for the two groups. If the calculated t value was greater than the critical value ($t_{0.05}$) obtained from a table of student's t's (10, 17), the difference between the two means was significant at the 5 per cent level and the procedure whose mean was nearer the theoretical value was considered to be the better.

Seven collaborators analyzed benzocaine (ethyl p-aminobenzoate) by the Clark method and reported a total of 36 determinations. The results are shown in Table 1, and gave these values: $\bar{\bar{x}} = 27.22$ per cent; $\bar{s} = 0.156$; $\bar{\bar{x}}$—theory $= -0.06$ per cent; $s_{\bar{x}} = 0.072$. Nine of the collaborators analyzed the sample by the Elek method (4, 5, 12) and reported a total of 54 determinations, the results of which are shown in Table 1, and gave these values: $\bar{\bar{x}} = 25.63$ per cent; $\bar{s} = 0.574$; $\bar{\bar{x}}$—theory $= -1.65$ per cent; $s_{\bar{x}} = 3.546$.

TABLE 1.—*Determination of ethoxyl in benzocaine (ethyl p-aminobenzoate); 27.28% exthoxyl*

Clark Method							
Collaborator No.	2	8	17	23	37	45	57
n	6	4	5	8	5	4	4
$\bar{x}$ (%)	27.27	27.07	27.25	27.19	27.25	27.27	27.25
s	0.413	0.227	0.021	0.226	0.064	0.045	0.093
$\bar{x}$−Theory (%)	−0.01	−0.21	−0.03	−0.09	−0.03	−0.01	−0.03

$\bar{\bar{x}}=27.22\%$; $\bar{\bar{x}}-\text{Theory}=-0.06\%$ $\quad\bar{s}=0.156$; $s_{\bar{x}}=0.072$

Elek Method									
Collaborator No.	15	29	30	40	46	71	77	79	82
n	8	4	7	8	5	9	5	6	2
$\bar{x}$ (%)	22.60	29.44	25.63	27.09	17.51	27.02	26.89	27.28	27.23
s	1.148	0.175	0.204	0.107	3.284	0.119	0.108	0.011	0.010
$\bar{x}$−Theory (%)	−4.68	+2.16	−1.65	−0.19	−9.77	−0.26	−0.39	0.00	−0.05

$\bar{\bar{x}}=25.63\%$; $\bar{\bar{x}}-\text{Theory}=-1.65\%$ $\quad s=0.574$; $s_{\bar{x}}=3.546$

TABLE 2.—*Determination of methoxyl in methyl p-aminobenzoate; 20.53% methoxyl*

Clark Method							
Collaborator No.	2	8	17	23	37	45	57
n	6	4	6	7	5	4	4
$\bar{x}$ (%)	20.35	20.50	20.55	20.53	20.56	20.44	20.22
s	0.333	0.046	0.110	0.048	0.136	0.061	0.050
$\bar{x}$−Theory (%)	−0.18	−0.03	+0.02	0.00	+0.03	−0.09	−0.31

$\bar{\bar{x}}=20.45\%$; $\bar{\bar{x}}-\text{Theory}=-0.08\%$ $\quad\bar{s}=0.112$; $s_{\bar{x}}=0.125$

Elek Method									
Collaborator No.	15	29	30	40	46	71	77	79	82
n	8	4	4	4	4	4	5	6	2
$\bar{x}$ (%)	20.49	20.48	20.40	20.43	18.85	20.33	20.31	20.51	20.31
s	0.259	0.227	0.094	0.086	0.575	0.102	0.110	0.009	0.141
$\bar{x}$−Theory (%)	−0.04	−0.05	−0.13	−0.10	−1.68	−0.20	−0.22	−0.02	−0.22

$\bar{\bar{x}}=20.23\%$; $\bar{\bar{x}}-\text{Theory}=-0.30\%$ $\quad\bar{s}=0.178$; $s_{\bar{x}}=0.525$

Figure 1 is a plot of the individual values. At a glance it can be seen that the results obtained by four of the collaborators who used the Elek method on benzocaine were greatly in error. The $\bar{x}$ − theory values for three of these were −1.65, −4.68, and −9.77, indicating a loss of ethanol during the determination. One collaborator (No. 46), using an unweighed sample, detected ethanol during the determination (by means of potassium dichromate). The fourth collaborator who was greatly in error

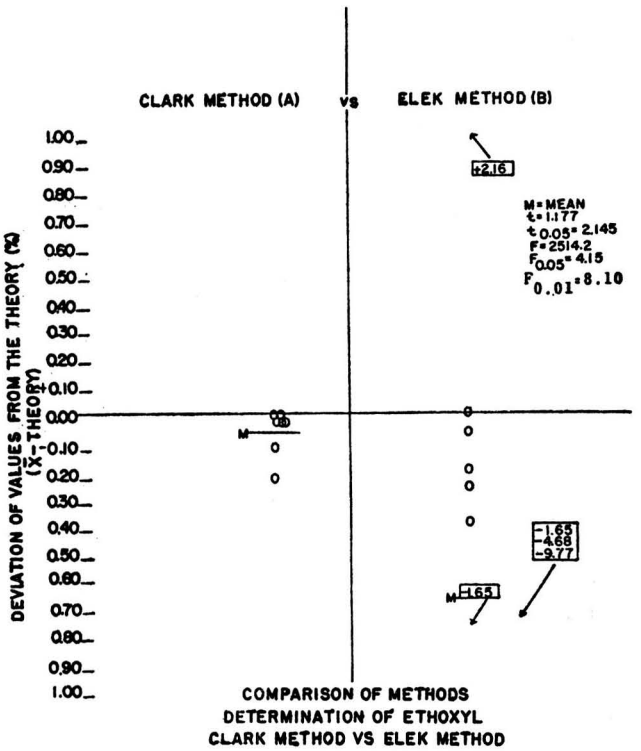


FIG. 1

obtained high results ($\bar{x}$ − theory = +2.16) which cannot be accounted for on this basis. Although it is obvious that the Clark method gave the better results, the F values were calculated to compare the precisions obtained by the two methods. The F value, 2514.2, was critical at the 2 per cent level ($F_{0.01} = 8.10$) and in favor of the Clark method. The t values are not discussed because the critical F value invalidates this measurement. However, the t values are included in Figs. 1 and 2.

Seven collaborators analyzed methyl p-aminobenzoate by the Clark method and reported a total of 36 determinations. The results are shown in Table 2, and gave the values: $\bar{\bar{x}} = 20.45$ per cent; $\bar{s} = 0.112$; $\bar{\bar{x}}$ − theory

$= -0.08$ per cent; $s_{\bar{x}} = 0.125$. Nine collaborators analyzed the sample by the Elek method and reported a total of 41 determinations. These results are shown in Table 2, and gave the values: $\bar{\bar{x}} = 20.23$ per cent; $\bar{s} = 0.178$; $\bar{\bar{x}}$ – theory $= -0.30$ per cent; $s_{\bar{x}} = 0.525$. A comparison was made between the Clark and Elek methods, giving an F value of 17.25, which was critical at the 2 per cent level ($F_{0.01} = 8.10$) and in favor of the Clark method (see Fig. 2, the plot of the individual values).

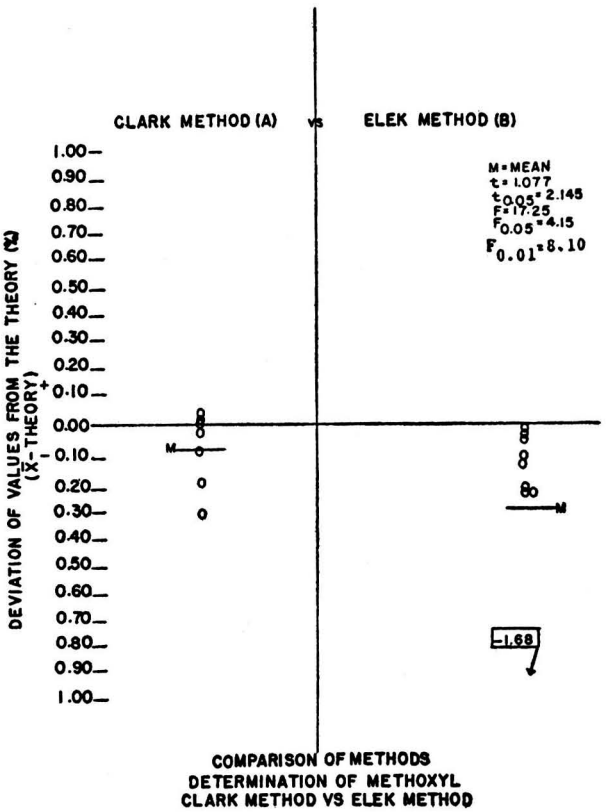


FIG. 2

Inspection of the results of this study without calculating either the t or the F values showed that the results reported by those who used the Clark method were superior. Apparently, the Elek method gave poor results because of the loss of ethanol prior to its being converted to ethyl iodide. In general, the results were in agreement with previously reported results for *p*-ethoxybenzoic acid, although with that compound the results with the Elek method were much more accurate.

The results obtained for methyl *p*-aminobenzoate were in complete agreement with those on vanillin reported previously.

As a result of this study and the one previously reported (15), a procedure was written for the Clark method and submitted for further collaborative study. The remainder of this report presents the data obtained from the study in which the collaborators, using a specific procedure and apparatus, analyzed both ethyl and methyl esters (benzocaine and methyl *p*-aminobenzoate, respectively) and ethers (*p*-ethoxybenzoic acid and vanillin, respectively). The apparatus used was similar to that described in *Official Methods of Analysis* of the Association of Official Agricultural Chemists (6).

B. RESULTS OBTAINED FOR ESTERS AND ETHERS BY THE TENTATIVE PROCEDURE

It was hoped that the results would permit an official procedure to be written with the Clark method as the basis, but from the results which follow, it appears that additional study is necessary before such action is taken. The results obtained by the various collaborators who used the tentative procedure are shown in Tables 3 and 4. (Compare References 7–9, 13–16.)

Sixteen collaborators reported a total of 78 determinations on benzocaine (ethyl *p*-aminobenzoate) for which $\bar{\bar{x}}=27.09$ per cent; $\bar{s}=0.268$; $\bar{\bar{x}}-\text{theory}=-0.19$ per cent; $s_{\bar{x}}=0.717$. The results are shown in Table 3; Figure 3A is a plot of the individual values. Twelve collaborators reported a total of 45 determinations on *p*-ethoxybenzoic acid for which $\bar{\bar{x}}=26.95$ per cent; $\bar{s}=0.489$; $\bar{\bar{x}}-\text{theory}=-0.17$ per cent; $s_{\bar{x}}=1.062$. Table 3 shows the results; Figure 3B is a plot of the individual values.

Twelve collaborators reported a total of 50 determinations on methyl *p*-aminobenzoate for which $\bar{\bar{x}}=20.16$ per cent; $\bar{s}=0.156$; $\bar{\bar{x}}-\text{theory}=-0.37$ per cent; $s_{\bar{x}}=0.893$. The results are shown in Table 4; a plot of the individual values is shown in Figure 3C.

Thirteen collaborators analyzed vanillin and reported a total of 58 determinations for which $\bar{\bar{x}}=19.99$ per cent; $\bar{s}=0.274$; $\bar{\bar{x}}-\text{theory}=-0.41$ per cent; $s_{\bar{x}}=0.725$. The results are shown in Table 4; a plot of the individual values is shown in Figure 3D.

One collaborator, No. 15, used a stainless steel spiral in the absorber instead of glass and it is quite possible that his low values resulted from reaction between the spiral and the bromine. Other collaborators (Nos. 0, 23, 46, 71) first attempted to use stainless steel but obtained precipitates when the acetic acid–potassium acetate–bromine mixture came in contact with the stainless steel spiral, and therefore substituted a glass spiral before doing the collaborative analyses.

In general, the results were disappointing. When analyzing benzocaine, 7 of the 16 collaborators obtained results which had a standard deviation of greater than 0.3, or $\bar{x}-\text{theory}$ of greater than ± 0.3 per cent, or both. For *p*-ethoxybenzoic acid, 5 out of 12 obtained either a standard deviation of greater than 0.3 or $\bar{x}-\text{theory}$ of greater than ± 0.3 per cent, or both.

TABLE 3.—*Determination of ethoxyl by the tentative procedure*

Sample: Benzocaine (ethyl *p*-aminobenzoate)—27.28% ethoxyl

Collaborator Number	0	2	8	15	23	29	30	37	40	45	46	49	62	71	77	79
n	12	4	4	4	4	4	7	6	4	4	4	4	3	4	4	6
$\bar{x}$ (%)	26.76	27.03	25.42	26.77	27.53	28.91	27.41	26.48	26.97	27.35	27.54	27.24	26.49	27.19	27.00	27.28
s	0.727	0.126	0.304	0.125	0.375	0.171	0.235	1.201	0.082	0.098	0.189	0.154	0.177	0.185	0.116	0.018
$\bar{x}$—Theory	−0.52	−0.25	−1.86	−0.51	+0.25	+1.63	+0.13	−0.80	−0.31	+0.07	+0.26	−0.04	−0.79	−0.09	−0.28	0

Total No. collaborators: 16
Total No. determinations: 78

$\bar{\bar{x}}$ = 27.09%
$\bar{\bar{x}}$—Theory = −0.19%

$\bar{s}$ = 0.268
$s_{\bar{x}}$ = 0.717

Sample: *p*-Ethoxybenzoic acid—27.12% ethoxyl

Collaborator Number	8	15	29	37	40	45	46	49	62	71	77	79
n	4	4	4	4	4	4	4	3	1	4	3	6
$\bar{x}$ (%)	26.81	25.53	28.75	26.90	27.26	27.07	24.50	27.15	27.20	27.83	27.22	27.19
s	0.221	0.122	0.074	0.369	0.108	0.059	3.895	0.146	——	0.301	0.071	0.012
$\bar{x}$—Theory (%)	−0.31	−1.59	+1.63	−0.22	+0.14	−0.05	−2.62	+0.03	+0.08	+0.71	+0.10	+0.07

Total No. Collaborators: 12
Total No. Determinations: 45

$\bar{\bar{x}}$ = 26.95%
$\bar{\bar{x}}$—Theory = −0.17%

$\bar{s}$ = 0.489
$s_{\bar{x}}$ = 1.062

TABLE 4.—*Determination of methoxyl by the tentative procedure*

Sample: Methyl *p*-aminobenzoate—20.53% methoxyl

Collaborator Number	2	8	15	23	29	37	40	45	46	71	77	79
n	4	4	4	4	4	4	5	4	4	4	3	6
$\bar{x}$ (%)	20.45	20.48	17.35	20.20	20.52	20.36	20.40	20.38	20.20	20.65	20.35	20.52
s	0.129	0.057	0.096	0.602	0.119	0.273	0.106	0.028	0.219	0.220	0.016	0.011
$\bar{x}$—Theory (%)	−0.08	−0.05	−3.18	−0.33	−0.01	−0.17	−0.13	−0.15	−0.33	+0.12	−0.18	−0.01

Total No. Collaborators: 12
Total No. Determinations: 50

$\bar{\bar{x}}$ = 20.16%
$\bar{\bar{x}}$—Theory = −0.37%

$\bar{s}$ = 0.156
$s_{\bar{x}}$ = 0.893

Sample: Vanillin—20.40% methoxyl

Collaborator Number	0	8	15	23	29	37	40	46	49	62	71	77	79
n	4	4	8	5	4	4	4	4	4	2	4	5	6
$\bar{x}$ (%)	20.27	20.38	17.77	19.37	20.56	20.07	20.21	19.99	20.19	20.16	20.27	20.26	20.39
s	0.277	0.124	0.158	0.950	0.171	0.369	0.086	0.682	0.055	0.290	0.135	0.249	0.15
$\bar{x}$—Theory (%)	−0.13	−0.02	−2.63	−1.03	+0.16	−0.33	−0.19	−0.41	−0.21	0.24	−0.13	−0.14	−0.01

Total No. Collaborators: 13
Total No. Determinations: 58

$\bar{\bar{x}}$ = 19.99%
$\bar{\bar{x}}$—Theory = −0.41%

s = 0.274
$s_{\bar{x}}$ = 0.725

Two of 12 collaborators analyzing methyl *p*-aminobenzoate and 4 of the 16 collaborators analyzing vanillin also obtained results of this type.

In addition to this, each group had a few borderline cases. For all of the results on benzocaine, the $s_{\bar{x}}$ was 0.717; for *p*-ethoxybenzoic acid, the $\bar{s}$ was 0.489 and the $s_{\bar{x}}$ was 1.062. For methyl *p*-aminobenzoate, the $\bar{\bar{x}}$—theory was −0.37 per cent and the $s_{\bar{x}}$ was 0.893. For vanillin, the $\bar{\bar{x}}$—theory was −0.41 per cent and the $s_{\bar{x}}$ was 0.725. It is possible that

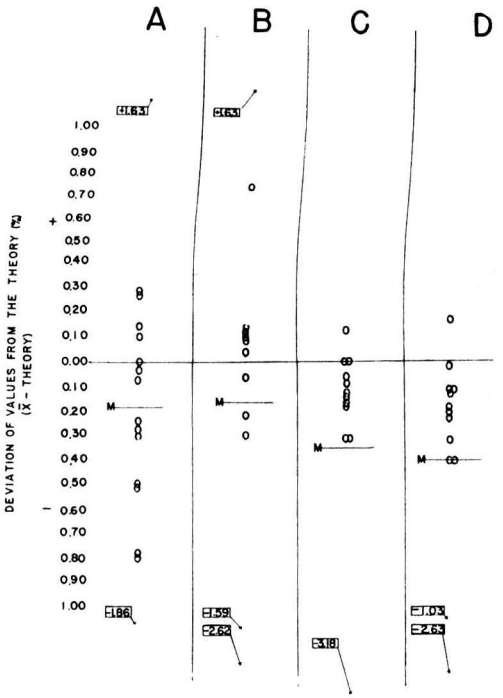


FIG. 3.—Plot showing deviations of values from theory for the various samples: **A**—Benzocaine (ethyl *p*-aminobenzoate); **B**—*p*-ethoxybenzoic acid; **C**—methyl *p*-aminobenzoate; **D**—vanillin. All analyses were by the tentative procedure.

some of the low findings resulted from a holdback of the alkyl iodide, particularly the ethyl, in the cold reflux condenser. The temperature of the water used by some collaborators might have been so low as to have caused this condition. For this reason, in future work this point will be studied. If it is the cause of error, it can be corrected by draining the water from the condenser during the latter part of the determination.

It should be noted that 4 collaborators (Nos. 15, 29, 46, 71) who obtained poor results with the tentative procedure had also obtained poor results when they analyzed the same samples by the Elek method previously. It is also of interest to note that 4 collaborators (Nos. 2, 8, 23, 37)

who reported poor results for this study obtained good results previously by the Clark method on the same samples (except collaborator No. 2 who had standard deviations of 0.413 for benzocaine and 0.333 for methyl *p*-aminobenzoate).

RECOMMENDATIONS

It is recommended*—

(1) That the present first action procedure in the Seventh Edition of *Official Methods of Analysis* be deleted as a general method and that it be restricted to the determination of methoxy groups in lignin and guaiacol.

(2) That additional work be done on the alkoxyl determination to obtain a procedure which will give more satisfactory results.

ACKNOWLEDGMENTS

The author is indebted to Esther A. Bass, June C. Dell, Marie W. Garner, and Margaret Sturtevant for the elementary analyses shown in footnote 1; and to Patricia Hillman for her assistance in preparing Figures 1, 2, and 3.

COLLABORATORS

Aluise, V. A., Hercules Experiment Station, Hercules Powder Company, Inc.
Bain, C. J., Ordnance Corps, Picatinny Arsenal
Bass, E. A., Hoffmann-La Roche Inc.
Boos, R. N., Merck & Company, Inc.
Brancone, L. M., Lederle Laboratories Division, American Cyanamid Co.
Brown, L. E., Southern Utilization Research Branch, United States Department of Agriculture
Brown, W. L., Eli Lilly & Company
Browning, B. L., The Institute of Paper Chemistry
Duiguid, L. I., Du-Good Chemical Laboratory
Feldman, J. R., General Foods Corporation
Huff, W. J., Ordnance Corps, Picatinny Arsenal
Johnson, S. H., Smith, Kline and French Laboratories
Jones, G. A., E. I. du Pont de Nemours & Company, Inc.
Kaup, R. M., Hoffmann-La Roche Inc.
Keller, K. P., Tennessee Eastman Company
Kelly, R., Eastern Utilization Research Branch, United States Department of Agriculture
Ketchum, D. F., Eastman Kodak Company
Ogg, C. L., Eastern Utilization Research Branch, United States Department of Agriculture
Paulson, R. A., National Bureau of Standards
Schranz, R. E., Hercules Experiment Station, Hercules Powder Company, Inc.
Shrader, S. A., Dow Chemical Company
Tassinari, S. J., National Dairy Research Labs., Inc.
VanEtten, C. H., Northern Utilization Research Branch, United States Department of Agriculture

---

* For report of Subcommittee C and action of the Association, see *This Journal*, 38, 77 (1955).

White, L. M., Western Utilization Research Branch, United States Department of Agriculture
Wilson, J. B., Tennessee Eastman Company

REFERENCES

(1) CLARK, E. P., *This Journal*, **51**, 136 (1932).
(2) ———, *ibid.*, **22**, 622 (1939).
(3) ———, *Semimicro Quantitative Organic Analysis*, Academic Press, Inc., New York, 1943.
(4) ELEK, A., *Ind. Eng. Chem., Anal. Ed.*, **11**, 174 (1939).
(5) NIEDERL, J. B., and NIEDERL, V., *Micromethods of Quantitative Organic Analysis*, Second Edition, John Wiley & Sons, Inc., New York, 1942.
(6) *Official Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Washington, D. C., 1950, p. 744.
(7) OGG, C. L., *This Journal*, **35**, 305 (1952).
(8) ———, *ibid.*, **36**, 335 (1953).
(9) ———, *ibid.*, **36**, 344 (1953).
(10) SNEDECOR, G. W., *Statistical Methods*, Fourth Edition, The Iowa State College Press, Ames, Iowa, 1946.
(11) STEYERMARK, A., *Anal. Chem.*, **20**, 368 (1948).
(12) ———, *Quantitative Organic Microanalysis*, The Blakiston Company, Philadelphia, 1951.
(13) ———, and FAULKNER, M. B., *This Journal*, **35**, 291 (1952).
(14) ———, and GARNER, M. W., *ibid.*, **36**, 319 (1953).
(15) ———, and LOESCHAUER, E. E., *ibid.*, **37**, 433 (1954).
(16) WILLITS, C. O., and OGG, C. L., *ibid.*, **32**, 56 (1949).
(17) YOUDEN, W. J., *Statistical Methods for Chemists*, John Wiley & Sons, Inc., New York, 1951.

REPORT ON THE GRAVIMETRIC MICRODETERMINATION OF SULFUR

By C. L. OGG (Eastern Regional Research Laboratory,* Philadelphia 18, Pa.), *Associate Referee*

The previous studies of the determination of sulfur[1] produced a volumetric method which was suitable as an official procedure, but the gravimetric method tested did not give sufficiently good between-laboratory precision to warrant recommending it as an A.O.A.C. method. A gravimetric method is required because sulfur should not be determined by the volumetric procedure if the material being analyzed contains phosphorus. Consequently a revised gravimetric procedure was subjected to a collaborative test in which the same two pure samples (benzyl-isothiourea hydrochloride and sulfanilamide) were employed that had been used in the two previous studies. The new tentative method described

* A laboratory of the Eastern Utilization Research Branch, Agricultural Research Service, U. S. Department of Agriculture.
[1] OGG, C. L., *This Journal*, **35**, 305 (1952); **36**, 335 (1953).

below was designed to test the need for a double washing and ignition of the precipitated barium sulfate. It had been assumed that such a procedure was unnecessary because in the present A.O.A.C. micromethods for sulfur the solutions from which the sulfate would be precipitated contain only small amounts of foreign ions which might be adsorbed or coprecipitated with the barium sulfate. To test this assumption and to try to find a suitable method, a copy of the following tentative gravimetric procedure was sent to each collaborator. Each analyst was asked to use either the Carius or the catalytic combustion methods (both first action) to convert the sulfur to sulfate and then to determine the sulfate by the tentative procedure.

GRAVIMETRIC PROCEDURE FOR CARIUS COMBUSTION METHOD

Dissolve residue in 3 ml distd H_2O, pour into previously ignited and weighed porcelain crucible, rinse beaker with four 2 ml portions H_2O, and place crucible on steam bath until soln is near boiling point. (If total vol. exceeds 10–11 ml, evap. to this vol.) Add, dropwise, 0.5 ml 10% $BaCl_2$ (for samples which might contain more than 5 mg S, use 1 ml), digest at least 15 min., and cool 15 min. Connect porcelain filter, previously ignited and weighed with crucible, to arm of siphon with rubber tubing; connect other arm of siphon to suction flask thru a rubber stopper. Lower filter into crucible; draw off soln slowly; rinse ppt, walls of crucible, and filter with three 3 ml portions 1:300 HCl, drawing off as much liquid as possible. Carefully detach filter, place in crucible, wipe outside of crucible and end of filter with moist chamois or cheesecloth, and handle thereafter with crucible tongs. Place crucible and filter in larger crucible and dry in oven at ca 110°C. for 10 min. Then ignite in muffle at 700–750°C. for 10 min. (ppt may be ignited by heating larger crucible contg crucible and stick to dull red heat with Meker burner), cool on metal block for 30 min. (or in desiccator for 1 hr), and weigh. Make blank run on reagents. Repeat treatment of ppt beginning, "rinse ppt, walls of crucible . . . ."

Calculation: (wt $BaSO_4$ − blank) × 0.1374 × 100/sample wt = % S.

GRAVIMETRIC PROCEDURE FOR CATALYTIC COMBUSTION METHOD

Rinse contents of absorber quantitatively into previously ignited and weighed porcelain crucible, using five 2 ml portions H_2O. Continue as under Carius method, beginning " . . . place crucible on steam bath . . . ."

RESULTS

Five collaborators reported 52 and 46 values for samples 1 and 2, respectively. These data are summarized in Table 1, where n is the number of values reported, $\bar{x}$ is the mean, and s is the standard deviation of the collaborator's individual values; $\bar{\bar{x}}$ is the mean and $s_{\bar{x}}$ is the standard deviation of the mean ($\bar{x}$) values.

Examination of the data shows that the single ignition treatment of the $BaSO_4$ from sample 1 gave the more accurate value, 15.85 versus the theoretical value of 15.82, whereas the value by double ignition was 15.63, or 0.19 per cent low.

The t value, 2.64, calculated for the difference between means exceeded the critical $t_{0.05}$ (2.37). The two treatments, therefore, produced significantly different means. The introduction of a constant error by this

TABLE 1.—*Results of sulfur determination by tentative micromethod (effect of single and double ignition)*

COLLABORATOR NO.	SINGLE IGNITION[a]			DOUBLE IGNITION[b]		
	n	$\bar{x}$	s	n	$\bar{x}$	s
Sample 1: Benzylisothiourea hydrochloride (15.82% S)						
9	4	15.70	0.15			
15	8	15.71	0.12	8	15.62	0.09
29	8	15.90	0.11	8	15.47	0.19
40	4	15.97	0.07	4	15.70	0.06
59	4	15.96	0.14	4	15.71	0.05
$\bar{\bar{x}}$		15.85	(0.12)		15.63	(0.10)
$s_{\bar{x}}$		0.13			0.11	
Sample 2: Sulfanilamide (18.62% S)						
9	6	18.69	0.14			
15	8	18.51	0.30	8	18.42	0.25
29	4	18.84	0.01	4	18.78	0.05
40	4	18.86	0.12	4	18.63	0.13
59	4	18.93	0.14	4	18.47	0.05
$\bar{\bar{x}}$		18.77	(0.14)		18.58	(0.13)
$s_{\bar{x}}$		0.17			0.16	

[a] $BaSO_4$ washed and ignited once.
[b] $BaSO_4$ from single treatment rewashed and reignited.

treatment is indicated by the fact that the theoretical value falls outside the 95 per cent confidence limits for the mean of the data from the double ignition.

The data for sample 2 show that the double ignition again lowered the values by the same amount, but in this case the mean for the single ignition is considerably higher and that for the double ignition only a little lower than the theoretical value. The t value of 1.71, however, did not exceed the critical $t_{0.05}$ (2.37), showing that the difference between means for sample 2 was not significant.

The theoretical value also was bracketed by the confidence limits of the mean for the single ignition data.

From these findings, it is concluded that the single ignition treatment following the proposed procedures is the better. The within-laboratory precision (s values) was generally good; the between-laboratory precision, ($s_{\bar{x}}$ values of 0.13 and 0.17 per cent for samples 1 and 2, respectively) seems quite satisfactory; and the agreement between the over-all mean ($\bar{\bar{x}}$) and the theoretical value is good for sample 1 (0.03 per cent) and acceptable for sample 2 (0.15 per cent).

There is evidence that values from the single ignition procedure tend to be high. To try to bring these values closer to the theoretical value, collaborator 59 proposed that the precipitated barium sulfate be washed 5 or 6 times with 1:300 hydrochloric acid instead of the 3 times specified in the method and, of course, that the double ignition be eliminated. Whether or not this will have a significant effect on the results is not certain, but such a change should not decrease the accuracy of the results. The Referee proposes that the procedure be changed accordingly.

RECOMMENDATIONS

It is recommended†—

(1) That the gravimetric method tested this year be changed as follows:

(a) Change sentence 8, above, from " . . . rinse . . . with three 3 ml portions 1:300 HCl . . . " to " . . . rinse . . . with five or six 3 ml portions 1:300 HCl . . ."

(b) Delete last sentence: "Repeat treatment of ppt beginning, 'rinse ppt, wall of crucible . . . '."

(2) That the amended procedure be adopted as first action.

COLLABORATORS

C. J. Bain, Picatinny Arsenal
W. L. Brown, Eli Lilly & Company
E. E. Gansel, General Aniline & Film Corporation
G. A. Jones, E. I. du Pont de Nemours & Company
R. A. Paulson, National Bureau of Standards

REPORT ON STANDARD SOLUTIONS

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

Sodium Thiosulfate Solutions.—This subject was reopened following a comment by V. A. Stenger (*Anal. Chem.*, **23,** 1543 (1951)) that in analyses of a standard iodate solution, various analysts obtained results deviating by ±0.2 per cent from those obtained with thiosulfate solutions standardized by A.O.A.C. methods **39.35** and **39.36.** He suggested it would be desirable to make a thorough comparison of dichromate and iodate as standards for thiosulfate under various conditions.

The preliminary study of the Associate Referee, reported in *This Journal,* **37,** 459 (1954), indicated that the present A.O.A.C. method gives results which compare favorably with those obtained by using iodine and

† For report of Subcommittee C and action of the Association, see *This Journal,* **38,** 77 (1955).

potassium iodate. Collaborative study the past year confirmed the preliminary study. The Associate Referee has recommended slight modification of the wording of the present method to provide for use of chlorine-free water and to insure that the hydrochloric acid is added immediately before the solution is placed in the dark for ten minutes. He has also recommended the addition of an equation to calculate the normality of the sodium thiosulfate solution. The Referee concurs in these recommendations.*

The Associate Referee recommends that the standardization be carried out in a "glass-stoppered iodine flask," rather than in the "glass-stoppered flask" specified in **39.36,** and that analytical grade potassium iodide be specified. The Referee, however, recommends that the first line of the revised directions for **39.36** read:

"Place 0.20–0.23 g $K_2Cr_2O_7$, accurately weighed (National Bureau of Standards std sample dried 2 hrs at 100°C.), in a glass-stoppered iodine flask (or glass-stoppered flask) and dissolve in 80 ml Cl-free distd H_2O contg 2 g KI."

The section "Definitions of Terms and Explanatory Notes" of *Official Methods of Analysis* requires use of analytical reagent grade potassium iodide, and the Referee does not believe that the recommended change in wording warrants changing the method from official to first action. The Referee recommends the subject be closed.

Buffer Solutions for Calibration of pH Equipment.—It is recommended that the first action methods for preparation of 0.05 *M* acid potassium phthalate, 0.025 *M* phosphate, and 0.01 *M* borax buffer solutions for use in calibration of *p*H equipment, **39.7** and **39.8,** adopted by the Association in 1949, be made official.

Hydrochloric Acid, Constant Boiling Method.—It is recommended that the first action method for standardization of hydrochloric acid by the constant boiling method (*This Journal,* **36,** 96 (1953); as revised, *This Journal,* **37,** 122 (1954)), adopted by the Association in 1953, be made official.

Potassium Dichromate.—It is recommended that the first action method for preparation of standard potassium dichromate solutions, **39.20, 39.21,** and **39.22,** adopted first action by the Association in 1949, be made official.

Titanium Trichloride.—It is recommended that the first action method for standardization of titanium trichloride solutions, **39.40, 39.41,** and **39.42,** adopted by the Association in 1948, be made official.

* For report of Subcommittee A and action of the Association, see *This Journal,* **38,** 68 (1955).

REPORT ON STANDARDIZATION OF 0.1 NORMAL SODIUM THIOSULFATE

By WILLIAM H. MUNDAY (Food and Drug Administration, Minneapolis, Minn.), *Associate Referee*

In accordance with the recommendations approved by the Association in 1953 (*This Journal*, **37**, 63 (1954)), the standardization of sodium thiosulfate was studied collaboratively.

In preliminary work, no difficulty was encountered with the present A.O.A.C. method when chlorine-free distilled water was used. However, it was found that as little as 0.5 p.p.m. of free chlorine will cause erratic results and an unstable end point (*This Journal*, **38**, 459 (1954)).

In collaborative work on this problem, T. S. Smith noted that if the potassium iodide-acid solution was made up before the determination and allowed to stand in the light for as little as ten to fifteen minutes, free iodine was released from the potassium iodide. If the potassium iodide-acid solution is used immediately or if the solution is kept in the dark, this interference is eliminated. The method was accordingly reworded before the instructions for further collaboration were sent out.

INSTRUCTIONS TO THE COLLABORATORS

Prepare a sufficient volume (ca 2 liters) of the sodium thiosulfate solution to be used for this collaborative study by method **39.35** (*Official Methods of Analysis*, 7th Ed.). Since each collaborator is using his own thiosulfate solution, he should make at least six determinations by each of the following three methods:

Method 1.—Use A.O.A.C. method **39.36**, modified as follows: Place the $K_2Cr_2O_7$ in a glass-stoppered flask and dissolve it in Cl-free distd H_2O contg analytical reagent grade KI. Next add the HCl, prepd from Cl-free H_2O, and immediately place the soln in the dark for 10 min. before titrating.

Method 2.—Use the method described in Willard-Furman's *Elementary Quantitative Analysis*, 2nd Ed., as follows: Dissolve 0.12–0.17 g analytical reagent grade KIO_3 (recrystd three times from Cl-free H_2O, dried at 180°C., and stored in a desiccator) in ca 50 ml recently boiled, Cl-free distd H_2O; dissolve 2 g analytical reagent grade KI in this soln, and then add 10 ml HCl (1+3). Titrate the I_2 immediately with Na thiosulfate soln, adding starch as indicator when the soln becomes faint yellow. Calc. the normality of the Na thiosulfate by the equation: $N = \text{Wt } KIO_3/\text{ml thiosulfate} \times 0.03567$.

Method 3.—Use the method described in Treadwell and Hall's *Analytical Chemistry*, 8th Ed., as follows: Mix 5–6 g analytical reagent grade iodine with ca 2 g analytical reagent grade KI, place mixt. in the bottom dish of a sublimator, and resublime until most of the free I_2 is gone from the bottom dish. Remove the resublimed I_2 from the condenser and repeat the sublimation at as low a temperature as possible, without addn of KI, to obtain a KI-free product. Grind the I_2 somewhat in a clean agate mortar and dry in a desiccator over $CaCl_2$ (*not* H_2SO_4). Avoid formation of hydriodic acid by not greasing the desiccator cover.

Place 2–2.5 g analytical reagent grade KI and no more than 0.5 ml Cl-free distd H_2O in a small weighing tube (thoroly cleaned inside and out) with a tightly fitting

glass stopper, close tube, and weigh accurately. Open tube, insert 0.4–0.5 g of the purified I_2, stopper tube, and again weigh. Obtain wt of I_2 by difference. (Moisture which collects on the outside of tube when iodine dissolves must be wiped off before weighing.) Place tube in the neck of a 500 ml erlenmeyer, held in inclined position, contg ca 200 ml Cl-free distd H_2O and ca 1 g analytical reagent grade KI. Allow tube to drop to bottom of flask, simultaneously removing stopper and allowing it to fall also, thereby avoiding loss of I_2 in transferring. Titrate I_2 immediately with Na thiosulfate soln, adding starch as indicator when soln becomes faint yellow. Calc. normality of the Na thiosulfate by equation: $N = \text{Wt } I_2/\text{ml thiosulfate} \times 0.12691$.[1]

Comments are solicited from the collaborators, with particular reference to any difficulty encountered or recommendations which may clarify the methods; whether any method is to be preferred; and whether more than one method for standardization of thiosulfate solutions should be included in *Official Methods of Analysis*.

Stenger (*Anal. Chem.*, **23**, 1543 (1951)) stated that the reaction was affected by the acid concentration of the $K_2Cr_2O_7$ solution. If any collaborator can confirm this observation, please give full details. The Referee varied the acid concentration by as much as 100% with no noticeable effect.

COMMENTS OF COLLABORATORS

Collaborator 1 found method 1 preferable, although he noted the end point was easily overrun. He suggested using 5 ml reagent grade HCl, as in the U.S.P. XIV method, which gives a green color and thus an easily discernible end point. He listed method 2 as second choice and found method 3 poorest.

Collaborator 2 felt that all three methods gave about equal precision but that method 1 seemed preferable because $K_2Cr_2O_7$ can easily be obtained in a pure state. He doubted if the acid concentration greatly affected the reaction, and suggested that the same effect would be obtained by an inexperienced analyst adding starch too soon and overrunning the end point.

Collaborator 3 did not experience difficulty with any of the methods. He suggested that it would be desirable to have an alternate method included in *Official Methods of Analysis*, and also thought the method of calculating the factor should be given.

Collaborator 4 preferred the use of an iodine flask (flanged) rather than an ordinary glass-stoppered flask in method 1. He considered method 2 the least desirable, but believed that method 3 could be included as an alternate.

Collaborator 5 preferred method 1 because of the ease of obtaining pure $K_2Cr_2O_7$. Collaborator 6 noted the decomposition of the KI in KI-acid solution when it was exposed to light.

CONCLUSIONS

A study of the results obtained by the collaborators (Table 1) and of previous work by the Associate Referee (*This Journal*, **37**, 459 (1954)) shows that the present A.O.A.C. method for standardizing sodium thiosulfate gives accurate and easily reproducible results. When the three methods used in this study were compared, it was noted that potassium dichromate can be more accurately weighed than the other reagents because of its higher equivalent weight. Further, it is a more convenient primary standard because it can be obtained in a pure form from the National Bureau of Standards. Both potassium iodate and iodine must be purified before use.

[1] Factor from 1954 atomic weight.

TABLE 1.—*Results of standardization of 0.1 N sodium thiosulfate by three methods*

COLLABORATOR	1	2	3	4	5	6	
Method 1 (potassium dichromate)	0.1010	0.1061	0.1002	0.1071	0.1079	0.1093	
	0.1010	0.1062	0.1003	0.1070	0.1080	0.1096	
	0.1010	0.1065	0.1003	0.1071		0.1094	
	0.1009	0.1059	0.1002	0.1072		0.1094	
	0.1011	0.1058	0.1003	0.1073		0.1095	
	0.1011	0.1063	0.1003	0.1071		0.1096	
			0.1002				
Method 2 (potassium iodate)	0.1011	0.1057	0.1004	0.1074	0.1081	0.1104	0.1096
	0.1012	0.1059	0.1005	0.1074	0.1082	0.1101	0.1096
	0.1012	0.1063	0.1004	0.1073		0.1101	0.1096
	0.1011	0.1061	0.1004	0.1076		0.1100	0.1103
	0.1011	0.1057	0.1004	0.1072		0.1099	0.1101
	0.1012	0.1055	0.1004	0.1074		0.1099	0.1094
			0.1003				0.1095
Method 3 (iodine)	0.1011	0.1054	0.1004	0.1073	0.1079	0.1096	
	0.1010	0.1055	0.1002	0.1071	0.1081	0.1096	
	0.1010	0.1059	0.1002	0.1073		0.1099	
	0.1011	0.1057	0.1005	0.1073		0.1101	
	0.1011	0.1058	0.1002	0.1071		0.1099	
	0.1011	0.1059	0.1002	0.1072		0.1099	
			0.1003				
			0.1002				

Distilled water which contains free chlorine yields erroneous results, and where potassium iodide-acid solution is specified (as in **39.36**), free iodine is liberated if the solution is exposed to light.

Two precautions were therefore found necessary: (1) Free chlorine must be eliminated from the distilled water; and (2) decomposition of the potassium iodide in the potassium iodide-acid solution must be avoided.

It is believed that only one standardization method need be included in *Official Methods of Analysis*.

RECOMMENDATION

It is recommended* that the potassium dichromate method for standardizing 0.1 *N* sodium thiosulfate be retained in the Eighth Edition of *Official Methods of Analysis* and that it be revised as follows to eliminate errors due to the use of distilled water containing free chlorine:

Place 0.20–0.23 g $K_2Cr_2O_7$, accurately weighed (National Bureau of Standards sample dried 2 hrs at 100°C.), in a glass-stoppered iodine flask and dissolve in 80

* For report of Subcommittee A and action of the Association, see *This Journal*, **38**, 68 (1955).

ml Cl-free distd H_2O contg 2 g analytical reagent grade KI. While swirling flask, add 20 ml ca normal HCl, prepd from Cl-free H_2O, and immediately place in the dark for 10 min. Then titrate with the $Na_2S_2O_3$ soln, **39.35,** adding starch soln after most of the I has been consumed. $N = \text{wt } K_2Cr_2O_7/0.04904 \times \text{ml}$ thiosulfate.

CAUTION: Distd H_2O contg as much as 0.5 ppm free Cl causes interference. If free Cl is present, remove by sufficient boiling or by some other method.

ACKNOWLEDGMENT

The Associate Referee is extremely grateful to the following collaborators, all of the Food and Drug Administration: Garland L. Reed, T. E. Byers, R. Edge, M. Harris, James H. Cannon, and Theodore S. Smith.

REPORT ON PLANTS

By E. J. MILLER (Michigan Agricultural Experiment Station, East Lansing, Mich.), *Referee*

A number of reports have been prepared by Associate Referees during the present year.

Erwin J. Benne, Associate Referee on Carotene in Plants, submitted recommendations relative to methods for carotene to be carried in Chapter 6 of the 8th Edition of *Official Methods of Analysis*, A.O.A.C.

Kenneth C. Beeson, Associate Referee on Cobalt and Copper in Plants, submitted a clarification of recommendations concerning methods for copper to be included in Chapter 6 of the 8th Edition of *Official Methods of Analysis.*

W. T. Mathis, Associate Referee on Potassium in Plants, reported on a general procedure for the flame photometric determination of potassium in plants, which had been developed with the help of a number of persons who are currently using this procedure.

Eunice J. Heinen, Associate Referee on Sodium in Plants, reported results of a collaborative study of a procedure involving the flame photometric evaluation of sodium extracted from plant tissue with ammonium acetate solution. On the basis of the results obtained in this study it was recommended that the procedure be included in Chapter 6 of the 8th Edition of *Official Methods of Analysis,* as an alternative method to the magnesium uranyl acetate gravimetric procedure.

Carrol L. Hoffpauir, Associate Referee on Starch in Plants, reported results obtained in a continuation of his study. The most promising method at present involves extraction of starch from the plant tissue with perchloric acid, purification by precipitation as the iodide, acid

hydrolysis of the starch to glucose, and determination of reducing sugars by copper reduction.

Kenneth T. Williams, Associate Referee on Sugars in Plants, reported that his study during the year centered chiefly around means of extracting sugars from plant materials without the use of Soxhlet extractors.

Erwin J. Benne, Associate Referee on Zinc in Plants, summarized his work and that of his associates on this subject during the past several years and made recommendations concerning methods for zinc to be carried in Chapter 6 of the 8th Edition of *Official Methods of Analysis*.

RECOMMENDATIONS

It is recommended*—

(1) That the Associate Referees on various constituents of plants, listed in *This Journal*, **37**, 5 (1954), continue with their respective assignments with the exception of Erwin J. Benne, Associate Referee on Carotene in Plants, whose subject is to be dropped.

(2) That another Associate Referee on Sodium in Plants be appointed to replace Miss Eunice J. Heinen, who recently resigned.

(3) That an Associate Referee on Molybdenum in Plants be appointed.

(4) That the following recommendations of the Associate Referees be accepted:

Carotene:

(*a*) That the method for mixed carotenes by phasic separation, **6.67**, be deleted.

(*b*) That the study be dropped.

(*c*) That a reference to carotene methods in the chapter on Nutritional Adjuncts be inserted in Chapter 6.

Cobalt and copper: That the method for copper in plants, **6.22** and **6.23**, be replaced by the method outlined in "Changes in Official Methods of Analysis" made at the Sixty-sixth Annual Meeting and reported in *This Journal*, **36**, 74 (1953), and be clarified by amending statements.

Sodium:

(*a*) That the procedure for the flame photometric determination of sodium in plant tissues in which 2 *N* ammonium acetate is used as the extractant be adopted as first action as an alternative procedure to the magnesium uranyl acetate gravimetric method.

(*b*) That the study be continued, especially in regard to (1) comparison of the use of an extraction procedure to dry ashing of plant tissue and (2) the use of 0.06 *N* ammonium oxalate as an extractant.

Starch: That the method described in the Associate Referee's report be subjected to collaborative study.

Sugars: That the study be continued.

Zinc:

(*a*) That the one-color procedure, as given in the report, be adopted as first action and that it be included in the 8th Edition of *Official Methods of Analysis* as an alternative procedure to the mixed-color method.

---

* For report of Subcommittee A and action of the Association, see *This Journal*, **38**, 66, 67 (1955).

(*b*) That the study be continued with special attention to the cause and correction of the variations in transmittance of the blank determinations.

(5) That the following first action methods be made official (references are to *Official Methods of Analysis*, 7th Ed.):

Titrimetric method for iron, **6.10**
Perchloric acid method for potassium, **6.18**
Magnesium uranyl acetate gravimetric method for sodium, **6.20–6.21**
Arsenic (already official in Chapter 34), **6.31** and **6.32**
Selenium, **6.47**
Sugars, **6.48–6.50**
Ether extract (already official in Chapter 22), **6.51**
Crude fiber (already official in Chapter 22), **6.52**
Total nitrogen (already official in Chapter 2), **6.53**
Organic and ammoniacal nitrogen (already official in Chapter 2), **6.54**

(6) That the following methods be adopted as first action:

Nitrosocresol method for cobalt
Nitroso-R-salt method for cobalt

(7) That the following methods be continued as first action:

Method II for copper, to replace **6.22** and **6.23**
Mixed-color dithizone method for zinc, **6.24–6.30**
Lignin, **6.55–6.59**
Total boron, **6.69**

(8) That the micro method for aluminum only, **6.11**, be deleted.

ACKNOWLEDGMENT

The Referee wishes to express his appreciation to the Associate Referees and all others who participated in the program for their cooperation and accomplishments during the year.

REPORT ON THE FLAME PHOTOMETRIC DETERMINATION OF POTASSIUM IN PLANT TISSUE

By W. T. MATHIS (Agricultural Experiment Station, New Haven, Conn.), *Associate Referee*

Comments were solicited on a draft of a method which gave rather detailed basic instructions for instrumentation and optional procedures for sample solution preparation. Copies went to members of a group which had previously participated in collaborative analysis of samples in which their own respective flame photometric techniques were used. As results obtained by these various techniques appeared to be about equally satisfactory in relation to results obtained by chemical methods, the method submitted for consideration was sufficiently general in design to permit use of any of these procedures.

On the basis of the excellent criticisms and suggestions received from the group the following conclusions were reached:

1. In view of the fact that specific instructions for the quantitative operation of each type and make of instrument are available from the manufacturer, it is neither necessary nor feasible to include such directions in a general method.

2. For the sake of uniformity and convenience the simplest possible procedure that proves to be satisfactory for preparation of sample solution should be adopted as standard.

With these points in mind the method was re-written as follows:

FLAME PHOTOMETRIC DETERMINATION OF POTASSIUM IN PLANT TISSUE

REAGENTS

(a) *KCl stock soln.*—Dissolve 1.907 g dry analytical reagent grade KCl in H_2O and dil. to 1 l. Contains 1000 ppm K.

(b) *LiCl stock soln.*—Dissolve 6.110 g analytical grade LiCl in H_2O and dil. to 1 l. Contains 1000 ppm Li.

(c) *Extractant.*—If internal std is to be used, dil. a portion of the LiCl stock soln to the concn required for the detn; otherwise, use distd H_2O as the extractant.

STANDARDS

Dil. appropriate aliquots of the stock solns to prep. series of stds contg K in stepped amounts (including zero) to cover the instrument range, and Li (if required) in the same concn as in the extractant.

SAMPLE EXTRACTION

Transfer a weighed portion (Note 1) of the finely ground and well-mixed plant tissue to an erlenmeyer of at least twice the capacity of the vol. of extractant to be used. Add measured vol. of the extractant, stopper flask, and shake vigorously at frequent intervals for at least 15 min. Filter thru dry, fast paper. (If paper clogs, pour the contents onto addnl clean filter and combine filtrates.) Use this soln for instrumentation.

NOTE 1: It is preferable for K concns in the exts to fall well within the instrument extremes. However, the ratio of sample weight (in g) to vol. of extractant (in ml) should not exceed 1:500, or slightly incomplete extn may result. Example: For samples contg 1–2.5% K, an instrument range of 0–35 ppm and a required Li level of 100 ppm, ext. 50 mg sample with 50 ml extractant contg 100 ppm Li.

ANALYSIS

It is assumed that the operator is thoroughly familiar with the quantitative manipulation of the instrument to be used. Such instructions are available from the manufacturers.

Be sure that the instrument has reached an operating equilibrium before running the sample solns. (Atomize portions of the stds toward the end of the warmup period until repeatable readings for the series are obtained.)

Run stds, covering the K range of the samples, at frequent intervals during atomization of series of sample solns. Repeat this operation with both std and sample solns sufficient number of times, depending upon precision of technique, to give a reliable average reading for each soln.

Plot analysis curve and compute % K in the samples.

The results of collaborative trial of this procedure on six samples of miscellaneous plant materials are shown in Table 1. The samples used had previously been submitted to the collaborators by Miss Eunice Heinen, Associate Referee on Sodium, for evaluation of a procedure for sodium determination. The materials, in numerical order in the series, were corn grain, oat grain, soybean grain, mixed hay, Ladino straw, and soybean plants. Collaborators 2, 3, 4, and 5 used water as the extractant, and did not use the internal standard. Collaborator 1 used 0.06 *N* ammonium oxalate which contained lithium for the internal standard; collaborator 6 used water which contained lithium for the internal standard.

TABLE 1.—*Collaborative analyses of six samples of miscellaneous plant materials by the flame photometric method; per cent K on "as received" basis*

COLLABORATOR	SAMPLE					
	1	2	3	4	5	6
1	0.41	0.41	1.72	1.41	1.66	0.65
2	0.41	0.39	1.64	1.46	1.77	0.57
3	0.40	0.38	1.50	1.50	1.65	0.69
4	0.39	0.37	1.61	1.49	1.69	0.68
5	0.37	0.33	1.53	1.42	1.65	0.65
6	0.39	0.38	1.76	1.48	1.70	0.64
Sample average	0.40	0.38	1.63	1.46	1.69	0.65
Chemical method[a]	0.40	0.40	1.73	1.43	1.62	0.63

[a] Performed by laboratory No. 6 according to the A.O.A.C. method: dry ashed, dissolved in HCl, etc.

The average coefficient of variation (for all samples) between the photometric laboratories in this study is 4.7 per cent. On the basis of previous collaborative studies, the agreement between laboratories appears to be about as good as can be expected for this instrumental determination, regardless of the procedure used for preparation.

The fact that the results on the water extracts for the six samples total exactly the same as the results by the chemical method, in which an acid solution of the ash is used, indicates that the water extraction as prescribed in the method is adequate. This conclusion is further confirmed by use of this method by collaborator No. 6 in analysis of a set of samples from a previous collaboration. These samples consisted of apple, cherry, citrus and peach leaves, and alfalfa, and were analyzed by a group of laboratories including six which used flame photometric methods and seven which employed chemical methods. All of the procedures involved dry ashing and subsequent solution in acid.

The study was jointly conducted in 1952 by Professor A. L. Kenworthy, of Michigan State College, and the writer. Flame photometric results

obtained in the Kenworthy study by five of the collaborators (or laboratories) who participated in the current investigation are shown in Table 2.

Inspection of the two tables shows that agreement between the laboratories and agreement of their averages with the results by the chemical method is closer with the proposed flame photometric method than with the more detailed procedures used in the Kenworthy study.

TABLE 2.—*Collaborative analyses of five plant samples in the Kenworthy study; per cent K on dry basis*

COLLABORATOR	SAMPLE				
	1	2	3	4	5
1	1.09	1.63	1.01	2.12	——
2	0.95	1.54	1.22	2.17	2.06
3	1.15	1.65	1.00	2.05	——
4	0.83	1.29	1.90	2.46	1.82
5	1.16	1.76	1.08	2.20	2.25
Sample average	1.04	1.57	1.24	2.20	2.04
Chemical methods[a]	1.14	1.66	1.06	2.15	2.23
Proposed method[b]	1.18	1.65	1.02	2.17	2.28

[a] Averages of the seven chemical laboratories.
[b] Currently performed by collaborator No. 6 (Laboratory 6 did not submit flame photometer results for the Kenworthy study).

COMMENTS OF COLLABORATORS

Collaborator 1 referred to an article by Attoe (*Soil Sci. Soc. Am. Proc.*, **12**, 131 (1948)) in which it was reported that a portion of the sodium and potassium in some plant tissues is in the exchangeable form. He suggested that this point should be studied further before the water extraction method is adopted.

Collaborator 2 felt that the simplicity of the method offered a great advantage, but thought that the small sample size introduced a source of error.

Collaborator 4 raised several objections, chiefly the suppressing effect of phosphate on potassium line intensity. He pointed out that the ratio of phosphorus to potassium in most plant materials gives rise to considerable suppression, even though the concentration of either is adjusted within wide limits. He stated that in his laboratory this effect has been greatly diminished by the addition of ammonium monohydrogen phosphate to all samples and standards.

Collaborator 5 experienced no difficulty with the method. He noted that the extract of sample 3 was quite murky after filtration; however, this apparently did not interfere with the operation of the instrument. He obtained very consistent results on triplicate extractions.

DISCUSSION

The comments of collaborator 4 are especially interesting because the current results from his laboratory when the proposed method was used are in much better agreement with the other laboratories than his previous results in the Kenworthy study, shown in Table 2. It is assumed that the precautionary measures with relation to phosphorus which he mentions in his comments were used at that time.

There are reports in the literature of inability to extract potassium from certain plant materials, including alfalfa, with water alone. These findings have not been confirmed in our laboratory. It has been found that extraction is incomplete when the ratio of sample weight to volume of extractant greatly exceeds the limits given in the proposed method (say, 1 g to 50 ml), but where these details of the method have been followed, satisfactory extraction has resulted in every case. Incidentally, boiling with the extractant does not change this situation.

This method is obviously not perfect but the results indicate that it is probably less likely to produce extremely erratic results when used by different analysts than flame photometric methods which employ more complicated preparation and standardization procedures. It is recommended* that the method be adopted as first action.

ACKNOWLEDGMENT

The Associate Referee wishes to thank the following collaborators for their kind and valuable assistance in this study: Miss Eunice Heinen, Michigan State College; W. G. Schrenk, Kansas State College; Alston W. Specht, Agricultural Research Service, Beltsville, Md.; E. E. Pickett, University of Missouri; J. G. Brown, University of California; and S. R. Squires, Connecticut Agricultural Experiment Station.

REPORT ON SODIUM IN PLANTS†

By Eunice J. Heinen (Michigan Agricultural Experiment Station, East Lansing, Mich.), *Associate Referee*

Following the recommendations in last year's report (1), the study this year centered primarily around (*a*) the effectiveness of different extractants for removing sodium from dry plant tissues and the subsequent evaluation of the sodium in the extracts with a flame photometer; (*b*) a comparison of the sodium values thus obtained with those by the A.O.A.C.

* For report of Subcommittee A and action of the Association, see *This Journal*, **38**, 66, 67 (1955).

† Published with the approval of the Director of the Michigan Agricultural Experiment Station as Journal Article No. 1671.

magnesium uranyl acetate gravimetric procedure (2); and (*c*) a collaborative study of a procedure involving the flame photometric evaluation of sodium extracted from plant tissues with ammonium acetate solution.

The initial study to determine the reliability of a direct extraction procedure for removing sodium from dried plant tissue, and the possible advantages of such a practice over dry ashing techniques, included the use of hot water and aqueous solutions of hot normal acetic acid, hot 0.25 *N* hydrochloric acid, hot 0.9 *N* nitric acid, and 2 *N* ammonium acetate at room temperature, respectively, as extractants. In the case of the first four extractants, weighed portions of finely ground plant tissue were transferred to 200 ml volumetric flasks, 100 ml of extractant was added, and the flasks were heated on a steam-bath for approximately 30 minutes. Then 10 ml of a saturated solution of ammonium oxalate was added to each flask (to remove calcium from solution), and each of the three acid solutions was nearly neutralized with ammonium hydroxide. The solutions were then permitted to cool to room temperature (overnight in this experiment), an appropriate quantity of lithium chloride was added as an internal standard, and the solutions were diluted to volume with distilled water. After the solutions were thoroughly mixed, portions of the supernatant liquid from each were filtered and their sodium contents were evaluated flame photometrically. The procedure used for the ammonium acetate extraction of the plant tissues was essentially that of Seay, Attoe, and Truog (3). The weighed plant tissue was transferred to an Erlenmeyer flask, a measured quantity of the 2 *N* ammonium acetate solution, which also contained ammonium oxalate and lithium chloride, was added, and the flask was shaken occasionally over a period of 1 hour. A portion of the supernatant liquid was filtered, and its sodium concentration was determined flame photometrically. In all cases the sodium values obtained were compared with those by the A.O.A.C. magnesium uranyl acetate gravimetric procedure. The results obtained in this study are given in Table 1.

TABLE 1.—*Sodium values by different procedures*

PLANT TISSUE ANALYZED	EXTRACTION-FLAME PHOTOMETRIC PROCEDURE					A.O.A.C. MAGNESIUM URANYL ACETATE METHOD
	SODIUM EXTRACTED WITH					
	HOT DISTD H_2O	1.0 *N* ACETIC ACID	0.25 *N* HCl	0.9 *N* HNO_3	2 *N* NH_4 ACETATE	
			per cent			*per cent*
Celery plants	0.98	1.01	1.03	1.04	1.00	0.98
Onion bulbs	0.026	0.026	0.027	0.027	0.024	0.024
Timothy hay	0.003	0.003	0.004	0.003	0.003	0.003
Wheat grain	0.002	0.003	0.001	0.002	0.001	0.001

This table shows that there was very good agreement in the sodium values obtained for each of the plant tissues by use of the various extractants, and that these values agree well with those obtained by the A.O.A.C. method. These results were encouraging; they indicated complete extraction of sodium by each of the five extracting solutions, the use of which is less time consuming than the dry ashing technique. However, the rate of filtration was rather slow in a few instances; this was especially true in the extraction of the onion bulbs and wheat grain with the acetic acid solution. In the author's opinion, the ammonium acetate solution is more advantageous to use than the other extractants because of the simplicity of its procedure and the somewhat faster rate of filtration.

Six plant tissues (later submitted for collaborative study, the results of which are given in this report) were then analyzed for sodium, both by use of the 2 *N* ammonium acetate extracting solution and by the A.O.A.C. magnesium uranyl acetate procedure. The results obtained are presented in Table 2 and show that the sodium values by the two procedures agree very well.

TABLE 2.—*Sodium values by the ammonium acetate extraction-flame photometric procedure and the A.O.A.C. gravimetric method*

PLANT TISSUE ANALYZED	NH_4 ACETATE EXTN-FLAME PHOTOMETRIC PROCEDURE	A.O.A.C. MG URANYL ACETATE METHOD
	per cent	*per cent*
Corn grain	0.270, 0.272	0.287, 0.264
Oat grain	0.005, 0.005	—
Soybean seed	0.002, 0.002	0.001, 0.003
Mixed hay	0.017, 0.017	0.015, 0.018
Ladino straw	0.117, 0.117	0.115, 0.112
Soybean plants	0.003, 0.003	0.001, 0.007

Attoe (4) and Seay, *et al.*, (3) have also published data illustrating the completeness of extracting sodium from plant tissues by salt solutions. The extracting solution used in the first instance was 2 *N* with respect to ammonium acetate and 0.2 *N* with respect to magnesium acetate, and in the latter work, the solution was 2 *N* with respect to ammonium acetate and contained ammonium oxalate for the removal of calcium. Attoe stated that sodium was found to be present in plant tissue entirely in water-soluble and exchangeable forms (4). He also presented a table containing the sodium values obtained for 10 different kinds of plant tissues when both an extraction of the plant tissue with a salt solution and an extraction of the plant ash were used. The sodium values obtained by these two procedures were in excellent agreement. The Associate Referee's additional experience with the 2 *N* ammonium acetate solution as extractant has likewise shown that it completely extracts the sodium. In

view of all this evidence that the sodium in plant tissue is completely extractable with a salt solution, and of the advantage of the procedure over the dry ashing technique, the Associate Referee submitted several samples of plant tissue for a collaborative study of the 2 *N* ammonium acetate extraction procedure and the subsequent flame photometric evaluation of the sodium.

A set of six plant tissues was sent to each of seven collaborators, together with a copy of the procedure to be followed. The collaborators were also asked to state the type of flame photometer used, whether the direct intensity or internal standard method of evaluation was used, and any comments concerning their experience with the method. The procedure which was followed in this study is given below.

PROCEDURE[1]

REAGENTS

(a) *4 N Ammonium acetate stock solution.*—Dissolve 308.3 g $NH_4C_2H_3O_2$ in H_2O and dil. to 1 l. Or prep. by dilg 228 ml glacial acetic acid with ca 300 ml H_2O, adding with agitation 270 ml concd NH_4OH, adjusting to *p*H 6.9 with NH_3 or acetic acid, and dilg to 1 l.

(b) *0.24 N Ammonium oxalate stock solution.*—Dissolve 17.0 g $(NH_4)_2C_2O_4 \cdot H_2O$ in H_2O and dil. to 1 l.

(c) *NaCl standard stock solution (containing 1000 ppm Na).*—Dissolve 2.542 g NaCl in H_2O and dil. to 1 l.

(d) *LiCl standard stock solution (containing 1000 ppm Li).*—(Needed only if the internal std method of evaluation is used.) Dissolve 6.110 g LiCl in H_2O and dil. to 1 l.

(e) *Extracting solution.*—(2 *N* with respect to NH_4 acetate and 0.06 *N* with respect to NH_4 oxalate, and contg 25 ppm Li if the internal std method is used). To 500 ml of the stock soln of NH_4 acetate add ca 175 ml H_2O and 250 ml of the 0.24 *N* NH_4 oxalate stock soln; if the internal std method of evaluation is used add 25 ml of the std LiCl stock soln (or a quantity found to be most satisfactory for the instrument used). Dil. to exactly 1 l with H_2O.

(f) *Standard NaCl calibrating solutions.*—To prep. the std solns, dil. appropriate aliquots of the stock soln contg 1000 ppm Na with 50 ml of the 4 *N* NH_4 acetate stock soln and 25 ml of the 0.24 *N* NH_4 oxalate stock soln. If the internal std is to be used, add 2.5 ml of the soln contg 1000 ppm Li (or a quantity to give the same final Li concn as that of the extg soln). Finally dil. to 100 ml with H_2O. (In the analysis of plant tissues, where a Perkin-Elmer model 52A flame photometer is used, 20 ppm Na is a convenient top standard.)

EXTRACTION AND DETERMINATION OF SODIUM IN PLANT TISSUE

Place a weighed sample (usually 1 g is appropriate) of finely ground plant tissue in a flask and add an accurately measured vol. of the extg soln (50 ml is generally suitable for most plant tissues when 1 g samples and a top std of 20 ppm Na are used). Stopper flask and swirl occasionally over a period of at least 1 hr. Filter a portion of the supernatant liquid, discarding the first few ml of filtrate. (Folded S&S No. 588-HF filter paper is very satisfactory and permits rapid filtration. Much difficulty has been experienced in using filter paper of finer porosity because of slow filtration; in some instances satisfactory filtration is nearly impossible.) Use filtrate in the flame photometer for evaluation of the Na content.

[1] See SEAY, ATTOE, and TRUOG, *Soil Sci.*, **71**, 83 (1951).

NOTE: it is very important that all glassware used in the detn of Na be previously rinsed with a dil. HNO_3 soln, followed by several rinsings with distd H_2O. Reasonable care must be exercised to protect the test solns from air-borne Na contamination.

The results obtained in this study are given in Table 3. Several collaborators also analyzed these samples for their sodium content by other techniques. These values are also shown in Table 3, and a description of the procedure used is included in the comments of the collaborators.

COMMENTS OF COLLABORATORS

Collaborator No. 1: The first comparisons were made by comparing ashed samples to those run with the Attoe extract. However, the samples were ashed in Pyrex beakers which gave very high results; the samples were then rerun in porcelain crucibles. As you will note, the soybean grain was very difficult to filter. If a good furnace is available it is quite easy to ash the samples and take up the sodium with HCl. In that way all of the sodium is obtained. In a run not reported, it appeared that the materials were not being completely wetted by the extracting solution; the cause of the trouble was that some of the tissue was quite oily, such as soybean seeds. Perhaps this is a factor with the Attoe extract and should be given some consideration. However, the analyses with second and third extractions checked fairly well.

We followed instructions explicitly with Attoe extract, and we found soybean grain very difficult to filter. The ashing procedure used was as follows: Heat 1 g tissue in porcelain crucible at 250°C. for 2 hrs and then at 525°C. for 2 hrs, until clean gray ash free of carbon is obtained (except for mixed hay). Transfer to filter paper with 50 ml 0.8 *N* HCl and wash with 40 ml distd H_2O, collecting in 100 ml vol. flask; then make up to vol. (Samples were ashed in the first run in 50 ml Pyrex beakers; results were not reported. The results in this detn were considerably higher than in cases where porcelain crucibles were used, indicating that Na contamination from Pyrex glass occurs when samples are dry ashed.) The flame photometer used for evaluating sodium concentration was a Beckman Model DU with photomultiplier No. 4300; direct intensity slit 0.01 mm; O_2, 10 lb; C_2H_2, 7 lb.

Collaborator No. 2: The check sets of determinations were run on different days, and one set was run by using extracting solution and standards in which the ammonium acetate and oxalate were deliberately omitted (second line of values for Collaborator No. 2 in Table 3) to evaluate the effect of these reagents. This work was done on a Barclay (filter) instrument, and 100 ppm of lithium was used as internal standard.

The use of a cold extraction appears to be completely satisfactory for sodium and should apply equally well to potassium. A few limited experiments indicate that these elements are extracted quantitatively with distilled water, and I have simplified the extractant to this medium (except where internal standard is required) in a proposed procedure for potassium determination. We do not encounter any appreciable interference from matrix elements with our technique under these extraction conditions.

Collaborator No. 3: The procedure seems to be quite satisfactory. We used a Beckman Model B flame photometer, without internal standard.

Collaborator No. 4: We attempted to follow the proposed method as closely as possible. Trials 1 and 2 were conducted on separate days with separately weighed samples. In order to determine how nearly these data agree with other techniques, we carried out two other experiments with these samples in which 1 g quantities of each of the Referee samples were ashed at 600°C. overnight. The ash was treated with a minimum amount of HCl and made up to a known volume. This solution was then read on the flame photometer and no attempt was made to compensate for the

TABLE 3.—*Results from the collaborative study expressed as per cent sodium (duplicate values are given in most instances)*

COLLABORATOR	METHODS USED	SAMPLES ANALYZED											
		CORN GRAIN		OAT GRAIN		SOYBEAN SEEDS		MIXED HAY		CLOVER STRAW		SOYBEAN PLANTS	
1	A[a]	0.261	0.264	0.004	0.005	0.004	0.003	0.021	0.020	0.095	0.112	0.005	0.004
	B[b]	0.275	0.272	0.005	0.005	0.004	0.006	0.022	0.022	0.132	0.135	0.006	0.006
2	A	0.273	0.287	0.010	0.012	0.005	0.008	0.021	0.023	0.113	0.114	0.009	0.011
	B	0.271		0.011		0.009	0.005	0.024		0.125		0.013	
3	A	0.270	0.274	0.005	0.005	0.002	0.002	0.018	0.018	0.119	0.115	0.004	0.004
4	A	0.295	0.293	0.015	0.010	0.011	0.008	0.026	0.025	0.140	0.140	0.011	0.009
	B	0.300		0.008		0.007		0.032		0.230		0.025	
	B	0.300		0.012		0.010		0.031		0.170		0.015	
5	A	0.300		0.013		0.010		0.025		0.150		0.010	
	B	0.190		0.007		0.005		0.014		0.110		0.014	
6	A	0.273	0.260	0.008	0.008	0.004	0.003	0.022	0.022	0.125	0.127	0.005	0.007
Author	A	0.270	0.272	0.005	0.005	0.002	0.002	0.017	0.017	0.117	0.117	0.003	0.003
	C[c]	0.287	0.264	—		0.001	0.003	0.015	0.018	0.115	0.112	0.001	0.007

[a] A: Ammonium acetate extraction-flame photometric procedure.
[b] B: See comments of collaborators for description of methods used.
C: A.O.A.C. magnesium uranyl acetate gravimetric procedure.

effect of interfering ions. The standards contained only sodium chloride. (The results obtained in this manner are shown in the second line of values for Collaborator No. 4 in Table 3.) In another trial each of the samples was ashed in the manner previously described, put in solution with a minimum amount of HCl, and made up to a known volume. To each sample an aliquot of ammonium oxalate was then added. These solutions were thoroughly stirred and the precipitate which formed was allowed to settle. Flame determinations were then made on the solution decanted from each sample. (The results obtained in this manner are shown in the third line of values for Collaborator No. 4 in Table 3.) In this case the standards were similar to those used in the previous trial. I believe these data are interesting for comparison purposes and definitely indicate the combined effects of calcium and magnesium ions on sodium determinations.

We used the Beckman Model DU Spectrophotometer with the newer model flame photometer attachment for these measurements. As a result we did not employ the internal standard technique. Sodium standards were prepared from reagent quality sodium chloride dried at 100°C. and stored in a desiccator until weighed.

This is our first attempt to use this method of analysis for sodium. It seems to be a relatively simple way to obtain data on the sodium concentration of plant tissues. The only criticism would be that extraction of sodium might not be complete. Although we did no work on these samples in regard to completeness of extraction, this point should be definitely established if the method is to be used widely.

Collaborator No. 5: The samples were prepared for the flame photometer according to the specified procedure furnished by the Referee. The determinations were made by using a Beckman Model DU spectrophotometer with a laboratory constructed flame unit. The flame unit is very similar to the one used in the Perkin-Elmer Model 52A flame photometer.

The spectrographic determinations were made with a Bausch & Lomb large Littrow spectrograph, according to Method 1, D.C. Arc Excitation, in *This Journal*, **37**, 83 (1954).

Collaborator No. 6: The samples were analyzed for sodium by the procedure outlined for the direct intensity method. No difficulties were encountered, and the values obtained are quite consistent and agree very well, considering the small amounts involved.

In general, as shown in Table 3, there is good agreement in the sodium values reported by the various collaborators, and these values in turn agree well with those obtained by the A.O.A.C. procedure. This flame photometric procedure is considerably less time consuming than the A.O.A.C. gravimetric procedure and is especially advantageous in the analysis of plant tissues which have a very low sodium content.

Following the suggestion of Collaborator No. 2, the Associate Referee undertook a limited study of the effectiveness of cold distilled water as an extractant for sodium in dry plant tissues. The six samples submitted to the collaborators, as well as the four plant tissues included in the initial study, were analyzed for their sodium content by this method. The procedure used was identical to that furnished the collaborators with the exception that reagent No. 1 (4 N ammonium acetate) was no longer used, and the extracting solution was again 0.06 N with respect to ammonium oxalate and contained lithium as an internal standard. The results obtained are given in Table 4.

TABLE 4.—*Sodium values obtained by the use of 0.06 N ammonium oxalate as the extractant*

PLANT TISSUE ANALYZED	SODIUM EVALUATED FLAME PHOTOMETRICALLY
	per cent
Celery plants	0.99
Onion bulbs	0.024
Timothy hay	0.006
Wheat grain	0.002
Corn grain	0.277
Oat grain	0.006
Soybean seed	0.003
Mixed hay	0.018
Ladino straw	0.122
Soybean plants	0.004

As can be seen from the tables, the sodium values obtained were in very good agreement with those obtained by use of the A.O.A.C. gravimetric procedure, the 2 *N* ammonium acetate solution, and the various other extractants, which indicates that in the plant tissues used in this study, the sodium present was completely soluble in these dilute salt solutions. If this should be universally true, the procedure submitted for collaborative study could be further simplified by the use of ammonium oxalate solution as the extractant.

ACKNOWLEDGMENT

The Associate Referee wishes to express sincere appreciation to the following collaborators for their fine cooperation in this study: L. E. Engelbert, Department of Soils, University of Wisconsin; W. T. Mathis, Connecticut Agricultural Experiment Station; E. E. Pickett, Department of Agricultural Chemistry, University of Missouri; W. G. Schrenk, Department of Chemistry, Kansas State College; Alston W. Specht, Horticultural Crops Research Branch, United States Department of Agriculture; J. G. Brown, Department of Pomology, University of California.

RECOMMENDATIONS

It is recommended*—

(1) That the procedure for the flame photometric determination of sodium in plant tissues in which 2 *N* ammonium acetate is used as the extractant be adopted as first action, as an alternative procedure for the A.O.A.C. magnesium uranyl acetate gravimetric method.

(2) That the study be continued, especially in regard to (*a*) the use of an extraction procedure as compared to dry ashing of plant tissues and (*b*) the use of 0.06 *N* ammonium oxalate as an extractant.

* For report of Subcommittee A and action of the Association, see *This Journal*, **38**, 66, 67 (1955).

REFERENCES

(1) HEINEN, E. J., *This Journal*, **37**, 714 (1954).
(2) *Official Methods of Analysis*, 7th Ed., A.O.A.C., Wash. 4, D. C., 1950.
(3) SEAY, W. A., ATTOE, O. J., and TRUOG, E., *Soil Sci.*, **71**, 83 (1951).
(4) ATTOE, O. J., *Soil Sci. Soc. Am. Proc.*, (1947), **12**, 131 (1948).

REPORT ON STARCH IN PLANTS

By CARROLL L. HOFFPAUIR (Southern Utilization Research Branch, New Orleans, La.), *Associate Referee*

The results of a collaborative study of a procedure for the determination of starch, involving the use of anthrone, were reported last year (1). The values reported by the collaborators showed poor precision and were appreciably higher than corresponding values obtained previously by other methods. The comments of several of the collaborators indicated they had encountered difficulty with the final color development. Consequently, an examination of the recovery of starch in this step of the procedure was undertaken. Weighed samples of sweet potato starch of known purity were gelatinized by heating in a boiling water bath with distilled water, dispersed in 4.8 *N* perchloric acid at room temperature, transferred to volumetric flasks, and diluted to volume. Recovery of this starch was determined by using several different lots of anthrone. Ten aliquots of the starch solution were allowed to react with each lot of the anthrone reagent as previously described (2) and results were compared with standards prepared at the same time from National Bureau of Standards glucose. It is seen from the data in Table 1 that widely varying values were obtained. Although all of the preparations melted at 154–155°C. (corr.) as reported for anthrone (4), only one lot showed suitably accurate and reproducible results. This preparation consisted of colorless crystals although all of the others, including one synthesized in the laboratory by Meyer's directions (4), were yellowish even after repeated recrystallizations. Most of the

TABLE 1.—*Influence of different lots of anthrone on recovery of sweet potato starch from a solution of known starch content*

SOURCE OF ANTHRONE	RECOVERY		
	HIGH	LOW	AVERAGE
	per cent	*per cent*	*per cent*
Commercial lot A	101.0	96.6	99.0
Commercial lot B	120.8	95.0	109.0
Commercial lot B (soln 1 day old)	140.9	116.8	126.7
Commercial lot C	160.8	119.3	143.4
Laboratory preparation	150.9	131.8	139.5
Laboratory preparation, recrystd	142.9	115.3	132.3

TABLE 2.—*Starch content of several plant materials as determined by the perchloric acid extraction procedure*

SAMPLE	TEST				AVERAGE
	A	B	C	D	
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Buckwheat leaves	8.6	8.5	8.4	7.9	8.35
Peanut meal	7.4	7.2	7.3	7.5	7.35
Alfalfa	0.07	0.07	0.04	0.05	0.06
Orange rind	0.4	0.5	0.3		0.4
Gladiolus leaves	0.0	0.0	0.0	0.0	0.0

individual recovery values obtained with these lots of anthrone not only varied widely, but were considerably above 100 per cent. Several of the collaborators in last year's study reported poor reproducibility when carbothrone was substituted for anthrone. This step in the procedure must therefore be considered unsatisfactory because of the difficulty of obtaining a suitable anthrone reagent.

Several other possible procedures for the determination of starch in plant materials were examined. The most promising of these appears to be a modification (3) of the Pucher, Leavenworth, and Vickery method (5). It involves extraction of starch with perchloric acid, purification by precipitation as the iodide, acid hydrolysis of the purified starch to glucose, and determination of reducing sugars by copper reduction. The recovery of starch from peanut meal and buckwheat leaves at each step of this procedure prior to acid hydrolysis was found to be satisfactory by colorimetrically measuring the absorbance at 565 mμ after color development with iodine.

Values obtained when this method was applied to several types of plant material, involving a number of constituents which might interfere (3), are reported in Table 2. These values, which include replicate determinations analyzed at different times, and in several instances by two analysts, are in reasonable agreement. Since the procedure appears to be reproducible, it is recommended* that it be subjected to collaborative study.

ACKNOWLEDGMENT

The Associate Referee wishes to thank Mr. R. M. H. Kullman for performing some of the analyses.

REFERENCES

(1) HOFFPAUIR, C. L., *This Journal*, **37**, 716 (1954).
(2) ———, *ibid,*. **35**, 398 (1952).
(3) ———, *ibid.*, **33**, 810 (1950).
(4) MEYER, K. H., in *Organic Syntheses*, Coll. Vol. I, 2nd Ed., ed. by Gilman and Blatt, John Wiley & Sons, Inc., New York, 1941, p. 60.
(5) PUCHER, G., LEAVENWORTH, C., and VICKERY, H., *Anal. Chem.*, **20**, 850 (1948).

* For report of Subcommittee A and action of the Association, see *This Journal*, **38**, 66, 67 (1955).

REPORT ON SUGARS IN PLANTS

By KENNETH T. WILLIAMS, *Associate Referee*, and EARL F. POTTER
(Western Utilization Research Branch, Agricultural Research Service, U. S. Department of Agriculture, Albany 6, Calif.)

The Associate Referee has begun an investigation of extraction procedures in the attempt to save the analyst's time and to maintain or improve the accuracy of the determination of sugar in plant materials. This report describes the first experiment, which was designed to show whether the volume occupied by the alcohol-insoluble material could be disregarded, and whether the sugar could be extracted without the use of Soxhlet equipment.

The materials were comminuted in an electric blender without addition of liquid. Then samples of equal weight were transferred to vessels containing sufficient 95 per cent ethyl alcohol to give an 80 per cent alcohol solution. The mixtures were heated one hour on the steam bath, transferred to a 500 ml, a 1000 ml, and a 2000 ml flask, respectively, and made to volume with 80 per cent alcohol. Samples of asparagus, potatoes, and peas were also taken from the blender for extraction by the present method.[1]

The alcoholic solutions were filtered, and aliquots of 250, 500, and 1000 ml were taken from the samples that had been diluted to 500, 1000, and 2000 ml, respectively. After evaporation of the alcohol and clarification, each aliquot was diluted to 250 ml and the analysis was completed.[2]

(Blending as described above is not recommended for analysis and was used in this experiment only to insure that uniform samples would be obtained. It is the practice of the authors to use a food grinder, vegetable slicer, vegetable dicer, or similar equipment to prepare a representative sample. A suitable portion of the sample is added to sufficient boiling 95 per cent alcohol to make an 80 per cent alcohol solution. After the solution is boiled for one hour, the liquid is decanted into a volumetric flask and the solid material is comminuted in an electric blender with 80 per cent alcohol. The mixture is added to the volumetric flask, the volume is adjusted with 80 per cent alcohol, and the analysis is completed as described above.)

The data given in Table 1 were calculated on the basis that the alcohol-insoluble material (A.I.M.) did not occupy a significant volume. Thus x equals the volume occupied by the A.I.M., and the aliquot taken, $250/(500-x) = 500/(1000-x) = 1000/(2000-x) = \frac{1}{2}$ of each sample, since x is insignificant. If, however, the volume occupied by the A.I.M. had been significant, the sugar values obtained in the analyses in which a 500 ml volumetric flask was used would be significantly higher than those ob-

[1] *Official Methods of Analysis*, 7th Ed., A.O.A.C., 6.2(b) and 6.48(a).
[2] *Ibid.*, 29.36 and 29.38.

TABLE 1.—*A comparison of the sugar content[a] of plants obtained by using a fixed sample size and varying the volume of 80% alcohol for extraction*

MATERIAL	SAMPLE	500 ML REPLICATES				AV.	1000 ML REPLICATES				AV.	2000 ML REPLICATES				AV.
	grams	*per cent*				*per cent*	*per cent*				*per cent*	*per cent*				*per cent*
Apple	12	7.49	7.38	7.44	7.45	7.44	7.53	7.51	7.49	7.49	7.50	7.40	7.42	7.40	7.43	7.41
Asparagus[b]	20	1.81	1.81	1.80	1.81	1.81	1.81	1.79	1.78	1.80	1.80	1.82	1.81	1.81	1.82	1.81
Cabbage	30	2.95	2.93	2.95	2.94	2.94	2.95	2.91	2.92	2.93	2.93	2.95	2.93	2.94	2.93	2.94
Carrot	50	2.15	2.14	2.14	2.13	2.14	2.12	2.11	2.12	2.12	2.12	2.15	2.13	2.12	2.12	2.13
Pea[b]	20	3.42	3.43	3.43	3.43	3.43	3.48	3.50	3.47	3.48	3.48	3.37	3.39	3.40	3.40	3.39
Potato[b]	100	0.20	0.20	0.20	0.19	0.20	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21

[a] The reducing sugar content of apple, cabbage, carrot, and potato and the total sugar content of asparagus and peas were determined. All values are expressed as per cent glucose.

[b] The asparagus, peas, and potatoes were extracted by the present method **6.2 (b)**; 1.79, 3.48, and 0.21% sugar, respectively, were found.

tained when a 2000 ml volumetric flask was used. The data do not show a significant difference.

The values obtained for asparagus, potatoes, and peas by the proposed type of extraction agree very well with those by the present Soxhlet method[1], as shown in Table 1.

It is recommended* that the study of extraction of sugar from plant material without Soxhlet equipment be continued.

REPORT ON ZINC IN PLANTS

By ERWIN J. BENNE (Michigan Agricultural Experiment Station, East Lansing, Mich.), *Associate Referee*

In 1941 Cowling and Miller (1) published a method for determining zinc in plants, in which the zinc was converted to the dithizonate and evaluated photometrically in carbon tetrachloride solution. Since the solution contained not only the colored zinc dithizonate but excess dithizone as well, the procedure has been referred to as the mixed-color method. During the same year, Cowling, who was then Associate Referee on Zinc in Plants, reported the results of a collaborative study of the mixed-color method (2) and recommended that it be included in *Official Methods of Analysis*. Later Cowling was succeeded as Associate Referee by the author, who, with his associates, has published a number of reports on the subject, including those of Shirley, *et al.* (3, 4) and those of Heinen and Benne (5–7).

These later studies have been largely directed toward simplification of Cowling's method without loss of accuracy. In the 1951 report (6) Heinen and Benne described a simplified one-color procedure which gave results in good agreement with those by the mixed-color method. Later this procedure was submitted to collaborative study, and the results obtained (7) showed that the values for zinc reported by the different collaborators who used the simplified procedure agreed well with those by the other procedures employed, which included the A.O.A.C. mixed-color method.

As pointed out in an earlier report (5), the one-color procedure possesses the disadvantage of difficulty in obtaining consistent transmittance readings for the blank determinations, even with a given set of reagents. The Associate Referee has never been able to detect and correct the cause, and unfortunately the same is true of the mixed-color procedure. Therefore, since the one-color procedure possesses the advantages of being simpler and less time-consuming than the original mixed-color procedure, the Associate Referee feels justified in recommending its adoption.

---

* For report of Subcommittee A and action of the Association, see *This Journal*, 38, 66, 67 (1955).

ONE-COLOR PROCEDURE

REAGENTS

(a) *Carbon tetrachloride.*—Reagent grade.

(b) *Standard zinc solutions.*—(1) *Solution containing 1 mg Zn per ml:* Place 0.25 g pure Zn in a 250 ml vol. flask, add ca 50 ml distd H_2O and 1 ml concd H_2SO_4, and heat on a steam bath until Zn is dissolved. Cool, dil. to mark with distd H_2O, mix, and store in a Pyrex vessel. (2) *Solution containing 10 mmg Zn per ml:* Dil. 10 ml of (1) to 1 l and store in a Pyrex vessel.

(c) *Ammonium hydroxide.*—(1) *Normal solution:* Prep. soln slightly stronger than normal from reagent grade concd NH_4OH, stdze against std acid, make necessary diln, and store in a Pyrex vessel. (2) *0.01 N solution:* Dil. 20 ml of (1) to 2 l.

(d) *Normal hydrochloric acid solution.*—Prep. soln slightly stronger than normal from reagent grade concd acid, stdze against std base, make necessary diln, and store in a Pyrex container.

(e) *Diphenylthiocarbazone (dithizone) reagent.*—(1) *Solution I:* Dissolve 0.2 g dithizone in 500 ml CCl_4, and filter to remove insoluble material. Place soln in a large separatory funnel, add 2 l 0.02 *N* NH_4OH soln (40 ml normal soln dild to 2 l), and shake vigorously to ext. dithizone into aq. phase. Allow layers to sep., and discard CCl_4 layer. Ext. ammoniacal soln of dithizone with a 100 ml portion of CCl_4 and discard CCl_4 layer. Continue extn with successive 100 ml portions of CCl_4 until ext. is pure green color. Add 500 ml CCl_4 and 45 ml normal HCl to the ammoniacal soln of dithizone and shake to ext. the dithizone into the CCl_4. Allow layers to sep. and discard the aq. layer. Dil. the CCl_4 soln of dithizone to 2 l with CCl_4, and store in a brown bottle in a dark, cool place. (2) *Solution II:* Dil. 1 vol. of Solution I with 4 vols of CCl_4.

(f) *Ammonium citrate solution.*—(1) *Solution I (0.5 M):* Dissolve 226 g $(NH_4)_2HC_6H_5O_7$ in 2 l H_2O. Add NH_4OH (80–85 ml) until *p*H of soln is 8.5–8.7. Add excess of the dithizone soln (orange-yellow color in aq. phase after shaking and sepn of phases), and ext. with 100 ml portions of CCl_4 until ext. is full green color, adding more dithizone if necessary. Sep. and discard the CCl_4 layer and store aq. soln in a Pyrex bottle. (2) *Solution II:* Add 70 ml normal NH_4OH to 500 ml Solution I and dil. to 2 l.

(g) *Carbamate solution.*—Dissolve 1.25 g diethyldithiocarbamate in distd H_2O and dil. to 1 l. Store in refrigerator (prep. fresh after long periods of storage).

DETERMINATION

(a) *Ashing and extraction of ash.*—Weigh 2 g portions of finely-ground plant material into suitable crucibles (well-glazed porcelain crucibles and Vycor vessels can be used as well as Pt dishes), include crucibles for blank detns, and heat in a muffle furnace at 500–550°C. until ashing is complete. Cool, moisten ash with a little H_2O, add 10 ml normal HCl (more if necessary to insure an excess of acid), and heat on a steam bath until all soluble material is dissolved. Add a few ml hot H_2O, and filter thru quantitative paper into a 200 ml vol. flask. Wash the filter with hot H_2O until washings are no longer acid to Me red indicator. Add 2 drops Me red soln to filtrate, neutralize with normal NH_4OH, add exactly 3.2 ml normal HCl, dil. to vol. with H_2O, and mix.

(b) *Removal of interferences, formation of zinc dithizonate, and separation of excess dithizone.*—Pipet an aliquot (25 ml is usually satisfactory) of the ash ext. contg not more than 15 mmg Zn into a 125 ml amber glass separatory funnel. (If necessary to use aliquot of different vol., add 0.4 ml 0.2 *N* HCl for each 5 ml less, or 0.4 ml 0.2 *N* NH_4OH for each 5 ml more than 25 ml. If less than 25 ml of the ext. is taken, add H_2O to bring vol. to 25 ml.)

Add 10 ml dithizone reagent, Solution I, to the aliquot in the separatory funnel, and shake vigorously for 1 min. Allow layers to sep., and discard CCl_4 layer. Add

2 ml CCl_4 to the aq. soln, permit layers to sep., and discard the CCl_4. Repeat this rinsing process once. Then add 5 ml CCl_4, shake vigorously for 15 sec., allow layers to sep., and discard CCl_4. Rinse once more with 2 ml CCl_4 as above. Discard the CCl_4 layer and permit CCl_4 remaining on surface of soln in funnel to evap. before proceeding.

Add 40 ml NH_4 citrate, Solution II; 5 ml carbamate solution (g); and 25 ml dithizone reagent, Solution II. (The carbamate and dithizone reagents should be accurately added from a pipet or buret.) Shake the funnel and contents vigorously for 1 min., allow layers to sep., and draw off aq. layer thru a fine-tip glass tube connected to an aspirator by rubber tubing. To remove the excess dithizone from the CCl_4 layer, add 50 ml 0.01 *N* NH_4OH and shake vigorously for 30 sec.

(c) *Evaluation of zinc present.*—Dry funnel stem with pipestem cleaner, and flush out with ca 2 ml of the Zn dithizonate soln. Collect an adequate portion of the remaining soln in a 25 ml erlenmeyer, or other suitable container, and stopper tightly. (Amber glass containers are convenient for this purpose, but colorless glassware will suffice if solns are kept in a dark place until transmittance readings are made.)

Measure the light transmittance of each soln with a photoelectric colorimeter equipped with a light filter which has max. transmittance near 535 mμ. (A Sextant Green Corning glass light filter No. 4010 is suitable.) Use CCl_4 as the transmission std and make an appropriate correction for the amount of Zn in the blank detns. Evaluate the quantity of Zn present in each soln from a curve relating concn and transmittance, prepd as follows:

Into 200 ml vol. flasks place 0, 2, 4, 6, 8, 10, 12, and 14 ml portions, resp., of the std soln contg 10 mmg Zn per ml. To each flask add 2 drops Me red soln, neutralize with normal NH_4OH, add 3.2 ml normal HCl, and dil. to vol. with distd H_2O. Pipet 25 ml aliquots of each of these solns, contg 0, 2.5, 5, 7.5, 10, 12.5, 15, and 17.5 mmg Zn, resp., into amber glass separatory funnels, and carry them thru the procedure as for the ash solns, beginning "Add 10 ml dithizone reagent, Solution I, to the aliquot in the separatory funnel . . . ." Det. transmittance of each soln and plot values against corresponding quantities of Zn.

RECOMMENDATIONS

It is recommended*—

(1) That the one-color procedure be adopted as first action and that it be included in the Eighth Edition of *Official Methods of Analysis* as an alternative procedure to the mixed-color method.

(2) That the study be continued with special attention to the cause of the variations in transmittance of the blank determination and their correction.

REFERENCES

(1) COWLING, H., and MILLER, E. J., *Ind. Eng. Chem., Anal. Ed.*, **13**, 145 (1941).
(2) COWLING, H., *This Journal*, **24**, 520 (1941).
(3) SHIRLEY, R. L., WALDRON, D. R., JONES, E. D., and BENNE, E. J., *ibid.*, **31**, 285 (1948).
(4) ———, BENNE, E. J., and MILLER, E. J., *ibid.*, **32**, 276 (1949).
(5) HEINEN, E. J., and BENNE, E. J., *ibid.*, **34**, 692 (1951).
(6) ———, *ibid.*, **35**, 397 (1952).
(7) ———, *ibid.*, **36**, 397 (1953).

No reports were given on boron in plants or sampling.

* For report of Subcommittee A and action of the Association, see *This Journal*, **38**, 66, 67 (1955).

TUESDAY—MORNING SESSION

REPORT ON FERTILIZERS

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

Reports were received from the Associate Referees on Boron, Inert Materials, Nitrogen, Phosphoric Acid, Potash, and Sampling and Preparation of Sample; their recommendations are approved. It is also recommended* that the methods for acid-soluble calcium, **2.53** and **2.54,** be made official.

E. W. Constable, the Associate Referee on Acid-Forming or Non-Acid Forming Quality, has recommended that the first action method, **2.67** and **2.68,** be made official, and the Referee concurs in this recommendation.

J. F. Fudge, the Associate Referee on Ammoniacal Solutions and Liquid Fertilizers, corresponded during the year with a number of State and industry control chemists, asking them to comment and suggest possible collaborative studies on the principles for collection and analysis of fertilizer solutions which contain important quantities of ammonia, as outlined by the Associate Referee last year (*This Journal,* **37,** 328 (1954)). Those who answered agreed with the Associate Referee that the basic principles have already been outlined and that the nature of the product and the area covered by the assignment are such as to preclude collaborative work. The Associate Referee therefore recommends that the subject be discontinued; the Referee, however, recommends that this work be continued.[1]

The Associate Referee on Sulfur, Gordon Hart, recommends that the first action method for free sulfur, **2.71,** be made official, and the Referee concurs in the recommendation.

"Report on Sampling and Preparation of Sample of Fertilizers" by Stacy B. Randle will be published in the August, 1955 issue of *This Journal.*

---

* For report of Subcommittee A and action of the Association, see *This Journal,* **38,** 63, 64 (1955).

[1] Because it was clear that a number of problems in regard to the assignment have still not been solved, Subcommittee A recommended that the subject be continued.

REPORT ON BORON IN MIXED FERTILIZERS

By RODNEY C. BERRY (Virginia Department of Agriculture, Richmond 19, Va.), *Associate Referee*

Four years of collaborative study on methods for the determination of boron in mixed fertilizers has produced two satisfactory procedures. Methods were developed for the determination of both water-soluble boron materials such as borax and acid-soluble boron materials like colemanite or howalite. The boron in some of the fritted materials now available can be determined by the acid-soluble method. The Associate Referee's report on both of these methods appears in *This Journal*, **37**, 330 (1954).

Only two collaborators experienced difficulties with the two methods this year. Neither laboratory had tried the methods before; one submitted very good results too late for inclusion in this report.

FORMULATION OF SAMPLES

Four samples of mixed fertilizers were formulated from different source materials, as shown below, in order to evaluate the adaptability of these methods to different mixtures. Borax ($Na_2B_4O_7 \cdot 10H_2O$) was added to one sample from each of these formulations, and the resulting samples were numbered 1B, 2B, 3B, and 4B, respectively. Colemanite as the source of boron was added to the other four samples, which were then numbered 1C, 2C, 3C, and 4C, respectively. Boron was added from these sources so that each sample should contain 0.454% B from borax or colemanite. The formulation of these fertilizers before the boron was added is as follows:

1	3–9–6	2	2–12–12
210 lb.	Process tankage	200 lb.	Sulfate of ammonia
210 lb.	Cottonseed meal	1300 lb.	Superphosphate (R.O.P.)
150 lb.	Sulfate of ammonia	400 lb.	Muriate of potash (60%)
1000 lb.	Superphosphate (R.O.P.) (run of the pile)	100 lb.	Filler
200 lb.	Muriate of potash (60%)	2000 lb.	Net
230 lb.	Filler		
2000 lb.	Net		

3	0–14–14	4	0–10–20
1500 lb.	Superphosphate (R.O.P.)	1100 lb.	Superphosphate (R.O.P.)
470 lb.	Muriate of potash (60%)	700 lb.	Muriate of potash (60%)
30 lb.	Filler	200 lb.	Filler
2000 lb.	Net	2000 lb.	Net

The borax mixtures were made up as follows:

1B: 2177.28 g 3– 9– 6 + 90.72 g borax
2B: 2177.28 g 2–12–12 + 90.72 g borax
3B: 2177.28 g 0–14–14 + 90.72 g borax
4B: 2177.28 g 0–10–20 + 90.72 g borax

TABLE 1.—*Collaborative analyses of per cent boron in mixed fertilizers with borax added*

COLLABORATOR	SAMPLE 1B		SAMPLE 2B		SAMPLE 3B		SAMPLE 4B	
	VIRGINIA MODIFIED METHOD	IDENTICAL *p*H METHOD	VIRGINIA MODIFIED METHOD	IDENTICAL *p*H METHOD	VIRGINIA MODIFIED METHOD	IDENTICAL *p*H METHOD	VIRGINIA MODIFIED METHOD	IDENTICAL *p*H METHOD
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	0.4795	0.4906	0.4665	0.4799	0.4536	0.4829	0.4579	0.4940
	.4838	.5018	.4665	.4853	.4536	.4906	.4579	.4919
	.4838	.4826	.4665	.4773	.4493	.4720	.4493	.4986
Average:	.4824	.4917	.4665	.4808	.4522	.4818	.4550	.4948
Deviation:	.0043	.0192	.0000	.0080	.0043	.0186	.0086	.0033
2	.460	.453	.460	.458	.452	.448	.453	.460
	.451	.460	.468	.457	.449	.458	.446	.454
	.452	.456	.458	.463	.453	.452	.446	.456
Average:	.454	.456	.462	.459	.451	.453	.450	.457
Deviation:	.009	.007	.010	.006	.004	.010	.007	.006
3	.4536	——	.4752	——	.4406	——	.4536	——
	.4665		.4665		.4493		.4579	
	.4665		.4665		.4363		.4493	
Average:	.4622		.4627		.4421		.4536	
Deviation:	.0129		.0013		.0130		.0086	
4	.500	.459	.485	.467	.429	.459	.440	.453
	.502	.451	.481	.459	.432	.465	.444	.453
	.492	.448	.477	.459	.432	.462	.434	.467
Average:	.498	.453	.481	.462	.431	.462	.439	.458
Deviation:	.010	.011	.006	.008	.003	.006	.010	.014
5	.458	.464	.462	.461	.438	.457	.429	.474
	.458	.461	.459	.462	.444	.464	.431	.474
	.458	.461	.464	.453	.438	.464	.426	.453
Average:	.458	.462	.462	.459	.440	.462	.429	.467
Deviation:	.000	.003	.005	.009	.006	.007	.005	.021
6	.4806	.3719	.4871	.3719	.4374	.3719	.4363	.3653
	.4752	.3719	.4871	.3653	.4385	.3762	.4488	.3653
	.4621	.3638	.4837	.3655	.4363	.3719	.4417	.3719
Average:	.4726	.3692	.4860	.3676	.4374	.3733	.4423	.3675
Deviation:	.0185	.0081	.0034	.0066	.0022	.0043	.0125	.0066
7	.487	.462	.520	.465	.435	.467	.442	.466
	.485	.460	.510	.458	.442	.467	.442	.463
	.470	.460	.497	.457	.433	.468	.442	.455
Average:	.481	.461	.509	.460	.437	.467	.442	.461
Deviation:	.017	.002	.023	.008	.009	001	.000	.011
8	.4622	.4590	.4666	.4671	.4646	.4617	.4385	.4671
	.4579	.4617	.4666	.4671	.4644	.4671	.4298	.4671
	.4579	.4590	.4730	.4725	.4644	.4671	.4277	.4644
Average:	.4593	.4599	.4687	.4689	.4645	.4653	.4320	.4662
Deviation:	.0043	.0027	.0064	.0054	.0002	.0054	.0108	.0027

TABLE 1—(*Continued*)

COLLABORATOR	SAMPLE 1B		SAMPLE 2B		SAMPLE 3B		SAMPLE 4B	
	VIRGINIA MODIFIED METHOD	IDENTICAL *p*H METHOD	VIRGINIA MODIFIED METHOD	IDENTICAL *p*H METHOD	VIRGINIA MODIFIED METHOD	IDENTICAL *p*H METHOD	VIRGINIA MODIFIED METHOD	IDENTICAL *p*H METHOD
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
9	.48 .45 .48	.39 .38 .40	.48 .41 .50	.43 .41 .39	.48 .39 .43	.39 .43 .41	.48 .41 .45	.45 .41 .41
Average:	.47	.39	.46	.41	.43	.41	.45	.43
Deviation:	.03	.02	.09	.04	.09	.04	.07	.04
10	.494 .484 .527	.464 .475 .466	.471 .494 .469	.474 .478 .477	.480 .422 .482	.476 .478 .474	.472 .479 .404	.476 .478 .476
Average:	.502	.468	.478	.476	.461	.476	.452	.477
Deviation:	.043	.011	.025	.004	.060	.004	.075	.002
11	.4701 .4742 .4618	.4420 .4420 .4420	.4451 .4451 .4510	.4407 .4329 .4407	.4222 .4295 .4306	.4420 .4459 .4407	.4451 .4436 .4482	.4407 .4420 .4368
Average:	.4687	.4420	.4471	.4381	.4274	.4429	.4456	.4398
Deviation:	.0124	.0000	.0059	.0078	.0084	.0052	.0046	.0052
12	.398 .473 .485	.456 .456 .457	.363 .377 .431	.457 .461 .467	.349 .349 .365	.460 .475 .483	.399 .399 .404	.460 .467 .487
Average:	.452	.456	.390	.462	.354	.473	.401	.471
Deviation:	.087	.001	.068	.010	.016	.023	.005	.027
13	.456 .458 .465	.457 .451 .467	.463 .471 .443	.463 .460 .454	.438 .436 .417	.455 .455 .445	.447 .453 .427	.462 .451 .475
Average:	.460	.458	.459	.459	.430	.452	.442	.463
Deviation:	.009	.016	.028	.009	.021	.010	.026	.024
14	.4884 .4862 .4906	.4689 .4632 .4660	.4884 .4950 .4950	.4660 .4689 .4689	.4641 .4641 .4663	.4689 .4675 .4660	.4442 .4531 .4442	.4746 .4746 .4746
Average:	.4884	.4660	.4928	.4679	.4648	.4675	.4472	.4746
Deviation:	.0044	.0057	.0066	.0029	.0022	.0029	.0089	.0000
15	.4687 .4449 .4320	.4343 .4402 .4284	.4298 .4104 .4147	.4905 .4964 .4934	.4320 .4363 .4298	.4875 .4462 .4402	.4622 .4428 .4298	.4934 .4432 .4373
Average:	.4485	.4343	.4183	.4934	.4327	.4580	.4449	.4580
Deviation:	.0367	.0118	.0194	.0059	.0065	.0473	.0324	.0561
Average all results:	.4705	.4476	.4629	.4581	.4370	.4567	.4417	.4561
Average deviation:	.0199	.0085	.0196	.0093	.0164	.0130	.0190	.0156

TABLE 2.—*Collaborative analysis of per cent boron in mixed fertilizers with colemanite added*

COLLABORATOR	SAMPLE 1C		SAMPLE 2C		SAMPLE 3C		SAMPLE 4C	
	VIRGINIA MODIFIED METHOD	IDENTICAL *p*H METHOD	VIRGINIA MODIFIED METHOD	IDENTICAL *p*H METHOD	VIRGINIA MODIFIED METHOD	IDENTICAL *p*H METHOD	VIRGINIA MODIFIED METHOD	IDENTICAL *p*H METHOD
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	0.3499	0.4430	0.3758	0.4324	0.3715	0.4547	0.3758	0.4534
	.3499	.4382	.3542	.4520	.3629	.4504	.3802	.4449
	.3542	.4454	.3758	.4480	.3672	.4427	.3780	.4534
Average:	.3513	.4422	.3686	.4441	.3672	.4493	.3780	.4506
Deviation:	.0043	.0072	.0216	.0198	.0086	.0120	.0044	.0085
2	.385	.437	.423	.444	.376	.443	.375	.445
	.388	.438	.417	.446	.367	.442	.385	.440
	.379	.432	.426	.449	.387	.450	.374	.438
Average:	.384	.436	.422	.446	.377	.445	.378	.441
Deviation:	.009	.006	.009	.005	.020	.008	.011	.007
3	.3672	—	.4104	—	.3672	—	.3672	—
	.3628	—	.4147	—	.3542	—	.3542	—
	.3628	—	.4060	—	.3542	—	.3628	—
Average:	.3643		.4104		.3585		.3614	
Deviation:	.0044		.0087		.0130		.0130	
4	.432	.428	.462	.437	.365	.437	.365	.437
	.438	.437	.472	.437	.354	.431	.350	.428
	.453	.431	.470	.442	.367	.434	.361	.431
Average:	.441	.432	.468	.439	.362	.434	.359	.432
Deviation:	.021	.009	.010	.005	.013	.006	.015	.009
5	.420	.426	.417	.428	.354	.438	.379	.436
	.418	.430	.417	.422	.352	.434	.377	.443
	.415	.428	.418	.430	.356	.434	.380	.424
Average:	.418	.428	.417	.427	.354	.435	.379	.434
Deviation:	.005	.004	.001	.008	.004	.004	.003	.019
6	.3488	.3522	.4104	.3456	.3510	.3566	.3715	.3456
	.3380	.3478	.4039	.3500	.3488	.3445	.3812	.3556
	.3380	.3456	.4104	.3555	.3564	.3566	.3737	.3500
Average:	.3416	.3485	.4082	.3504	.3521	.3526	.3755	.3504
Deviation:	.0108	.0066	.0065	.0099	.0076	.0121	.0097	.0100
7	.380	.442	.465	.438	.407	.434	.370	.432
	.375	.443	.467	.435	.408	.443	.370	.432
	.384	.440	.473	.434	.370	.435	.364	.423
Average:	.380	.442	.468	.436	.395	.437	.368	.429
Deviation:	.009	.003	.008	.004	.038	.009	.006	.009
8	.3780	.4482	.3618	.4617	.3305	.4509	.3391	.4347
	.3758	.4563	.3618	.4590	.3262	.4482	.3348	.4428
	.3780	.4428	.3618	.4590	.3305	.4509	.3391	.4428
Average:	.3773	.4491	.3618	.4599	.3291	.4500	.3380	.4401
Deviation:	.0022	.0135	.0000	.0027	.0043	.0017	.0043	.0081

TABLE 2—(*Continued*)

COLLABORATOR	SAMPLE 1C		SAMPLE 2C		SAMPLE 3C		SAMPLE 4C	
	VIRGINIA MODIFIED METHOD	IDENTICAL *p*H METHOD	VIRGINIA MODIFIED METHOD	IDENTICAL *p*H METHOD	VIRGINIA MODIFIED METHOD	IDENTICAL *p*H METHOD	VIRGINIA MODIFIED METHOD	IDENTICAL *p*H METHOD
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
9	.37 .35 .34	.39 .36 .41	.36 .39 .39	.37 .39 .37	.37 .37 .35	.38 .40 .39	.39 .40 .41	.40 .41 .42
Average:	.35	.39	.38	.38	.36	.39	.40	.41
Deviation:	.03	.05	.03	.02	.02	.02	.02	.02
10	.400 .378	.445 .449 .461	.395	.444 .441 .448	.380 .368	.440 .452 .440	.370 .390	.447 .446 .448
Average:	.389	.452	.395	.444	.374	.443	.380	.447
Deviation:	.022	.016	—	.007	.012	.012	.020	.002
11	.3058 .3110 .3170	.3991 .4121 .4090	.3744 .3765 .3723	.4256 .4355 .4251	.3162 .3266 .3390	.4508 .4302 .4460	.3515 .3430 .3452	.4410 .4502 .4340
Average:	.3113	.4034	.3744	.4287	.3273	.4423	.3466	.4417
Deviation:	.0112	.0130	.0042	.0104	.0228	.0206	.0085	.0162
12	.375 .380 .406	.420 .430 .432	.335 .367 .387	.431 .432 .439	.293 .296 .321	.408 .410 .417	.332 .332 .332	.407 .408 .420
Average:	.387	.427	.363	.434	.303	.412	.332	.412
Deviation:	.031	.012	.052	.008	.028	.009	.000	.013
13	.3367 .3341 .3341	.423 .435 .409	.4163 .4163 .4112	.451 .427 .473	.3264 .3290 .3264	.428 .441 .428	.3906 .3906 .3932	.421 .442 .454
Average:	.3350	.422	.4146	.450	.3273	.432	.3915	.439
Deviation:	.0026	.026	.0051	.046	.0026	.013	.0026	.033
14	.3558 .3536 .3536	.4376 .4289 .4346	.4155 .4133 .4155	.4356 .4417 .4388	.3553 .3540 .3542	.4317 .4317 .4332	.3653 .3691 .3674	.4346 .4386 .4356
Average:	.3543	.4337	.4148	.4387	.3545	.4322	.3673	.4363
Deviation:	.0022	.0087	.0022	.0061	.0013	.0015	.0038	.0040
15	.4082 .3736 .3607	.4875 .4225 .3575	.3931 .3823 .3823	.4609 .4462 .4668	.4384 .3672 .3520	.4757 .4462 .4609	.4644 .4428 .4039	.4728 .4196 .4378
Average:	.3842	.4225	.3859	.4580	.3858	.4609	.4370	.4434
Deviation:	.0475	.1300	.0108	.0206	.0864	.0295	.0605	.0532
Average all results:	.3712	.4235	.4156	.4311	.3551	.4295	.3728	.4276
Average deviation:	.0141	.0203	.0113	.0123	.0188	.0113	.0121	.0151

The colemanite mixtures were made up as follows:

1C: 2171.84 g 3– 9– 6+96.16 g colemanite
2C: 2171.84 g 2–12–12+96.16 g colemanite
3C: 2171.84 g 0–14–14+96.16 g colemanite
4C: 2171.84 g 0–10–20+96.16 g colemanite

The methods used were the Virginia Modified Method as described in *This Journal*, **36**, 623 (1953) and the Identical *p*H Method, the details of which appeared in *This Journal*, **32**, 422 (1949). Results of the collaborative analyses are shown in Table 1 (borax added) and Table 2 (colemanite added).

COLLABORATORS

Collaborators are not given in the same order as results are listed in Tables 1 and 2.

W. R. Austin, Madalane C. Buford, and W. P. Goins, Armour Fertilizer Works, Box 1088, Nashville 2, Tenn.

Allen B. Lemmon and Vasily Kniaseff, Bureau of Chemistry, Department of Agriculture, State Office Building 1, Sacramento 14, Calif.

C. V. Marshall and W. H. Hollington, Plant Products Division, Canadian Department of Agriculture, 79 Sussex St., Ottawa, Canada

J. J. Taylor and Dan Long, Florida Department of Agriculture, Box 408, Tallahassee, Fla.

F. W. Quackenbush and O. W. Ford, Purdue University Agricultural Experiment Station, Lafayette, Ind.

H. R. Allen and C. R. Ball, Kentucky Agricultural Experiment Station, University of Kentucky, Lexington, Ky.

M. P. Etheredge, Sam Few, and O. Walling, Mississippi Department of Agriculture, Box 1536, State College, Miss.

Harry A. Miller, Z. B. Bradford, and B. A. Britt, North Carolina Department of Agriculture, Raleigh, N. C.

S. B. Randle and Leo J. Faneuf, New Jersey Agricultural Experiment Station, New Brunswick, N. J.

W. J. Ingram, Oregon Department of Agriculture, Salem, Ore.

Dr. E. W. Fajans, Pacific Coast Borax Co., Research Laboratory, 630 Shatto Place, Los Angeles 5, Calif.

W. A. Ryder, F. S. Royster Guano Co., Norfolk, Va.

H. J. Webb and Mr. Leslie, Clemson Agricultural College, Clemson, S. C.

R. T. Wetherbee and E. F. Boyce, Agricultural Experiment Station, Burlington, Vt.

Rodney C. Berry, J. H. Elder, and J. M. Van Pelt, Virginia Department of Agriculture, Richmond 19, Va.

RECOMMENDATIONS

It is recommended*—

(1) That the first action Method I for boron, **2.45–2.47,** be made official, and that the title be expanded to include the phrase "Acid-Soluble Boron" or a similar phrase to indicate that the method is applicable to boron materials which are not soluble in water.

(2) That the first action Virginia Modified Method, *This Journal*, **36,** 623 (1953), be made official, and that the phrase "Water-Soluble Boron"

or a similar phrase be added to the title to indicate that the method is applicable to boron materials which are soluble in water.

(3) That Method II, **2.48** and **2.49,** be deleted.

(4) That work on methods for the determination of boron in fritted materials be continued.

REPORT ON INERT MATERIALS IN FERTILIZERS

CARBONATE CARBON OR CALCIUM CARBONATE EQUIVALENT AND ACID-INSOLUBLE ASH

By K. G. CLARK, *Associate Referee,* and V. L. GADDY (Fertilizer and Agricultural Lime Section, Soil and Water Conservation Research Branch, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Md.)

This report presents the results of the 1954 collaborative studies on methods for determination of carbonate carbon as CO_2 and of acid-insoluble ash. Reports on the development of these methods and their application to commercial fertilizers have been presented previously (1–3).

Samples.—The formulation of the samples submitted to the collaborators is given in Table 1, which also shows the CO_2, $CaCO_3$ equivalent and the acid-insoluble ash contents of the ingredient materials. Table 2 shows the estimated CO_2, $CaCO_3$ equivalent and the acid-insoluble ash contents of the samples based on the data of Table 1.

Collaborators' Directions.—Samples of 0–20–0, 3–12–6, 3–12–12, 4–10–6, and 5–10–5 grade materials were provided. These samples were ground to pass a 35-mesh sieve during preparation so that grinding would not be necessary before sampling for analysis.

Collaborators were requested to make three replicate determinations of carbonate carbon and of acid-insoluble ash on each sample in accordance with the following methods. They were asked to make comments and observations for improvement of the proposed analytical procedures.

METHODS

Carbonate Carbon or Calcium Carbonate Equivalent

APPARATUS

Knorr alkalimeter.—With upper end of condenser connected to a CO_2 absorption train. Guard tube of alkalimeter is filled with Ascarite (NaOH-asbestos mixt.).

* For report of Subcommittee A and action of the Association, see *This Journal,* **38,** 63, 64 (1955).

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

MATERIAL	CO_2	$CaCO_3$ EQUIV.	ACID-INSOLUBLE ASH	SAMPLE NUMBER AND GRADE				
				1	2	3	4	5
				0-20-0	3-12-6	3-12-12	4-10-6	5-10-5
	per cent	*per cent*	*per cent*	*lbs per ton*				
Ammonium sulfate	—	—	—	—	40	40	180	275
Ammoniated superphosphate	—	—	4.53	—	1340	1340	1115	1115
Normal superphosphate	—	—	4.52	2000	—	—	—	—
Potassium chloride	—	—	.12	—	200	400	200	170
Dolomite	45.92	104.43	2.67	—	175	125	200	275
Sand	—	—	99.43	—	195	45	255	115
Peanut hull bran	—	—	6.46	—	50	50	50	50
Total	—	—	—	2000	2000	2000	2000	2000

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

MATERIAL	SAMPLE NUMBER AND GRADE				
	1	2	3	4	5
	0-20-0	3-12-6	3-12-12	4-10-6	5-10-5
Calcium carbonate equivalent, per cent					
Dolomite, CO_2	—	4.02	2.87	4.59	6.31
Dolomite, $CaCO_3$ equiv.	—	9.14	6.53	10.44	14.35
Acid-insoluble ash, per cent					
Ammonium sulfate	—	0.00	0.00	0.00	0.00
Ammoniated superphosphate	—	3.04	3.04	2.53	2.53
Normal superphosphate	4.52	—	—	—	—
Potassium chloride	—	.01	.02	.01	.01
Dolomite	—	.23	.17	.27	.37
Sand	—	9.69	2.24	12.68	5.72
Peanut hull bran	—	.16	.16	.16	.16
Total	4.52	13.13	5.63	15.65	8.79

Absorption train consists of 5 or 6 U-shaped glass-stoppered drying tubes, or equiv., joined in series. First 2 tubes in train remove acidic gases other than CO_2. First tube is charged with H_2SO_4 (sp. gr. 1.84) and second with Ag_2SO_4–H_2SO_4 soln (10 g Ag_2SO_4 in 100 ml H_2SO_4). Third tube is charged with anhyd. Mg perchlorate for moisture removal. Inlet ⅔ of fourth and succeeding tubes is filled with Ascarite for CO_2 absorption, and outlet third of each tube is filled with anhyd. Mg perchlorate. Last or guard tube in train is connected with aspirating bottle or suction source. App. is conditioned before use each day, and also when a freshly filled tube is introduced into train, by aspirating a stream of air at rate of 2–3 bubbles per sec. thru dry alkalimeter assembly and absorption train until CO_2 absorption tubes attain constant wt (usually 20–30 min.). (Some analysts recommend a standard procedure for wiping tubes with dry lint-free cloth before each weighing and weighing against similarly packed tare.)

DETERMINATION

Weigh and place 2 g sample in dry alkalimeter flask. Open stopcocks of first two CO_2 absorption tubes to air for instant to equalize internal and external pressures; weigh tubes separately and place in position in train. With assembled alkalimeter connected to absorption train, adjust rate of aspiration of air thru system to ca 2 bubbles per sec. Close funnel stopcock, remove alkalimeter guard tube, fill funnel with 50 ml HCl (1+4), and replace guard tube. Open funnel stopcock and allow acid to run slowly into the flask, taking care that evolution of gas is so gradual that the flow thru the tubes is not materially increased. After all acid has been added, agitate alkalimeter assembly for complete dispersion of sample in acid soln. Continue aspiration, gradually heat contents of the flask to boiling, and allow to boil 2–3 min. after the H_2O has begun to condense. Discontinue heating and continue aspiration for 15–20 min. or until app. cools. Remove, equalize internal and external pressures, and reweigh absorption tubes. Increase in wt is due to CO_2. (A material increase in wt of second tube usually indicates exhaustion of the first tube but may result from too rapid evolution of CO_2 in relation to the rate of aspiration.) Report wt % CO_2 in sample.

Acid-insoluble Ash

DETERMINATION

Weigh and place 2 g sample in 400 ml beaker. Add 100 ml HCl (1+4), cover with watch glass, and immerse 30 min. in steam or hot H_2O bath (98°–100°C.), keeping liquid level in beaker below that of H_2O in bath. Stir at 10 min. intervals, and after 30 min. remove from bath and filter thru 11 or 12.5 cm medium filter paper, transferring insol. residue to filter with stream of H_2O. Fold filter paper contg residue, place in porcelain crucible, and ignite in muffle furnace at 800°C. for 1 hr. Cool, transfer contents of crucible to original beaker with 50 ml HCl (1+4), cover, and again immerse in steam or hot H_2O bath 30 min. with occasional stirring. After 30 min. remove from bath and filter thru tared gooch contg acid-washed asbestos mat on filter paper disk. Wash insol. residue several times with H_2O, dry crucible 1 hr at 125°C., cool in desiccator, and weigh. Report net increase in wt of crucible as % acid-insoluble ash.

COLLABORATORS

Everard G. Baker, Mississippi State Chemical Laboratory, State College, Miss.

E. F. Boyce, Regulatory Service, Related Services Division, University of Vermont and State Agricultural College, Burlington, Vt.

TABLE 3.—*Carbonate carbon expressed as CO_2*

COLLABORATOR	SAMPLE NUMBER AND GRADE				
	1	2	3	4	5
	0-20-0	3-12-6	3-12-12	4-10-6	5-10-5
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
3	0.02	4.23	2.91	4.64	6.32
	0.02	4.09	2.89	4.61	6.32
	0.10	4.05	2.91	4.58	6.32
Av.	0.05	4.12	2.90	4.61	6.32
4	0.00	4.05	2.87	4.54	6.24
	0.00	4.04	2.88	4.56	6.23
	0.00	3.98	2.89	4.54	6.25
Av.	0.00	4.02	2.88	4.55	6.24
5	0.00	4.00	3.00	4.50	6.33
	0.00	4.08	3.00	4.52	6.33
	0.00	4.04	2.95	4.60	6.33
Av.	0.00	4.04	2.98	4.54	6.33
6	0.02	3.98	2.93	4.61	6.28
	0.02	4.00	2.89	4.62	6.29
	0.04	4.01	2.93	4.61	6.28
Av.	0.03	4.00	2.92	4.61	6.28
7	0.20	4.16	3.03	4.54	6.53
	0.29	4.09	2.93	4.53	6.53
	0.17	4.10	2.91	4.62	6.32
Av.	0.22	4.12	2.96	4.56	6.46
8[a]	0.00	3.86	2.74	4.32	6.13
	0.00	3.86	2.77	4.24	6.16
	0.00	3.87	2.74	4.26	6.09
Av.	0.00	3.86	2.75	4.27	6.13
9	0.04	4.00	2.82	4.54	6.24
	0.05	4.03	2.82	4.64	6.02
	0.01	3.92	2.85	4.53	6.20
Av.	0.03	3.98	2.83	4.57	6.15

[a] Values converted to CO_2 from $CaCO_3$ equivalent.

TABLE 3—(*Continued*)

COLLABORATOR	SAMPLE NUMBER AND GRADE				
	1	2	3	4	5
	0-20-0	3-12-6	3-12-12	4-10-6	5-10-5
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
10	0.07	4.08	2.93	4.59	6.17
	0.095	4.04	2.88	4.66	6.25
	0.01	4.04	2.87	4.73	6.35
Av.	0.06	4.05	2.89	4.66	6.26
11	0.13	3.94	2.32	4.50	6.21
	0.29	3.89	2.22	4.32	5.94
	0.10	3.88	2.33	4.48	6.15
Av.	0.17	3.90	2.29[b]	4.43	6.10
12	0.11	3.97	2.98	4.48	6.15
	0.11	3.96	2.88	4.65	6.19
	0.11	3.88	2.68	4.51	6.12
Av.	0.11	3.94	2.85	4.55	6.15
Group av.	0.07	4.00	2.88	4.54	6.25
Observed range	0–0.22	3.86–4.12	2.75–2.98	4.27–4.66	6.10–6.46
Estimated value	—	4.02	2.87	4.59	6.13
L.S.D. $P=0.01$	0.10	0.11	0.14	0.14	0.18

[b] Omitted from group average and analysis of variance.

Dorothy M. Carroll, Fertilizer and Agricultural Lime Section, Soil and Water Conservation Research Branch, Agricultural Research Service, Beltsville, Md.

Joseph G. Cummings, Fertilizer and Agricultural Lime Section, Soil and Water Conservation Research Branch, Agricultural Research Service, Beltsville, Md.

Leo J. Faneuf, New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick, N. J.

V. L. Gaddy, Fertilizer and Agricultural Lime Section, Soil and Water Conservation Research Branch, Agricultural Research Service, Beltsville, Md.

Curtis L. Godfrey, Texas Agricultural Experiment Station, Agricultural and Mechanical College of Texas, College Station, Texas.

Vasily Kniaseff, Bureau of Chemistry, Department of Agriculture, State of California, Sacramento, Calif.

W. A. Morgan, Polychemicals Department Research Division, E. I. du Pont de Nemours & Company, Inc., Wilmington, Del.

M. T. Murrill and S. J. Barden, Division of Chemistry, Virginia Department of Agriculture, Richmond, Va.

TABLE 4.—*Acid-insoluble ash*

COLLABORATOR	SAMPLE NUMBER AND GRADE				
	1	2	3	4	5
	0-20-0	3-12-6	3-12-12	4-10-6	5-10-5
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
1	4.37	13.28	5.89	15.37	8.55
	4.40	13.59	5.98	15.28	8.51
	4.65	13.43	5.97	15.49	8.80
Av.	4.47	13.43	5.95	15.38	8.62
2	4.00	12.94	5.18	16.17	8.48
	4.03	13.53	5.34	16.41	8.95
	4.00	13.14	5.31	16.17	8.95
Av.	4.01[a]	13.20	5.28	16.25	8.79
3	4.57	14.03	5.83	15.62	9.19
	4.58	13.49	5.67	16.00	9.19
	4.62	14.16	5.82	15.52	9.37
Av.	4.59	13.89	5.77	15.71	9.25
4	4.59	13.70	5.53	16.09	8.98
	4.51	13.81	5.71	15.78	8.87
	4.56	13.81	5.74	15.98	9.01
Av.	4.55	13.77	5.66	15.95	8.95
5	4.77	13.82	5.87	16.52	9.22
	4.75	13.82	5.87	16.17	9.42
	4.70	14.00	5.89	16.40	9.18
Av.	4.74	13.88	5.88	16.36	9.27
6	4.59	13.58	5.73	15.85	9.14
	4.49	13.66	5.71	15.78	9.13
	4.49	13.73	5.68	15.73	9.00
Av.	4.52	13.66	5.71	15.79	9.09
7	4.79	13.98	5.91	15.78	9.74
	4.62	13.47	5.91	16.51	9.85
	4.77	14.24	6.34	17.13	9.83
Av.	4.73	13.90	6.05	16.47[a]	9.81

[a] Omitted from average and analysis of variance.

TABLE 4—(*Continued*)

COLLABORATOR	SAMPLE NUMBER AND GRADE				
	1	2	3	4	5
	0-20-0	3-12-6	3-12-12	4-10-6	5-10-5
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
8	4.48	13.70	5.80	16.08	9.18
	4.45	13.84	5.84	16.13	9.15
	4.40	13.93	5.92	16.24	9.09
Av.	4.44	13.82	5.85	16.15	9.14
9	4.65	13.44	5.76	16.26	9.27
	4.61	13.61	5.79	16.08	9.34
	4.52	13.81	5.81	16.09	8.94
Av.	4.59	13.62	5.79	16.14	9.18
10	4.63	14.21	5.69	16.08	9.39
	4.75	14.61	5.83	15.99	9.29
	4.51	14.54	5.53	15.84	9.32
Av.	4.63	14.45	5.68	15.97	9.33
11	4.54	14.12	5.41	16.89	9.62
	4.39	13.58	5.48	16.25	9.06
	4.45	13.80	5.51	16.43	8.80
Av.	4.46	13.83	5.47	16.52[a]	9.16
12	4.60	14.39	6.08	16.41	9.25
	4.60	14.53	6.26	16.29	9.43
	4.66	14.46	6.20	16.19	9.30
Av.	4.62	14.46	6.18	16.30	9.33
Group av.	4.58	13.83	5.77	16.00	9.16
Observed range	4.01–4.74	13.20–14.46	5.28–6.18	15.38–16.36	8.62–9.81
Estimated value	4.52	13.13	5.63	15.65	8.79
L.S.D. $P=0.01$	0.17	0.50	0.24	0.33	0.40

E. C. Petrie, Chemical Division, Florida Department of Agriculture, Tallahassee, Fla.

Robert P. Reynolds, Roger Armstrong, and P. A. Lang, Chemical Control Department, The American Agricultural Chemical Company, New York, N. Y.

COMMENTS OF COLLABORATORS

Carbonate Carbon or Calcium Carbonate Equivalent

Collaborators 3 and 6 considered the method satisfactory.

Collaborator 4 found that best results were obtained when the system was swept between determinations with the CO_2 absorption bulb replaced by a glass tube.

Collaborator 9 used 2 scrubbers containing concentrated H_2SO_4 to remove moisture instead of the specified anhydrous magnesium perchlorate. Anhydrous $CaSO_4$ was used at the outlet end of the CO_2 absorption bulb instead of the magnesium perchlorate.

Collaborator 10 weighed all tubes in CO_2 determination in an air-conditioned room, and put the tubes in a desiccator ½ hour before weighing. When CO_2 tubes were left in the desiccator overnight, no appreciable change was found in the tubes after conditioning. However, when the tubes were left attached to the apparatus overnight, the change was appreciable after conditioning. Side-arm test tubes were used for H_2SO_4 and Ag_2SO_4 solutions.

Collaborator 12 used 2 Fleming jars in place of 2 U-tubes for H_2SO_4–Ag_2SO_4 solutions. A National Bureau of Standards sample of dolomite was found to contain 47.22% CO_2 by this method in comparison to the stated value of 47.25%.

Before introducing the Ascarite tubes into the system, the apparatus containing the dry sample was aspirated for 5 minutes through the magnesium perchlorate tube to remove any CO_2 introduced into the flask with the sample.

Acid-Insoluble Ash

Collaborators 3 and 6 considered the method satisfactory.

Collaborator 4 thought that 5 g samples appeared to be too large in the case of materials containing more than about 10% acid-insoluble ash.

Collaborator 8 observed that although the procedure calls for transferring the insoluble residue to the filter paper with a stream of water, particles of silica and silicic acid could not be removed from the beaker without the aid of a rubber policeman. He suggested a change of wording to include a quantitative transfer to the filter paper.

Collaborator 12 ignited sample 1 in a muffle furnace and checked it over a Meker burner; he found it more convenient to burn off samples 2, 3, 4, and 5 over a Meker burner.

DISCUSSION OF RESULTS

Carbonate Carbon as CO_2 or Calcium Carbonate Equivalent.—The values obtained by the collaborators for triplicate determinations of the CO_2 contents of the samples are presented in Table 3. It is apparent that the group averages very closely approximate the estimated CO_2 contents in all cases. Although the observed range of the averages reported by the several collaborators exceeded the least significant difference at $P=0.01$ for all samples, replicate analyses by the individual analysts were in good agreement.

Acid-insoluble Ash.—As shown in Table 4, the variation between the estimated values and those found by the individual collaborators were significant at the 1 per cent level for all samples. In some cases the variations among replicates were quite small, while in others they were con-

siderable. The principal sources of the larger variations presumably were associated with incomplete ignition of the sample and filter paper and with mechanical losses.

RECOMMENDATIONS

It is recommended*—

(1) That the method presented for carbonate carbon as CO_2 be adopted as first action.

(2) That the method presented for acid-insoluble ash be adopted as first action.

REFERENCES

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

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

(3) ———, and GADDY, V. L., *This Journal*, **36**, 655 (1953).

REPORT ON PHOSPHORIC ACID IN FERTILIZERS

DIRECT DETERMINATION OF AVAILABLE PHOSPHORIC ACID

By K. D. JACOB, *Associate Referee*, and W. M. HOFFMAN (Fertilizer and Agricultural Lime Section, Soil and Water Conservation Research Branch, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Md.)

The report on phosphoric acid presented at the 1953 meeting of this Association (3) dealt with the direct determination of available phosphorus in fertilizers by volumetric and photometric methods. Further investigation of the volumetric method was made in a study of 3 samples by 32 collaborators, the results of which are reported herein. Study of the photometric method was held in abeyance because of its unsatisfactory precision and the poor agreement of the results with those by the official method.

As determined by official methods, the collaborators' results for total, citrate-insoluble, and available P_2O_5 in the samples, all ground to pass the 35-mesh Tyler sieve, are summarized in Table 1. Here, as in the preceding study (3), there was a wide range in the average values for each of the three determinations—considerably wider, in fact, than one would expect for such well-known and extensively used methods. It may well be asked

* For report of Subcommittee A and action of the Association, see *This Journal*, **38**, 63, 64 (1955).

TABLE 1.—*Total, citrate-insoluble, and available P_2O_5 in fertilizer samples, as determined by official methods*

SAMPLE	TOTAL P_2O_5		CITRATE-INSOLUBLE P_2O_5		AVAILABLE P_2O_5	
	RANGE[a]	AVERAGE	RANGE[a]	AVERAGE	RANGE[a]	AVERAGE
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1[b]	20.16–21.83	20.56	0.00–0.50	0.11	19.95–21.34	20.45
2[c]	50.30–53.36	51.29	0.65–1.79	1.15	49.07–52.13	50.13
3[d]	18.88–20.58	19.38	1.73–4.78	2.67	14.55–17.79	16.71

[a] Averages of triplicate determinations by individual collaborators.
[b] Normal superphosphate.
[c] Triple superphosphate.
[d] Ammoniated superphosphate.

whether these discrepancies result wholly or in part from either deficiencies in the methods themselves, their inadequate delineation in the *Official Methods of Analysis*, failure of the analyst to adhere strictly to the methods as outlined, poor techniques, or other causes.

Samples 1 and 2 (normal and triple superphosphates, respectively) were commercial, well-cured materials made from Florida land-pebble phosphate.

Sample 3 (ammoniated normal superphosphate) was a laboratory prepared, thoroughly aged material made from commercially produced Tennessee brown-rock superphosphate. It contained approximately 3.6 per cent of nitrogen, supplied entirely from anhydrous ammonia.

As determined in the writers' laboratory, the samples contained, respectively, 17.1, 46.3, and 9.2 per cent of water-soluble P_2O_5 and 3.0, 3.9, and 7.2 per cent of citrate-soluble P_2O_5.

INSTRUCTIONS TO COLLABORATORS

Without further grinding, mix each sample thoroughly and analyze by the following methods. Make all determinations in triplicate, each on a separate portion of the sample, and report the individual results on the form enclosed with these instructions. If for any reason it is necessary or desirable to repeat a determination the repetition should be made in triplicate and the three results reported should be those obtained in simultaneous replications. Your comments and observations concerning this investigation are requested.

TOTAL P_2O_5

Prep. the soln as in *Official Methods of Analysis*, 7th Ed., 1950, p. 8, sect. **2.9(a)**. Det. P_2O_5 as on pp. 9–10, sect. **2.13(a)**.

AVAILABLE P_2O_5

Preparation of solution.—Place 1 g sample in a 250 ml erlenmeyer, add 50 ml H_2O (room temp.), and shake or stir continuously 15 min. Transfer contents of flask to filter (Whatman No. 2 paper or equiv.), and filter by suction into a 1 l filter

flask contg 10 ml HNO_3 (1+1). Wash residue with successive small portions of H_2O (room temp.) to total vol. of ca 125 ml. After the final washing, remove filter from funnel and rinse funnel with 10 ml H_2O with drainage into filter flask. Within 1 hr and using same erlenmeyer as for the H_2O extn of the sample, digest the filter and H_2O-insol. residue with neutral NH_4 citrate soln as on p. 11, sect. **2.17(a)**, either with shaking at 5 min. intervals or preferably with continuous agitation during the citrate digestion. Filter the citrate ext. into the flask contg the H_2O ext., and wash the residue with H_2O at 65°C. to a total vol. of 450 ml in the filter flask. Reserve the citrate-insol. residue. Transfer the contents of the filter flask to a 500 ml vol. flask, cool to room temp., adjust the vol. to 500 ml, and mix thoroly.

VOLUMETRIC METHOD

Use aliquots of the soln corresponding approximately to 0.10, 0.05, and 0.025 g sample for materials contg less than 25, 25 to 50, and more than 50% of available P_2O_5, resp., and proceed as follows:

Transfer the appropriate aliquot to a 250 ml erlenmeyer or other suitable vessel, add 15 ml NH_4NO_3 soln contg 10 g P-free NH_4NO_3, adjust the vol. to 75–100 ml with H_2O, and heat to 50°C. Then add slowly, with agitation, 60 ml hot (50°C.) molybdate soln contg the supplemental HNO_3 (5 ml/100 ml molybdate) customarily added in prepg the volumetric molybdate soln. Without further application of heat, shake or stir continuously for 30 min., and complete the detn as on pp. 9–10, sect. **2.13(a)**.

Procedure 2.—With another aliquot (same vol.) of soln, proceed as under Procedure 1 thru the point where the molybdate soln is added, and then shake or stir continuously for 30 min. while maintaining the temp. at 50°C. Complete the detn as before.

Omit the determinations by Procedure 2 if apparatus for continuous agitation at 50°C. is not available or cannot be assembled readily.

CITRATE-INSOLUBLE P_2O_5

Procedure A.—Digest the citrate-insol. residue (obtained in prepg the citrate ext. for direct detn of available P_2O_5 as described above) as on p. 8, sect. **2.9(a)**, and det. P_2O_5 as on pp. 9–10, sect. **2.13(a)**.

Procedure B (official).—Prep. citrate-insol. residue as follows: Remove H_2O-sol. P_2O_5 as in **2.14,** using Whatman No. 2 paper or equiv.; digest H_2O-insol. residue with neutral NH_4 citrate soln as in **2.17,** using the same agitation procedure as in obtaining citrate-insol. residue, and det. P_2O_5 in the citrate-insol. residue as in *Procedure A.*

NOTES ON THE PROCEDURES

In the preparation of the citrate extracts of the samples, 19 of the 32 collaborators used continuous agitation devices of one kind or another, whereas 12 (Nos. 4, 5, 6, 12, 14, 16, 17, 19, 22, 25, 30, 31) employed manual agitation at intervals of 5 minutes or less. Both methods of agitation are officially permitted. The method of agitation used by Collaborator 32 was not stated. In agreement with previous experience, the trend was toward higher values for citrate-insoluble P_2O_5 with intermittent agitation. Thus, with intermittent agitation the values for citrate-insoluble P_2O_5 in sample 1 by the official method averaged 0.13 per cent as compared to 0.10 per cent with continuous agitation. Similarly, the respective values were 1.20 and 1.13 per cent for sample 2, and 2.71 and 2.54 per cent for sample 3.

It has been shown (4, 5) that failure to effect substantially complete removal of water-soluble phosphate prior to the citrate digestion usually results in lower values for available P_2O_5. In the preceding study (3), therefore, water-soluble phosphate was removed as in the official method. The work of Allen (1) and Allen, *et al.*, (2) has indicated, however, that the determination can be shortened, with little effect on the values for available P_2O_5, by extracting the water-soluble phosphate in the manner outlined above for *Preparation of solution* in the instructions to collaborators.

In the present study, the procedures for determining P_2O_5 in the combined water-citrate extracts differed from those used in the preceding study (3) in the following respects only: The molybdate solution was fortified with HNO_3 (5 ml/100 ml of molybdate) as in the official volumetric method, whereas the supplemental HNO_3 was omitted in the preceding study; the volume of molybdate solution added in each determination was reduced from 60 ml to 50 ml; and the time of agitation was increased from 30 minutes to 45 minutes. Treatment of the water-citrate extracts to convert the P_2O_5 to the ortho form was not necessary with the samples used in the present study.

TABLE 2.—*Percentages of available P_2O_5 as determined by several methods*

COLLABORATOR	METHOD[a]				DIFFERENCE IN RESULTS BY METHOD I AND METHOD:		
	I[b]	II	III	IV	II[c]	III[c]	IV[c]
Sample 1: Normal Superphosphate							
1	20.15	20.16	20.23	20.39	0.01	0.08	0.24
2	20.17	20.16	20.18	20.38	−0.01	0.01	0.21
3	20.42	20.39	20.03	20.12	−0.03	−0.39	−0.30
4	20.48	20.48	20.40	——	0.00	−0.08	——
5	20.16	20.17	20.04	——	0.01	−0.12	——
6	19.95	20.12	20.12	——	0.17	0.17	——
7	20.28	20.26	20.28	20.48	−0.02	0.00	0.20
8	20.46	20.47	20.54	20.50	0.01	0.08	0.04
9	20.20	20.24	20.68	——	0.04	0.48	——
10	20.16	20.17	20.10	20.21	0.01	−0.06	0.05
11	20.33	20.33	20.18	20.08	0.00	−0.15	−0.25
12	20.59	20.59	20.43	——	0.00	−0.16	——
13	20.16	20.02	20.11	20.04	−0.14	−0.05	−0.12
14	20.48	20.48	20.48	——	0.00	0.00	——
15	20.50	20.50	20.25	20.31	0.00	−0.25	−0.19
16	20.52	20.55	20.55	——	0.03	0.03	——
17	20.21	20.22	20.02	19.90	0.01	−0.19	−0.31
18	21.34	21.30	20.88	20.72	−0.04	−0.46	−0.62
19	20.87	20.91	21.72	21.37	0.04	0.85	0.50
20	21.10	21.12	20.90	——	0.02	−0.20	——
21	20.23	20.23	20.25	20.13	0.00	0.02	−0.10
22	20.46	20.43	20.17	——	−0.03	−0.29	——
23	20.49	20.52	20.35	20.55	0.03	−0.14	0.06
24	20.19	20.17	20.41	20.27	−0.02	0.22	0.08
25	20.59	20.55	20.63	——	−0.04	0.04	——
26	20.59	20.62	20.67	20.48	0.03	0.08	−0.11
27	20.36	20.34	20.05	20.08	−0.02	−0.31	−0.28
28	20.65	20.63	18.97	20.23	−0.02	−1.68	−0.42
29	20.23	20.19	20.27	20.26	−0.04	0.04	0.03
30	20.42	20.40	20.30	——	−0.02	−0.12	——
31	21.30	21.43	20.91	——	0.13	−0.39	——
32	20.21	20.15	20.30	20.20	−0.06	0.09	−0.01

[a] Average results of 3 replications.
[b] Official method.
[c] The minus sign denotes that the result by this method is lower than that by Method I.

COLLABORATIVE RESULTS FOR AVAILABLE P_2O_5

For convenience the several methods used for determination of available P_2O_5 in this investigation are designated as follows:

Method I.—The available P_2O_5 is the difference between the total and the citrate-insoluble P_2O_5 (Procedure B), as determined by the official procedures indicated in the instructions to collaborators.

Method II.—The available P_2O_5 is the difference between the total P_2O_5 (official) and the citrate-insoluble P_2O_5 (Procedure A) determined on the water-insoluble residue prepared by the modification outlined under *Available P_2O_5* in the instructions.

Method III.—The combined water-citrate extract is analyzed by Procedure 1, as outlined in the instructions.

Method IV.—The combined water-citrate extract is analyzed by Procedure 2, as outlined in the instructions.

TABLE 2—(*Continued*)

COLLABORATOR	METHOD [a]				DIFFERENCE IN RESULTS BY METHOD I AND METHOD:		
	I[b]	II	III	IV	II[c]	III[c]	IV[c]
	Sample 2: Triple Superphosphate						
1	49.20	49.38	49.86	49.94	0.18	0.66	0.74
2	49.32	49.45	49.49	49.42	0.13	0.17	0.10
3	49.88	50.13	49.79	49.75	0.25	−0.09	−0.13
4	50.27	50.32	50.20	——	0.05	−0.07	——
5	49.73	49.75	49.63	——	0.02	−0.10	——
6	49.42	49.67	49.67	——	0.25	0.25	——
7	49.30	49.60	50.21	50.56	0.30	0.91	1.26
8	50.26	50.64	50.65	50.73	0.38	0.39	0.47
9	49.90	50.35	50.28	——	0.45	0.38	——
10	50.47	50.49	49.93	49.90	0.02	−0.54	−0.57
11	49.65	49.65	49.83	49.97	0.00	0.18	0.12
12	50.29	50.47	50.53	——	0.18	0.24	——
13	50.01	50.07	49.69	49.65	0.06	−0.32	−0.36
14	50.12	50.05	50.00	——	−0.07	−0.12	——
15	50.27	50.67	50.83	50.63	0.40	0.56	0.36
16	49.42	50.13	50.02	——	0.71	0.60	——
17	49.07	49.13	49.03	49.03	0.06	−0.04	−0.04
18	51.90	52.02	50.57	51.80	0.12	−1.33	−0.10
19	51.11	51.12	51.90	51.70	0.01	0.79	0.59
20	52.13	52.53	52.13	——	0.40	0.00	——
21	50.38	50.17	50.28	50.22	−0.21	−0.10	−0.16
22	49.63	49.74	49.75	——	0.11	0.12	——
23	50.19	50.37	50.40	50.50	0.18	0.21	0.31
24	49.83	50.00	49.89	50.10	0.17	0.06	0.27
25	49.92	49.81	49.70	——	−0.11	−0.22	——
26	50.08	50.28	50.73	50.67	0.20	0.65	0.59
27	50.14	50.40	50.40	50.40	0.26	0.26	0.26
28	50.92	50.98	51.27	50.00	0.06	0.35	−0.92
29	49.93	50.08	49.60	49.90	0.15	−0.33	−0.03
30	50.51	50.70	51.31	——	0.19	0.80	——
31	51.57	51.97	53.65	——	0.40	2.08	——
32	49.32	49.32	49.60	50.13	0.00	0.28	0.81

(*Continued on next page*)

TABLE 2—(*Continued*)

COLLABORATOR	METHOD[a]				DIFFERENCE IN RESULTS BY METHOD I AND METHOD:		
	I[b]	II	III	IV	II[c]	III[c]	IV[c]
	Sample 3: Ammoniated Superphosphate						
1	16.47	16.12	16.19	16.31	−0.35	−0.28	−0.16
2	16.32	16.06	16.33	16.33	−0.26	0.01	0.01
3	17.57	17.53	17.30	17.21	−0.04	−0.27	−0.36
4	16.45	16.53	16.40	——	0.08	−0.05	——
5	16.57	16.26	15.99	——	−0.31	−0.58	——
6	16.05	16.05	16.05	——	0.00	0.00	——
7	16.73	16.58	16.67	16.65	−0.15	−0.06	−0.08
8	16.86	16.54	16.44	16.44	−0.32	−0.42	−0.42
9	16.69	16.56	16.20	——	−0.13	−0.49	——
10	16.54	16.36	16.20	16.27	−0.18	−0.34	−0.27
11	16.57	16.54	16.23	16.18	−0.03	−0.34	−0.39
12	16.61	16.70	16.90	——	0.09	0.29	——
13	16.80	16.38	16.28	16.10	−0.42	−0.52	−0.70
14	16.55	16.10	16.10	——	−0.45	−0.45	——
15	17.12	17.12	17.12	16.92	0.00	0.00	−0.20
16	16.98	17.05	16.87	——	0.07	−0.11	——
17	16.45	16.55	16.20	16.07	0.10	−0.25	−0.38
18	17.79	17.97	16.60	16.53	0.18	−1.19	−1.26
19	17.55	17.37	17.77	17.60	−0.18	0.22	0.05
20	17.28	17.07	16.97	——	−0.21	−0.31	——
21	16.77	16.55	16.47	16.30	−0.22	−0.30	−0.47
22	16.45	16.24	16.15	——	−0.21	−0.30	——
23	16.72	16.47	16.25	16.38	−0.25	−0.47	−0.34
24	17.39	17.06	16.29	16.36	−0.33	−1.10	−1.03
25	16.38	16.32	16.37	——	−0.06	−0.01	——
26	16.71	16.30	16.47	16.38	−0.41	−0.24	−0.33
27	16.74	16.33	16.36	16.28	−0.41	−0.38	−0.46
28	17.32	17.05	16.27	16.37	−0.27	−1.05	−0.95
29	16.35	16.37	16.16	16.36	0.02	−0.19	0.01
30	16.58	16.30	16.89	——	−0.28	0.31	——
31	16.78	16.97	17.16	——	0.19	0.38	——
32	14.55	13.19	16.40	16.53	−1.36	1.85	1.98

The averages of the individual collaborator's replicated results for available P_2O_5 in each of the samples by the several methods are shown in Table 2, which also indicates the individual differences between the results by the official method (I) and those by each of the other methods. The data are summarized in Tables 3 and 4.

Taking into consideration the signs of the differences (Table 2), the average net differences between the values for available P_2O_5 by the official method and those by Method II, which differs from the official method only in the procedure used for removing the water-soluble P_2O_5, range from −0.19 per cent for sample 3 to 0.17 per cent for sample 2 and average −0.01 per cent for all the samples (Table 4). Disregarding signs, the range is 0.03 (sample 1) to 0.24 per cent (sample 3) and the average is 0.15 per cent. Among the individual collaborators (Table 2) the differences are equally distributed between plus and minus values in the case of sample 1. On the other hand, the official method gave pre-

TABLE 3.—*Summary of average percentages of available P_2O_5*[a]

SAMPLE[b]	32 COLLABORATORS: METHOD			20 COLLABORATORS: METHOD	
	I[c]	II	III	I[c]	IV
1	20.45	20.45	20.36	20.40	20.33
2	50.13	50.30	50.34	50.06	50.24
3	16.71	16.52	16.50	16.77	16.48
Average	29.10	29.09	29.07	29.08	29.02

[a] Average of comparative results by all collaborators.
[b] Sample 1, normal superphosphate; Sample 2, triple superphosphate; Sample 3, ammoniated superphosphate.
[c] Official method.

dominantly lower values with sample 2 but predominantly higher values with sample 3.

In comparing the results by the official method with those by Methods III and IV it should be remembered that the latter methods involve not only the direct determination of available P_2O_5 in the combined water-citrate extract but also a different procedure in extracting the water-soluble phosphate. Although the trends for the respective samples are similar, these procedural differences are reflected in greater divergences in the average results by Methods III and IV than by Method II, in comparison with the results by the official method (Table 4). It will be noted, however, that the average differences with the official method *vs.* Method III are generally close to those for the official method *vs.* Method IV, whether or not the signs of the differences are taken into consideration. Among the individual collaborators (Table 2) the differences in the results for available P_2O_5 with the official method *vs.* Methods III and IV generally show higher proportions of minus values than with the official method *vs.* Method II.

TABLE 4.—*Summary of average differences in percentages of available P_2O_5*[a]

SAMPLE[b]	METHOD I[c] VS.:					
	METHOD II[d] WITH SIGNS:		METHOD III[d] WITH SIGNS:		METHOD IV[e] WITH SIGNS:	
	CONSIDERED[f]	DISREGARDED	CONSIDERED[f]	DISREGARDED	CONSIDERED[f]	DISREGARDED
1	0.00	0.03	−0.09	0.23	−0.07	0.20
2	0.17	0.19	0.21	0.41	0.18	0.41
3	−0.19	0.24	−0.21	0.40	−0.29	0.49
Average	−0.01	0.15	−0.03	0.35	−0.06	0.37

[a] Average of comparative results by all collaborators.
[b] Sample 1, normal superphosphate; Sample 2, triple superphosphate; Sample 3, ammoniated superphosphate.
[c] Official method.
[d] 32 comparisons on each sample.
[e] 20 comparisons on each sample.
[f] The minus sign denotes that Method I gave the higher result.

TABLE 5.—*Summary of differences in average results for available P_2O_5*

METHOD I[a] VS. METHOD:	NUMBER OF COMPARISONS		DIFFERENCE IN AVAILABLE P_2O_5[c]	
	TOTAL	SELECTED[b]	RANGE	AVERAGE
		Sample 1: Normal Superphosphate		
			per cent	*per cent*
II	32	—	−0.14, 0.17	0.00
	—	32	−0.14, 0.17	0.00
III	32	—	−1.68, 0.85	−0.09
	—	30	−0.46, 0.48	−0.07
IV	20	—	−0.62, 0.50	−0.07
	—	18	−0.42, 0.24	−0.07
		Sample 2: Triple Superphosphate		
II	32	—	−0.21, 0.71	0.17
	—	31	−0.21, 0.45	0.15
III	32	—	−1.33, 2.08	0.21
	—	22	−0.33, 0.39	0.07
IV	20	—	−0.92, 1.26	0.18
	—	13	−0.36, 0.47	0.08
		Sample 3: Ammoniated Superphosphate		
II	32	—	−1.36, 0.19	−0.19
	—	31	−0.45, 0.19	−0.15
III	32	—	−1.19, 1.85	−0.21
	—	26	−0.49, 0.38	−0.16
IV	20	—	−1.26, 1.98	−0.29
	—	15	−0.47, 0.05	−0.25
		Samples 1 to 3		
II	96	—	−1.36, 0.71	−0.01
	—	94	−0.45, 0.45	0.00
III	96	—	−1.68, 2.08	−0.03
	—	78	−0.49, 0.48	−0.06
IV	60	—	−1.26, 1.98	−0.06
	—	46	−0.47, 0.47	−0.09

[a] Official method.
[b] In which the average of 3 determinations differed from that by Method I by less than 0.5% of available P_2O_5.
[c] *Range* of the difference in the average replicated results of the individual comparisons; *average* of the difference in all the comparisons. The minus sign denotes that Method I gave the higher result.

Table 5 summarizes the numerical values of the differences in the average results of the individual collaborators for available P_2O_5 by the official method in comparison with the other methods. The distribution of the differences according to their magnitude and the number showing plus and minus values, respectively, is shown in Table 6. These data afford an indication of the precision of the results among the collaborators.

TABLE 6.—*Distribution of differences in average results for available P_2O_5*

DIFFERENCE	COMPARISONS OF METHOD I[a] WITH:								
	METHOD II			METHOD III			METHOD IV		
	PLUS[b]	MINUS[c]	TOTAL	PLUS[b]	MINUS[c]	TOTAL	PLUS[b]	MINUS[c]	TOTAL
per cent	*number*	*number*	*number*	*number*	*number*	*number*	*number*	*number*	*number*
Sample 1: Normal Superphosphate									
<0.10	11	12	29[d]	9	3	14[e]	5	1	6
0.10–0.24	2	1	3	2	7	9	3	4	7
0.25–0.49	0	0	0	1	6	7	0	5	5
0.50–1.00	0	0	0	1	0	1	1	1	2
>1.00	0	0	0	0	1	1	0	0	0
——	13	13	32[d]	13	17	32[e]	9	11	20
Sample 2: Triple Superphosphate									
<0.10	7	1	10[e]	1	3	5[f]	0	2	2
0.10–0.24	10	2	12	5	4	9	2	3	5
0.25–0.49	9	0	9	6	2	8	5	1	6
0.50–1.00	1	0	1	7	1	8	4	2	6
>1.00	0	0	0	1	1	2	1	0	1
——	27	3	32[e]	20	11	32[f]	12	8	20
Sample 3: Ammoniated Superphosphate									
<0.10	4	3	9[e]	1	3	6[e]	3	1	4
0.10–0.24	3	7	10	1	3	4	0	2	2
0.25–0.49	0	12	12	3	13	16	0	9	9
0.50–1.00	0	0	0	0	2	2	0	2	2
>1.00	0	1	1	1	3	4	1	2	3
——	7	23	32[e]	6	24	32[e]	4	16	20
Samples 1 to 3									
<0.10	22	16	48[g]	11	9	25[h]	8	4	12
0.10–0.24	15	10	25	8	14	22	5	9	14
0.25–0.49	9	12	21	10	21	31	5	15	20
0.50–1.00	1	0	1	8	3	11	5	5	10
>1.00	0	1	1	2	5	7	2	2	4
——	47	39	96[g]	39	52	96[h]	25	35	60

[a] Official method.
[b] Method I gave the lower result.
[c] Method I gave the higher result.
[d] Including 6 comparisons in which the methods gave the same result.
[e] Including 2 comparisons in which the methods gave the same result.
[f] Including 1 comparison in which the methods gave the same result.
[g] Including 10 comparisons in which the methods gave the same result.
[h] Including 5 comparisons in which the methods ave the same result.

For all comparisons the differences among the individual collaborators' average results for available P_2O_5 by the official method and by Method II range from −1.36 to 0.71 per cent (average, −0.01 per cent) (Table 5). With Method III the range is −1.68 to 2.08 (average, −0.03 per cent), and with Method IV, −1.26 to 1.98 per cent (average, −0.06 per cent). The minus sign denotes that the official method gave the higher result. Excluding the comparisons in which the values differ by ±0.5 per cent or more, the respective average differences are 0.00, −0.06, and −0.09 per cent. Differences of ±0.5 per cent or more of P_2O_5 are shown in 2 per cent of all the comparisons of the official method with Method II, 19 per cent with Method III, and 23 per cent with Method IV (Tables 5 and 6). For differences of less than 0.25 per cent of P_2O_5, the comparable figures are 76, 49, and 43 per cent with Methods II, III, and IV, respectively (Table 6). In 41 per cent of all the comparisons, the values by the official method are higher than those by Method II (Table 6). The values by the official method are higher in 54 per cent of the comparisons with Method III and in 58 per cent of those with Method IV.

TABLE 7.—*Summary of differences in the high and low results in triplicate determinations of available P_2O_5*

SAMPLE[a]	METHOD I[b,c]		METHOD II[c]		METHOD III[c]		METHOD IV[d]	
	RANGE	AVERAGE	RANGE	AVERAGE	RANGE	AVERAGE	RANGE	AVERAGE
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	0.00–0.52	0.12	0.00–0.45	0.13	0.00–0.70	0.16	0.00–0.50	0.14
2	0.02–0.78	0.21	0.04–0.85	0.21	0.00–0.80	0.26	0.00–0.43	0.23
3	0.00–0.74	0.16	0.00–0.55	0.19	0.00–1.50	0.17	0.00–0.41	0.13
1 to 3[e]	0.00–0.78	0.16	0.00–0.85	0.18	0.00–1.50	0.20	0.00–0.50	0.17
1 to 3[f]	0.00–0.49	0.14[g]	0.00–0.45	0.16[h]	0.00–0.49	0.16[g]	0.00–0.50	0.17[i]

[a] Sample 1, normal superphosphate; Sample 2, triple superphosphate; Sample 3, ammoniated superphosphate.
[b] Official method. [c] 32 triplicated determinations on each sample.
[d] 20 triplicated determinations on each sample. [e] All analyses.
[f] Excluding analyses in which the difference between the high and low results in triplicate determinations was more than 0.50% of available P_2O_5.
[g] 91 triplicated determinations. [h] 92 triplicated determinations.
[i] 60 triplicated dermination.

The differences between the high and low results in triplicate determinations of available P_2O_5 by the several methods are summarized in Table 7. For all the samples the range is 0.00 to 0.78 per cent of P_2O_5 (average, 0.16 per cent) with Method I (official), 0.00 to 0.85 per cent (average, 0.18 per cent) with Method II, 0.00 to 1.50 per cent (average, 0.20 per cent) with Method III, and 0.00 to 0.50 per cent (average, 0.17 per cent) with Method IV. Excluding analyses in which the triplicate values differ by more than 0.50 per cent of P_2O_5, the average differences are 0.14, 0.16, 0.16, and 0.17 per cent with Methods I, II, III, and IV, respectively.

TABLE 8.—*Distribution of differences in the high and low results in triplicate determinations of available P_2O_5*

DIFFERENCE	METHOD I[a]		METHOD II		METHOD III		METHOD IV	
	ANALYSES	PROPORTION	ANALYSES	PROPORTION	ANALYSES	PROPORTION	ANALYSES	PROPORTION
per cent	*number*	*per cent*	*number*	*per cent*	*number*	*per cent*	*number*	*per cent*
Sample 1: Normal Superphosphate								
<0.10	17	53	15	47	8	25	9	45
0.10–0.24	10	31	13	41	19	59	8	40
0.25–0.49	4	13	4	12	4	13	2	10
>0.49	1	3	0	0	1	3	1	5
——	32	100	32	100	32	100	20	100
Sample 2: Triple Superphosphate								
<0.10	6	19	7	22	3	9	1	5
0.10–0.24	19	59	15	47	16	50	11	55
0.25–0.49	4	13	7	22	10	32	8	40
>0.49	3	9	3	9	3	9	0	0
——	32	100	32	100	32	100	20	100
Sample 3: Ammoniated Superphosphate								
<0.10	15	47	9	28	14	44	10	50
0.10–0.24	9	28	12	37	12	37	6	30
0.25–0.49	7	22	10	32	5	16	4	20
>0.49	1	3	1	3	1	3	0	0
——	32	100	32	100	32	100	20	100
Samples 1 to 3								
<0.10	38	40	31	32	25	26	20	33
0.10–0.24	38	40	40	42	47	49	25	42
0.25–0.49	15	15	21	22	19	20	14	23
>0.49	5	5	4	4	5	5	1	2
——	96	100	96	100	96	100	60	100

[a] Official method.

The difference between the high and low results in triplicate determinations exceeds 0.49 per cent in 5 per cent of all the analyses by Method I (official), as compared with 4 per cent by Method II, 5 per cent by Method III, and 2 per cent by Method IV (Table 8). For differences of less than 0.25 per cent of P_2O_5, the figures are 80, 74, 75, and 75 per cent for Methods I, II, III, and IV, respectively.

The data indicate, in general, that the precision of the results among replicates of an analysis is highest with the official method, but is not greatly different from that with the other methods.

SELECTED COMMENTS OF COLLABORATORS

Collaborators 4 and 26 stated that the direct volumetric method appears to be impractical for routine work involving large numbers of samples. *Collaborator 27* indicated that the direct method does not effect much saving of either time or labor, whereas *Collaborator 31* reported a saving of time with this method.

Collaborator 5 expressed the opinion that, as compared with the official method, reversion of the P_2O_5 to citrate-insoluble forms is probably favored by the procedure used for extracting water-soluble P_2O_5 in the direct method, especially with materials such as ammoniated superphosphate (Sample 3).

Collaborator 10 suggested that specification of a maximum amount of P_2O_5 in an aliquot for the volumetric determination, preferably not more than 40 mg, would improve the directions.

Collaborator 14 felt that the volume of 450 ml in the filter flask did not leave sufficient room for the washings in removing the water- and citrate-soluble portions of the sample.

Collaborator 17 brought up the question as to whether some very finely divided insoluble phosphate may pass through the filter paper when vacuum is used in washing the sample with water, as in the direct volumetric method. Precipitation of the the phosphomolybdate in the presence of citrate, as in the direct method, seems to result in a finer grained precipitate than in the official method.

Collaborator 21 believed that the procedure used for removing water-soluble P_2O_5 in the direct method may not be as efficient as that in the official method. His analyses show slightly higher results for citrate-insoluble P_2O_5 in samples 2 and 3 by Method II than by the official method. No significant differences were found in the results for available P_2O_5 by Methods III and IV. He considered the transfer of the combined water-citrate extract from the filter flask to the volumetric flask to be time consuming, and thought that filtration directly into the volumetric flask is advantageous.

Collaborator 28 suggested that with samples containing small percentages of P_2O_5, it may be necessary to make a supplemental addition of nitric acid in order to effect complete precipitation of the P_2O_5 as phosphomolybdate in the direct method.

Collaborator 30 stated that with materials containing 40 per cent or more of P_2O_5, the aliquots for volumetric determination of total P_2O_5 and of available P_2O_5 by the direct method should be equivalent to not more than 0.05 g of the sample.

SUMMARY

Two variations of a direct volumetric method for available P_2O_5, involving a modification of the official procedure for extracting water-soluble phosphate, were compared with the official method by 32 collaborators in some 450 analyses in triplicate of 1 sample each of normal and triple superphosphates and ammoniated normal superphosphate.

The official method gave the higher result for available P_2O_5 in the majority of the individual comparisons. Thus, on the three materials the result by this method was higher in 54 and 58 per cent of the comparisons with the two variations of the direct method. For all the comparisons

and with consideration of the signs of the differences, the weighted average values for P_2O_5 by the official method were 0.03 and 0.06 per cent higher than by the variations of the direct method. Disregarding signs, the average differences in the results by the official method and those by the direct methods were 0.35 and 0.37 per cent of P_2O_5, respectively. Differences of less than 0.25 per cent of P_2O_5 were shown in 49 and 43 per cent of the comparisons.

Differences between the results for available P_2O_5 by the official method and the variations of the direct method appear to be due in part to conditions, as yet unexplained, brought about by the modification in the procedure for extracting water-soluble phosphate as used in the latter method.

The data do not indicate definite advantages of the one variation of the direct method over the other. The average precision in the analysis of the samples, as indicated by the difference between the high and low values in triplicate determinations, did not vary greatly among the methods. It ranged from 0.16 to 0.20 per cent of available P_2O_5. The difference was less than 0.25 per cent of P_2O_5 in 80 per cent of the individual analyses by the official method and in 75 per cent of those by the direct method.

Further study needs to be made of the direct volumetric method for available P_2O_5, in order to define more clearly the conditions under which this method may be expected to give results that are in better agreement with those by the official method.

SUGGESTED EDITORIAL CHANGES IN METHODS FOR P_2O_5

For many years it has been the custom in fertilizer circles to designate phosphorus pentoxide (P_2O_5) by the term "phosphoric acid." This practice has been followed by the Association of Official Agricultural Chemists since 1884, the year in which the Association was formed. Such use of the term, a survival of the older chemistry, is followed in most if not all countries, and it is chronicled in the dictionaries. In the parlance of modern chemistry, however, the term "phosphoric acid" properly refers to the compound H_3PO_4, not to P_2O_5. Thus we have the anomaly of two different compounds being designated by the same name.

With the recent advent of liquid phosphoric acid (H_3PO_4) as a fertilizer and as an article of commerce in the fertilizer industry, this situation is causing a great deal of confusion. It is suggested, therefore, that in the chapter on fertilizers (*Official Method of Analysis*) the use of the term "phosphoric acid" to designate P_2O_5 be discontinued and that this term be reserved solely for the compound H_3PO_4. It is further suggested (1) that the compound P_2O_5 be designated as phosphorus pentoxide and (2) that the more general term "phosphorus" be used wherever this can be done without danger of ambiguity—as is now the case in the chapters on

feeds, gelatin, meat and meat products, plants, soils, vitamin preparations, and wheat and wheat flour. Thus, for example, the center heading "TOTAL PHOSPHORIC ACID" (*Official Methods of Analysis*, 7th Ed.,1950, p. 8) would be changed to "TOTAL PHOSPHORUS."[1]

For the sake of uniformity throughout the volume, similar changes should be made in other chapters where appropriate.

ACKNOWLEDGMENT

The Associate Referee wishes to express his appreciation of the fine cooperation given by the collaborators and their respective organizations.

COLLABORATORS

The following alphabetical listing of the collaborators does not correspond with the numbered designations used in this report.

W. R. Austin, C. Madalane Buford, and W. P. Goins, Armour Fertilizer Works, Nashville, Tenn.

Everard G. Baker, Mississippi State Chemical Laboratory, State College, Miss.

C. R. Ball and H. R. Allen, Department of Feed and Fertilizer, Kentucky Agricultural Experiment Station, Lexington, Ky.

R. E. Baumgardner, Maryland Inspection and Regulatory Service, College Park, Md.

P. R. Bidez and R. W. Richburg, State Chemical Laboratory, Alabama Department of Agriculture and Industries, Auburn, Ala.

A. T. Blackwell and F. Hoffmaster, Davison Chemical Co., Division of W. R. Grace & Co., Baltimore, Md.

R. D. Caldwell and G. F. Henry, Armour Fertilizer Works, Atlanta, Ga.

Dorothy M. Carroll, Soil and Water Conservation Research Branch, U. S. Department of Agriculture, Beltsville, Md.

R. Lindsey Dunn, Division of Chemical Development, Tennessee Valley Authority, Wilson Dam, Ala.

C. W. Garland, Davison Chemical Co., Division of W. R. Grace & Co., Nashville, Tenn.

Charles W. Gehrke and J. M. Baker, Department of Agricultural Chemistry, University of Missouri, Columbia, Mo.

Bertram Gersten, Feed and Fertilizer Regulatory Service, Massachusetts Agricultural Experiment Station, Amherst, Mass.

Curtis L. Godfrey, State Chemical Laboratory, Texas Agricultural Experiment Station, College Station, Tex.

W. M. Hoffman, Soil and Water Conservation Research Branch, U. S. Department of Agriculture, Beltsville, Md.

W. H. Hollington, Plant Products Division, Canada Department of Agriculture, Ottawa, Ontario, Canada

Wm. J. Ingram, Division of Foods and Dairies, Weights and Measures, Oregon Department of Agriculture, Salem, Oreg.

Helen Kocaba, Department of Analytical Chemistry, Connecticut Agricultural Experiment Station, New Haven, Conn.

Leo F. Krzeminski, Department of Biochemistry, Purdue University Agricultural Experiment Station, Lafayette, Ind.

---

[1] See REINDOLLAR, WM. F., *This Journal*, **38**, 61 (1955).

J. K. Lawton, and G. S. Roberts, Jr., Chemical Division, Florida Department of Agriculture, Tallahassee, Fla.

E. E. Leslie, Department of Fertilizer Inspection and Analysis, Clemson Agricultural College, Clemson, S. C.

E. L. Nelson and H. B. Baird, Nitrogen Division, Allied Chemical & Dye Corp., Hopewell, Va.

Boyce M. Olive, Soil and Water Conservation Research Branch, U. S. Department of Agriculture, Beltsville, Md.

Chas. E. Olney, Department of Chemistry, Rhode Island Agricultural Experiment Station, Kingston, R. I.

J. S. Pittard, R. T. Teague, Jr., and Isabell Smith, Analytical Division, North Carolina Department of Agriculture, Raleigh, N. C.

Wm. A. Ryder, F. S. Royster Guano Co., Norfolk, Va.

Philip McG. Shuey, Shuey & Co., Inc., Savannah, Ga.

Marvin H. Snyder and Sue Alexander, West Virginia Department of Agriculture, Charleston, W. Va.

A. J. Sterges, Department of Chemistry, Tennessee Agricultural Experiment Station, Knoxville, Tenn.

Chas. E. Weber, Department of Chemistry, New Jersey Agricultural Experiment Station, New Brunswick, N. J.

Rhodella Wilkins and Harlan Specht, Bureau of Chemistry, California Department of Agriculture, Sacramento, Calif.

Robert G. Wood and James L. McClaran, Tennessee Department of Agriculture, Nashville, Tenn.

W. S. Yowell, American Agricultural Chemical Co., New York, N. Y.

RECOMMENDATIONS

It is recommended*—

(1) That further study be made of:

(*a*) Methods for direct determination of available P_2O_5 in fertilizers.

(*b*) The use of perchloric acid in preparation of phosphate fertilizers for analysis.

(2) That the following editorial change be made in the Eighth Edition of *Official Methods of Analysis:*

In the chapter on fertilizers, discontinue the use of the term "phosphoric acid" to designate P_2O_5, and reserve this term solely for the compound H_3PO_4. Designate the compound P_2O_5 as phosphorus pentoxide, but use the more general term "phosphorus" wherever this can be done without danger of ambiguity. For the sake of uniformity throughout the volume, similar changes should be made in other chapters as may be appropriate.

REFERENCES

(1) ALLEN, H. R., *This Journal*, **36**, 872 (1953).
(2) ———, SWIFT, E., HAYES, R., and KAUFMAN, Z. F., *ibid.*, **35**, 764 (1952).
(3) JACOB, K. D., and HOFFMAN, W. M., *ibid.*, **37**, 334 (1954).
(4) ———, HOFFMAN, W. M., and SCHRAMM, F. C., *ibid.*, **36**, 632 (1953).
(5) ———, and TREMEARNE, T. H., *ibid.*, **20**, 277 (1937).

* For report of Subcommittee A and action of the Association, see *This Journal*, **38**, 63, 64 (1955).

REPORT ON NITROGEN IN FERTILIZERS

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

No definite recommendations for collaborative work based on previously reported problems were proposed in the 1953 report of the Associate Referee.

The question was discussed of having only one Kjeldahl method appear in *Official Methods of Analysis*. The General Referee on Fertilizers, together with the various Associate Referees, the American Association of Cereal Chemists, and other interested persons developed a plan of collaborative work on this subject during 1954. The recommendations of this committee are presented as a part of this report.

It was suggested to the Associate Referee that a chemical method for measuring the relative availability of nitrogen in urea-formaldehyde compounds be studied collaboratively. It was observed that the present method of determining nitrogen activity or availability (solubility in neutral permanganate) apparently can not be satisfactorily applied to urea-formaldehyde (U-F) materials. A method developed by Morgan and Kralovec appeared in *This Journal*, **36**, 907 (1953). Morgan and Kralovec presented the problem before a committee of fertilizer referees at the 1953 A.O.A.C. convention and it was agreed that the Associate Referee should arrange for a collaborative study of the Morgan-Kralovec method of determining an "availability index," AI, to indicate in a relative manner the availability of nitrogen in urea-formaldehyde compounds. It was later decided that "activity index" was a more suitable term for use in *Official Methods of Analysis*, and "AI" in the present report is thus used to refer to activity index.

It was pointed out that nitrification studies should be made on the samples submitted for chemical analysis to relate the "activity index" obtained by chemical analysis to the availability to plants of the nitrogen present in the materials. W. A. Morgan, R. D. Kralovec, and D. W. Kolterman of the Polychemicals Department, E. I. du Pont de Nemours & Company, Inc., Wilmington, Del., cooperated very closely with the Associate Referee in drawing up the plan.

Four samples were submitted for collaborative work:

Sample A: Urea formaldehyde containing about 38 per cent total nitrogen with an activity index (AI) of about 62.

Sample B: Urea formaldehyde containing about 38 per cent total nitrogen with an AI of about 33.

Sample C: Urea formaldehyde resin scrap with a total nitrogen content of about 19 per cent.

Sample D: Castor pumice, a common source of organic nitrogen; total nitrogen content about 6 per cent.

Each sample was to be analyzed as follows:

(1) Neutral Permanganate Method, *Official Methods of Analysis*, 7th Ed., **2.36.**

(2) Availability Index (AI) Method (Morgan and Kralovec, *This Journal*, **36**, 907 (1953)).

If possible, the nitrification rate (nitrate analyses at 3, 6, and 12 weeks) should also be determined.

INSTRUCTIONS TO COLLABORATORS

Instructions in considerable detail were sent to collaborators. Methods given in the 7th Ed. of *Official Methods of Analysis* are referred to by number.

(1) Determine the water-insoluble nitrogen, soluble in neutral permanganate, by use of methods **2.34, 2.35,** and **2.36.**

(2) Determine the AI (activity index) applied to U-F polymers as follows:

NITROGEN ACTIVITY INDEX (AI) OF UREA-FORMALDEHYDE COMPOUNDS

(Applicable to urea-formaldehyde compounds only)

REAGENTS AND APPARATUS

(a) *Phosphate buffer solution (0.063 M).*—Dissolve 14.3 g KH_2PO_4 and 91.0 g K_2HPO_4 in 1 l H_2O. Dil. 100 ml of the soln to 1000 ml. Adjust *p*H of dild soln to 7.5.

(b) *Water bath.*—With circular openings in the cover thru which 400 ml beakers may be suspended. Temp. of 99–100°C. should be maintained.

DETERMINATION

Crush sample to pass 20-mesh std sieve (avoid fine grinding). Det. cold H_2O-insoluble nitrogen (WIN) as in **2.34.** (Temp. of H_2O used in extn should be 25° ±2°C.) Det. hot H_2O-insoluble nitrogen (HWIN) in phosphate buffer. Place accurately weighed sample contg 0.3000 ± .005 g WIN_{25} in a 400 ml beaker and add 250 ml boiling buffer soln, *p*H 7.5. Stir, cover, and immerse in a boiling H_2O bath so that the liquid level in the beaker is below the water line in the bath. Stir the soln gently for ca 5 sec. after 10, 20, and 30 min. intervals; then remove from bath and filter at once thru 15 cm Whatman No. 12 fluted filter. Wash insol. residue onto the filter with nearly boiling H_2O and continue washing 4 times from the top down (total vol. of wash water should be 75–100 ml). Det. the total N in the wet paper and residue as under **2.23** or **2.24.**

$$\text{Activity Index} = \frac{(WIN_{25} - HWIN)}{WIN_{25}} \times 100$$

WIN_{25} = % water-insoluble nitrogen; HWIN = % hot water (buffer) insoluble nitrogen.

KJELDAHL METHOD FOR ORGANIC AND AMMONIACAL NITROGEN ONLY

REAGENTS AND APPARATUS

(a) *Sulfuric acid.*—96–98% H_2SO_4, reagent grade.

(b) *Mercuric oxide or metallic mercury.*—Reagent grade.

(c) *K_2SO_4 or Na_2SO_4.*—Anhyd. N-free.

(d) *Sulfide or thiosulfate solution.*—40 g K_2S, or 40 g Na_2S, or 80 g $Na_2S_2O_3 \cdot 5H_2O$, in 1 l H_2O.

(e) *Sodium hydroxide solution.*—Ca 450 g NaOH, free from nitrates, in 1 l H_2O (soln with sp. gr. of 1.36 or higher may be used).

(f) *Granulated zinc.*—30 mesh or suitable "bump" preventative.

(g) *Standard sulfuric acid solution (0.5 N).*—Chapter 39.

(h) *Standard sodium hydroxide solution (0.1 N).*—Chapter 39.

(i) *Methyl red indicator.*—1 g Me red in 200 ml alcohol.

(j) *Kjeldahl flasks.*—Of suitable size; usually ca 500 or 800 ml capacity. Suitable digestion and distn racks, hoods, and trap systems should be employed. Either gas or electric heat may be used.

Solutions (g) and (h) should be stdzd with primary standards and checked against each other.

Before using reagents, test by blank detn with sugar, which insures partial reduction of any nitrates possibly present.

DETERMINATION

Place sample (0.7–2.2 g, according to N content) in digestion flask. Add 0.7 g HgO (or 0.65 g Hg), 10 g powd. K_2SO_4 (or anhyd. Na_2SO_4), and 25 ml concd H_2SO_4. If necessary to use larger sample than 2.2 g, increase H_2SO_4 10 ml for each g of sample. Place flask in inclined position, heat gently until frothing ceases (if necessary, add small amount of paraffin to reduce frothing), and boil briskly until soln clears and then for at least 30 min. longer.

Cool, add ca 200 ml H_2O, cool below 25°C., add 25 ml sulfide or thiosulfate soln to ppt Hg, and mix. (Sulfide or thiosulfate may be mixed with the NaOH before addition to flask). Add few Zn granules to prevent bumping, tilt flask, and add layer of NaOH (sufficient soln to make contents strongly alk.; ca 60 ml usually sufficient) without agitation. Immediately connect to still fitted with a suitable scrubber bulb (to prevent carryover of alkali during distn) and a suitable receiver contg std acid and fitted with a trap or delivery tube to receive NH_3 as it is distd over. Rotate to mix contents thoroly and heat until all NH_3 has distd (at least 150 ml distillate). Titr. excess std acid in distillate with std alkali soln, using Me red indicator.

PROCEDURE FOR DETERMINING NITRIFICATION RATE

(To be performed by collaborators having suitable facilities)

Weigh 100 g portions of the std air-dry soil provided (passing 2 mm sieve and contg sufficient $CaCO_3$ to bring *p*H to 7), and transfer each to a tared 500 ml erlenmeyer. For each test weigh amount of the cold H_2O-washed sample equiv. to 20 mg H_2O-insol. N and transfer to flask contg the soil. (Prewashed samples were supplied each collaborator to save time). Mix sample and soil thoroly by rolling; then level the soil in the flask. Pipet tap H_2O equal to 50% of the moisture-holding capacity of the soil and contg 1 ml inoculum per flask onto surface of soil in flasks (ca 21.7 ml H_2O per 100 g is required to bring the std soil to 50% moisture capacity). (The inoculum is made from the decantate of a fertile soil suspension obtained by mixing equal parts by wt of tap H_2O and fresh soil.) Then plug flasks with nonabsorbent cotton, record gross wts without the plugs (weight of flask, soil, treatment, and H_2O) to nearest 0.5 g, and incubate at 30°C. Bring flasks to gross wt twice weekly by adding distd H_2O. Analyze duplicate or triplicate flasks of each treatment for nitrate content at intervals of 3, 6, and 12 weeks. Directions for the nitrate detn are as follows:

DETERMINATION OF NITRATE NITROGEN IN SOIL NITRIFICATION STUDIES

REAGENTS

(a) *Aluminum sulfate, 8%.*—$Al_2(SO_4)_6 \cdot 18H_2O$.

(b) *Ammonium hydroxide.*—100 ml NH_4OH, sp. gr. 0.9, +200 ml H_2O.

(c) *Phenol disulfonic acid.*—Prepd as in **31.5**(a).

PROCEDURE

To the nitrification flask contg 100 g soil, add from graduated cylinder 240 ml of soln made by mixing 235 ml distd H_2O with 15 ml 8% Al sulfate. Stopper and place in a horizontal shaker and shake for 10 min. Add ca 2 g $Ca(OH)_2$, and shake again for 3 min. Remove from shaker and let settle for 0.5 hr. Filter thru 15 cm folded filters into dry flasks, discarding first 20 ml. Collect 50 ml filtrate.

(If necessary to remove chloride, follow **31.6,** *Official Methods of Analysis,* 7th Ed. (1950). By using a soil contg only a few ppm chloride, there is no interference.)

Transfer 5 ml aliquot with a pipet to a small porcelain dish, add 50 mg $CaCO_3$, and evap. to dryness on steam bath. Remove dish, allow to cool, and add 2 ml phenol disulfonic acid soln to the center of the dish from a pipet whose tip has been cut off for rapid delivery. Rub around at once with short glass rod to wet all dry material, otherwise losses of nitrate may result from CO_2 evolution.

After 0.5 hr or more, add 20 ml H_2O and mix. When cool, add 20 ml NH_4OH (1+2), in excess, to develop yellow color. Transfer to 100–200 ml graduated cylinder (size depends upon depth of color), make to vol., and mix. If turbid, filter thru folded filter.

Det. concn in the photocolorimeter, using Corning Pyrex No. 554 light filter, and read results from graph prepd with known solns of $NaNO_3$. Set reading for max. transmission, I_0, with distd H_2O. Calc. ppm nitrate nitrogen in the soil to the air-dried basis. Then calc. % added nitrogen nitrified as follows:

$$\frac{\text{(ppm nitrate N in treated soil)} - \text{(ppm nitrate N in control soil)}}{\text{ppm added nitrogen}} \times 100 = \% \text{ added N nitrified}$$

COLLABORATORS

Sixteen laboratories signified that they would participate in the chemical analysis part of this program; four of them stated they would also make the nitrification tests. Reports were received from thirteen laboratories, of which three did both the chemical and nitrification tests. They are as follows: (order in which they are listed is not related to the order in which the data are presented in the tables):

C. V. Marshall, Department of Agriculture, Ottawa, Canada

Frances L. Bonner, Feed & Fertilizer Laboratory, Louisiana State University, Baton Rouge, La.

G. M. Brooks and M. P. Etheredge, Mississippi State Chemistry Laboratory, State College, Miss.

V. L. Gaddy,[1] Soil and Water Conservation Branch, U. S. Department of Agriculture, Beltsville, Md.

J. B. Smith,[1] Rhode Island Agricultural Experiment Station, Kingston, R. I.

Gordon Hart and E. C. Petrie, Chemical Division, Department of Agriculture, Tallahassee, Fla.

Vesta Staab, New Hampshire Agricultural Experiment Station, Durham, N. H.

A. C. Wark[1] and Robert Leydon,[1] New Jersey Agricultural Experiment Station, New Brunswick, N. J.

L. D. McKillop and R. C. Berry, Division of Chemistry, Department of Agriculture, Richmond, Va.

E. Kuta and O. W. Ford, Purdue University, Lafayette, Ind.

[1] Made the nitrification test in addition to the chemical tests.

TABLE 1.—*Results obtained by Morgan-Kralovec method (expressed as per cent N "AI" calculated)*

COLLABORATOR NO.	SAMPLE A HIGH GRADE PRODUCT			SAMPLE B MEDIUM GRADE PRODUCT			SAMPLE C POOR GRADE, RESIN SCRAP			SAMPLE D ORGANIC N SOURCE CASTOR PUMICE		
	WIN[a]	HWIN[b]	AI[c]	WIN	HWIN	AI	WIN	HWIN	AI	WIN	HWIN	AI
1	29.08	14.09	51.5	32.30	25.44	21.2	19.85	18.83	5.1	5.09	3.34	34.4
2	28.42	10.55	62.9	32.03	21.62	32.5	19.45	18.47	5.0	5.03	1.29	74.4
3	28.90	13.79	52.2	32.04	23.90	25.4	19.40	0.36[d]	98.0[d]	5.12	2.13	58.4
4	28.13	14.63	48.0	31.46	26.61	79.1	19.41	0.84[d]	95.7[d]	5.24	0.36[d]	93.1
5	29.40	11.60	60.5	32.85	24.90	24.2	19.90	19.00	4.5	5.19	4.61	11.2
6	28.56	10.40	63.6	32.44	22.21	31.5	20.07	18.55	7.6	5.10	3.44	32.5
7	28.49	12.42	56.4	32.08	32.29	27.4	19.67	18.44	6.3	5.08	—	—
8	27.98	19.39	30.7	30.85	28.09	8.9	19.16	19.40	—	2.49[d]	2.96	—
9	28.70	11.99	58.2	32.02	22.61	29.4	19.79	18.54	6.3	5.13	3.70	27.9
10	28.23	7.83	72.3	32.06	17.98	43.9	19.85	8.89[d]	55.2[d]	5.08	0.45[d]	91.1
11	28.90	13.00	55.0	32.10	23.30	27.4	19.80	18.60	6.1	5.00	3.10	28.0
12	28.54	13.64	52.2	32.15	22.00	31.6	19.72	18.53	6.0	5.14	4.34	15.6
13	28.22	13.87	50.8	32.23	25.23	21.7	19.87	18.71	5.8	5.10	2.84	44.3
Average:	28.58	12.86	55.0	32.05	22.09	31.1	19.68	18.71	5.8	5.10	3.18	37.4
Standard Deviation:	0.32	2.61	9.46	0.45	4.98	15.84	0.25	0.28	0.86	0.06	0.97	17.6

[a] WIN: Water insoluble nitrogen.
[b] HWIN: Hot buffer insoluble nitrogen
[c] $AI = \frac{WIN - HWIN}{WIN} \times 100$
[d] Omitted from average.

Allen B. Lemmon, Bureau of Chemistry, Department of Agriculture, Sacramento, Calif.

D. N. Willett and W. B. Griem, Department of Agriculture, Madison, Wis.

John Thompson and Willis Richerson, Chemistry Laboratory, State Department of Agriculture, Oklahoma City, Okla.

COMMENTS OF COLLABORATORS

The most frequent comment was that sample D was difficult to handle. Typical of other remarks was the statement that only on the cold water-insoluble nitrogen did the results show fair agreement. It was also noted that the determinations of water-insoluble organic nitrogen soluble in neutral permanganate gave poor results. Several collaborators had serious difficulty in filtration of the solution obtained on digesting sample D in the phosphate buffered solution.

DISCUSSION OF RESULTS

The results submitted by the collaborators are shown in Tables 1, 2, and 3. In most cases, each result represents an average of several determinations.

TABLE 2.—*Water-insoluble organic nitrogen soluble in neutral permanganate (expressed as per cent N)*

COLLABORATOR NO.	SAMPLE A	SAMPLE B	SAMPLE C	SAMPLE D
1	20.56	14.63	4.59	4.56
2	26.98	24.52	8.17	4.67
3	24.05	21.69	6.97	4.57
4	20.81	17.72	7.38	4.79
5	5.13	14.40	15.60	0.53
6	26.22	24.20	7.68	4.66
7		Reported as % AI, does not apply		
8	10.49	6.07	1.81	1.45
9	23.63	21.97	7.74	4.70
10	0.29	0.64	1.93	0.32
11	24.70	20.10	6.10	4.60
12	24.29	24.35	8.17	4.78
13	3.58	20.43	6.44	0.39

The average of the three collaborators reporting rate of nitrification of the urea-formaldehyde mixtures is shown in Figure 1.

The results of the collaborators, with one exception, agree very well in the determination of water-insoluble nitrogen. The variations among results for the hot water-insoluble nitrogen are considerable when measured by the usual standards for nitrogen. With the exception of two or three collaborators, the agreement is good in the case of the urea-formaldehyde samples. Variation is much greater in the case of the castor

TABLE 3.—*Results of nitrification test (expressed as per cent of added nitrogen converted to nitrate during the time indicated)*

SAMPLE	INTERVAL	COLLABORATOR NO.			AVERAGE FOR 3 COLLABORATORS
		2	7	9	
	weeks				
A	3	4.9	—	19.0	8.0
	6	22.5	18.8	31.5	24.3
	12	42.1	27.5	54.5	41.4
B	3	3.1	—	8.5	3.9
	6	10.8	15.0	12.5	12.8
	12	21.9	20.0	30.0	24.0
C	3	1.2	—	0.0	0.4
	6	−1.1	1.3	1.5	0.6
	12	1.5	5.0	1.0	2.8
D	3	56.9	34.3	66.5	52.6
	6	58.4	35.0	60.5	51.3
	12	63.7	47.5	60.5	57.2
$(NH_4)_2SO_4$	3	74.4	29.3	80.5	61.4
	6	89.7	38.8	90.0	72.8
	12	90.9	55.0	81.0	75.6

pumice, typical of natural organic nitrogen sources. It should be pointed out that the Morgan-Kralovec method is not designed for this type of material, a fact emphasized by the comments of several of the collaborators. The extreme variation in the results reported by two or three collaborators has caused the Associate Referee concern as to whether the procedure was clear to the analyst.

All data reported are shown in the tables. In arriving at averages, no data were omitted in the case of samples A and B. In the case of samples C and D, it appeared to the Associate Referee that in a very few instances, as indicated, it was advisable to omit certain results in the calculation of averages. The wide variations between replicate determinations in some cases by the same analyst was cause for some concern. However, variations should decrease greatly as the analyst becomes more familiar with the method, an observation borne out by comments of several collaborators.

The standard deviation is shown in each instance. If two values were omitted where results differ greatly from the average, the standard deviation would be much less.

The analysis of these samples for the water-insoluble organic nitrogen soluble in neutral permanganate did not give even reasonable agreement. The data are presented in Table 2; no averages are given because of wide

variations. Replicate determinations by the same analyst often varied greatly. The data indicate that this method is not applicable to urea-formaldehyde mixtures, since in general no great difference is indicated between the medium and good grade product, samples B and A. This observation confirms one of the principal reasons for this study.

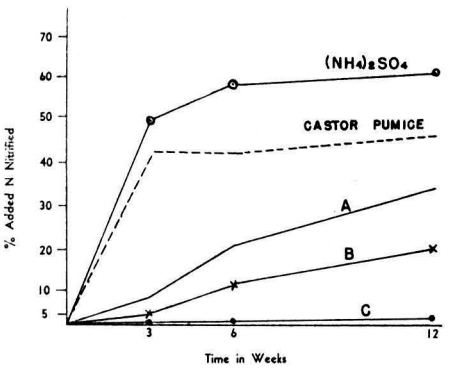


FIG. 1.—Average results of nitrification test.

The results of all three collaborators in the nitrification test show the same trends although the exact values vary widely. In all instances, the result shows that the nitrogen in the U-F resin scrap is nitrified very little and that this material is of little or no value as a source of nitrogen for plants. Sample B is a fair source in that approximately 25 per cent of its nitrogen was nitrified over a twelve week period. Sample A is about twice as good a product as sample B, as shown by these tests. This study demonstrates that since most of the nitrogen in ammonium sulfate and the usual organics such as castor pumice is nitrified within a comparatively short time, the nitrification of nitrogen in the urea-formaldehyde resins proceeds more slowly and steadily over a considerable period of time.

ACKNOWLEDGMENT

The Associate Referee wishes to thank Dr. Morgan and Dr. Kolterman and the E. I. du Pont de Nemours and Company for their help in supplying materials for this collaborative study.

The work of the various collaborators is sincerely appreciated. Their sincere and constructive efforts insure progress in the development and testing of analytical methods.

SUMMARY

1. An activity index (AI) as determined by the Morgan-Kralovec method provides a means of indicating the availability of nitrogen in urea-formaldehyde compounds.

2. The method is not applicable to the natural organics such as castor pumice.

3. The neutral permanganate method usually employed to determine the availability or "activity" of nitrogen in natural organics is not applicable in the case of urea-formaldehyde compounds.

4. The nitrogen in urea-formaldehyde compounds, if it is available as shown by the index, is made available to plants by nitrification at a steady rate over a considerable period of time under the proper soil and season conditions. This situation is in contrast with the usual rapid nitrification of the available nitrogen in natural organics and ammonium sulfate.

RECOMMENDATIONS

It is recommended*—

(1) That the term "Availability Index" as described in the Morgan-Kralovec method for determining the quality of urea-formaldehyde compounds be changed to "Activity Index" to maintain uniformity in terminology, and that the method as described here be adopted as first action.

(2) That further collaborative work with the Morgan-Kralovec "AI" method be delayed until its use indicates that modification may be desirable.

(3) Based upon the report of collaborative work on total nitrogen by the Joint A.O.A.C.–A.O.C.S. Committee,† it is recommended:

(*a*) That the use of copper as a catalyst in official methods for total N be discontinued.

(*b*) That the directions for preparing standard acid and standard alkali, **2.20,** be deleted; that new directions refer to Chapter 39 and require the use of a primary standard for each solution; that the acid and alkali, so prepared, be finally checked one against the other.

(*c*) That the collaborative mercury procedure be revised to omit reference to boric acid and that the revised procedure be adopted as first action with a view toward its replacement of **2.20–2.24** and its incorporation into **2.25** and **2.26.**

(*d*) That further collaborative work be done on the relation of the boil-test to the time necessary for digestion of the sample.

---

* For report of Subcommittee A and action of the Association, see *This Journal*, 38, 63, 64 (1955).
† See *This Journal*, 38, 56 (1955).

REPORT ON POTASH IN FERTILIZERS

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

In accordance with the recommendations approved by this Association in 1953 (*This Journal*, **37**, 318 (1954)), additional collaborative studies were made on methods for the determination of potash in fertilizers. An outline of the proposed work for 1954 was sent to each analyst who had expressed a willingness to collaborate. Reports were returned from 24 collaborators in the fertilizer industry and in commercial and control laboratories.

The primary objective of the study was the evaluation of the flame photometer procedure as a method for determining potash in fertilizers. Results obtained by this method were compared with those obtained by the official gravimetric procedures used on the same samples (Table 1). In addition, some samples prepared from chemically pure salts were included so that the true potash content was known within narrow limits, and a check upon the reliability of all methods used in the study was thus available.

An attempt was made to select samples that would represent a cross section of the samples normally encountered in a fertilizer laboratory. The potash content ranged from 6 to 63 per cent (the latter was reagent grade potassium chloride), and the phosphoric acid ranged from 0 to 52 per cent. Some samples were also included for an investigation of the effectiveness of the proposed flame photometer procedure in overcoming cation or anion interference. Since urea is now used widely in fertilizers, a sample containing this compound was used to determine whether urea interference could be expected.

COMPOSITION OF SAMPLES

Five pounds of each sample was prepared by grinding in a Micro Samplmill (⅛ inch screen) and mixing thoroughly in a twin shell dry blender. Collaborators were furnished with 2 ounce portions of each sample, of the following compositions:

No. 1: Commercial sulfate of potash-magnesia (approximately 21.5% K_2O).

No. 2: Reagent grade urea (0.5 lb.), rock phosphate (3.5 lb.), and reagent grade potassium chloride (1 lb.) Calculated analysis: 12.64% K_2O.

No. 3: Commercial fertilizer, 10–52–17.

No. 4: Commercial fertilizer, 4–16–16.

No. 5: Commercial fertilizer, 2–12–16.

No. 6: Reagent grade potassium chloride. Calculated analysis: 63.17% K_2O.

No. 7: Reagent grade ammonium sulfate (4 lb.) and potassium sulfate (1 lb.) Calculated analysis: 10.81% K_2O.

No. 8: Commercial fertilizer, 3–10–30.

DIRECTIONS TO COLLABORATORS

In analyzing the samples gravimetrically, use the method (Perrin or Lindo-Gladding) normally employed in your laboratory. Prepare all sample solutions ac-

TABLE 1.—*Per cent potash found by gravimetric and flame photometric methods*

COLLABORATOR[a]	INSTRUMENT[b]	SAMPLE NO. 1		SAMPLE NO. 2		SAMPLE NO. 3		SAMPLE NO. 4		SAMPLE NO. 5		SAMPLE NO. 6		SAMPLE NO. 7		SAMPLE NO. 8	
		GRAVIMETRIC	FLAME PHOTOMETER	GRAVIMETRIC	FLAME PHOTOMETER	GRAVIMETRIC	FLAME PHOTOMETER	GRAVIMETRIC	FLAME PHOTOMETER	GRAVIMETRIC	FLAME PHOTOMETER	GRAVIMETRIC	FLAME PHOTOMETER	GRAVIMETRIC	FLAME PHOTOMETER	GRAVIMETRIC	FLAME PHOTOMETER
1	A	22.61	——	12.77	——	17.30	——	17.09	——	6.86	——	63.27	——	10.48	——	29.48	——
2	B	22.92	22.38	12.98	12.78	17.43	17.28	17.23	17.45	6.93	7.13	63.57	63.88	10.48	10.42	29.96	29.88
3	C	22.86	22.59	12.91	13.09	17.31	16.80	17.04	16.65	6.90	6.84	63.61	63.38	10.61	10.57	29.71	29.43
4	A	22.74	23.78	12.93	12.98	18.03	17.44	17.17	17.18	6.81	6.79	62.98	63.48	10.75	10.47	29.80	29.38
5	B	22.46	23.22	12.82	12.54	17.26	17.95	17.07	16.88	6.80	7.09	63.12	62.16	10.75	11.00	29.16	29.15
6	C	23.17	23.39	12.98	13.13	17.49	17.61	17.44	17.59	7.03	7.09	64.32	64.38	10.62	10.94	30.07	30.62
7	B	18.35	18.35	13.04	12.96	17.69	17.06	17.49	17.05	6.95	6.92	63.47	63.95	10.49	10.33	30.29	30.01
8	C	22.61	22.74	12.67	13.19	16.99	17.18	16.84	17.21	6.78	6.98	62.89	62.90	10.03	10.29	29.27	29.45
11	B	22.80	23.21	12.69	12.91	17.52	17.54	16.90	17.16	6.66	7.00	62.97	63.07	10.29	10.49	29.38	29.69
12	C	22.83	22.66	12.96	12.69	17.22	17.12	17.09	17.07	6.85	6.90	62.88	61.55	10.51	10.28	29.59	29.54
13	A	24.11	21.78	12.64	12.63	17.08	17.08	16.88	16.35	6.53	6.75	61.53	62.20	10.44	10.33	29.03	28.85
14	B	23.03	22.64	12.96	12.61	17.44	17.15	17.27	17.11	6.98	6.93	62.71	61.50	10.60	10.45	29.82	29.13
15	B	22.43	21.49	12.84	13.17	17.75	17.58	17.30	17.29	7.01	7.15	63.94	63.64	10.84	10.64	30.35	30.49
16	C	25.29	16.92	13.02	13.21	17.35	17.44	17.00	16.91	6.85	6.75	63.42	63.73	10.59	10.43	30.00	29.60
17	C	22.96	22.96	13.06	12.86	17.54	17.53	17.22	17.07	6.92	6.96	63.42	62.83	10.59	10.67	29.79	29.83
18	A	18.27	17.69	12.62	12.74	16.67	16.56	16.23	16.21	6.46	6.57	61.92	61.70	9.97	9.90	29.92	29.90
19	B	18.60	22.12	13.03	13.23	17.30	16.83	17.25	16.93	6.98	6.65	63.30	61.75	10.38	10.45	29.95	29.13
20	B	23.15	22.53	13.44	13.27	17.79	16.94	17.72	17.10	7.27	6.98	63.05	62.78	10.84	10.61	30.14	29.91
21	D	13.89	12.43	12.91	12.79	17.22	17.17	16.95	16.73	6.68	6.70	63.15	63.05	10.49	10.52	29.42	29.57
22	D	22.58	22.55	13.01	13.18	17.38	17.08	17.11	17.05	6.90	6.83	63.17	63.70	10.86	10.84	29.49	29.80
23	B	19.55	19.30	12.95	12.94	17.49	17.26	17.04	17.19	6.92	7.03	63.44	62.40	10.43	10.88	29.56	29.98
24	B	22.43	22.04	12.51	12.18	17.03	15.80	16.91	16.99	6.64	6.79	62.57	63.11	10.58	10.65	29.26	29.55
25	D	17.16	17.23	12.79	12.88	17.19	17.18	16.93	17.06	6.78	6.82	62.74	62.78	10.33	10.35	29.17	29.28
26	A	13.54	11.38	12.99	13.05	17.39	16.66	16.74	16.64	6.94	6.68	63.08	63.27	10.63	10.49	29.76	29.85
Average:		——	——	12.90	12.91	17.37	17.14	17.08	16.99	6.85	6.88	63.11	62.92	10.52	10.52	29.68	29.65
High:		——	——	13.44	13.27	18.03	17.58	17.72	17.45	7.27	7.15	63.94	63.88	10.86	11.00	30.35	30.49
Low:		——	——	12.51	12.18	16.67	15.80	16.23	16.21	6.46	6.57	61.53	61.53	9.97	9.90	29.03	28.85
Maximum Variation:		——	——	0.93	1.09	1.36	1.78	1.49	1.24	0.81	0.58	2.41	2.38	0.89	1.10	1.32	1.64
Standard Deviation:		——	——	0.19	0.27	0.29	0.44	0.29	0.32	0.17	0.16	0.60	0.82	0.23	0.25	0.37	0.42

[a] Gravimetric values of collaborators 1–8 were by the Perrin Method and 11–26 were by Lindo-Gladding Method.
[b] A: Beckman DU, hydrogen, direct intensity. B: Beckman DU, acetylene, direct intensity. C: Beckman, B, acetylene, direct intensity. D: Perkin-Elmer, propane, internal standard.

cording to the directions in section **2.40**, *Official Methods of Analysis*, 7th Ed., with these exceptions:

For potash salts (muriate and sulfate of potash), add 50 ml of saturated ammonium oxalate before diluting with water.

For sulfate of potash and magnesia, add ammonium oxalate but omit the addition of ammonium hydroxide to permit precipitation of magnesium as the oxalate.

Withdraw two aliquots from each sample solution. Use one aliquot for the gravimetric analysis and dilute the other for flame analysis.

To provide a basis for statistical analysis of normal variations between duplicates and between different times of analysis, duplicate samples should be weighed and analyzed in parallel and the procedure repeated at least 1 week later. (Four gravimetric and four flame results should thus be recorded for each sample.) If more than one analyst performs the work, the results of each should be reported separately.

Make a separate analysis by the flame photometric procedure as follows:

FLAME PHOTOMETER DETERMINATION

REAGENTS

(a) *Potassium chloride.*—Recrystallize KCl 3 times from H_2O soln and dry at 110° to constant wt.

(b) *Phosphate-sulfate interference buffer solution.*—Dissolve 55.80 g $(NH_4)_2HPO_4$ and 13.75 g $(NH_4)_2SO_4$ in H_2O, and dil. to 1 l. Contains 30,000 ppm P_2O_5 and 10,000 ppm SO_4.

STANDARD POTASH SOLUTIONS

(a) *Potash stock solution.*—Dissolve 1.5830 g KCl in H_2O and dil. to 1 l. Contains 1000 ppm K_2O.

(b) *Potash standard solutions (for direct intensity instruments).*—To 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 20.0, 22.5, and 25.0 ml soln (a) add 5 ml phosphate-sulfate interference buffer soln and 1.0 ml satd NH_4 oxalate soln, and dil. to 250 ml with H_2O. Stds contain 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 ppm K_2O, resp.

(c) *Potash standard solutions (for internal standard instruments only).*—Prep. solns as in (b) but add sufficient $LiNO_3$ soln before dilg to vol. to give final soln contg 100 ppm Li for an acetylene flame, or 800 ppm Li for a propane flame.

DILUTION OF SAMPLE SOLUTION

(a) *Mixed fertilizers.*—(1) Less than 15% K_2O: Transfer 5 ml aliquot to 100 ml vol. flask, add 2 ml interference buffer soln, dil. to vol. with H_2O, and mix. (2) 15–30% K_2O: Transfer 5 ml aliquot to 250 ml vol. flask, add 5 ml interference buffer soln, dil. to vol. with H_2O, and mix. (3) More than 30% K_2O: Transfer 5 ml aliquot to 500 ml vol. flask, add 10 ml interference buffer soln, dil. to vol. with H_2O, and mix.

(b) *Potash salts (muriate and sulfate of potash).*—(1) Transfer 5 ml aliquot to 500 ml vol. flask, add 10 ml interference buffer soln, dil. to vol. with H_2O, and mix.

(c) *Sulfate of potash and magnesia.*—(1) Proceed as in (a), step (2), above.

NOTE: For internal standard instruments, the dild soln should also contain lithium (100 ppm for acetylene flame; 800 ppm for propane flame).

DETERMINATION

With the wavelength set at 767 mμ (or with K filter in position), adjust flame photometer to read mid-scale with the 40 ppm standard. Det. emission of remaining stds and prep. std curve of relative emission intensity *vs.* concn. Det. relative emission intensity of sample and calc. K_2O concn from std curve.

$$\text{Per cent } K_2O \text{ in sample} = \frac{\text{ppm } K_2O \text{ found} \times \text{vol. final soln} \times 50}{\text{wt of sample} \times 10^6} \times 100$$

Example: 2.5 g sample (250 ml); dil. 5 ml to 100; 60 ppm K_2O found.

$$\text{Per cent } K_2O = \frac{60 \times 100 \times 50 \times 100}{2.5 \times 10^6} = 12.0\%$$

COMMENTS OF COLLABORATORS

Of the 24 collaborators who returned reports, 12 stated that they were interested in sodium tetraphenyl-boron studies. Eight reported low or erratic results with sample 1 and suggested that the preparation of the sample solution should be modified to include boiling. One collaborator believed the difficulty was due to presence of undecomposed langbeinite in the residue from the 1 hour cold extraction.

No. 4 complained that the solution bumped excessively so that the boiling time had to be shortened and low results were obtained on the first samples. However, the addition of a few granules of Ottawa sand eliminated the bumping and later results were higher.

No. 6 listed clogging of the burner as the main objection, and adopted the practice of rinsing after each reading. No. 16 reported difficulty with the operation of the photometer atomizer because of the addition of large amounts of the phosphate-sulfate interference buffer. He felt that a procedure for removal of the interference would be preferable to compensation.

No. 5 stated that a flame photometric determination had been made on the T.P.A. solutions of about 50 samples of commercial fertilizer. Good agreement with results from analyses of the oxalate solutions was achieved.

Some difficulty was reported by collaborators who used the internal standard method to establish a standard curve by adjusting the photometer to mid-scale with the 40 p.p.m. standard. They found it desirable to adjust the instrument to read 100 with the highest standard and then determine the remaining points on the standard curve.

DISCUSSION

Sample No. 1 was selected because of its high magnesium-potash ratio. Since magnesium alters the emission of potassium if it is present in sufficient concentration, it was anticipated that results of this analysis would indicate if magnesium interference might be a factor in the flame photometric procedure. Unfortunately, the directions for preparation of the solution of this sample specified that boiling should be omitted, which led to very erratic results, and thus no conclusions could be drawn in regard to magnesium interference. The results clearly indicate the necessity of boiling this type of sample when preparing solutions for analysis by either gravimetric or flame photometric procedures. To facilitate the precipitation of magnesium as the oxalate, the boiled solution should be cooled to room temperature and allowed to stand one hour before it is diluted to volume. Ammonium hydroxide should be omitted.

Sample No. 2 was a mixture of urea, rock phosphate, and potassium chloride. It was included to determine whether the urea in commercial fertilizers would affect the accuracy of the flame photometric method. The average values obtained by the gravimetric and flame photometric procedures were surprisingly close, indicating that urea interference is not a factor.

Sample No. 3 was a water-soluble 10–52–17 and was included as an example of a highly soluble phosphate sample for determining if phosphate interfered with the flame photometric determination. Results (Table 1) show good agreement between the two methods. Although the average flame values are slightly lower, the difference is small (0.23 per cent K_2O) and probably is not significant. Several analysts reported flame values equal to or higher than gravimetric results. Since the P_2O_5 to K_2O ratio of 3:1 in this sample is higher than that normally encountered in most fertilizers, it appears that phosphate interference is not significant if the standards and sample solutions are prepared according to directions.

Samples No. 4, 5, and 8 were commercial fertilizers included as examples of various potash levels (6, 16, and 30 per cent). Agreement between gravimetric and flame photometric results was excellent for all three samples.

Sample No. 6, reagent grade potassium chloride, was selected for its high potash content and its purity. Thus it served as a check on the gravimetric method as well as on the flame photometric procedure. Results by the two methods are in very good agreement, although the average values are slightly below theory in both cases. The only apparent advantage of the gravimetric method is its lower standard deviation (.59 *vs* .82).

Sample No. 7 was a mixture of ammonium sulfate and potassium sulfate with a sulfate-to-potash ratio of approximately 7:1. Although the ratio was considerably higher than that usually encountered in normal fertilizers, the sample was included for an investigation of the effectiveness of adding sulfate to the flame photometer standards to overcome the interference of this anion. Results in Table 1 show the effectiveness of the procedure. The average values are identical for both methods and the standard deviations are not significantly different.

Three models of commercial flame photometers were represented in this study, the Beckman Model DU, the Beckman Model B, and the Perkin-Elmer. The direct intensity method, using either hydrogen or acetylene, was employed by all collaborators with the Beckman instruments, and the internal standard method, with propane as the fuel, was employed by all collaborators using the Perkin-Elmer instrument. Comparable results were reported in all cases, indicating that the selection of the instrument is a matter of personal preference where potassium is concerned.

CONCLUSIONS

The samples analyzed in this study covered the range of potash levels normally found in commercial fertilizers (6 to 63 per cent). Samples were also included in the attempt to determine if interference from urea, phosphate, or sulfate could be expected. The conditions in each case represented extremes, since it was felt that interference effects were not likely to appear in the analysis of normal fertilizer samples if they did not appear under these conditions.

The maximum variation and the standard deviation of results for each sample by the two methods were of the same magnitude and were as good as, or better than, those normally obtained by this number of laboratories. Since there was no significant difference in the results by gravimetric and flame photometric methods for these widely varying samples, the Associate Referee was at first inclined to recommend the adoption of the flame photometric method. However, in view of the continuous improvement of this method, it appears desirable to continue study for another year.

COLLABORATORS

George J. Allen, New Orleans 24, La.

H. R. Allen and Rose J. Ruh, Kentucky Agricultural Experiment Station, Lexington, Ky.

W. C. Geagley and Virginia A. Thorpe, Department of Agriculture, Lansing, Mich.

Sherril S. Gibbs, New York Agricultural Experiment Station, Geneva, N. Y.

R. M. C. King and C. H. Perrin, Canada Packers, Toronto, Canada

Cecil Pinkerton, College Park, Md.

Paul R. Rexroad, Farm Bureau Cooperative Association, Columbus, Ohio

William J. Tucker, Soil Building Laboratory, Ithaca, N. Y.

F. L. Aldrich, Department of Biochemistry, Purdue University, Lafayette, Ind.

D. B. Bates, Smith-Douglass Co., Inc., Norfolk, Va.

B. W. Beadle, G. W. Gooch Laboratories, Los Angeles 23, Calif.

R. C. Berry, L. D. McKillip, Mary T. Murrill, and J. H. Elder, Richmond, Va.

J. F. Fudge, Edward Ibert, and Curtis L. Godfrey, College Station, Texas.

C. W. Gehrke, Agricultural Laboratory Building, University of Missouri, Columbia, Mo.

W. S. Hager, H. Y. Fisher, and D. H. Myers, Bureau of Foods and Chemistry, Harrisburg, Pa.

William M. Hoffman, Soils Building, Plant Industry Station, Beltsville, Md.

Charles V. Marshall, Plant Products Laboratory, Ottawa, Canada

Henry A. Miller and Wilbur P. Matthews, Raleigh, N. C.

C. R. Phillips, Darling & Co., East St. Louis, Ill.

Stacy B. Randle and Leo J. Faneuf, New Jersey Agricultural Experiment Station, New Brunswick, N. J.

Richard M. Smith, and R. C. Crooks, Department of Agriculture, Tallahassee, Fla.

Charles W. Urquhart, Spencer Chemical Co., Pittsburgh, Kans.

W. D. Wilhide, Tennessee Valley Corp., Wilson Dam, Ala.

A. T. Blackwell and Mr. Diffenbaugh, Davison Chemical Co., Baltimore, Md.

ACKNOWLEDGMENT

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

RECOMMENDATIONS

It is recommended*—

(1) That in view of continuous improvements now being made in the flame photometric method for the determination of potash, its adoption be postponed for another year and further collaborative work be done.

(2) That studies be initiated on the use of sodium tetraphenylboron for the determination of potash in fertilizers.

No reports were given on copper and zinc, free water, or magnesium and manganese.

ANNOUNCEMENTS

DRUGS IN FEEDINGS STUFFS:

A new section, "Drugs in Feeding Stuffs," has been established under Subcommittee B with the following appointments:

FURAZOLIDONE:

Chester A. Luhman, Department of Agriculture, State Office Building 1, Sacramento, Calif.

NICARBAZIN:

W. J. Mader, Analytical Research Department, Chemical Control Division, Merck & Co., Inc., Rahway, N. J.

NITROFURAZONE:

W. R. Flach, Eastern States Farmers Exchange, P. O. Box 948, Buffalo, N. Y.

NITROSAL:

GUMS IN FOODS:

Sam H. Perlmutter, Food and Drug Administration, Minneapolis 1, Minn., has been appointed Associate Referee on Cacao Products.

METALS, OTHER ELEMENTS, AND RESIDUES IN FOODS:

G. E. Keppel, Food and Drug Administration, Minneapolis 1, Minn., has been appointed Associate Referee on Tetramethyl Thiuram Disulfide.

R. A. Baxter, Food and Drug Administration, Los Angeles 15, Calif., has been appointed Associate Referee on Biphenyl.

PRESERVATIVES AND ARTIFICIAL SWEETENERS:

W. H. Munday, Food and Drug Administration, Minneapolis 1, Minn., has been appointed Associate Referee on Hydrogen Peroxide in Dairy Products.

FLAVORS AND NON-ALCOHOLIC BEVERAGES:

J. C. M. Griffin, Food and Drug Administration, Atlanta 3, Ga., has been appointed Associate Referee on Propylene Glycol.

* For report of Subcommittee A and action of the Association, see *This Journal*, **38**, 63, 64 (1955).

CONTRIBUTED PAPERS

A SIMPLE APPARATUS FOR AUTOMATICALLY CONTROLLING THE DISTILLATION OF FLUORINE AS HYDROFLUOSILICIC ACID*

By E. D. SCHALL and H. G. WILLIAMSON† (Department of Biochemistry, Purdue University, Lafayette, Ind.)

The Willard and Winter (5) procedure has been widely accepted as an efficient means of isolating fluorine from accompanying compounds which interfere with its quantitative determination. In this procedure fluorine is volatilized as hydrofluosilicic acid from a sulfuric or perchloric acid solution of the sample. Temperature control is required and is obtained normally by replacing the water lost through distillation. Willard and Winter accomplished this control by adding water manually from a dropping funnel. Later, other workers modified the original apparatus to achieve semi-automatic temperature control by introducing a regulated flow of steam into the distilling flask placed in a temperature-controlled oil bath (3) or in a bath of refluxing tetrachloroethylene, b.p. 121°C. (1, 2). Willard, Toribara, and Holland (4) described a fully automatic apparatus in which temperature control was obtained by regulating the addition of water through a solenoid valve actuated by a platinum resistance thermometer. All component parts of the unit were made by the authors and required skills and facilities not universally available.

The apparatus described below is simple to construct and with the exception of the mercury thermoregulator was assembled from readily available parts. The operation is fully automatic with a temperature regulation of ± 1.0°C. The rate of distillation is approximately 200 ml per hour with a 350 watt heater.

The assembled unit is shown in Fig. 1. When the temperature rises above the selected value, the mercury thermoregulator closes the input circuit of the relay, energizing the solenoid valve and admitting water to the distillation mixture. As the solution cools, the relay input circuit is opened and the valve closes. Repetition of this cycle maintains the desired temperature.

Details of the assembly are illustrated in Fig. 2. The Claisen distilling head was shortened to reduce the over-all height of the unit and the auxiliary neck was sealed off above the side arm. Water is admitted to the flask through a 0.2 ml measuring pipet, although any similar capillary tubing capable of delivering 15 to 20 ml per minute under full flow would

* Journal Paper No. 807 of the Purdue University Agricultural Experiment Station, Lafayette, Indiana. Presented at the Sixty-eighth Annual Meeting of the Association of Official Agricultural Chemists. Oct. 11, 12, and 13, 1954, at Washington, D. C.

† *Present address*: Mead Johnson Company, Evansville, Indiana.

be equally satisfactory. The 110 volt, 60 cycle solenoid valve (Model V-5, $\frac{3}{8}$ inch inlet and outlet) was obtained from the Skinner Electric Valve Division, Skinner Chuck Co., Norwalk, Conn., and the relay was obtained from the Fisher Scientific Co., Pittsburgh, Pa. The heater (350 watt with Cenco Hotcone) was obtained from the Central Scientific Co., Chicago, Ill.

The mercury thermoregulator was made by blowing a bulb 1 cm in diameter on one end of a 30 cm length of 5 mm capillary tubing. A 2 cm piece of No. 25 platinum wire was sealed into the tubing approximately 25 cm above the bulb. The regulator was filled with mercury to a point slightly above the platinum contact.

The temperature adjusting mechanism was made from a polystyrene

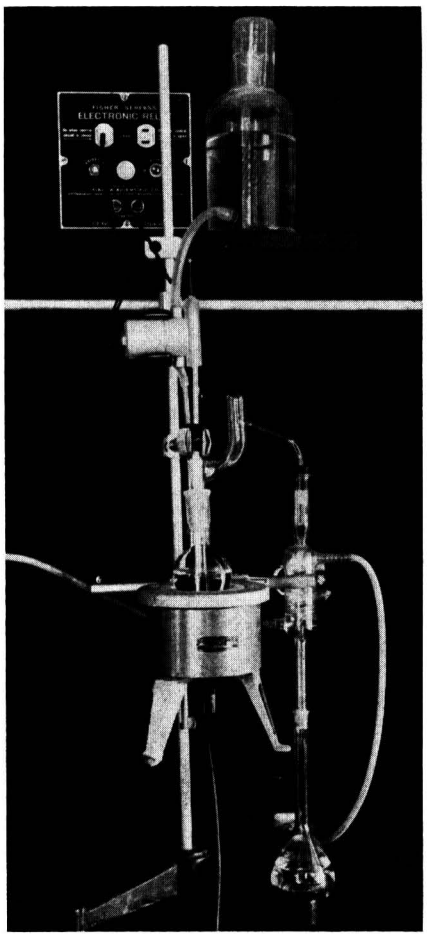


FIG. 1.—Automatic distillation apparatus.

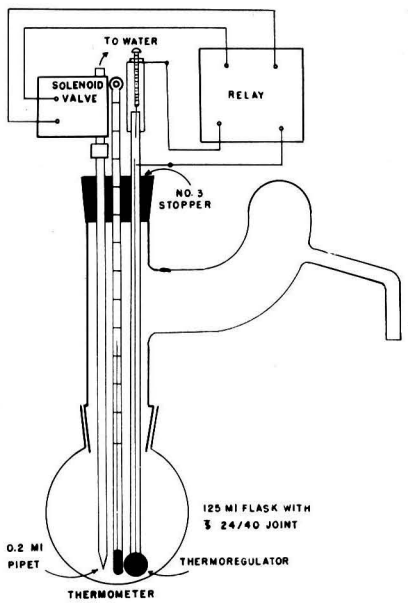


FIG. 2.—Schematic drawing of automatic distillation unit.

rod 1.2 cm in diameter and 7 cm in length. A center hole was drilled from one end and tapped to take a 12–24×2 inch machine screw. A 5 mm hole 3 cm deep was drilled from the opposite end, connecting with the threaded hole. The regulator was assembled by inserting the top of the capillary into the polystyrene rod and securing it with cement. A 5 cm length of No. 32 nichrome wire, silver soldered to the end of the machine screw, extends into the capillary as the adjustable contact. Its position in the capillary determines the temperature at which the regulator functions, and is controlled by the machine screw. This screw is adjusted to close the relay input circuit when the desired temperature is indicated on the thermometer.

DISCUSSION

The degree of temperature regulation is dependent, to a certain extent, upon the rate at which water is delivered to the distilling solution. If the rate is too fast, the solution is overcooled before the thermoregulator detects the change. If it is too slow, control is lost and the temperature rises unduly. A delivery rate of 15 to 20 ml per minute was found adequate to give a control of ±1.0°C. Closer control could be obtained by reducing the flow to 10 ml per minute but is unnecessary for this application where a temperature range of 10° is permissible.

Four of the units have been used without difficulty in this laboratory for three years.

REFERENCES

(1) Gilkey, W. K., Rohs, H. L., and Hansen, H. V., *Ind. Eng. Chem., Anal. Ed.*, **8**, 150 (1936).
(2) Huckabay, W. B., Welch, E. T., and Metler, A. V., *Anal. Chem.*, **19**, 154 (1947).
(3) Rempel, H. G., *Ind. Eng. Chem., Anal. Ed.*, **11**, 378 (1939).
(4) Willard, H. H., Toribara, T. Y., and Holland, L. N., *Anal. Chem.*, **19**, 343 (1947).
(5) Willard, H. H., and Winter, O. B., *Ind. Eng. Chem., Anal. Ed.*, **5**, 7 (1933).

SPECTROPHOTOMETRIC DETERMINATION OF FLUORIDE*

By E. D. Schall and H. G. Williamson† (Department of Biochemistry, Purdue University, Lafayette, Ind.)

Among the many methods that have been advanced for determining fluoride are gravimetric, volumetric, amperometric, polarographic, fluorometric, and colorimetric procedures. Since the literature contains

* Journal Paper No. 808 of the Purdue University Agricultural Experiment Station, Lafayette, Indiana. Presented at the Sixty-eighth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 11, 12, and 13, 1954, at Washington, D. C.
† *Present address*: Mead Johnson Company, Evansville, Indiana.

several excellent reviews of this subject, including the comprehensive annotated bibliography prepared by McKenna (7) covering the period from 1816 to 1950, the history of this determination will not be repeated here.

Since the advent of the familiar Willard and Winter method (13) in 1933, attention has focused primarily upon colorimetric procedures, and many of those proposed have been adaptations of this general method to particular analyses. These modifications usually depend upon the titration of fluoride with thorium or zirconium salts in the presence of an indicator, usually a dye, which forms a colored lake with these salts. Fluoride bleaches this lake and the degree of bleaching is related to the fluoride content. The sensitivity and effectiveness of a large number of indicator compounds was studied by Willard and Horton (12).

Recently considerable attention has been given to adapting either existing or new methods to instrumental procedures in order to eliminate errors and difficulties associated with the visual matching of colors. Among those reported are the spectrophotometric determination of the bleaching effect of fluoride on the following complexes: iron-salicyclic acid (10), aluminum-hemotoxylin (8), zirconium-alizarin (1), thorium-amaranth (6), thorium-thoron reagent (4), thorium-alizarin (5), and thorium-azurol-S (9). In many cases the response did not obey Beer's law but was satisfactory for use.

Hines and Boltz (3) recently reported a spectrophotometric method for determining titanium based upon the colored complex which it forms with ascorbic acid. Fluoride was found to interfere seriously with the color development. This paper reports a further investigation of this reaction and its application to the determination of fluoride.

The procedure outlined has been applied successfully to rock phosphate and defluorinated rock phosphate, and to mineral feed supplements containing these ingredients. It should also be applicable to the analysis of other materials of similar fluoride content.

METHOD

APPARATUS AND SOLUTIONS

Absorbance measurements were made with a Beckman Model DU spectrophotometer equipped with 1.00 cm Corex cells. *p*H measurements were made with a Beckman Model H-2 *p*H meter equipped with a glass electrode.

Prep. ascorbic acid reagent by dissolving 6.50 g L-ascorbic acid (Merck) and 5 g reagent grade sodium bisulfite in water and dilg to 500 ml. The sodium bisulfite inhibits the oxidation of the ascorbic acid (3).

Prep. the titanium reagent by dissolving 10 g C. P. potassium titanium oxalate in water and dilg to 1 l.

The standard fluoride solution was prepared by dissolving 2.21 g reagent grade sodium fluoride, dried 5 hours at 110°C., in water and dilg to 1 liter. This solution contained 1 mg of fluoride per ml.

The buffer solution was prepared by dissolving 30.6 g potassium biphthalate in water, adding 26.2 ml 1 *N* sodium hydroxide, and dilg to 1 liter with water.

REFERENCE CURVE

Prep. reference solns contg from 0 to 5 mg fluoride per 100 ml (0 to 50 p.p.m.) by transferring appropriate aliquots of the standard fluoride soln to 100 ml vol. flasks; adding 10 ml of the titanium reagent, 10 ml of the ascorbic acid reagent, and 25 ml buffer solution; and dilg to vol. with water. Adjust the instrument to 100% T at 360 mμ with the standard contg the largest amount of fluoride (50 p.p.m.) in the reference cell. Det. the absorbance of the remaining standards and plot *vs.* fluoride concn (Fig. 1).

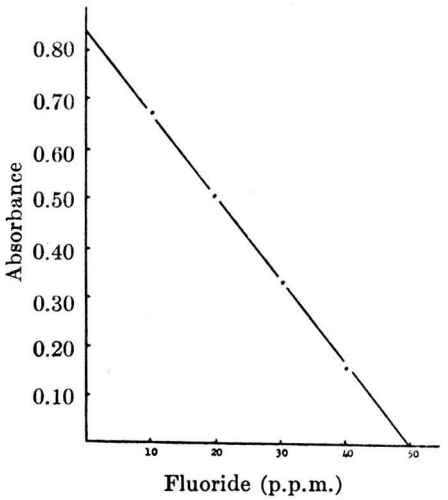


FIG. 1.—Reference curve.

PROCEDURE

Place 1 g sample in a 200–300 ml nickel crucible and add 5 ml satd aq. magnesium acetate soln as a fixative (2), making certain that the entire sample is moistened. Dry thoroly on a hot plate and then ignite at 600° for 2 hrs. Transfer ash quickly to a 125 ml round bottom flask with several 5 ml portions of 60% perchloric acid, using 25 to 30 ml in all. Add sufficient solid silver carbonate to the flask to ppt all chloride. Distil fluoride at 140°C. (11), collecting ca 190 ml distillate. Dil. to 200 ml with water and mix thoroly.

Transfer an aliquot of the distillate contg not more than 5 mg fluoride to a 100 ml vol. flask; add 10 ml titanium reagent, 25 ml buffer, and 10 ml ascorbic acid reagent. Dil. to vol. with water, mix thoroly, and det. absorbance (against cell contg 50 p.p.m. fluoride reference soln) within 1 hr. Calc. fluoride content from standard curve.

RESULTS

The efficiency of the proposed method in detecting known amounts of fluoride added to a fluoride-free mineral feed sample was evaluated by adding aliquots of the standard fluoride solution to 1 gram samples of feed contained in a nickel crucible. Magnesium acetate was added as a fixative, and the sample was ignited and distilled as described above. The results shown in Table 1 are averages of four determinations in each case.

TABLE 1.—*Recovery of added fluoride*

FLUORIDE ADDED	FLUORIDE FOUND
per cent	*per cent*
0	0
0.25	0.26
0.50	0.50
0.75	0.72
1.00	0.99
1.50	1.44
2.00	2.02

For the analysis of 24 commercial mineral feeds, 1 gram samples were weighed and treated as described above in all cases. To eliminate as many variables as possible, two appropriate aliquots were withdrawn from the distillate of each sample; the first was analyzed by the proposed method and the second by the Willard and Winter (13) method. The results are shown in Table 2.

TABLE 2.—*Comparison of methods for determining fluoride*

SAMPLE NUMBER	PROPOSED METHOD	WILLARD AND WINTER METHOD (13)	SAMPLE NUMBER	PROPOSED METHOD	WILLARD AND WINTER METHOD (13)
	per cent	*per cent*		*per cent*	*per cent*
1	.54	.57	13	.58	.41
2	.46	.43	14	1.60	1.40
3	.46	.48	15	.52	.56
4	1.28	1.15	16	.50	.42
5	.54	.58	17	.40	.38
6	.48	.50	18	.30	.28
7	.00	.00	19	.16	.17
8	.51	.52	20	.34	.37
9	.02	.02	21	.18	.22
10	.46	.47	22	.26	.30
11	.56	.58	23	.18	.20
12	.26	.25	24	1.08	1.12
			Average (24)	.486	.474

EFFECT OF VARIABLES

Titanium and Ascorbic Acid Concentration.—The intensity of the color developed in the absence of fluoride is proportional to both the titanium and the ascorbic acid concentrations. At titanium concentrations much higher than recommended the curve tends to lose linearity at the higher fluoride concentrations. Increasing the ascorbic acid concentration to double that recommended results in the curve falling off at low fluoride

concentrations. A linear response was found for fluoride concentrations between 0 and 50 p.p.m. when the recommended titanium and ascorbic acid concentrations were employed.

pH.—The intensity of the color is influenced markedly by the acidity of the solution but exhibits a maximum between *p*H 4.0 and 4.5 (Fig. 2). However, *p*H is controlled easily by use of buffers, and biphthalate, acetate, citrate, and phosphate systems were all found to be satisfactory. The biphthalate buffer system was employed in this investigation and the *p*H of the spectrophotometric solution was 4.2.

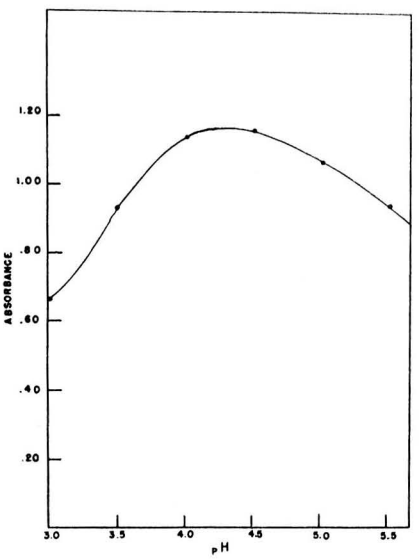


FIG. 2.—Influence of *p*H on the absorbance of the titanium-ascorbic acid complex at 360 mμ.

Contaminant Ions.—Any cations which form insoluble oxalates interfere by causing cloudiness in the solution. Fortunately, these cations are separated by distilling the fluoride from perchloric or sulfuric acid in the usual manner. Chloride ions also interfere but can be eliminated by adding silver carbonate to the sample before distilling the fluoride. Phosphate interferes in concentrations above 100 p.p.m. but it is also eliminated in the distillation.

Time.—The colored titanium-ascorbic acid complex is stable for an hour but begins to fade very gradually after this time. Acidic distillates may also lose fluoride upon standing. Therefore, the analysis should be completed as soon as possible after distillation.

DISCUSSION

The procedure outlined above differs from the usual spectrophotometric procedure in that the standard containing the highest concentration of the substance being determined is used in the reference cell to adjust the instrument to 100 per cent transmission. This is consistent, however, with the fact that fluoride bleaches the color developed by the titanium-ascorbic acid reagent; the absorbance of the solution decreases with increasing fluoride concentration.

If it is desirable to work with a narrower range, a standard containing less than 50 (e.g., 30 or 40) p.p.m. fluoride can be used in the reference cell. The range cannot be extended much beyond 50 p.p.m., since the absorbance of the low-fluoride solutions becomes too great for accurate reading.

Spectrophotometer cells must be clean and evenly matched. Ten ml semimicro burets are recommended for dispensing the titanium and ascorbic acid reagents. The reference curve should be checked frequently, and should be re-plotted when new reagents are prepared.

This method will detect approximately 1 p.p.m. of fluoride. Although slightly less sensitive than some other methods, it is more adaptable to the analysis of mineral feed supplements since a larger sample (1 g) and/or larger aliquots of the distillate can be taken. Sampling and aliquoting errors are thus reduced.

ACKNOWLEDGMENT

The authors are indebted to Mr. K. W. Kirby who assisted in obtaining the analytical data.

REFERENCES

(1) Bumstead, H. E., and Wells, J. C., *Anal. Chem.*, **24**, 1595 (1952).
(2) Crutchfield, W. E., Jr., *Ind. Eng. Chem., Anal. Ed.*, **14**, 57 (1942).
(3) Hines, E., and Boltz, D. F., *Anal. Chem.*, **24**, 947 (1952).
(4) Horton, A. D., Thomason, P. F., and Miller, F. J., *ibid.*, **24**, 548 (1952).
(5) Icken, J. M., and Blank, B. M., *ibid.*, **25**, 1741 (1953).
(6) Lambert, J. L., *ibid.*, **26**, 558 (1954).
(7) McKenna, F. E., *Nucleonics*, **8**, (No. 6), 24; **9** (No. 1), 40; **9** (No. 2), 51 (1951).
(8) Price, M. J., and Walker, O. J., *Anal. Chem.*, **24**, 1593 (1952).
(9) Revinson, D., and Harley, J. H., *ibid.*, **25**, 794 (1953).
(10) Rickard, R. R., Ball, F. L., and Harris, W. W., *ibid.*, **23**, 919 (1951).
(11) Schall, E. D., and Williamson, H. G., *This Journal*, **38**, 452 (1955).
(12) Willard, H. H., and Horton, C. A., *Anal. Chem.*, **22**, 1190 (1950).
(13) Willard, H. H., and Winter, O. B., *Ind. Eng. Chem., Anal. Ed.*, **5**, 7 (1933).

USE OF A MECHANICAL MIXER IN PREPARING FERTILIZER SAMPLES FOR ANALYSIS

By H. R. ALLEN (Kentucky Agricultural Experiment Station, Lexington, Ky.)*

A motor-driven mixing apparatus was made for the Department of Feed and Fertilizer by Mr. T. A. Kendall of the Mining Engineering Laboratories, University of Kentucky. This paper describes the machine and reports on tests to evaluate its mixing efficiency. In addition, the paper describes a project to determine how accurately our laboratory analyses represent the entire sample submitted by the inspectors.

DESCRIPTION OF MIXER

The mixer (Fig. 1) was designed to hold four mixing containers: two large ones for mixing the unground sample, and two smaller ones for mixing smaller portions after grinding. The mixer is used to mix both feed and fertilizer samples. At present, fertilizer samples are mixed only before reduction in size, and the ground portion is usually mixed by hand.

A one-fourth h.p. motor on the bottom shelf of a table is connected by belt and pulley with a shaft on the table top. Four pulleys are attached to this shaft. Each of these pulleys is connected by belt to another pulley which is attached to a shaft bolted to the device for holding the sample container. Thus on the table top there are four belt and pulley systems; each system is connected to the central shaft and to a shaft attached to the container holder. This holder is attached at an angle of 45° with the horizontal. When the machine is in motion the sample is subjected to an end-over-end motion which also tends to move it from the top to the bottom of the opposite side.

The larger sample container is a cylinder of stainless steel 10⅜ inches high and 7¼ inches in diameter. The holder for the sample container is made of two stainless steel plates held about eleven inches apart by two rods. The top side of the upper plate is attached to the shaft which is connected to the pulley. The bottom side of this plate is covered with a smooth rubberized material which acts as a cover for the sample container. The lower plate can be raised or lowered by means of a threaded screw through a horizontal rod beneath the plate and the sample container can thus be held rigid while the machine is in motion.

The four belts connecting the pulleys to the shafts which rotate the sample containers are engaged by means of a separate lever for each container and the rotation of any container is started by pulling back the lever which engages the belt. If the lever is tripped, a braking effect disengages the pulley and stops the motion. The sample containers rotate 60 times per minute.

EXPERIMENTAL

Reducing Inspection Sample, Routine Procedure.—The whole sample, about three quarts for a 20-core sampling, is placed in the container, which is clamped in place on the mixer, and the sample is mixed for at

* This investigation is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director. Presented at the Sixty-eighth Annual Meeting of the Association of Official Agricultural Chemists, October 11–13, 1954, at Washington, D. C.

least one minute (60 revolutions). The container is placed on a rack which holds it at a convenient angle and portions of the sample are taken with a scoop from three places and placed in a labeled quart jar. The volume of this portion is about one pint. This amount is ground in the Mikro-Samplmill and remixed on oilcloth, and a 4 ounce portion is bottled for analysis. The remainder of the ground sample is held in reserve. The efficiency of the mixer in producing a uniformly mixed sample was tested by analyzing portions of the sample obtained in different ways.

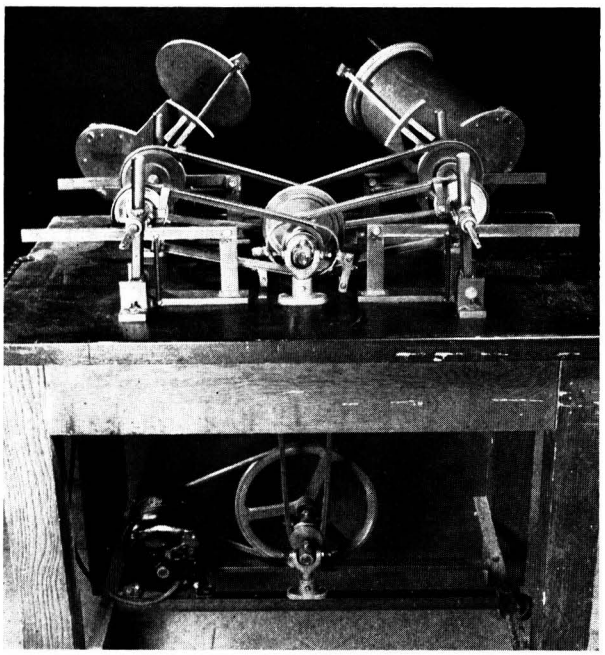


FIG. 1.—Mechanical mixer for fertilizer samples.

Procedure 1.—The sample was rotated in the mixer and a one-pint portion was obtained as described in the preceding paragraph. In addition two other portions of about 6 ounces each were obtained with the scoop. The three portions were ground and analyzed for nitrogen, available phosphoric acid, and potash. If the analyses of the different portions agreed within the limits of experimental error it was assumed that the sample was uniformly mixed. Results are shown in Table 1.

Procedure 2.—Some divergent results were obtained by Procedure 1 on high grades such as 0–20–20 and 5–20–20, so the following modification was tried on a group of samples, each of which contained more than 30 units of plant food. The sample was mixed in the mixer, transferred to oilcloth, and spread out with a spatula. A one-pint portion was obtained

TABLE 1.—*Comparison of analyses of different portions of samples after mixing in mechanical mixer*[a]

GRADE	NO. PORTIONS ANALYZED	MAXIMUM DIFFERENCE BETWEEN PORTIONS, PER CENT		
		NITROGEN	AVAILABLE P_2O_5	POTASH
4–12–4	3	.03	.10	.08
4–10–8	3	.01	.20	.21
0–14–14	3	0	.15	.01
8– 6–10	3	.09	.15	.11
3–12–12	3	.02	.05	.09
0–14–14	3	0	.10	.61
10–10–10	3	.12	.30	.11
5–20–20	3	.04	.05	.12
4–24–12	2	.05	.20	.28
0–20–20	2	0	.45	.16
4–16–16	3	.01	.50	.42
0–20–20	3	0	.15	.10
5–10–10	2	.01	.05	.18
5–10–10	2	.01	.00	.25
8– 8–8	2	.02	.25	.00
4–24–12	2	.01	.10	.06

[a] Portions taken from mixing container with scoop.

by taking portions from at least ten places in the outside perimeter and from four places inside; a flat aluminum boat with turned-up sides was used to obtain these portions. This one-pint portion was ground and remixed on oilcloth, and a 4 ounce portion was bottled for analysis. The remainder of the sample, two quarts or more, was ground, remixed in the mixer, and spread out on oilcloth, and a 4 ounce portion obtained. These samples were analyzed for nitrogen, available phosphoric acid, and potash. Results are shown in Table 2.

Procedure 3.—Four samples were treated as follows: The sample was mixed in the mixer and quartered by putting it through the Jones sample

TABLE 2.—*Comparison of analyses of portions of samples obtained by procedure 2*[a]

GRADE	DIFFERENCE BETWEEN PORTIONS, PER CENT		
	NITROGEN	AVAILABLE P_2O_5	POTASH
4–16–16	.02	.10	.05
4–16–16	.02	.08	.09
14–14–14	.07	.15	.02
0–20–20	0	.10	.03
0–20–20	0	.17	.16

[a] Whole sample mixed in mixer, spread on oilcloth, and 1-pint portion obtained for grinding and analysis. Remainder of sample ground, remixed in mixer, spread out, and portion obtained for analysis.

TABLE 3.—*Comparison of analyses of one-fourth and three-fourths portions through sample riffle*[a]

GRADE	DIFFERENCE BETWEEN PORTIONS, PER CENT		
	NITROGEN	AVAILABLE P_2O_5	POTASH
4–16–16	.03	.23	.07
13–13–13	.08	.00	.05
0–20–20	0	.20	.62
0–20–20	0	.50	.35

[a] After mixing in mechanical mixer.

riffle; one-fourth and three-fourths portions were ground and analyzed for nitrogen, available phosphoric acid, and potash. Results are shown in Table 3.

Revised Routine Procedure.—As a result of experience gained in these tests, this procedure is now followed: The sample is mixed in the mixer and a one-pint portion is taken with the scoop for all mixtures containing less than 30 units of plant food. Mixtures containing 30 units or more are transferred to oilcloth after mixing and spread out, and a one-pint portion is obtained as described in Procedure 2.

If the plant food content of a mixture is more than 30 units, the remainder of the unground sample is transferred to a one-half gallon glass jar which is labeled and set aside until the official sample has been analyzed. If this analysis is below guaranty in any nutrient, this one-half gallon portion is remixed and another one-pint sample is obtained and analyzed.

DISCUSSION OF RESULTS

Results shown in the tables indicate that Procedure 1 (mixing samples in mixer and taking samples with a scoop to be ground for analysis) is usually satisfactory if the mixture contains less than 30 units of plant food. This procedure is not satisfactory for higher analysis mixtures if there is considerable difference in the particle size of the components of the mixture. Satisfactory results are obtained if all mixtures containing more than 30 units of plant food are mixed in the mixer and spread out on oilcloth, and the portions for grinding are obtained as described in Procedure 2. Use of the Jones sample riffle to reduce the sample size did not give quite as good results as Procedure 2 but the number of samples divided by this method was not large enough to warrant a definite conclusion. If a riffle is used it should be an enclosed type, otherwise the dust problem is a serious one.

Recheck analyses have been made on a number of the one-half gallon reserve samples, and 28 analyses for nitrogen, 41 analyses for available phosphoric acid, and 48 analyses for potash have been made. Of the

nitrogen analyses, 86 per cent were within 0.1 per cent and 93 per cent were within 0.2 per cent of the original result. Of the phosphoric acid analyses, 59 per cent were within 0.1 per cent, 76 per cent were within 0.2 per cent, and 88 per cent were within 0.3 per cent of the original result. Of the potash analyses, 42 per cent were within 0.1 per cent, 63 per cent were within 0.2 per cent, and 85 per cent were within 0.3 per cent of the original result.

Study of some changes in the use of the mixer is contemplated. Among these are reduction in mixing speed, use of baffles in the mixing container, and use of a core-type sampler instead of a scoop.

SUMMARY

A motor-driven mixing apparatus for mixing fertilizer samples before reduction in size is described, together with procedures to test its mixing efficiency. With mixtures containing less than 30 units of plant food, the one-pint sample could be obtained directly from the mixing container with a scoop. When higher analysis mixtures contained ingredients varying greatly in particle size, it was necessary to spread out the sample on oilcloth after mixing, and to obtain the one-pint sample by taking portions from a number of places. This procedure gave somewhat better results than use of a sample riffle.

Results in this study[1] point to two conclusions: (*a*) It is more difficult to obtain uniform portions in subdividing high-analysis fertilizer mixtures in the laboratory, and special attention should be given to this point; (*b*) if manufacturers use materials for high analysis mixtures which differ greatly in particle size, the batch should be reground after mixing.

The mixer has facilitated transfer of inspection samples from the inspection container (special paper bag) to quart jars. The entire portion of sample placed in the jar is ground for analysis without further mixing.

ACKNOWLEDGMENT

Analyses reported in this paper were made by the following members of the laboratory staff: Chester Ball, Louise Jett, Joe Koles, and Rose Run.

[1] See also *Reliability of Chemical Analyses for Fertilizers and Feeds*, by S. R. Miles and F. W. Quackenbush, *This Journal*, **38**, 108 (1955).

THE PHENOL COEFFICIENT NUMBER AS AN INDEX TO THE PRACTICAL USE-DILUTION FOR DISINFECTION*

By L. S. STUART, L. F. ORTENZIO, and J. L. FRIEDL (Pesticide Regulation Section, Plant Pest Control Branch, Agricultural Research Service, U. S. Department of Agriculture, Washington 25, D. C.)

It is especially important to both regulatory and research bacteriologists to be able to estimate from laboratory data the potential practical value of a disinfectant. However, factual information on this score is so meager that proper developments in the field of chemical disinfection have been seriously impeded. Tests now commonly employed in compiling data for this purpose include the phenol coefficient method and its various modifications, numerous use-dilution procedures, concentration–death time measurements for exponent determinations, and so-called dilution tube capacity techniques.

Either in theory or practice all of the above mentioned methods have certain limitations which are not fully understood. This is particularly true of the phenol coefficient method in which the result must be converted by a calculation into the concentration safe for actual use. Therefore, discussion covering some of the limitations of this particular procedure is pertinent at this time.

The phenol coefficient procedure was initially described in 1903 (1). It has been employed with numerous minor modifications continuously for the past 50 years. Values obtained therewith by using *S. typhosa* at 20°C. are multiplied by the figure 20 to determine the maximum number of parts of water in which 1 part of product may be incorporated and still provide a solution strong enough to disinfect in actual use.

If it were true that the activity of germicides increased directly as the concentration increased, or decreased as the dilution was increased, there could be no objection to the fixed formula outlined above. It is based on the knowledge that a 1 per cent concentration (a 1:100 dilution) of phenol is approximately the critical killing concentration in the method and that a 5 per cent concentration of phenol (a 1:20 dilution) will provide an adequate margin of safety for disinfection under adverse conditions. In addition, it is assumed that the resulting solution will have germicidal activity equivalent to a 5 per cent solution of the pure chemical, phenol.

Unfortunately, this assumption is not true because the activity of germicides does not increase directly as the concentration increases. The relation between concentration and germicidal activity was shown as early as 1908 by both Chick (2) and Watson (3) to be exponential in

* Presented at the Sixty-eighth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 11–13, 1954, at Washington, D. C.

character, and their findings have been confirmed by many other workers since that time.

Numerous investigators have pointed to this error in the phenol coefficient procedure and some have claimed that it is serious enough to justify discarding the method entirely. However, Varley and Reddish (4), Reddish (5), and Stuart, Ortenzio, and Friedl (6) have all shown that the use of this procedure with many commonly encountered commercial preparations will usually provide for effective disinfection. Such observations, combined with the fact that no alternative procedure of equivalent precision has ever been advanced, account for its continued use.

The simplest interpretation of the initial observation of Chick and of Watson would be that disinfectant activity varies as a logarithmic function of concentration (temperature, time, and other factors being constant). Assuming for the moment that this is true, that 5 per cent phenol is the satisfactory standard of germicidal activity for actual use and that a 1.11 per cent solution (1:90 dilution) of phenol is the critical killing concentration in the method, the following equation would be justified:

$$\frac{\log C\equiv_{5\%p}}{\log C_{ckc}} = \frac{\log 0.05}{\log 0.0111},$$

in which $C\equiv_{5\%p}$ designates the concentration of the unknown germicide equivalent in activity to 5 per cent phenol and C_{ckc} designates the critical killing concentration of the unknown germicide.

If figures are substituted in this equation for C_{ckc} which correspond to percentages necessary to yield increasing coefficient values where a 1:90 phenol control is employed, it is possible to calculate theoretical $C\equiv_{5\%p}$ values for unknown germicides which possess increasing coefficient values. From such data ratios of the concentrations of unknown germicides theoretically equivalent to 5 per cent phenol and the critical killing concentrations in the phenol coefficient method have been determined. Typical results are listed in Table 1.

TABLE 1.—*Calculated ratios of $C\equiv_{5\%p}$ phenol to C_{ckc} based on the assumption that germicidal activity varies as a simple logarithmic function of concentration*

PHENOL COEFFICIENTS	C_{ckc} OF GERMICIDES BY PHENOL COEFFICIENT METHODS	$C\equiv_{5\%P}$	RATIO $C\equiv_{5\%P}$ TO C_{ckc}
1	0.011	0.05	4.5
10	0.00111	0.0108	9.7
100	0.000111	0.00232	20.8
1000	0.0000111	0.000502	45.1

It can be seen from Table 1 that if a simple exponential relation did exist between concentration and germicidal activity, the ratio of concen-

tration equivalent in activity to 5 per cent phenol would increase as the critical killing concentration in the phenol coefficient method decreased. This, of course, means that it would be necessary to employ concentration conversion factors of increasing magnitude as the phenol coefficient values increased. The data in Table 1 have been used to construct Figure 1 which illustrates this point quite clearly.

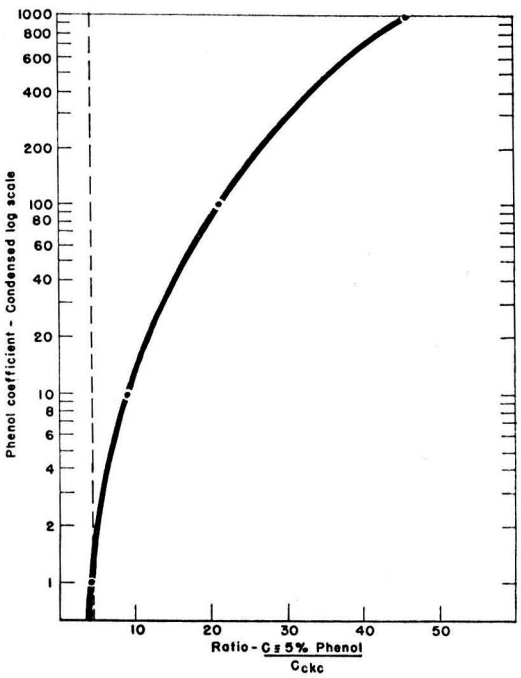


FIG. 1.—Relation of concentration conversion factors to phenol coefficient values.

The vertical light, broken line in Fig. 1 at the 4.5 $C \equiv_{5\%Phenol} : C_{ckc}$ ratio represents the correct constant concentration multiple which should be employed, according to conventional practice. The heavy, curved line illustrates the rate at which this concentration multiple should be increased as the phenol coefficient value increases from 1 to 1000, if simple exponential relationship is assumed. It can be seen from this figure that the heavy, curved line begins to pull away from the light, broken vertical line with products which have a phenol coefficient of 2.0 and shows serious departure therefrom with products which have a phenol coefficient of 6.0. Most of the commercial disinfectant preparations examined by regulatory bacteriologists in the past have had phenol coefficient values ranging from 1.0 to 6.0. According to Fig. 1, conversion of the C_{ckc} values to relatively safe use-dilutions should be achieved by using the constant

concentration multiple of 5 with products within this range of activities. However, similar conversions attempted with products above this range of activities might be expected to fail.

It is not possible to check the validity of the curve shown in Fig. 1 by direct experimental measurements, for no convenient method for measuring the germicidal activity of 5 per cent phenol is available. However, use-dilution methods have been proposed for directly measuring the maximum dilution of germicides which will assure disinfection under commonly encountered conditions. One of these methods has recently

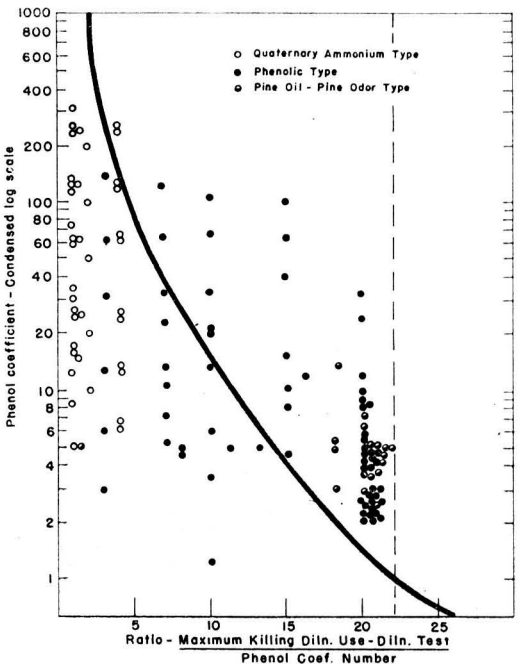


FIG. 2.—Results to determine validity of dilution conversion factors.

been adopted as first action by the Association of Official Agricultural Chemists (6). It was believed that an analysis of results obtained in this method by using *S. choleraesuis* with products possessing varying phenol coefficient values with *S. typhosa* might indirectly yield evidence bearing on this point.

In Fig. 2, the reciprocal of the exponential curve shown in Fig. 1 has been plotted. The intersection points for the horizontal lines representing the phenol coefficient values listed on the left-side vertical scale with the curve may be checked and a perpendicular line dropped to the base scale. By this method it should be feasible to determine a theoretically possible multiple to employ with increasing phenol coefficient values to determine the number of parts of water in which 1 part of product could be incor-

porated to disinfect in actual use. It should be borne in mind that the figure 20 is commonly employed for this purpose, but that the figure 22.2 represented by the light, broken vertical line in Fig. 2 would be the correct value if a culture were employed which had a resistance in the method to a 1:90 dilution of phenol.

By using this reciprocal curve as a background, a scattergram has been constructed in which the denominator of the fraction representing the maximum killing dilution in the A.O.A.C. use-dilution procedure is divided by the known phenol coefficient number, and the points represented by these calculations are located according to the ordinate phenol coefficient scale and then marked with small circles. Data from 125 commercial and laboratory compounded preparations were employed. Open circles were used for products of the quaternary ammonium type, solid circles for products of the phenolic type, and half-solid circles for pine oil and pine type disinfectants.

The wide scattering of the various points in Fig. 2 indicates quite clearly that neither the constant conversion factor of 20 now commonly employed nor a conversion formula based on a simple exponential relationship between concentration and germicidal activity can be relied upon to convert phenol coefficient numbers to safe dilutions for use in the absence of other information. Further study of the data employed revealed that all those points below and to the left of the exponential curve represented products containing relatively high percentages of water. When recalculations were made on the basis of the percentage of active ingredients actually present, all points were located near the exponential curve or scattered above and to the right of it. It was also found that constant conversion factors were required for products made with varying concentrations of the same germicidal chemical to give different phenol coefficient values. For example, a 100 per cent preparation of alkyl (C_8H_{17}–$C_{18}H_{37}$) dimethyl benzyl ammonium chloride was found to have a phenol coefficient of 250 and to kill in the use-dilution procedure at a dilution of 1:1000. By dividing the figure of 1000 by 250 a factor of 4 was obtained which fell near but slightly to the right of the exponential curve in Fig. 2. Products made to contain 50 per cent, 25 per cent, 10 per cent, and 5 per cent of this chemical gave phenol coefficient values of 125, 62.5, 25.0, and 12.5. The maximum safe dilutions found in the use-dilution method were 1:500, 1:250, 1:100, and 1:50, respectively. Thus the conversion factor was 4 in all instances. Obviously, the points for those products containing less than 100 per cent of the germicidal chemical fell farther below the exponential curve in Fig. 2 as the concentration of the active ingredient decreased. Likewise, a preparation of the pure phenol 4, N-octylresorcinol which had a phenol coefficient of 122 gave an end point in the use-dilution test at a dilution of 1:366. This provided a factor of 3 which gave a point slightly to the left but in close proximity to the exponential curve in Fig. 2. Products made to contain decreasing concentrations of this chemical

which had phenol coefficients of 61, 30.5, 12.2, and 6.1 gave end points at dilutions of 1:180, 1:90, 1:35, and 1:18, respectively, in the use-dilution test. These results provided a constant factor of 3 for all preparations containing this single active ingredient. Thus the points on Fig. 2 for this group of products fell farther and farther below the exponential curve as the concentration of the active ingredient decreased.

If, as such data indicate, all deviations below and to the left of the exponential curve in Fig. 2 can be dismissed as resulting from low concentrations of active ingredients, this curve might have some practical value in interpreting phenol coefficient numbers in terms of maximum safe use-dilutions, provided a calculation based on a multiple derived from the phenol coefficient of the active ingredient present was employed. In fact, it is quite surprising how accurately such calculations work out. This emphasizes the importance of information on chemical composition to the interpretation of a phenol coefficient number.

The deviations shown in Fig. 2, above and to the right of the exponential curve, appear to be primarily the result of one of two factors or a combination of both. These are (*a*) a discrepancy between the degree of activity required to kill in the use-dilution test employed and the actual killing potential of a solution of 5 per cent phenol, and (*b*) variation according to specific activities of the chemical germicide or germicides present.

Phenol will actually kill in the use-dilution method if it is employed at a dilution of 1:80 or at a concentration of 1.25 per cent. It might be supposed that if a use-dilution test procedure could be adjusted through the introduction of organic matter, decreasing the volume of germicide used with the individual carrier or increasing the number of organisms adsorbed on the carrier so that 5 per cent phenol would be necessary to kill the test organism, all points on the scattergram would thus have location at a considerably lower level. It has been observed that products and preparations with the same active ingredients gave a constant ratio between the maximum killing dilution in the use-dilution test and the phenol coefficient number. This observation strongly indicates that the correct multiple to employ for converting a phenol coefficient number to a safe use-dilution may be directly related to some specific property of each germicidal chemical. Now this specific property was identified in the early studies of Chick and of Watson as the concentration exponent. Both recognized that the relation between activity and concentration was considerably more complex than that illustrated by the simple exponential curve in Fig. 1. It was found from concentration–death time studies that the relation between concentration of a germicide and death time was not only exponential but that the exponent varied with each disinfectant and each test organism. Watson (3) and Tilley (7) have outlined procedures for determining this exponent which is supposed to provide an

index to the effect that dilution has upon the germicidal efficiency of a substance. For this reason, it has frequently been suggested that this exponent should be employed in the standardization of germicides, although no specific application procedure has been advanced.

Specifically, it would seem that there should be a fixed relation between the *S. typhosa* concentration exponent and the reciprocal concentration curve in Fig. 2. If this could be established, it should be possible to estimate from the phenol coefficient number and the concentration exponent value the maximum killing dilution in the use-dilution test or the maxi-

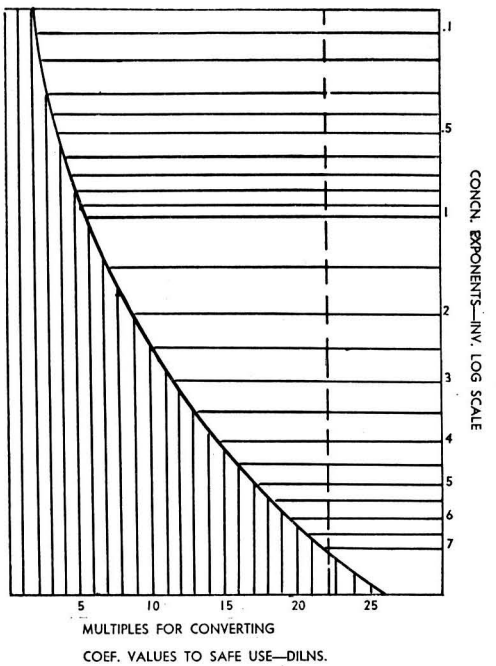


FIG. 3.—Nomograph for converting phenol coefficient numbers to effective dilutions for disinfecting.

mum killing dilution for actual use. Studies of the data available indicate that as the concentration exponent value as determined by the method of Tilley decreases, the ratio of the maximum killing dilution in the use-dilution test to the phenol coefficient number also decreases. The rate at which this decrease takes place does not appear to be arithmetic. However, it does appear to be logarithmic. A nomograph has been constructed by reproducing Fig. 2 and superimposing thereon an inverted vertical log scale in which the interception of the vertical line 22.2 and the exponential curve is assigned a value of 7.0. This nomograph, designated herein as Fig. 3, has proved to be a most useful laboratory guide for converting phenol coefficient numbers to safe use-dilutions.

When both the concentration exponent and phenol coefficient numbers are known, even though the information on chemical composition is incomplete, the approximate safe use-dilution may be determined by multiplying the phenol coefficient number found by the nearest figure in the arithmetic base scale of Fig. 3 to a perpendicular line dropped from the point of interception of the horizontal line corresponding to the exponents listed in the left-side vertical scale with the curve. This will provide with a fair degree of accuracy the number of parts of water in which 1 part of product may be incorporated to kill in the use-dilution procedure.

In this nomograph, the point of interception of the curve and the vertical line 22.2 was assigned a value of 7.0 because this is approximately the correct concentration exponent for the pure chemical phenol which has a phenol coefficient of 1.0 against *S. typhosa*. The left-side scale above this point was segmented by the use of parallel intercepts from a standard log scale.

An example of the type of data employed to arrive at the relationship suggested in Fig. 3 is listed as follows: A 10 per cent preparation of alkyl (C_8H_{17}–$C_{18}H_{37}$) dimethyl benyzl ammonium chloride was found to have an *S. typhosa* coefficient of 25.0. The maximum killing dilution for *S. choleraesuis* in the use-dilution test was found to be 1:150. The maximum 10 minute killing dilution in the phenol coefficient method was 1:22,500, whereas the 5 minute killing dilution was 1:15,000.

By the use of Tilley's equation:

$$N = \frac{\log t_2 - \log t_1}{\log C_1 - \log C_2},$$

and designating 10 minutes as t_2, 44 p.p.m. as C_2, 5 minutes as t_1, and 67 p.p.m. as C_1, we find that:

$$N = \frac{\log 10 - \log 5}{\log 67 - \log 44}; \qquad N = \frac{0.30303}{0.18255}; \qquad N = 1.5 +$$

From Fig. 3, the correct multiple for use with the phenol coefficient is 6 and $6 \times 25 = 150$, corresponding to the maximum safe use-dilution found in the use-dilution test for this particular preparation. Other examples are listed in Table 2.

The examples shown in Table 2 were selected simply because complete data were available for drawing comparisons. They are not necessarily representative of the usual run of commercial products in each class. They suggest a very close correlation between conversions of *S. typhosa* phenol coefficient numbers to safe use-dilutions through the use of the *S. typhosa* concentration exponent and Fig. 3, and those dilutions found to be safe against *S. choleraesuis* in the A.O.A.C. use-dilution test.

It has been previously pointed out that the concentration exponent will vary with the test organism as well as with changes in germicidal chemicals due to dilution. Thus, a preliminary analysis of the available data

TABLE 2.—*Examples of the use of Figure 3 in converting S. typhosa coefficient values to presumed safe use-dilutions for disinfecting*

SAMPLE NO.	TYPE OF DISINFECTANT	S. TYPHOSA COEFFICIENT	MIN. KILLING CONCN IN COEFFICIENT METHOD		S. TYPHOSA CONCNS EXPONENT	MULTIPLE INDICATED BY EXPONENT IN FIGURE 3	USE-DILN AS CALCD USING COEFFICIENT × FIGURE 3 MULTIPLE	DILN FOUND EFFECTIVE IN USE-DILUTION TEST (*S. choleraesuis*)
			5 MIN. AT 20°C.	10 MIN. AT 20°C.				
			ppm	*ppm*				
1	Quaternary	100.0	167	111	1.3	5.5	1:550	1:500
2	Quaternary	24.4	1111	436	0.7	3.5	1:85	1:80
3	Pine Oil	5.5	2222	2111	6.6	20.0	1:110	1:120
4	Pine Oil	2.8	4444	4000	6.7	21.0	1:59	1:60
5	Coal-Tar Em.	5.0	2500	2222	5.9	18.0	1:90	1:100
6	Coal-Tar Em.	13.3	1111	833	2.4	8.5	1:113	1:110
7	Synthetic Phenol	7.2	1868	1523	3.5	11.5	1:83	1:85
8	Synthetic Phenol	17.0	909	667	2.2	7.5	1:128	1:160

was made to determine the possibility of employing the nomograph shown in Fig. 3 for arriving at safe use-dilutions for hospitals and similar places which use the *S. typhosa* coefficient number and the lowest concentration exponent found with other types of test organisms which would have to be destroyed in such places. Considerable evidence was found to support this possibility. The data shown in Table 3 for one commercial disinfectant of the synthetic phenol type illustrate this point fairly well.

TABLE 3.—*Use of concentration exponents found with different organisms in converting the S. typhosa coefficient number to safe use-dilutions*

Phenol Coefficient (*S. typhosa*)	20.0
Concentration exponents:	
S. typhosa	2.7
M. pyogenes var. *aureus*	1.3
Use-dilutions indicated by conversions employing Fig. 3:	
Using *S. typhosa* exponent	1:180
Using *M. pyogenes* var. *aureus* exponent	1:100
Safe use-dilutions by A.O.A.C. method:	
Using *S. choleraesuis*	1:180
Using *M. pyogenes* var. *aureus*	1:100

Table 3 shows that the safe use-dilution in ordinary premise disinfection where enteric organisms are of primary significance according to the A.O.A.C. use-dilution procedure is 1:180 instead of 1:400 as would be indicated by use of the conventional method of multiplying the *S. typhosa* phenol coefficient by the figure 20 to obtain the number of parts of water in which 1 part of germicide should be incorporated. This is also the dilution which would be arrived at by employing the *S. typhosa* exponent found and the nomograph identified herein as Fig. 3. However, it was found that a dilution of 1:100 was required in the A.O.A.C. use-dilution method if *M. pyogenes* var. *aureus* was used as the test organism. This lower dilution corresponds exactly with the safe use-dilution calculated by using Fig. 3, the *S. typhosa* coefficient number, and the *M. pyogenes* var. *aureus* concentration exponent.

Phenol coefficient work sheet data are not particularly adapted to the accurate determination of concentration exponents because the number of dilutions employed are not usually spaced sufficiently close over a wide enough range to supply the necessary information. Thus, it was believed that the validity of the observations concerning Fig. 3 should be established by employing a standardized procedure which would provide more accurate data on killing times at specific concentrations.

For this work, the 10 minute time interval and the minimum killing concentrations at this interval in the phenol coefficient method for *S. typhosa* and *M. pyogenes* var. *aureus* were used at t_2 and c_2 values. These concentrations were doubled for the c_1 values and the killing time for the

c_1 values in seconds was determined at 20°C. for t_1 values. This required the use of one death time measurement in the A.O.A.C. phenol coefficient procedure in addition to each phenol coefficient determination. Each product selected for this study was also tested by the A.O.A.C. use-dilution procedure. The results for one commercial quaternary ammonium germicide, one commercial cresylic acid germicide, and one commercial synthetic phenol mixture are summarized in Table 4 as typical of all of the results obtained. They do not show the exact agreement indicated in Table 3, but they do illustrate the approximate precision which can be expected and the value of the simple procedure outlined in arriving at concentration exponent values for use in estimating safe use-dilutions from *S. typhosa* coefficient numbers employing Fig. 3. In Table 4, the greatest differences between the dilutions indicated in Fig. 3 to be safe and those found to be safe by the A.O.A.C. use-dilution procedure are shown with the quaternary ammonium germicide. In considering these differences, it should be noted that with products possessing very high coefficient numbers and concentration exponents of a low order, small variations in the exponent values will produce the largest variations in the indicated safe use-dilutions, according to the relation indicated in the nomograph.

COMMENTS AND CONCLUSIONS

The foregoing will undoubtedly be considered by those interested primarily in the dynamics of germicidal activity as an oversimplified version of a complex problem. On the other hand, the chemical formulator whose primary interest lies in the solution of problems by the most simple procedures may look upon it as introducing additional complexities in a problem where further simplification is desirable. No apologies are offered to either group, since the information developed does have practical value to the laboratory worker and may eventually prove useful to the consumer of commercial disinfectants.

The logarithmic relation found between the concentration exponent and the reciprocal of the curve for the simple exponential relation between concentration and activity in Fig. 1 provides a clear cut explanation for the long-time successful use with many types of germicides of the constant multiple of 20. It can be seen from Fig. 3 that serious deficiencies should not appear in such a procedure when the concentration exponent is 4.0 or higher, and most of the older commonly used disinfectants active at low dilution possess concentration exponents higher than 4.0. The heavy clumping of points on Fig. 2 immediately above the curve and just to the left of the light, broken, multiple line of 22.2 provides factual evidence on this particular point. The existence of this relation also provides an insight into the reason for the apparent failure of the method with many of the newer germicides active in very high dilutions, since these chemicals usually possess concentration exponents of a relatively low order, and dis-

TABLE 4.—*Use of concentration exponents determined by standardized procedure in converting S. typhosa coefficient values to safe use-dilutions*

TYPE GERMICIDE	S. TYPHOSA COEFFICIENT NO.	CONCN EXPONENT		SAFE USE-DILUTIONS INDICATED BY CONVERSIONS USING FIGURE 3		SAFE USE-DILUTIONS FOUND BY A.O.A.C. METHOD	
		S. TYPHOSA	M. PYOGENES VAR. AUREUS	S. TYPHOSA	M. PYOGENES VAR. AUREUS	S. CHOLERAESUIS	M. PYOGENES VAR. AUREUS
Quaternary ammonium	250.0	0.6	0.8	1:750	1:875	1:500	1:750
Cresylic acid–soap	7.7	4.3	4.0	1:108	1:100	1:154	1:100
Synthetic phenol–soap	6.6	4.2	3.6	1:92	1:80	1:100	1:80

crepancies in the use of the constant conversion factor of 20 could be expected to become greater and greater as the concentration exponent decreases below 4.0.

Further study should be made on the use of concentration exponents obtained with different test organisms in correcting the dilution indicated to be safe by the *S. typhosa* coefficient and the *S. typhosa* exponent, according to Fig. 3, in line with the types of organisms against which the product might be used. The preliminary data shown indicate that this procedure might be the most practical available in arriving at a safe use-dilution for a bacterial spectrum (8) of any desired width.

It should be emphasized that the nomograph identified as Fig. 3 is not offered as a replacement for the direct measurement of the safe use-dilution by the A.O.A.C. use-dilution method. As a laboratory tool for converting phenol coefficient data and concentration exponent data to approximate safe use-dilutions, it has been found to have considerable value. It is especially useful in this respect where the information on chemical composition is incomplete. The formula 2.5 [coef. $\times(N+1)$], in which the coefficient is the value found for *S. typhosa* at 20°C. and N is the concentration exponent for the specific organism against which the germicide is to be employed, will give approximately the same result as the nomograph conversion described.

On the whole, the results emphasize the unreliability of phenol coefficient numbers as the sole index to practical germicidal values and clearly point to the necessity of employing analytical chemical data and/or concentration exponent data in making estimates of the dilutions suitable for actual use in disinfecting. When adequate supporting information is available, the phenol coefficient number can probably be employed effectively in making estimations of practical disinfecting values. However, the high incidence of error from the use of the constant factor 20 in converting phenol coefficient numbers to safe use-dilutions clearly identifies this practice as the major deficiency in the method. A result is provided which at best is highly presumptive. More reliable estimates can be made by employing either of two procedures. The phenol coefficient found may be converted by a multiple located on the base scale of Fig. 2 from the interception of the left-side vertical phenol coefficient scale value corresponding to the phenol coefficient number converted to a 100 per cent active ingredient base and the curve shown in Fig. 2. Alternately, the multiple for use may be located on the base scale of Fig. 3 from the interception of the concentration exponent value according to the right-side vertical scale shown and the same curve. When sufficient information is available to make both types of conversions, the laboratory worker will find that the maximum killing dilution in the A.O.A.C. use-dilution method falls within the range indicated by the highest and lowest values obtained.

REFERENCES

(1) RIDEAL, S., and WALKER, J. T. A., *J. Roy. Sanit. Inst.*, **24**, 424 (1903).
(2) CHICK, H., *J. Hyg.*, **8**, 92 (1908).
(3) WATSON, H. E., *ibid.*, **8**, 536 (1908).
(4) VARLEY, J. C., and REDDISH, G. F., *J. Bact.*, **32**, 215 (1936).
(5) REDDISH, G. F., *Soap Sanit. Chemicals*, **11**, 95 (1935).
(6) STUART, L. S., ORTENZIO, L. F., and FRIEDL, J. L., *This Journal*, **36**, 466 (1952).
(7) TILLEY, F. W., *J. Bact.*, **38**, 499 (1939).
(8) OSTROLENK, M., and BREWER, C. M., *J. Am. Pharm. Assoc., Sci. Ed.*, **38**, 95 (1949).

INFRARED DETERMINATION OF DIELDRIN AND DDT IN MIXTURES*

By GLENN E. POLLARD, WILLIAM M. SALTMAN,† and PETER YIN
(Shell Chemical Corporation, Denver, Colo.)

Several methods are known for the analysis of DDT and dieldrin. Dieldrin is defined as an insecticidal product containing not less than 85 per cent of 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-endo, exo-5,8-dimethanonaphthalene (HEOD) and not more than 15 per cent of insecticidally active related compounds. Garhart, *et al.*, (1) determined dieldrin by infrared analysis, but the method is applicable only to micro amounts. HEOD may be analyzed by a hydrogen bromide volumetric method (2) which, however, is not specific for dieldrin. The method involves the reaction of HBr in dioxane with HEOD to form its bromohydrin and the titration of the excess HBr with alcoholic alkali. DDT may be determined by setting point (3), by solubility in 75 per cent (by volume) aqueous ethanol (4), or by several colorimetric methods (9–11); however, these methods are not readily applicable to formulated materials. Downing, *et al.*, (6) have studied the application of infrared spectroscopy to DDT but have not applied this technique to formulated materials.

In present use, insecticide formulations are very often combinations of two or more insecticides, and a method of analysis is of greater value if the active ingredients can be determined simultaneously. The Stepanow method for chlorine (5) will determine total chlorine for dieldrin and DDT but will not distinguish between the two toxicants. Beckman (7) has employed partition chromatography for the analysis of dieldrin and DDT in combinations. None of these methods is specific for dieldrin or DDT.

---

* The information presented in this paper is based on data available to the authors and is believed to be correct. However, no warranty is expressed or implied regarding the accuracy of these data, the results to be obtained from their use, or that any use will not infringe any patent. Presented at the Sixty-eighth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 11–13, 1954, at Washington, D. C.

† Present address: Export Chemical Corporation, Denver, Colo.

The present procedure may be used to determine dieldrin and DDT simultaneously in insecticidal dusts. The method is rapid and accurate, and is specific for each component. Normally, mixtures of dieldrin and DDT in formulations will contain diluents that may interfere with the analysis. These diluents may be largely eliminated by extracting the original sample and analyzing the dieldrin and DDT in carbon disulfide solution.

PROCEDURE

APPARATUS AND REAGENTS

(a) *Spectrophotometer.*—Perkin-Elmer Model 12-C infrared spectrophotometer, with sodium chloride optics, or equivalent equipment.

(b) *Cell.*—Sealed 0.2 mm sodium chloride cell with syringe fittings.

(c) *Chromatographic column.*—Approx. 360 mm long and 20 mm inside diam., contg 5.5 g absorbent clay (Celite).

(d) *Solvent.*—Reagent grade carbon disulfide.

(e) *Calibration standards.*—Recrystd HEOD and *p,p'*-DDT.

CALIBRATION

Infrared spectra from 2 to 15μ were obtained on HEOD and *p,p'*-DDT in carbon disulfide solutions. Analysis peaks were selected by comparing these two spectra and selecting a wavelength where HEOD has an absorption band but *p,p'*-DDT has only slight absorption; a satisfactory band was found at 10.96μ. Similarly, a wavelength where *p,p'*-DDT has an absorption band but HEOD has only slight absorption was found at 14.06μ. At least five solutions of pure standard HEOD in carbon disulfide covering a concentration range from 1.5 to 6% w/v were prepared and the absorbances obtained at 10.96 and 14.06μ. Prep. solns of pure std *p,p'*-DDT in CS_2 covering range from 5 to 12% (w/v) and obtain absorbances at 10.96μ and 14.06μ. Calc. absorptivities by dividing the absorbance values by the concn of the soln: K = Absorbance/% (w/v) of soln. Substitute these absorptivities in the following equation:

$$A_1 = K_1^1 C_1 + K_1^2 C_2;\ A_2 = K_2^1 C_1 + K_2^2 C_2 \qquad (1)$$

where A_1 = absorbance at 10.96 μ; A_2 = absorbance at 14.06 μ; K_1^1 = absorptivity for HEOD at 10.96 μ; K_2^1 = absorptivity for *p,p'*-DDT at 10.96 μ; K_1^2 = absorptivity for HEOD at 14.06 μ; K_2^2 = absorptivity for *p,p'*-DDT at 14.06 μ; C_1 = concentration of HEOD, % (w/v); and C_2 = concentration of *p,p'*-DDT, % (w/v).

These equations were presolved by simultaneous equations, or by matrix inversion as discussed by Whittaker and Robinson (8). The solution of the foregoing equations gave the following equations:

$$C_1 = F_1^1 A_1 + F_1^2 A_2\ ;\ C_2 = F_2^1 A_1 + F_2^2 A_2 \qquad (2)$$

where F = constant obtained in the solution of equations (1).

DETERMINATION OF ABSORBANCE

For the Perkin-Elmer Model 12-C instrument, det. the absorbance of sample soln relative to a blank by the usual "cell in and out" technique, using the average of 30 sec. (or any other convenient time) for the I, I_0, and zero point readings. I and I_0 may be expressed in any convenient units. Absorbance, (A) = $\log_{10} I_0/I$. When several samples are to be analyzed, one solvent value is usually sufficient for each wavelength band (Fig. 1).

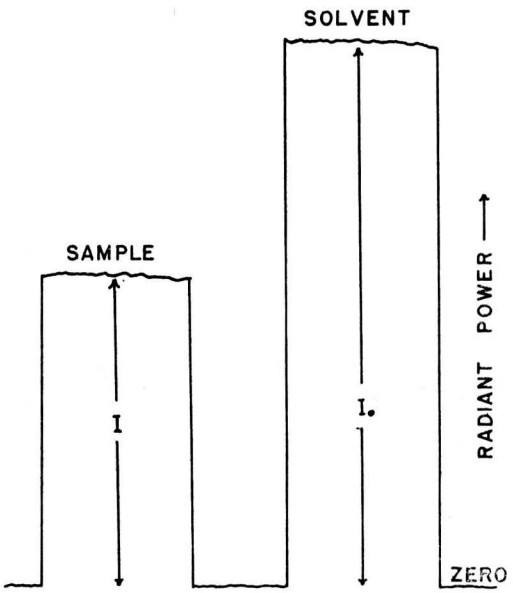


FIG. 1.—Determination of absorbance at a single wavelength.

ANALYSIS PROCEDURE

Add ca 5.5 g absorbent clay to the chromatographic column to produce a column 25 to 50 mm long. Add a weighed amount of sample, contg ca 10 g total toxicant, to the column; tamp the column slightly to settle the contents of the tube. Add CS_2, contg 5% by vol. acetone, in 50 ml increments until 150 ml has been collected from the bottom of the column. During percolation of the first 50 ml portion of CS_2, agitate the sample and the upper half of the absorbent clay column with a metal helix to prevent channeling. Evap. the ext. to dryness on steam bath, and place the residue in an oven at 75°C. for 15 min. Cool the residue, dissolve in CS_2, transfer to a 100 ml vol. flask, and dil. to vol. with CS_2. If substantial amounts of sulfur are present, ext. the sample with acetone (sulfur is highly soluble in CS_2); evap. the sample and dissolve in CS_2 in the usual manner. Place portion of the CS_2 soln in the cell and det. the absorbance at 10.96 and 14.06 μ as above. Substitute these absorbance values in equations (2) to obtain % (w/v) of HEOD and DDT. Convert HEOD to dieldrin by multiplying by the factor 1.175 (dieldrin is defined as 85% HEOD).

DISCUSSION

Table 1 shows the results of analysis of synthetic mixtures of HEOD and *p,p'*-DDT in carbon disulfide. Table 2 gives the results for several synthetic mixtures of commercial samples of dieldrin and DDT, the HEOD and DDT contents of which were known. The data in these two tables indicate that the accuracy of the method (95 per cent confidence level) should be ± 0.10 per cent for HEOD and ± 0.34 per cent for *p,p'*-DDT in the solution analyzed.

The procedure has been used for formulated dust samples containing from 1.5 to 5 per cent dieldrin and from 2 to 10 per cent by weight DDT,

TABLE 1.—*Synthetic mixtures of pure HEOD and p,p'-DDT in CS_2*

HEOD, PER CENT			*p,p'*-DDT, PER CENT		
PRESENT	FOUND	DIFFERENCE	PRESENT	FOUND	DIFFERENCE
4.06	3.92	−0.14	9.95	9.64	−0.31
2.33	2.31	−0.02	5.10	5.16	+0.06
2.51	2.44	−0.07	9.64	9.31	−0.33

and also for samples containing 40 per cent sulfur. The sulfur, even if extracted, causes no interference in the analysis since sulfur has no absorption bands in the region of analysis. The 14.06 μ band of *p,p'*-DDT used for analysis is subject to interference from *o,p'*-DDT, and analysis of several commercial samples of DDT indicate that the present method analyzes for both *p,p'*-DDT and *o,p'*-DDT.

In general, the analysis is specific for HEOD and DDT; however, if interfering materials are present or suspected, then a complete spectrum (2–15 μ) can be obtained and the approximate percentage of toxicants can be calculated from absorption bands that have no interference. The spectrum of HEOD has been presented by Garhart, *et al.*, (1) and that of *p,p'*-DDT by Downing, *et al.*, (6). The use of a single absorbance value for a standard has been found to yield results that are correct to ±10 per cent.

Preliminary experiments indicate that the saponification of the mixture may effect a better analysis procedure. Dieldrin is unchanged, but the dehydrochlorination product of DDT is more suitable for simultaneous analysis than DDT itself.

TABLE 2.—*Synthetic mixtures of technical dieldrin and DDT in CS_2*

HEOD, PER CENT			DDT, PER CENT		
PRESENT	FOUND	DIFFERENCE	PRESENT	FOUND	DIFFERENCE
0.00	0.09	+0.09	10.00	9.62	−0.38
0.00	0.13	+0.13	10.00	9.61	−0.39
0.00	0.08	+0.08	10.00	9.60	−0.40
0.99	1.05	+0.06	9.98	9.78	−0.20
1.94	2.01	+0.07	7.52	7.45	−0.07
2.50	2.46	−0.04	0.00	0.14	+0.14
2.50	2.48	−0.02	0.00	0.00	0.00
2.50	2.43	−0.07	0.00	0.14	+0.14
2.50	2.47	−0.03	10.00	9.77	−0.23
2.93	2.93	0.00	5.05	5.25	+0.20
3.93	4.04	+0.11	0.00	0.18	+0.18
4.04	3.91	−0.13	2.03	2.24	+0.21

SUMMARY

The increased use of mixtures of insecticides in solid and liquid formulations has presented a need for specific methods for analysis of the active components. The present paper describes a procedure for the simultaneous determination of dieldrin and DDT in insecticidal dusts by infrared analysis. The sample is extracted to remove the toxicants from the formulation, and the solution is analyzed by infrared spectrophotometry. A procedure for recording absorbances at a single wavelength on a Perkin-Elmer Model 12-C spectrophotometer is described. Accuracy at the 95 per cent confidence level is 0.34 per cent for DDT and ± 0.10 per cent for dieldrin. With proper extraction procedures or spectrophotometric corrections, the method can also be applied to liquid formulations.

REFERENCES

(1) GARHART, M. D., WITMER, F. J., and TAJIMA, Y. A., *Anal. Chem.*, **24**, 851 (1952).
(2) *Insecticides*, Manual of Specifications for Insecticides and for Spraying and Dusting Apparatus, World Health Organization, Geneva, 1953, WHO/SITECH/6-2.2.
(3) *Ibid.*, WHO/SITECH/1-2.1.
(4) *Ibid.*, WHO/SITECH/1-2.9.
(5) *Ibid.*, WHO/SITECH/1-2.2.1.
(6) DOWNING, J. R., FREED, W. V., WALKER, I. F., and PATTERSON, G. D., *Anal. Chem.*, **18**, 461 (1946).
(7) BECKMAN, H. F., *Anal. Chem.*, **26**, 922 (1954).
(8) WHITTAKER and ROBINSON, *The Calculus of Observations*, Chapter IX, D. Van Nostrand, Inc., New York, 1944.
(9) BAILES, E. L., and PAYNE, M. C., *Ind. Eng. Chem., Anal. Ed.*, **17**, 438 (1945).
(10) SCHECHTER, M. S., and HALLER, H. L., *J. Am. Chem. Soc.*, **66**, 2129 (1944).
(11) SCHECHTER, M. S., SOLOWAY, S. B., HAYES, R. A., and HALLER, H. L., *Ind. Eng. Chem., Anal. Ed.*, **17**, 704 (1945).

A MODIFIED CORNELL PHOSPHATASE TEST FOR THE ANALYSIS OF BLUE MOLD AND AGED CHEESES*

By JOHN H. MAHON, CONSTANCE ANGLIN, and ROSS A. CHAPMAN
(Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Canada)

Kay and Graham (4) were the first to develop a phosphatase test to detect the incomplete pasteurization of milk. Their test was based upon the fact that pasteurization inactivates the phosphatase enzyme of milk.

In the Kay-Graham procedure (4), any residual phosphatase enzyme

* Presented at the Sixty-eighth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 11–13, 1954, at Washington, D. C.

in the milk was allowed to hydrolyze sodium β-glycerophosphate in an alkaline medium; the liberated phosphate, which was determined colorimetrically, served as an index of phosphatase activity. However, because milk has a relatively high phosphate blank, Kay and Graham later employed disodium phenylphosphate as the substrate and estimated the liberated phenol with the Folin-Ciocalteu reagent (5). Gilcreas (3) employed this modification of the Kay-Graham test (5) to estimate the phosphatase activity of hard cheese and reported that the blank values, especially for aged cheese, were so high that the utility of this test for detecting phosphatase activity is limited.

Tyrosine, tyramine, and other constituents of aged cheese react with the Folin-Ciocalteu reagent to produce highly colored blanks (7, 10). Kosikowsky and Dahlberg (6, 7) developed a procedure for extracting these interfering substances from the cheese sample before estimating the liberated phenol with the Folin-Ciocalteu reagent. Although this procedure is time consuming and requires additional manipulation, it was effective in reducing the non-phenol blank.

In order to avoid the inherent difficulties of the Folin-Ciocalteu reagent, Scharer (13) employed the Gibbs reagent (2), 2,6-dibromoquinonechloroimide (BQC), for the colorimetric estimation of the phenol liberated by phosphatase activity. BQC and its chlorine analog, 2,6-dichloroquinonechloroimide (CQC), are sensitive reagents for phenol but do not react with tyrosine, tyramine, or other cheese constituents to produce false blue colors. Consequently, either BQC or CQC is employed in the Cornell (8), Sanders-Sager (12), and Sharer (14) procedures to estimate the phenol liberated from disodium phenylphosphate by phosphatase activity.

The Cornell phosphatase procedure (8) has been used in this laboratory for over two years to estimate the phosphatase activity of cheese. Although the Sanders-Sager procedure (12) yields comparable results and also employs one hour incubation, the reagents for the Cornell procedure are easier to prepare. In addition, the age or history of the cheese must be known for the proper use of the Sanders-Sager procedure (12); this information is not required for the Cornell procedure.

In this paper, several factors that affect the Cornell phosphatase procedure are elucidated and discussed. In addition, the Cornell procedure has been modified to improve the reproducibility of results, to increase the linear range of the phenol calibration line, and to remove some anomalies encountered in the determination of phosphatase activity of blue mold and aged cheeses.

EXPERIMENTAL RESULTS

Effect of Different Types of Cheese upon the Slope of the Phenol Calibration Line.—Experiments in this laboratory have shown that when the Cornell

or Sanders-Sager procedures were used, the slope of the phenol calibration line was always depressed by the presence of cheese. Depending upon the age and type of the cheese, this depression ranged from 16 to 90 per cent when the original Cornell procedure (8) was employed, and similar values were found with the Sanders-Sager procedures. It is possible, therefore, to have 12 to 100 "units" of phenol liberated by phosphatase activity and yet find only 10 "units" of phenol-BQC color. The danger of such a discrepancy is obvious.

To confirm this observation, 10, 20, and 30 microgram portions of phenol were added to 0.5 gram portions of different cheeses and the slope of the phenol calibration line was determined by the Cornell procedure (8). The per cent depression of the slope of the phenol calibration line was determined for each type of cheese by comparison with the phenol calibration line derived in the absence of cheese. Representative results are given in Table 1.

TABLE 1.—*Depressing effect of different cheeses upon the slope of the phenol calibration line by the original Cornell procedure*

TYPE OF CHEESE	SLOPE OF PHENOL CALIBRATION LINE ABSORBANCE PER MMG PHENOL	DEPRESSION OF SLOPE
		per cent
No cheese	0.0400 (Evelyn)	—
Cream cheese	0.0334	16
Cheddar	0.0288–0.0128	28–58 (depending on age)
Gouda or limburger	0.0268	33
Brick	0.0257	36
Swiss	0.0161	60
Roquefort	0.0102	74
Danish blue	0.0108–0.0041	73–90

The results in Table 1 illustrate the depressing effect of cheese on the slope of the phenol calibration line.

Effect of Cheese Concentration on the Slope of the Phenol Calibration Line.—In their present form, the Cornell (8) and Sanders-Sager (11) procedures do not take into consideration the possible effect of any dilution that may be necessary to bring the intensity of the colorimetric solution within the range of accurate measurement on the slope of the phenol calibration line. Experiments confirmed that the greater the dilution of a cheese-containing sample, the more nearly the slope of the phenol calibration line approached that obtained in the absence of cheese. This concept is not new (11); Gilcreas noted it when he employed the Sanders-Sager procedure (12) to estimate the phosphatase activity of cheese (3). The following selected data from Gilcreas (3) illustrate this point.

Cheese Dilution Factor Employed	*Measured Phenol Value*	*Measured Phenol Value × Cheese Dilution Factor*
1 to 5	14	70
1 to 8	17	136
1 to 16	20	320

Gilcreas found that the greater the dilution factor (up to 1:16), the greater the measured value became, and that the apparent phenol concentration increased almost five-fold as a result of a three-fold dilution. This phenomenon is undoubtedly due to the fact that dilution increased the slope of the phenol calibration line in the presence of this cheese more rapidly than it decreased the phenol concentration.

Gilcreas (3) dismissed the foregoing discrepancies as being of "no practical importance" on the grounds that precise measurement of high phenol concentrations are not significant since they represent gross underpasteurization. Gilcreas did not give the phenol value obtained without dilution, but this value would probably have been much lower than 70. Since the depressing effect of cheese upon the slope of the phenol calibration line is equally great at low phenol concentrations, failure to dilute all samples may lead to gross underestimation of phosphatase activity. To illustrate this effect, different amounts of a blue mold cheese were added to 10 micrograms of phenol and the slopes of the resulting phenol calibration lines were determined by the Cornell procedure (8). These results are given in Table 2.

TABLE 2.—*Effect of cheese concentration on the slope of the phenol calibration line*

WEIGHT OF BLUE MOLD CHEESE PER TUBE	SLOPE OF PHENOL CALIBRATION LINE, ABSORBANCE PER MMG PHENOL	DEPRESSION OF SLOPE OF PHENOL CALIBRATION LINE
mg		*per cent*
0	0.0400	0
100	0.0224	44
200	0.0136	66
400	0.0072	82
500 (usually employed)	0.0024	94
1000	0.0000	100

The results in Table 2 illustrate the increased slope of the phenol calibration line as the amount of cheese is reduced. Similar data were obtained with other cheeses, although the effect was less pronounced. In addition, the depressing effect of the cheese can be reduced still further by increasing the amount of BQC or CQC employed in the color-developing step. BQC and CQC reagents react with phenol in an identical manner (2) to produce blue indophenol complexes. However, since CQC is

more stable, it was employed in the subsequent modified Cornell procedure.

Effect of Dilution and CQC Concentration upon the Slope of the Phenol Calibration Line.—Results in this laboratory indicated that dilution of the cheese filtrate and the simultaneous increase in the CQC concentration might overcome much of the depressing effects of cheese upon the slope of the phenol calibration line. Similar effects had previously been demonstrated by Sanders and Sager (12) and Gilcreas (3).

Twenty micrograms of phenol was added to 0.5 gram portions of blue mold cheese and the slope of the phenol calibration line was determined by the basic principles of the Cornell procedure (8), in which two dilution factors were used, together with four CQC concentrations. In each case,

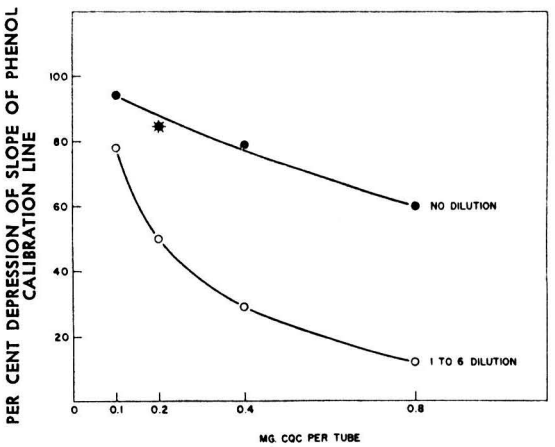


FIG. 1.—Effect of dilution factor and CQC concentration upon the slope of the phenol calibration line.

the per cent depression of the phenol calibration line was calculated by comparison with a phenol calibration line determined in the absence of cheese. These results are given in Fig. 1; the asterisk corresponds to the original Cornell procedure.

The data in Fig. 1 show that doubling the amount of color-forming reagent prescribed in the Cornell procedure, and employing a 1:6 dilution factor increased the slope of the phenol calibration line in the presence of cheese from 15 to 71 per cent of the value obtained in the absence of cheese. Although the use of 0.8 mg of CQC per tube further reduced the depression of the phenol calibration line, it was not practical to employ this amount of CQC, because the decomposition of the excess CQC causes the color of the blank to become too dark.

The following modified Cornell procedure was used:

MODIFIED CORNELL PHOSPHATASE TEST FOR CHEESE

REAGENTS

(a) *Buffer reagent.*—Dissolve 23.0 g anhyd. Na_2CO_3 and 20.3 g anhyd. $NaHCO_3$ in H_2O and dil. to 2000 ml.

(b) *Buffer substrate reagent.*—Dissolve 0.27 g disodium phenylphosphate (phenol-free) in 250 ml of the buffer reagent. Prep. just before use.

(c) *Sodium carbonate reagent.*—Dissolve 8 g anhyd. Na_2CO_3 in H_2O and dil. to 100 ml.

(d) *Trichloracetic-hydrochloric acid reagent.*—Dissolve 12.5 g trichloracetic acid crystals in H_2O, dil. to 25 ml with H_2O, add 25 ml concd HCl (ca 36% HCl), and mix.

(e) *Calgon reagent.*—Dissolve 10 g sodium hexametaphosphate in H_2O and dil. to 100 ml (the *p*H should be 6.3 for this soln).

(f) *2,6-Dichloroquinonechloroimide reagent (CQC).*—Dissolve 20 mg CQC in 25 ml absolute ethyl alcohol, dil. to 50 ml with H_2O, and store in a dark bottle. Prep. just before use for best results.

(g) *Stock phenol reagent.*—Dissolve 100 mg dry phenol crystals in H_2O and dil. to 100 ml (1000 mmg phenol/ml).

(h) *Dilute phenol reagent.*—Dil. 3 ml stock phenol reagent to 1000 ml with the buffer reagent (3 mmg phenol/ml).

PROCEDURE

Phenol calibration line.—Place 1 to 10 ml aliquots of the dil. phenol reagent into test tubes. Dil. all aliquots to 10 ml with the buffer reagent. Add 1 ml of the Calgon reagent and 1 ml of the CQC reagent with gentle blowing, and mix. Similarly treat a blank contg 10 ml of the buffer reagent only.

Place all tubes in a 37°C. H_2O-bath for 15 min. to completely develop the blue color. Measure the intensity of this color relative to the blank at 620 mμ. Calc. the slope of the phenol calibration line (i.e., absorbance (2 − log % I) divided by the micrograms of phenol present) over its linear range and employ this figure to calculate the phenol content of unknown samples.

Sampling and incubation.—Place 2 representative 0.5 g portions of cheese (taken at least ½" below an exposed surface) into 25×150 mm test tubes. Add 0.5 ml H_2O, allow to soak for 10 min., and stir the cheese into a paste with a heavy glass rod. Heat one cheese sample, to be employed as the blank, for 3 min. in a boiling H_2O bath with occasional stirring. Add 10 ml of the buffer substrate reagent to each tube, mix thoroly, and incubate for 1 hr in a 37°C. H_2O bath with occasional swirling.

Protein precipitation.—After the 1 hr incubation, slowly add 1 ml of the trichloracetic-hydrochloric acid reagent down the side of each tube. Mix, and filter off the resulting ppt thru an 11 cm Whatman No. 42 paper.

Color development and calculations.—Pipet 1 ml of the clear cheese filtrate into a test tube; add 1 ml of the Calgon reagent, 1 ml of the sodium carbonate reagent, and 8 ml of the buffer reagent; and mix. Add 1 ml of the CQC reagent with gentle blowing, mix, and place the tube in a 37°C. H_2O bath for 15 min. to completely develop the color. Measure the color intensity relative to the appropriate heat-treated blank at a wavelength of 620 mμ. Multiply the observed absorbance by a factor of 6, divide this value by the slope of the phenol calibration line, and express the results in terms of mmg phenol per 0.25 g cheese.

COMPARISON OF THE CORNELL, MODIFIED CORNELL, AND SANDERS-SAGER PHOSPHATASE TESTS

(1) *Effect of Age of Cheddar Cheese upon the Slope of the Phenol Calibration Lines.*—The slopes of the phenol calibration lines were determined

by adding phenol to 0.5 g portions of cheddar cheese, ranging in age from 2 to 11 months, by the Cornell (8), modified Cornell, and Sanders-Sager (12) procedures. Enough phenol was added to each cheese to ensure 10 micrograms in the volume of filtrate used. The per cent depression of the phenol calibration line was found by comparing it with the slope of the corresponding line determined in the absence of cheese. The cheddar cheeses used in this experiment were different cheeses manufactured by the same company and stored for 2 to 11 months under identical conditions. These cheeses were then analyzed; the results are shown in Fig. 2.

The data in Fig. 2 show that aged cheddar cheese depressed the slope of the phenol calibration line to the least extent when the modified Cornell

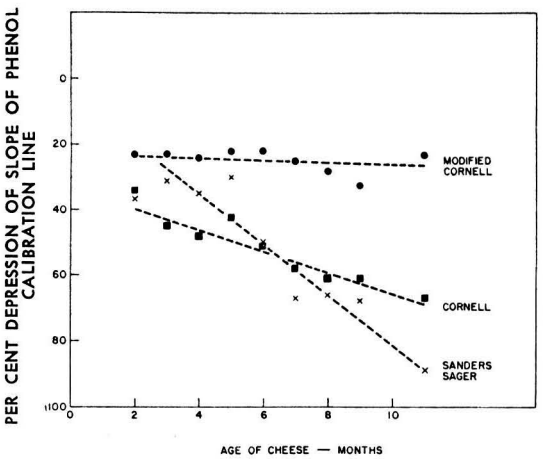


FIG. 2.—Effect of age of cheddar cheese upon the slope of the phenol calibration lines by three analytical procedures.

procedure was used. In addition, the modified Cornell procedure yielded consistently greater slopes for the phenol calibration lines in the presence of cheese, and greater sensitivity was then obtained.

The apparent loss of phosphatase activity during the storage of cheese (1) may be due to the increased depression of the phenol calibration line with the increasing age of the cheese. And the apparent loss of phosphatase during the storage of cheese may be much less than currently indicated by the literature.

(2) *Effect of Roquefort and Blue Mold Cheeses upon the Slope of the Phenol Calibration Lines.*—The depressing effect of eleven Roquefort and four blue mold cheeses upon the slope of the phenol calibration line was determined by employing three analytical procedures. Enough phenol was added to each cheese to ensure 10 micrograms in the volume of filtrate used. The results are given in Table 3.

TABLE 3.—*Effect of Roquefort and blue mold cheeses upon the slope of the calibration line*

CHEESE USED	AVERAGE PER CENT DEPRESSION OF THE SLOPE OF THE PHENOL CALIBRATION LINE IN THE PRESENCE OF CHEESE		
	CORNELL	MODIFIED CORNELL	SANDERS-SAGER
Blue mold	81 ± 6	30 ± 3	81 ± 13
Roquefort	73 ± 10	25 ± 3	65 ± 10

Data in Table 3 indicate that the slope of the phenol calibration line is depressed to the least extent by these cheeses when the modified Cornell procedure is employed.

The average depression of the slope of the phenol calibration line by using the modified Cornell procedure was 25 per cent for cheddar (see Fig. 2), 30 per cent for blue mold, and 25 per cent for Roquefort cheeses. This depression is relatively small and uniform when compared with the results from the Cornell and Sanders-Sager procedures. It would appear, employing the modified Cornell procedure, that the depression of the slope of the phenol calibration line is approximately 25 per cent irrespective of the age or type of cheese. To obtain the most accurate results, therefore, the phenol conversion factor determined in the absence of cheese could be reduced by 25 per cent and this corrected value employed when calculating the phenol produced by the phosphatase activity of cheese.

(3) *Relative Sensitivity.*—Unpasteurized cheddar cheese was carefully homogenized with the appropriate buffer solutions. Varying quantities of this cheese suspension were added to 0.5 gram portions of the same cheese which had previously been heat-treated to destroy all phosphatase activity. These cheese samples contained 0.01 to 2.0 per cent of unpasteurized cheese. They were then analyzed for phosphatase activity by five different methods to determine the relative sensitivity of these procedures. The results are given in Table 4.

The data in Table 4 indicate that the Cornell (8) and Sanders-Sager (12) procedures yielded similar results with a one-hour incubation period. However, the modified Cornell procedure gave results approximately 37 per cent higher than those obtained by the Cornell procedure with a one hour incubation period. When an 18 hour incubation period was used, the modified Cornell procedure yielded results about 50 per cent higher than the Cornell procedure up to 0.1 per cent raw cheese. In the case of fresh cheddar cheese, these differences are not really significant because the percentage of raw cheese detectable by these procedures is essentially the same. Only in the case of aged cheese or blue mold cheese is there a significant difference in the percentage of raw cheese that can

TABLE 4.—*Relative sensitivity of five phosphatase procedures*

RAW CHEDDAR CHEESE PRESENT IN CHEESE	PHOSPHATASE ACTIVITY,[a] MMG PHENOL PER 0.25 G CHEESE				
	CORNELL	MODIFIED CORNELL	SANDERS-SAGER	CORNELL	MODIFIED CORNELL
	1 HR	1 HR	1 HR	18 HRS	18 HRS
per cent					
2.0	15.6	20.4	18.7	70.0	240.0
1.0	7.5	11.0	8.3	40.0	127.7
0.4	3.4	4.6	3.5	21.7	49.0
0.2	1.5	2.4	2.0	12.7	26.0
0.1	0.9	1.2	0.4	6.6	10.2
0.07	—	—	—	5.1	7.3
0.04	0.3	0.5	0.1	3.3	4.6
0.02	—	—	—	1.6	2.6
0.01	—	—	—	0.7	1.0

[a] CQC was employed for all these comparative tests.

be detected. Above 0.2 per cent raw cheese, the difference rapidly increased because of the nonlinearity of the phenol calibration line for the Cornell procedure above this level. These data also indicate that the phenol calibration line for the modified Cornell procedure is linear up to 200 micrograms of phenol per 0.25 gram of cheese or 200/6 = 33 micrograms of phenol per tube. The greater linear range of the modified Cornell procedure is due in part to the larger CQC concentration employed.

In addition, the phosphatase activity of raw cheddar cheese with the one hour and 18 hour incubation periods and by the modified Cornell procedure is approximately 1150 and 12,000 micrograms of phenol per 0.25 gram of cheese, respectively.

(4) *Phosphatase Values for a Number of Cheeses by the Cornell and Modified Cornell Procedures.*—The phosphatase activity of a number of cheeses was determined by the Cornell and modified Cornell procedures. No dilutions other than those contained in these procedures were employed. These results are given in Table 5.

TABLE 5.—*Phosphatase activity of different cheeses as determined by the Cornell and modified Cornell procedures*

NAME OF CHEESE	PHOSPHATASE ACTIVITY, MMG PHENOL PER 0.25 G CHEESE (1 HR)	
	CORNELL PROCEDURE	MODIFIED CORNELL PROCEDURE
Blue mold (5 cheeses)	14 to 27	210 to 350
Roquefort (3 cheeses)	33 to 38	430 to 500++
Swiss	36.2	176.0
Gouda	1.0[a]	1.4[a]
Cheddar (process cheese)	0.7[a]	1.0[a]

[a] Pasteurized.

The data in Table 5 show that the modified Cornell procedure yields phenol values from 5 to 20 times greater than those by the Cornell procedure when the cheeses are unpasteurized. However, for pasteurized Gouda and cheddar cheeses the values obtained by these procedures are comparable.

CAUSE OF DEPRESSION OF THE SLOPE OF THE PHENOL CALIBRATION LINE

The large depression of the slope of the phenol calibration line, in the presence of blue mold or other aged cheeses, is attributed to the relatively high free amino acid content of these cheeses. When blue mold cheeses

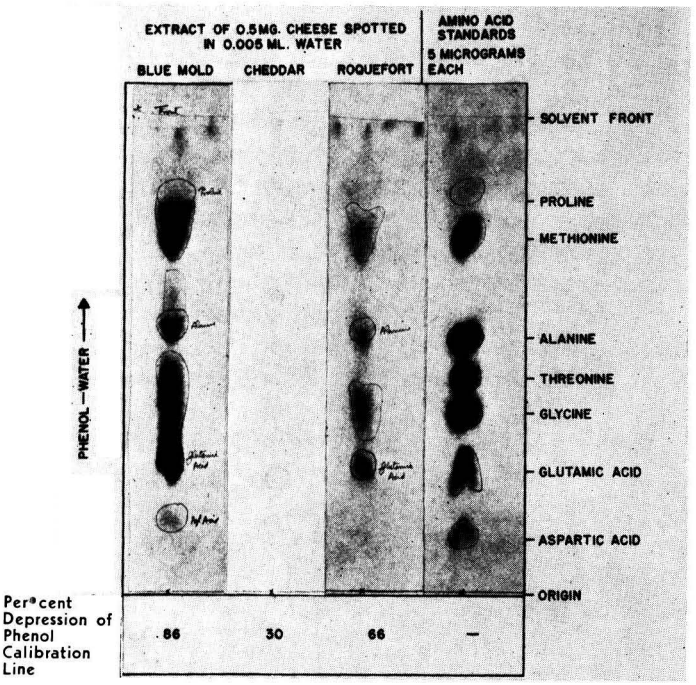


FIG. 3.—One-dimensional chromatograms of water-soluble cheese components.

are added to phenol-containing solutions, the cheese proteins are precipitated and filtered off, and the phenol-BQC or phenol-CQC color is developed, the final color is a yellowish-blue or brownish-blue, instead of the usual clear blue. A similar color can be obtained by adding small amounts of reducing materials such as sodium sulfite to the blue phenol-BQC or phenol-CQC complex. Further, the addition of free amino acids, especially cysteine, causes the blue phenol-BQC or phenol-CQC color to become yellowish-blue, whereas most other amino acids induce a

brownish-blue color similar to that obtained with blue mold cheeses. The formation of these off-colors is coincident with a marked reduction in color intensity at 620 mμ, hence the depression of the slope of the phenol calibration line. To substantiate further the role of free amino acids in depressing the slope of the phenol calibration line, one-dimensional chromatograms were made of the water-soluble extracts of different cheeses. In addition, the depressing effect of each cheese upon the phenol calibration line by the Cornell procedure was also determined. These results are presented in Fig. 3.

The data in Fig. 3 indicate a strong correlation between the depression of the slope of the phenol calibration line and the free amino acid content of the cheese, as judged by the intensity and distribution of the ninhydrin amino acid spots. These results are in good agreement with the literature (9).

MOLD PHOSPHATASE IN BLUE MOLD CHEESE

Blue mold cheeses possess a considerable alkaline mold-phosphatase activity. This phosphatase activity may be attributed to incompletely pasteurized cheese when the phosphatase procedures are used. In order to estimate the alkaline mold-phosphatase activity of blue mold cheese, the mold was isolated and grown on a broth medium. This cultured mold was lifted from the broth, washed with water, and dabbed dry between filter papers, and its phosphatase activity was determined by both the Cornell and modified Cornell procedures with the results shown in Table 6.

TABLE 6.—*Phosphatase activity of blue mold isolated from cheese*

PROCEDURE EMPLOYED	PHOSPHATASE ACTIVITY, MMG PHENOL/0.25 G MOLD
Cornell	37
Modified Cornell	390

The mold content of blue mold cheeses has been found to be of the order of 5 to 10 per cent of the cheese. Assuming a similar phosphatase activity for the mold in cheese as that for the mold cultured on a broth medium, the alkaline mold-phosphatase activity of blue mold cheeses should be of the order of 2 to 4 micrograms of phenol per 0.25 gram of cheese by the Cornell or Sanders-Sager procedures, and 19 to 39 micrograms of phenol per 0.25 gram of cheese by the modified Cornell procedure.

SUMMARY

(1) It has been established that all cheeses depress the slope of the phenol calibration line. This effect is especially marked in the case of blue mold and aged cheese. The slope has been found to range from 10 to 88 per cent of the theoretical value.

(2) The depressing effect of cheddar cheese upon the slope of the phenol calibration line becomes more pronounced with the age of the cheese. The apparent loss of phosphatase activity during the storage of cheese is attributed at least in part to this phenomenon.

(3) A correlation has been shown between the depressing effect of cheese upon the slope of the phenol calibration line and the free amino acid content of the cheese.

(4) The addition of the CQC reagent from a pipet, with gentle blowing to facilitate rapid mixing with the sample, ensures more reproducible results.

(5) The use of an increased amount of CQC increases the slope of the phenol calibration line especially in the presence of blue mold and aged cheese, thereby increasing the phosphatase values obtained.

(6) The use of a 1:6 dilution factor for all cheese samples increases the slope, the analytical range, and the linearity of the phenol calibration line. Employing a single dilution factor ensures that all results are comparable.

ACKNOWLEDGMENT

The authors wish to express their thanks to F. V. Kosikowsky of Cornell University for reviewing this work and for his many constructive suggestions.

REFERENCES

(1) CAULFIELD, W. J., and MARTIN, W. H., *J. Dairy Sci.*, **29**, 155 (1945).
(2) GIBBS, H. D., *J. Biol. Chem.*, **72**, 649 (1927).
(3) GILCREAS, F. W., *This Journal*, **30**, 422 (1947).
(4) KAY, H. D., and GRAHAM, W. R., JR., *J. Dairy Research*, **5**, 63 (1933).
(5) ———, and ———, *ibid.*, **6**, 191 (1935).
(6) KOSIKOWSKY, F. V., and DAHLBERG, A. C., *J. Dairy Sci.*, **32**, 751 (1949).
(7) ———, and ———, *ibid.*, **31**, 561 (1948).
(8) KOSIKOWSKY, F. V., *ibid.*, **34**, 1151 (1951).
(9) ———, and DAHLBERG, A. C., *ibid.*, **37**, 167 (1954).
(10) LEAHY, H. W., SANDHOLZER, L. A., and WOODSIDE, M. R., *J. Milk and Food Technol.*, **3–4**, 183 (1940–1941).
(11) SANDERS, G. P., and SAGER, O. S., *This Journal*, **28**, 656 (1945).
(12) *Official Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Box 540, Benjamin Franklin Station, Wash. 4, D. C., 1950, **15.144–15.147.**
(13) SCHARER, H., *This Journal*, **24**, 76 (1941).
(14) ———, *J. Milk and Food Technol.*, **16**, 86 (1953).

VARIATION IN FAT CONSTANTS OF GENUINE CANADIAN MILK FAT*

By R. R. RIEL (Chemistry Division, Science Service, Canada Department of Agriculture, Ottawa, Canada)

The properties of milk fat are being approached more and more closely in cheaper synthetic fat mixtures. The increasing availability of these mixtures renders adulteration of milk fat a greater probability, and inasmuch as the dairy industry is concerned with the problem of preserving the integrity of all dairy products the situation creates a need for more exact characterization of milk fat.

The analytical constants of milk fat have never been studied intensively in Canada. The ranges of values reported in the literature are wide and it is often claimed that they are probably not representative of the modern milk supply. In most cases they are given for milk fat from a single cow or a single herd. Nowadays the fat in most dairy products is representative of pooled milk, or milk from several herds representing a large number of cows. Such fats should have constants with narrower ranges than the so-called "normal ranges." If narrower ranges can be established, it will be more difficult to simulate milk fat by blending other fats in semblance of its physico-chemical characteristics.

Reichert-Meissl and Polenske values are still considered among the most useful constants in the examination of milk fat. Milk fat is different from all other edible fats because of its high Reichert-Meissl value. However, this characteristic is not specific because foreign fats may be enriched in volatile and water-soluble fatty acids by addition of tributyrin or similar substances. Furthermore, detection of the added substance by extraction procedures may be prevented by processes of rearrangement whereby the acid radical is randomly distributed among the triglycerides.

Milk fat has many properties which are intermediate between oils of the coconut family on one side, and most other edible fats on the other. Hence coconut oil is widely used in combination with other fats in compounding mixtures whose properties approach those of milk fat. Coconut oil is characterized by its very high Polenske value, which limits the amount which can be incorporated into such a mixture.

The refractive index has been widely used in the past to detect adulteration. This constant has lost a great deal of significance since mixtures of foreign fats may be prepared to have indices well within the range of those for milk fat. Nevertheless, the refractive index has the advantages of simplicity and accuracy of measurement.

---

* Presented at the Sixty-eighth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 11, 12, and 13, 1954, at Washington, D. C., as Contribution No. 271, Chemistry Division, Science Service, Ottawa, Canada.

The object of this study was to evaluate the seasonal variations in Reichert-Meissl values, Polenske values, and refractive indices of Canadian milk fat.

SAMPLING AND ANALYSIS

Provincial Dairy Inspectors cooperated by obtaining genuine seven-pound butter samples from 29 butter factories throughout Canada (2 in British Columbia, 3 in New Brunswick, and 4 in each of 6 other provinces). The factories were chosen to represent cream and water supplies of different quality and a cross-section of the dairy-producing areas in Canada. Monthly samples were collected from June 1953 through May 1954.

The butter oil was prepared from a representative sample of the 7 lb. block by melting the butter at 50°C. and filtering the oil layer at the same temperature. The Reichert-Meissl and Polenske values were determined by the A.O.A.C. method (1). The refractive indices were measured at 40°C. ($\pm 0.02°$) with a Zeiss refractometer (heatable measuring prism, L4) and monochromatic sodium light.

The average number of cows represented in each individual sample was estimated to be 1,400 (200–100,000), and the number of herds to be 200 (20–1,900). The monthly average values for all factories are representative of an estimated 39,000 cows and 5,300 herds.

In June 1953, all of the cows were on pasture. The summer of 1953 was dry and pastures started drying out in July. The transition from pasture feeding to winter feeding took place in December. In November, most of the cows were on late pasture but received some supplemental feeding. In 1954, the transition from winter feeding to pasture feeding took place in May with approximately 50 per cent of the cows on winter feed and 50 per cent on pasture feeding. This, of course, is a general statement, and does not apply to every herd.

RESULTS AND DISCUSSION

Reichert-Meissl Values.—The monthly variations of Reichert-Meissl values are reported in Table 1. In most cases, each individual value reported in the body of the table is an average for four factories. The average Reichert-Meissl value of the 327 samples is 27.8 with extreme values of 24.6 and 31.4. The 95 per cent fiducial limits are 25.1 to 30.5. This range is in fairly close agreement with the Reichert range (25.5–32.3) of New Zealand factory butterfat reported by Cox and McDowall (2).

Statistical analysis (Table 2) indicates that the differences between months, and between provinces, are significant. The monthly differences are greater than the province differences. Therefore, the monthly ranges are appreciably narrower than the yearly range. Monthly ranges within provinces would be still narrower; for instance, values below 25.0 occur in three provinces, and during October and November only. In British

TABLE 1.—*Average*[a] *Reichert-Meissl values*

PROVINCE	MONTH												AV. BY PROVINCE	RANGE[b]
	(1953) JUNE	JULY	AUG.	SEPT.	OCT.	NOV.	DEC.	(1954) JAN.	FEB.	MAR.	APR.	MAY		
N.S.	30.1	28.9	28.1	27.7	27.3	26.7	27.9	28.4	28.4	28.5	29.5	29.1	28.4	25.7–30.7
N.B.	29.3	28.0	27.7	26.8	26.4	26.3	26.5	27.3	27.5	30.1	29.6	29.9	27.8	25.4–30.9
Que.	28.7	28.9	27.9	27.2	25.5	25.8	—	25.7	26.7	29.7	31.0	29.2	27.8	24.6–31.4
Ont.	28.3	28.1	27.1	26.2	26.5	26.1	27.1	28.5	28.8	28.9	29.3	29.2	27.9	25.2–30.4
Man.	28.6	29.4	27.5	26.2	25.6	26.0	26.2	27.6	27.6	29.0	28.4	28.6	27.6	24.6–31.1
Sask.	29.5	29.7	27.7	26.4	26.2	25.7	26.3	27.1	27.4	27.9	28.0	28.2	27.5	24.7–30.1
Alta.	28.7	29.0	27.2	27.4	26.2	25.2	26.4	27.0	27.7	27.5	27.7	27.1	27.3	25.1–29.5
B.C.	—	27.2	29.4	27.8	27.8	26.8	27.8	27.6	28.6	29.5	29.4	29.7	28.4	26.7–30.1
Av. by month	29.0	28.8	27.7	26.9	26.4	26.0	26.8	27.5	27.8	28.7	29.0	28.7	27.8	
No. of samples	27	28	29	29	29	28	24	27	27	27	27	25	327	
S.D.	0.86	0.96	0.74	0.81	0.93	0.87	1.12	1.12	0.98	1.14	1.11	1.08	1.4	
Range[b]	27.0–30.7	27.2–31.3	26.2–29.5	25.2–29.2	24.6–28.0	24.7–28.0	25.2–29.4	25.2–29.5	26.3–29.5	26.5–30.9	27.3–31.4	27.0–30.6	24.6–31.4	
95% fiducial limits	27.3–30.7	26.9–30.7	26.3–29.1	25.3–28.5	24.6–28.2	24.3–27.7	24.6–29.0	25.3–29.7	25.9–29.7	26.5–30.9	26.8–31.2	26.6–30.8	25.1–30.5	

[a] For four factories with few exceptions.
[b] From individual values.

TABLE 2.—*Analysis of variance of Reichert-Meissl values*

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE
Months	11	31.95[a]
Provinces	7	6.07[a]
Months × provinces	77	1.32[a]
Between factories within provinces	21	1.57
Error (M × factories within provinces)	210	0.80

[a] Significant at $P = 0.01$, using "factories within provinces" as error for provinces, and the remainder error term for months and for months × provinces.

Columbia, the minimum value is 26.7. Furthermore, no value below 26.0 was found from February to August, inclusive. Figure 1 shows that 98.8 per cent of all values are above 25.0.

Low Reichert-Meissl values have been attributed to such factors as end of lactation, pasture feeding, sub-maintenance feeding, and general adverse conditions. The pattern of the monthly variations seems to reflect to a considerable degree the generally accepted influences of lactation period and feed quality; the values decrease gradually from June to November and then increase gradually from November to April. The months of April, May, and June presumably reflect the beginning of the average lactation period. The low values in the late fall may reflect the end of the average lactation period, and may also coincide with poor quality feed and adverse climatic conditions.

Polenske Values.—The monthly variations of the Polenske values are presented in Table 3. The average Polenske value of the 327 samples is 2.0 (1.4–3.0), with only one value below 1.5. The 95 per cent fiducial limits are 1.4–2.6.

Statistical analysis (Table 4) also indicates that the values are different between months and between provinces. The monthly variations of Polenske values, however, are less extensive than those of Reichert-Meissl values, with the highest values in June and July. It may be of interest to point out that 92.6 per cent of the values are in the range 1.6–2.6, 95.3 per cent are above 1.6, and 97.3 per cent are below 2.6 (Fig. 2).

Refractive Indices.—The monthly variations of refractive indices are presented in Table 5. The average refractive index of the 327 samples is 1.45418, and the range is 1.45295 to 1.45531. This range is appreciably narrower than the reported range of 1.4527 to 1.4560. The 95 per cent fiducial limits are 1.45326 to 1.45512.

As with the Reichert-Meissl and Polenske values, statistical analysis (Table 6) indicates significant differences between months and between provinces. The refractive indices appear to be strikingly correlated with the season, being high in summer (June to October) and low in winter

FIG. 1.—Frequency bar chart of Reichert-Meissl values.

FIG. 2.—Frequency bar chart of Polenske values.

FIG. 3.—Frequency bar chart of refractive indices.

TABLE 3.—*Average[a] Polenske values*

PROVINCE	MONTH												AV. BY PROVINCE	RANGE[b]
	(1953) JUNE	JULY	AUG.	SEPT.	OCT.	NOV.	DEC.	(1954) JAN.	FEB.	MAR.	APR.	MAY		
N.S.	2.3	2.0	1.8	1.9	2.1	2.1	2.1	2.2	2.1	2.1	2.1	1.8	2.1	1.6–2.6
N.B.	2.4	2.2	2.1	1.9	2.0	2.3	2.1	2.3	1.9	2.3	1.7	1.6	2.1	1.6–2.7
Que.	2.0	2.3	2.1	1.7	2.1	2.0	—	1.9	2.0	1.8	2.1	2.1	2.0	1.6–2.5
Ont.	2.1	2.1	1.9	1.9	2.1	1.8	1.9	1.9	1.8	2.0	1.9	2.2	2.0	1.6–2.6
Man.	2.2	2.4	2.2	1.8	2.0	2.0	2.0	2.0	1.9	2.0	2.0	1.8	2.0	1.5–2.8
Sask.	2.4	2.5	2.3	2.1	2.3	1.9	1.9	2.0	1.7	1.8	1.6	1.8	2.0	1.4–3.0
Alta.	2.2	2.1	1.9	2.0	2.1	1.8	1.8	1.8	1.7	1.7	1.7	1.7	1.9	1.5–2.3
B.C.	—	2.6	2.4	2.5	2.1	1.8	2.3	2.4	2.1	2.5	1.7	2.3	2.2	1.7–2.8
Av. by month	2.2	2.2	2.1	2.0	2.1	2.0	2.0	2.1	1.9	2.0	1.8	1.9	2.0	
No. of samples	27	28	29	29	29	28	24	27	27	27	27	25	327	
S.D.	0.20	0.31	0.27	0.25	0.19	0.26	0.21	0.25	0.21	0.28	0.26	0.31	0.28	
Range[b]	1.8–2.7	1.8–3.0	1.7–2.9	1.6–2.6	1.8–2.4	1.6–2.8	1.6–2.3	1.5–2.6	1.5–2.5	1.6–2.8	1.4–2.5	1.5–2.6	1.4–3.0	
95% fiducial limits	1.8–2.6	1.6–2.8	1.6–2.6	1.5–2.5	1.7–2.5	1.5–2.5	1.6–2.4	1.6–2.6	1.5–2.3	1.5–2.5	1.3–2.3	1.3–2.5	1.4–2.6	

[a] For four factories with few exceptions.
[b] From individual values.

TABLE 4.—*Analysis of variance of Polenske values*

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE
Months	11	0.44[a]
Provinces	7	0.34[a]
Months × provinces	77	0.09[a]
Between factories within provinces	21	0.11
Error (*M* × factories within provinces)	210	0.05

[a] Significant at $P = 0.01$, using "factories within provinces" as error for provinces, and the remainder error term for months and for months × provinces.

(November to May). The monthly variations are in close agreement with those reported from England by Davis and Macdonald (3) and from New Zealand by McDowell (4). The observed Canadian range (1.45295–1.45531) is somewhat lower than the range found in New Zealand (1.4535–1.4571) on mixed herd milk, and is within the range found in England (1.4522–1.4565) where the types of milk are not given. No value above 1.45469 was observed between November and April, and no value below 1.45357 was observed between June and September. The extreme values are 1.45357 and 1.45531 in summer, and 1.45295 and 1.45496 in winter. Furthermore, no value below 1.45371 was observed in the prairie provinces (Manitoba, Saskatchewan, Alberta). Figure 3 shows that 97.9 per cent of the values are higher than 1.45320.

CORRELATIONS

The interaction between months and provinces was significant for the three constants (Tables 2, 4, 6). This indicates that factors causing variations (lactation period, feed, breed, size of herds, adverse conditions, etc.) do not operate at the same time in all provinces. For example, the high Reichert-Meissl values occurred earlier in the dairy provinces (Ontario, Quebec, New Brunswick) than in the other provinces. Thus, monthly variations would have still more meaning on a provincial than on a national basis.

The coefficients of correlation between the three constants were calculated and tested for significance. The coefficient of 0.33 between the Reichert-Meissl values and the Polenske values and the coefficient of 0.31 between the Reichert-Meissl values and the refractive indices were significant. The Polenske values and the refractive indices were not significantly correlated.

The regression equations for the significant correlations were:

Polenske $= 0.23 + 0.0645$ *Reichert-Meissl.*

Refraction reading $(40°C.) = 32.9 + 0.3526$ *Reichert-Meissl.*

The Polenske values were calculated as percentages of the Reichert-Meissl values. The average percentage ratio was 7.3 with a range of 5.3

TABLE 5.—*Average[a] refractive indices at 40°C.*

(Each R.I. figure in the body of the table is preceded by 1.45)

PROVINCE	MONTH												AV. BY PROVINCE	RANGE[b]
	(1953) JUNE	JULY	AUG.	SEPT.	OCT.	NOV.	DEC.	(1954) JAN.	FEB.	MAR.	APR.	MAY		
N.S.	392	444	461	452	413	359	349	353	337	345	355	392	389	295–481
N.B.	398	454	478	473	435	368	346	355	375	358	351	360	402	321–496
Que.	433	442	465	465	453	389	—	388	394	373	351	408	420	339–508
Ont.	460	488	514	505	478	408	384	385	395	378	388	412	433	342–531
Man.	452	452	488	484	430	411	393	391	411	390	401	454	430	371–528
Sask.	420	447	471	474	422	398	388	392	416	390	405	458	423	371–508
Alta.	438	470	501	492	424	419	414	408	432	419	429	474	444	386–525
B.C.	—	422	449	437	408	377	355	348	345	354	376	400	386	330–449
Av. by month	429	456	481	475	435	394	380	380	393	379	385	428	419	
No. of samples	27	28	29	29	29	28	24	27	27	27	27	25	327	
S.D.	.00029	.00026	.00030	.00031	.00029	.00033	.00036	.00030	.00036	.00029	.00032	.00040	.00047	
Range[b]	357–481	416–508	422–531	419–528	316–514	295–469	324–443	309–428	295–443	306–428	339–434	345–496	295–531	
95% fiducial limits	371–486	404–507	421–540	414–536	378–492	328–459	308–451	321–439	322–464	322–436	323–448	350–505	326–512	

[a] For four factories with few exceptions.
[b] From individual values.

TABLE 6.—*Analysis of variance of refractive indices*

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE
Months	11	45.21[a]
Provinces	7	18.85[a]
Months × provinces	77	0.96[a]
Between factories within provinces	21	3.78
Error (M × factories within provinces)	210	0.40

[a] Significant at $P = 0.01$, using "factories within provinces" as error for provinces, and the remainder error term for months and for months × provinces.

to 10.5. Values above 10.0 (10.4 and 10.5) occurred only twice out of 327 values. This observation would justify the maximum limit of 10.0 per cent set forth by the Canadian Department of National Health and Welfare (Food and Drugs Laboratories) (5).

SUMMARY

A study of Reichert-Meissl, Polenske, and refractive index values indicated that the normal ranges for these constants were appreciably narrower in Canadian milk fat than are those reported in the literature. The average values and 95 per cent fiducial limits were:

Reichert-Meissl	27.8	(25.1–30.5)
Polenske	2.0	(1.4–2.6)
Refractive Index	1.45419	(1.45326–1.45512)

The Reichert-Meissl values decreased from June to November and then increased from November to April. The Polenske values were higher during June and July than during the other months. The refractive indices were high in summer and low in winter.

There were significant differences between months and between provinces. The interaction between months and provinces was also significant. Therefore, the ranges were significantly narrower when established for each month than for the year. It is believed that these constants would gain further significance if monthly ranges were established within each province.

Low but significant correlations were found between Reichert-Meissl and Polenske values ($r = 0.33$) and between Reichert-Meissl values and refractive indices ($r = 0.31$). The Polenske values expressed as percentage of the Reichert-Meissl values averaged 7.3 with 99.4 per cent of the values below 10.0.

ACKNOWLEDGMENT

The cooperation of A. H. White, Bacteriology Division, and of the various Provincial Dairy Commissioners is gratefully acknowledged.

REFERENCES

(1) *Official Methods of Analysis*, 7th Ed., Association of Official Agricultural Chemists, Washington, D. C., 1950.

(2) Cox, G. A., and McDowall, F. H., *J. Dairy Research*, **15**, 377 (1948).
(3) Davis, J. G., and Macdonald, F. J., *Richmond's Dairy Chemistry*, 5th Ed., Charles Griffin & Co. Ltd., London, 1952.
(4) McDowell, A. K. R., *J. Dairy Research*, **21**, 383 (1954).
(5) *Canadian Trade Information Letter*, No. 92, August 10, 1953.

IDENTIFICATION OF RODENT FUR HAIRS*

By Dorothy B. Scott (Division of Microbiology, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.)

Several investigators (1–5) report characteristics that can be used in the microscopic identification of rodent hairs. This paper reports a study of fur hairs from the rat, mouse, rabbit, squirrel, and muskrat. All have a ladder-like medulla structure and certain similarities in over-all microscopic characteristics. Fur hairs with ladder-like medullas are found in food and drug products. They may be pigmented or unpigmented; air may be entrapped in the medulla or the air may be replaced with liquids. The latter are more apt to be found in processed foods. A method for mounting hairs in glycerine jelly which eliminates the air from the air vesicles of the medulla was described by Bachrach (2) and has been used in this study.

Diagnostic structures in the medulla of fur hairs are revealed when the hairs are mounted in glycerine jelly and examined at 400–800×. The distinction is based on the structure of the medullary segment and the adjacent air vesicle which have been classed as a "medullary unit" for diagnostic purposes. The medullary unit comprising the medullary segment and adjacent air vesicle will be found in most scalariform hair fragments, regardless of size. The structure of the medullary unit in conjunction with other characteristics makes possible the identification of many hair fragments. The fur hairs of the rat and mouse are very similar in structure and are considered together.

The diagnostic characteristics of the medullary unit are found between the internodes in the rat-mouse and muskrat fur hairs, and throughout most of the length of the rabbit and squirrel fur hairs. At the proximal or distal ends, or at the internodes, there may be a change in the medullary units, which limits the usefulness of the "medullary unit" structure as a criterion in identification of fragments from these regions. The air vesicle is not to be confused with the hyaline structure sometimes seen in the medullary segment. The hyaline structure will transmit light with the same intensity as the cortex, although diffraction may result in color

* Presented at the Sixty-eighth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 11–13, 1954, at Washington, D. C.

differences. The air vesicle appears in the photographs as a less dense area than the hyaline area and does not show any internal structure.

METHOD FOR MOUNTING HAIRS IN GLYCERINE JELLY

Place the hairs on a microscopic slide with a small piece of glycerine jelly. Place a cover glass over the jelly and warm the slide gently until the jelly melts. The air is thus at least partially removed from the hair. Examine microscopically. When necessary, remelt the glycerine jelly to remove the air completely. (Overnight standing will also result in the removal of the air.) To make the mount permanent, ring the cover slip with a plastic mounting or sealing compound to prevent bleeding of the glycerine from the jelly. Examine at 400–800× for the characteristics given below.

Reference is made to the terms "distal" and "proximal" sides in describing air vesicles. The free edges of the scales, which can be seen in glycerine jelly mounts, always point toward the distal end or tip of the hair.

DESCRIPTION OF AIR VESICLES IN GLYCERINE JELLY MOUNTS

(1) *Rat or mouse fur hairs.*—Air vesicles are roughly "I" shaped across the hair. The measurement of the air vesicles across is much greater than the distance along the hair length (Fig. 1).

(2) *Rabbit fur hair.*—The side on the distal end is concave. The side on the proximal end may be concave or almost straight. In some hairs, the longitudinal sides (in the direction of hair elongation) are slightly rounded or bulged. The measurement along the hair length is long, often the same as that of the medullary segment (Fig. 2).

(3) *Squirrel fur hair.*—The most pronounced feature is the sharply rounded side on the proximal end which gives the air vesicle a bowl shape. If pigment is present, the rounded end may be hidden, leaving varying amounts of the air vesicle which may then resemble the rat-mouse vesicle. The length of the parallel portion of the sides, along the direction of hair elongation, is usually less than the distal side and about equal to the "base of the cup" (Fig. 3).

(4) *Muskrat fur hair.*—The distal end of the air vesicle is concave with either one or two prongs which extend from one segment almost to the next and almost completely through the clear area of the medullary segment. The proximal side is straight or slightly concave and sometimes has short prongs (Fig. 4).

CONCLUSION

Glycerine mounts of some hairs found in food and drug products are an aid in their identification. The "medullary unit" is characteristic for each type of hair studied. The use of glycerine jelly permits detailed microscopic observation without the loss of the sample that accompanies use of sodium hydroxide or other swelling procedures.

REFERENCES

(1) *Microanalysis of Food & Drug Products*, U. S. Government Printing Office, Washington, D. C., 1944.

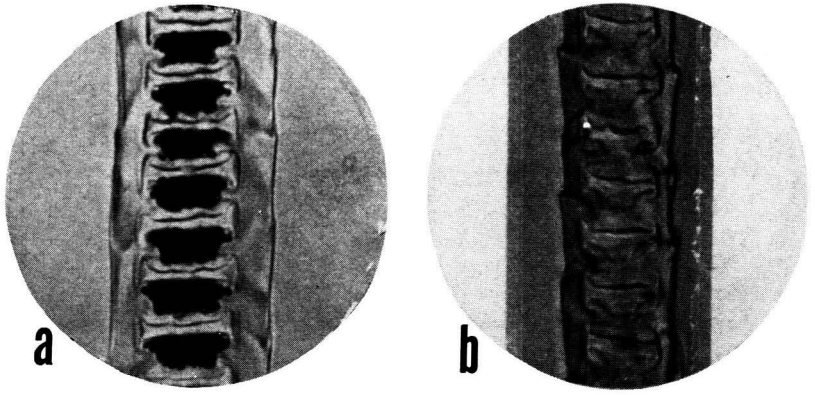


FIG. 1.—Rat fur hair. **a:** Pigmented; **b:** Unpigmented.

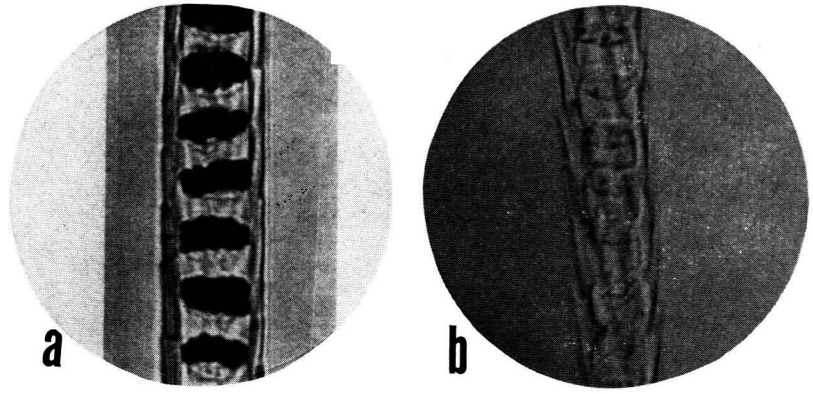


FIG. 2.—Rabbit fur hair. **a:** Pigmented; **b:** Unpigmented.

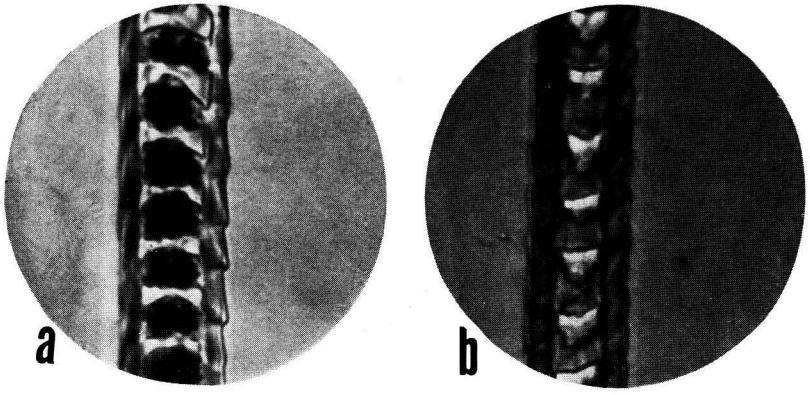


FIG. 3.—Squirrel fur hair. **a:** Pigmented; **b:** Unpigmented.

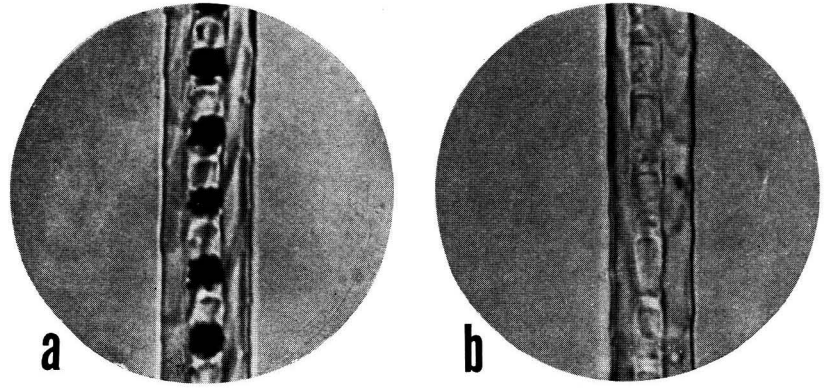


FIG. 4.—Muskrat fur hair. **a:** Pigmented; **b:** Unpigmented.

(2) BACHRACH, M., *Fur*, Prentice-Hall, New York, 1947.
(3) HAUSMAN, L. A., *Sci. Monthly*, **30**, 258 (1930).
(4) HARDY, J. I., *J. Textile Inst.*, **23**, 1 T (1932).
(5) STOVES, J. L., *Analyst*, **67**, 801 (1942).

A STUDY OF THE YEAST METHOD FOR VITAMIN B_6*

By WILLIAM P. PARRISH,† HENRY W. LOY, JR., and O. L. KLINE‡
(Division of Nutrition, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.)

It was concluded by this Administration in 1950 that the need for vitamin B_6 in human nutrition had been established. This judgment was based in part upon the studies of McHenry and associates (1), which revealed an effectiveness of the vitamin in the treatment of the nausea of pregnancy, the report from Holt's laboratory (2), in which it was shown that infants maintained on a diet deficient in vitamin B_6 developed specific symptoms that were relieved when the vitamin was administered, and that of Mueller and Vilter (3), in which it was observed that deficiency symptoms occurred in persons to whom pseudopyridoxine was administered. Under practical conditions of infant feeding, there has been a more recent, dramatic demonstration of the need for vitamin B_6. Cases of convulsions in infants which were reported late in 1952 and during the early months of 1953 were finally associated with a specific commercially available liquid infant formula. After a number of case reports had been reviewed, the Food and Drug Administration suggested that the infant convulsions were similar to those observed in rats suffering from a vitamin B_6 deficiency (4). Dr. C. D. May at the University of Iowa soon demonstrated the effectiveness of pyridoxine in treating the convulsion syndrome (5). This observation was followed by others which fully corroborated his findings. The infant formula in question has now been fortified with pyridoxine, and no additional cases of infant convulsions have been observed.

These developments have focused attention on the place of vitamin B_6 in nutrition and have emphasized the importance of accurate and specific methods for its determination. Chemical methods that have been in use for a number of years are adequate for pharmaceutical preparations, but are of questionable accuracy and specificity in application to lower potency materials such as food products. The microbiological method of assay described by Atkin, *et al.*, (6) in 1943, in which *S. carlsbergensis* A.T.C.C. No. 9080 is used as the test organism, has had no published

* Presented at the Sixty-eighth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 11–13, 1954, at Washington, D. C.
† Taken in part from work applicable to the degree of Doctor of Philosophy in the Department of Chemistry, Georgetown University, Washington, D. C.
‡ The authors wish to express their appreciation to Dr. M. X. Sullivan, Chemo-Medical Research Institute, Georgetown University, for his valuable aid and suggestions in this work.

critical evaluation since its appearance. Its general applicability and the equivalence of the three forms and their presumed stability have been widely accepted. Certain observations on these points have led us to a closer scrutiny of this method.

Originally applied to pyridoxine, the method was later used by Snell and Rannefeld (7) in the estimation of pyridoxal and pyridoxamine, then newly-recognized forms of the vitamin. They found great differences in the response of certain bacteria to the three forms. But with *S. carlsbergensis*, the ratio of response to pyridoxine of pyridoxal and pyridoxamine varied between 0.8 to 1.3 and 0.9 to 1.4, respectively. They therefore assumed approximately equal activity for this organism to the three forms of vitamin B_6. In preliminary work in this laboratory important differences in response among the three forms were observed, and for precise analysis a re-evaluation of this point was deemed necessary.

This paper sets forth certain modifications of the Atkin, *et al.*, yeast method that improve its sensitivity; it also defines certain details of procedure more closely.

EXPERIMENTAL AND RESULTS

The yeast method is similar in many respects to other microbiological assays. The sample is prepared by extraction and dilution. A fresh yeast inoculum is grown for use in seeding the assay tubes. The inoculated tubes are incubated for a specified period and the growth response is determined by measuring the turbidity in a suitable instrument. The steps of the assay were examined in order.

(A) *Preparation of Sample*

Vitamin B_6 occurs naturally as pyridoxine, pyridoxal, and pyridoxamine, together with the phosphorylated derivatives of each form. It has been reported that the yeast, *S. carlsbergensis*, cannot utilize the phosphorylated forms of the vitamin (8). Therefore, to obtain an accurate value for total vitamin B_6 content of natural materials, dephosphorylation must first be carried out. The literature is not in agreement as to the most satisfactory method of dephosphorylating the vitamin, and certain personal communications indicate that this is still an open question. Work is now being conducted to establish the effectiveness of acid hydrolysis and enzyme digestion in the dephosphorylation of vitamin B_6. Final verification of the vitamin B_6 content of some foods will depend upon a satisfactory solution of this problem. In the case of pharmaceutical preparations, where the phosphorylated forms do not occur, autoclaving with 0.06 *N* HCl at 121°–123° for 30 minutes is a satisfactory procedure for extracting the vitamin.

(B) *Preparation of Inoculum*

In any microbiological procedure the preparation of the inoculum is an important step. In the original method the inoculum was prepared by

growing the yeast on an agar slant and then transferring sufficient yeast cells to sterile saline to give a suspension of 1 mg of cells per ml. Finally this suspension was diluted 1:10 and 1 ml was used to seed each assay tube; this introduced 0.1 mg of moist yeast cells into each tube. The sensitivity of this assay may be expressed in terms of the concentration of the vitamin B_6 standard solution which was 10 millimicrograms per ml.

Rabinowitz and Snell (8) improved the sensitivity of the assay by diluting the yeast cell suspension 1:100 instead of 1:10, and by using the basal medium modification suggested by Hopkins and Pennington (9). Their standard solution contained 5 millimicrograms per ml.

On the basis of experience in this laboratory with *Lactobacilli* test organisms (10), we attempted to increase the sensitivity of the yeast culture by growing it in a liquid medium. A wire loopful of yeast cells from a malt agar slant was suspended in 10 ml of sterile inoculum broth prepared by diluting 5 ml of assay basal medium with 5 ml of water containing 10 millimicrograms of each of the three forms of vitamin B_6. This was incubated with constant shaking for 20 hours at 25°. The yeast cells were centrifuged and resuspended in 10 ml of suspension medium consisting of 5 ml of assay basal medium diluted with 5 ml of water. One drop (1/40 ml) of this inoculum, equivalent to 0.008 mg of moist yeast cells, was introduced into each tube. The concentration of the standard solution for a critical range of the response curve was 1.0 millimicrogram of vitamin B_6 per ml. The comparative responses obtained with these three procedures are shown in Fig. 1. Although there was an increase in sensitivity when a greater dilution of the inoculum was used, an even greater increase was obtained when the yeast was grown in an inoculum medium. This is in keeping with observations of *Lactobacilli*, where frequent transfer of the culture and the adaptation of the organism to the assay environment by growing the inoculum in the assay medium have greatly improved the growth response. Whether the lag phase of growth is reduced or whether selection of rapidly-growing yeast cells for the inoculum is responsible for improved rate of growth is not clear.

(C) *Basal Medium Sterilization*

When vitamin B_6 is heated in the presence of amino acids or sugars, its activity for certain test organisms is reduced (4, 11–14). In this laboratory reduced yeast response has been observed with the use of pyridoxal or pyridoxamine that had been heated with lactose or casein-hydrolysate. Since the basal medium used in this assay contains glucose and casein-hydrolysate, reduced response would be expected if the assay solution containing vitamin B_6 and basal medium were combined before sterilization. Therefore, separate sterilization of basal medium and assay solution is essential. This fact was pointed out by Snell and Rannefeld (7).

Furthermore, the time and temperature of the basal medium sterilization were found to be critical. The comparative response of the three forms

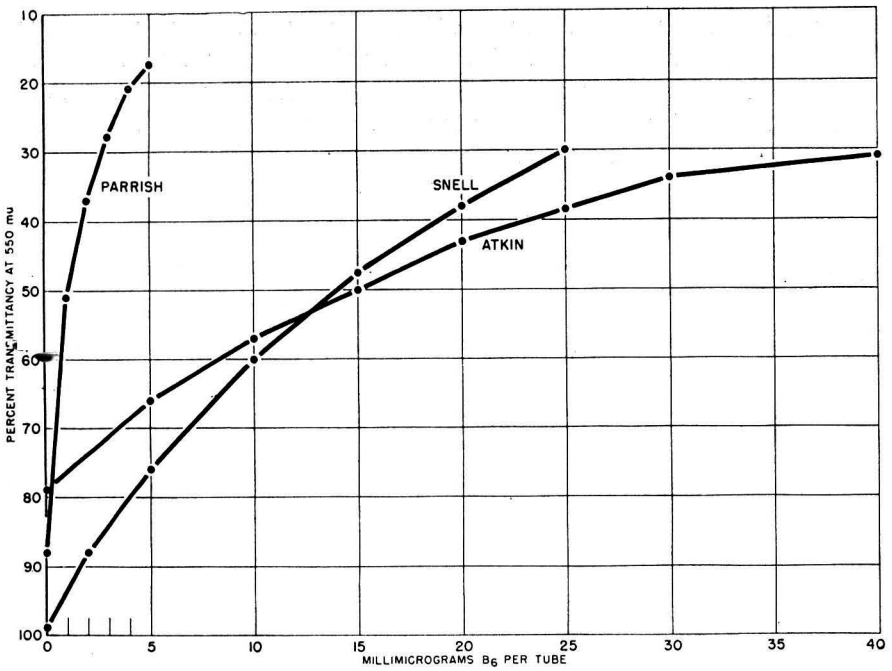


FIG. 1.—Effect of inocula on the growth response of *S. carsbergensis* to pyridoxine.

of the vitamin in basal media steamed at 100° for 10 minutes, or autoclaved at 121°–123° for 15 minutes was studied. Results are presented in Table 1. Not only were the responses to pyridoxal and pyridoxamine reduced, but that of pyridoxine was enhanced. In the medium steamed at 100° for 10 minutes the responses were those expected. In a later study it was observed that responses after autoclaving at 121° for 5 minutes were comparable to those when the medium was steamed at 100° for 10 minutes. Either of these two sterilization conditions is acceptable for the assay.

The variation caused by the more drastic conditions of heating is probably due to the combination of certain amino acids with glucose in the

TABLE 1.—*Effect of procedure for basal medium sterilization upon response to molecular equivalents of the 3 forms of vitamin* B_6[a]

VITAMIN B_6 FORM	STERILIZATION PROCEDURE	
	STEAMED AT 100° FOR 10 MINUTES	AUTOCLAVED AT 121° FOR 15 MINUTES
	per cent	*per cent*
Pyridoxine	100	131
Pyridoxal	99	88
Pyridoxamine	76	60

[a] Pyridoxine with steamed basal medium = 100% *S. carlsbergensis* assay after 24 hours' incubation at 25°.

medium in the browning reaction (15). Since many amino acids, such as L-histidine, L-valine, L-methionine, L-isoleucine, and others, have been shown to be vital for the adequate growth of *S. carlsbergensis* (16), their loss by combination with the sugar molecule induces inhibition. The stimulation of pyridoxine under these conditions is unexplained. However, it is feasible to believe that one or two amino acids found in casein-hydrolysate, acting individually or as a system, may induce yeast inhibition (17). When these amino acids are heated with sugar, they may become involved in the browning reaction, leaving the yeast free of their inhibiting effects.

(D) *Incubation Period*

(1) *Agitation during incubation.*—The rate of growth of yeast in the assay tubes is greatly stimulated by constantly agitating the tube contents throughout the incubation period. This agitation is ordinarily accomplished by means of a mechanical shaking device. The speed should be great enough to keep the yeast cells suspended. This effect can be augmented by inserting a glass bead into each assay tube prior to sterilization (18). By this procedure the new cells are kept constantly exposed to optimum nutrient conditions. Also, the added turbulence created by the movement of the bead aids in the removal of CO_2 which is antagonistic to fermentation. It was curious that in our comparisons, little difference was observed with pyridoxine, but as shown in Fig. 2, the growth with the

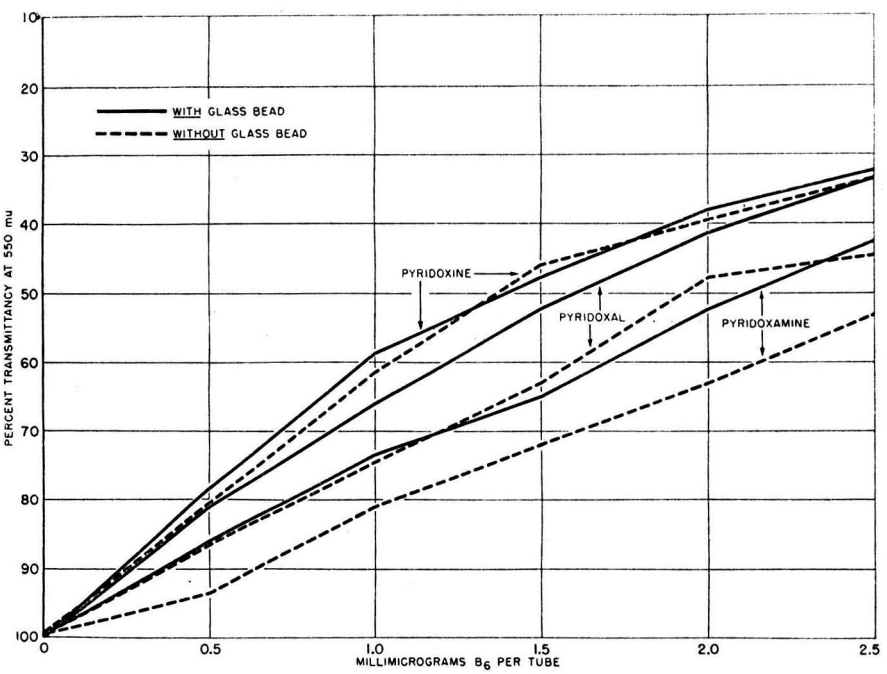


FIG. 2.—Effect of glass bead agitation on the growth response of *S. carlsbergensis* to vitamin B_6.

other two forms of the vitamin without the bead was only 70 to 75 per cent of that obtained when the glass bead was used.

(2) *Temperature of incubation.*—In their early work Atkin, Schultz, and Frey (19) designated 30°C. as the temperature of incubation, although there was no indication that the selection was based upon experimentation. Since *S. carlsbergensis* has been classified as a bottom yeast by Lindegren (20) who also stated that bottom yeast gives optimum response at 25–26°, it follows that use of this temperature should lead to optimum growth in an assay.

In this laboratory very little difference in response has been observed between 25° and 30°. However, it is known that if the temperature rises above 30°, yeast activity is impaired. If the temperature fluctuates above 30°, this reduced response will also be variable. Therefore, to establish a margin of safety and at the same time utilize the optimum temperature, $25° \pm 0.5°$ has been selected as a satisfactory temperature for incubation.

(3) *Optimum incubation time.*—In attempting to find conditions under which the yeast would respond equally to the three forms of vitamin B_6, the effect of various incubation periods ranging from 18 to 36 hours was studied (see Fig. 3).[1] From this study it is evident that after 22 hours of incubation, the responses to pyridoxine and pyridoxal are equal and maximum; this maximum response holds constant through a 28 hour incubation period. After this time, certain factors set in to disrupt the growth uniformity. With pyridoxamine, less activity is obtained for the yeast throughout the period studied. It is approximately 30 per cent lower than with pyridoxine and pyridoxal at 24 hours, but it approaches their maximum in 36 hours. However, in 36 hours the yeast response to pyridoxine and pyridoxal is erratic and stimulated. It is obvious that the yeast requires more time to utilize pyridoxamine than it does for the other two forms of the vitamin.

At a standard solution concentration of 1 millimicrogram per ml, and with the temperature at $25° \pm 0.5°$, the incubation time should not be less than 22 hours, nor more than 28 hours.

The response to be expected is that shown in Fig. 3 for the times specified. With this incubation time the responses of pyridoxine and pyridoxal are equal, but that of pyridoxamine is only 50 to 60 per cent of this value. Modifications that will allow increased response to pyridoxamine are the subject of further investigation.

After incubation is completed, the yeast cells should be inactivated to stop their growth. This is conveniently carried out by steaming at 100° for 5 minutes. Chilling the tubes for a time at low temperatures will not insure complete inactivation, since *S. carlsbergensis* can grow at 5° (20). It has been noted in this laboratory that agar slants of yeast cells show added growth after storage at 8° for 2 weeks.

[1] Owing to physical limitations this phase of the study was conducted in two experiments, 18 to 24 hours and 24 to 36 hours, respectively.

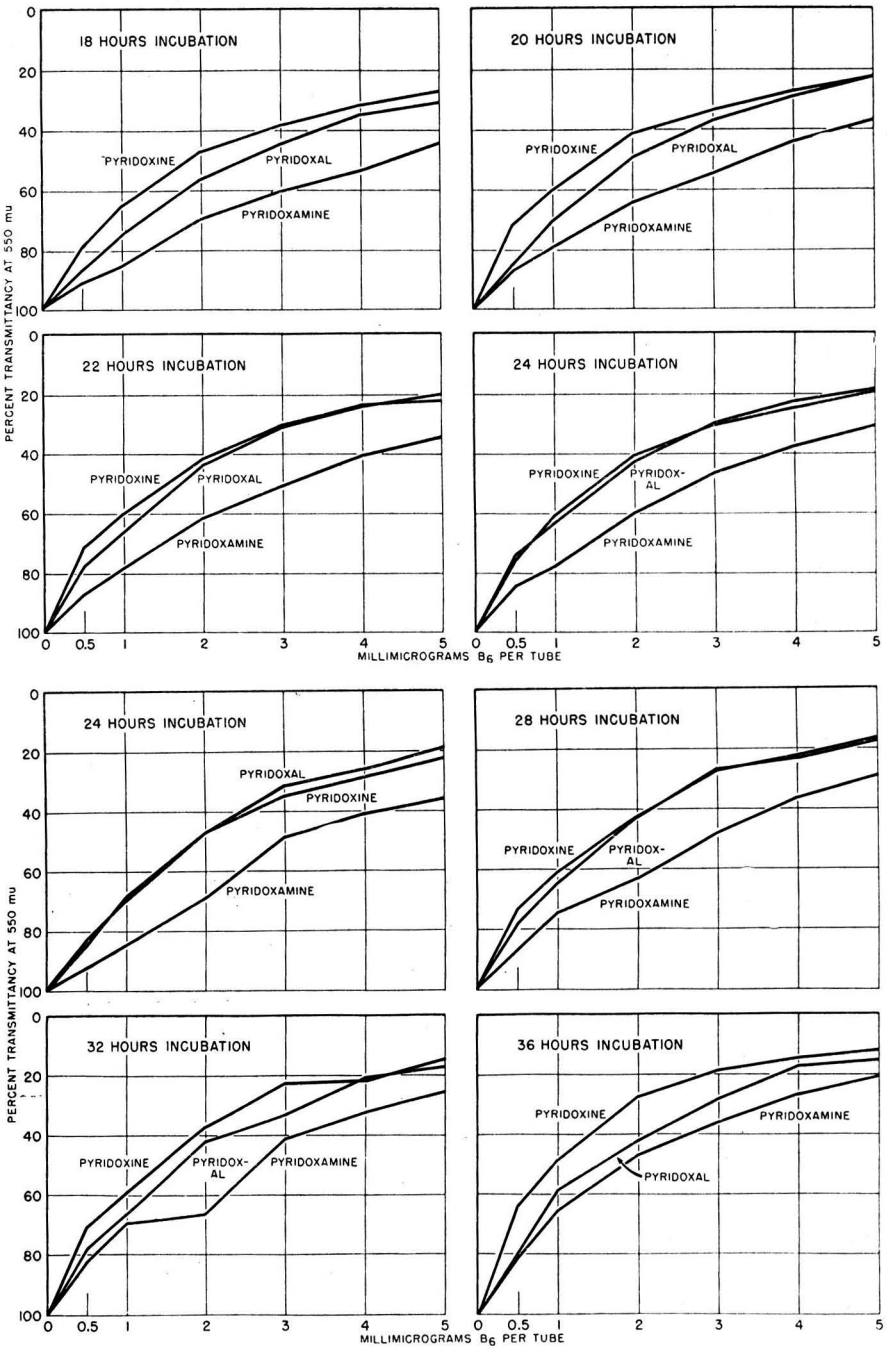


FIG. 3.—Effect of incubation time on growth response of *S. carlsbergensis* to three forms of vitamin B_6 (incubated at 25°).

SUMMARY

The *S. carlsbergensis* method of assay for vitamin B_6 has been studied and modified to give a more sensitive response.

The inoculum preparation has been changed to provide a more active organism. This is done by growing the inoculum in the assay medium, under the conditions of the assay, and by using a more dilute inoculum.

To assure accurate results, the assay solutions and the basal medium must be sterilized separately and then added together under aseptic conditions. This is necessary since both pyridoxal and pyridoxamine are highly reactive when heated with sugars and amino acids. The basal medium is sterilized by autoclaving for not more than 5 minutes at 121°, or by steaming at 100° for 10 minutes. As the products of the browning reaction increase, the response to pyridoxal and pyridoxamine decreases, while that to pyridoxine increases.

Uniformity of agitation during the incubation period was increased by placing a glass bead in each assay tube. With this change less variation between replicate tubes was observed. Best conditions for incubation were found to be a temperature of 25° and a period of 22 to 28 hours. Under these conditions the response of yeast to pyridoxal was equal to that of pyridoxine, but growth with pyridoxamine was approximately 50 to 60 per cent of that with pyridoxine.

REFERENCES

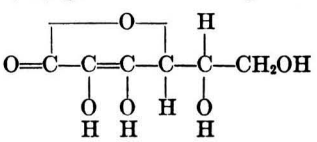
(1) McGanity, W. J., McHenry, E. W., Van Wyck, H. B., and Watt, G. L. *J. Biol. Chem.*, **178**, 511 (1949).
(2) Snyderman, S. E., Holt, L. E., Jr., Carretero, R., and Jacobs, K., *J. Clin. Nutrition*, **1**, 200 (1953).
(3) Mueller, J. F., and Vilter, R. W., *J. Clin. Invest.*, **29**, 193 (1950).
(4) Daniel, E. P., Kline, O. L., and Tolle, C. D., *J. Nutrition*, **23**, 205 (1942).
(5) May, C. D., *Proc. Inst. Med. (Chicago)*, **20**, 83 (1954).
(6) Atkin, L., Schultz, A. S., Williams, W. L., and Frey, C. N., *Ind. Eng. Chem.*, **15**, 141 (1943).
(7) Snell, E. E., and Rannefeld, A. N., *J. Biol. Chem.*, **157**, 475 (1945).
(8) Rabinowitz, J. C., and Snell, E. E., *Anal. Chem.*, **19**, 277 (1947).
(9) Hopkins, R. H., and Pennington, R. J., *Biochem. J.*, **41**, 110 (1947).
(10) Loy, H. W., Jr., Haggerty, J. F., and Kline, O. L., *This Journal*, **35**, 161 (1952).
(11) Friedman, L., unpublished data.
(12) Harris, S. A., Heyl, D., and Folkers, K., *J. Biol. Chem.*, **154**, 315 (1944).
(13) Rabinowitz, J. C., Mondy, N. I., and Snell, E. E., *ibid.*, **175**, 147 (1948).
(14) Snell, E. E., *ibid.*, **154**, 313 (1944).
(15) Friedman, L., and Kline, O. L., *ibid.*, **184**, 599 (1950).
(16) Parrish, W. P., in preparation for publication.
(17) Thorne, R. S. W., *J. Inst. Brewing*, **52**, 15 (1946).
(18) Rice, E. E., unpublished data, quoted in *Methods of Vitamin Assay*, 2nd Ed.. Interscience Publishers, Inc., New York, 1951, p. 225.
(19) Atkin, L., Schultz, A. S., and Frey, C. N., *Arch. Biochem.*, **1**, 9 (1942).
(20) Lindegren, C. C., *The Yeast Cell, Its Genetics and Cytology*, 1st Ed., Educational Publishers, Inc., St. Louis, Mo., 1949, Chapter 2.

A HIGHLY SPECIFIC PROCEDURE FOR ASCORBIC ACID*

By M. X. SULLIVAN and H. C. N. CLARKE† (Georgetown University, Washington, D. C.)

For the estimation of the antiscorbutic factor, ascorbic acid or vitamin C, in foodstuffs, tissues, etc., many methods have been employed, all of which depend on the strong reducing capacity of the vitamin. Ehrlich (1) in 1886 noted that some tissue would not reduce indophenol or alizarin blue, that other tissues would reduce the indophenol but not alizarin blue, while a few (lungs, liver, and mucosa of stomach) would reduce the difficultly reducible alizarin blue. In 1895 Smith (2) reported a peculiar hemorrhagic condition in guinea pigs that had been restricted to a cereal diet without grass, clover, or succulent vegetables. The importance of this observation was not appreciated until 1912 when Holst and Frölich (3) pointed out the similarity between this disease in guinea pigs and scurvy in man. By 1918 it was well recognized that scurvy was a deficiency disease (4–6). In 1919, Drummond (7) designated the antiscorbutic substance as "Vitamin C."

In 1928 Szent-Györgyi (8) isolated a reducing substance from the adrenal cortex and from various plant sources. He gave it the formula $C_6H_8O_6$ and thought he had a hexuronic acid. Then, independently, Waugh and King (9) isolated the crystalline antiscorbutic substance, and about the same time, and again independently, Svirbely and Szent-Györgyi (10) recognized the identity of the hexuronic acid with vitamin C. Haworth and Szent-Györgyi (11) named the substance ascorbic acid, and Herbert and associates (12) gave it the accepted formula:

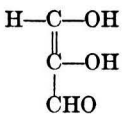


In 1911, while studying oxidation and reduction in plants and soils, Schreiner and Sullivan (13) came to the conclusion that the power of plants to reduce dyestuffs and other reducible compounds was due to unsaturated organic material. At that time some attention was given to Ehrlich's alizarin blue findings but with no satisfactory results. Some years later Nelson and Browne (14), following the procedure of Winter (15) on invert sugar, isolated a material of high reducing power from dextrose treated with calcium hydroxide; this material was first named glucic

* Presented at the Sixty-eighth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 11–13, 1954, Washington, D. C. Taken in part from the dissertation submitted by H. C. N. Clarke to the Department of Chemistry in partial fulfillment of the requirements for the degree of Master of Science in the Graduate School of Georgetown University. A report on this work was presented at the meeting of the American Society of Biological Chemists, Chicago, Illinois, April 1953.

† Present address: Herbert Reddy Memorial Hospital, Montreal, Canada.

acid by Dumas (16) and was subsequently named reductone by von Euler and Martius (17) who proved that it had the structure:



Since reductone, although inactive physiologically, reacts in practically every chemical test recommended for the determination of ascorbic acid (iodine titration, 2,6-dichloroindophenol, and phenylhydrazine) a procedure was sought in which reductone does not interfere significantly with the determination of ascorbic acid.

Such a procedure is presented here. It depends on the reduction of ferric chloride by ascorbic acid, the estimation of the ferrous iron by α,α'-dipyridyl, and inhibition of other reducers by the presence of orthophosphoric acid and a high degree of acidity (pH 1–2).

The use of α,α'-dipyridyl for the determination of ferrous iron was introduced by Hill (18). Albert and Gledhill (19) found α,α'-dipyridyl to be highly specific for ferrous iron.

In 1936 McFarlane (20) made a study of the relative capacities of tissue extracts to reduce ferric iron and 2,6-dichloroindophenol, respectively. He employed dipyridyl to determine the quantity of ferrous iron produced when ferric iron was reduced by the biological material, and he computed the amount of ascorbic acid required for the reduction. McFarlane demonstrated that the amount of ascorbic acid determined was always lower than that obtained by the dye titration. However, he did not consider the reductone which would be estimated by his procedure.

Von Euler and Martius found that reductone has a reducing capacity twice that of ascorbic acid as determined by 2,6-dichloroindophenol. Miller (21), by a formaldehyde procedure, found reductone present in all processed foods studied; the foods included preserved foods, honey, canned concentrates, and dehydrated foods. Rehberg and Lindberg (22) emphasized that part of the indophenol-reducing substances normally excreted originates from roasted matter in the diet.

In the early work on the differentiation of vitamin C from reductone, a reductone solution was made according to Kertesz (23) as follows: Five ml of a 0.5% glucose solution and 0.5 ml of normal NaOH were placed in a test tube closed by a Bunsen valve. The mixture was heated at 80°C. for 12 minutes and was then cooled and acidified with 1 ml of 10% HCl. According to Kertesz, 1 ml of the reaction mixture equals 0.25 mg of ascorbic acid in its action on 2,6-dichlorindophenol. It was tested in the dipyridyl reaction against ascorbic acid and other reducing compounds. Thus to 1 ml of each of the following substances were added 1 ml of 3% $FeCl_3$ and 5 ml of 0.1% α,α'-dipyridyl:

Ascorbic acid (0.05 g/100 ml H_2O)	immediate pink color
Glucoreductone solution	immediate pink color
Glutathione (0.1 g/100 ml H_2O)	no color in 30 minutes
Cysteine · HCl (0.1 g/100 ml H_2O)	no color in 30 minutes
Glyoxal (0.1% aqueous solution)	no color in 30 minutes
Methyl glyoxal (40% solution)	no color in 30 minutes
Acetol (50% solution in MeOH)	no color in 30 minutes
Uric acid (0.1 g/100 ml H_2O)	no color in 30 minutes
Creatinine (0.1 g/100 ml H_2O)	no color in 30 minutes

Since reductone was the only one of the reducing substances that gave an immediate reaction like ascorbic acid, attempts were made to eliminate the reductone. After some success with other relatively tedious procedures, we tried orthophosphoric acid (employed by Snell and Snell (24) to eliminate a number of interfering substances in the determination of iron by means of α,α'-dipyridyl). When a sufficient amount of orthophosphoric acid was used, it entirely inhibited reductone, and though it retarded the action of ascorbic acid somewhat, a very sensitive reaction for ascorbic acid was still obtained. Neither glucosone (25) nor reductic acid (26) gave a positive test by this procedure. For the reductone work we used a lead salt prepared according to von Euler and Martius (17), since the reductone lead salt was quite stable. Reductone without phosphoric acid was strongly positive but it was negative in the presence of the phosphoric acid. These conclusions are exemplified in the following experiment in which 1 ml of 20% $FeCl_3$, freshly prepared, and 5 ml of 0.5% α,α'-dipyridyl were added to each of the solutions shown in Table 1. It is noted that phosphoric acid entirely eliminates the interference of reductone.

TABLE 1.—*Effect of orthophosphoric acid*

SOLUTIONS TESTED	COLORIMETRIC READINGS (KLETT-SUMMERSON)	
	AFTER 60 MIN.	AFTER 90 MIN.
1.0 ml H_2O +0.3 ml H_3PO_4[a]	27	27
1.0 ml reductone lead salt (25 mg/100 ml H_2O; no H_3PO_4)	600	605
1.0 ml reductone lead salt (25 mg/100 ml H_2O) +0.3 ml H_3PO_4	25	26
1.0 ml ascorbic acid solution (0.125 mg) +0.3 ml H_3PO_4	219	232
1.0 ml of mixture of 1.0 ml reductone lead salt (50 mg/100 ml) +1 ml ascorbic acid solution (0.250 mg) +0.3 ml H_3PO_4	218	230

[a] 85 per cent orthophosphoric acid used in all cases.

EXPERIMENTAL

REAGENTS

(a) *Distilled water.*—H_2O (and glassware) must be free from oxidizing or reducing agents. Redistil H_2O twice out of glass.

(b) *Orthophosphoric acid.*—85% (Merck's reagent grade or equivalent). Add 0.3 ml to test solutions to give a *p*H of 1–2.

(c) *Ferric chloride.*—1% and 20% aq. solns. Use for not longer than 1 day.

(d) *α,α'-Dipyridyl.*—Weigh 0.5 g into 50 ml H_2O in a 100 ml vol. flask. Shake while warming under a stream of hot H_2O. When the dipyridyl is dissolved, dil. to 100 ml.

(e) *Ascorbic acid standard solution.*—Prep. fresh at each determination (can be kept in refrigerator until used). The concn of the standard should be as close as possible to that of the soln being tested. For urine, dissolve 20 mg ascorbic acid in 100 ml H_2O. Dil. 10 ml of this soln to 100 ml with H_2O in a 100 ml vol. flask.

GENERAL PROCEDURE

The best procedure is as follows: To 1 ml of a soln contg ca 0.02 mg ascorbic acid are added 0.3 ml 85% H_3PO_4 to pH 1–2, 5 ml 0.5% aq. α,α'-dipyridyl, and 1 ml 1% $FeCl_3$.

APPLICATIONS

Application to urine.—A part-day sample of freshly voided urine was used. The indophenol titration gave a value of 1.369 mg ascorbic acid per 100 ml. For comparison with the dipyridyl method the following experiment was arranged (Table 2) in which 0.3 ml 85% H_3PO_4, 5 ml 5% α,α'-dipyridyl, and 1.0 ml 20% $FeCl_3$ were added to each of the solutions *except* the blanks. The α,α'-dipyridyl method gives a slightly lower value than does the indophenol titration. Added ascorbic acid is recovered quantitatively.

TABLE 2.—*Application to urine*

SOLUTIONS TESTED	READING (15 MIN.)	READING (CORR.)	ASCORBIC ACID (MG/100 ML)
1 ml urine+7.3 ml H_2O	3	0	—
1 ml urine+1 ml ascorbic acid solution (0.01 mg) +6.3 ml H_2O	3	0	—
2 ml H_2O	6	0	—
1 ml ascorbic acid solution (0.01 mg)+1 ml H_2O	52	46	1.00
1 ml urine+1 ml H_2O	69	60	1.304
1 ml urine+1 ml ascorbic acid solution (0.01 mg)	116	107	2.326

Application to orange juice.—The dipyridyl method was applied satisfactorily to canned orange juice that had been diluted 10 to 20 times, mixed well, and centrifuged. The method was modified by using 1% $FeCl_3$ in place of the higher concentration of the ferric salt. A sample of orange juice gave a value of 33.8 mg per 100 ml undiluted juice by the indophenol method and 33.16 mg per 100 ml by the dipyridyl method. Later, fresher samples gave higher values.

Application to honey.—Ten ml of glass-packed honey was diluted with an equal volume of water. The indophenol titration gave an average value of 1.35 mg ascorbic acid per 100 ml. The α,α'-dipyridyl procedure gave a value of 0.774. If the difference is accepted as due to reductone-like material, the present work verifies the statement of Miller (21) that honey contains reductone.

Application to tissues.—No application has yet been made to tissues, since a deproteinizing agent that would not cause interference would have to be found.

A later paper will cover this phase of the problem; another article will compare the α,α'-dipyridyl procedure to iodine titration and indophenol, and possibly will describe a direct estimation of reductone.

SUMMARY

A highly specific test for ascorbic acid has been developed that depends on the reduction of ferric chloride in the presence of orthophosphoric acid at *p*H 1–2 and estimation of the ferrous iron by α,α'-dipyridyl. In the presence of phosphoric acid and at this *p*H, reductone, glucosone, reductic acid, and α-tocopherol do not interfere, nor do glutathione, cysteine, acetol, methyl glyoxal, or creatinine. To date the test has been applied to orange juice, honey, and urine. The test is sensitive to 5 micrograms of ascorbic acid per ml.

REFERENCES

(1) EHRLICH, P., *Das Sauerstoffbedürfniss des Organismus*, Berlin, 1885.
(2) SMITH, T., *U. S. Dept. Agr. Bureau Animal Industry*, Annual Report, p. 172, 1895–1896.
(3) HOLST, A., and FRÖLICH, T., *Z. Hyg.*, **72**, 1 (1912).
(4) CHICK, H., and HUME, E. M., *J. Soc. Trop. Med. Hyg.*, **10**, 141 (1917).
(5) COHEN, B., and MENDEL, L. B., *J. Biol. Chem.*, **35**, 425 (1918).
(6) HARDEN, A., and ZILVA, S. S., *Biochem J.*, **12**, 408 (1918).
(7) DRUMMOND, J. C., *ibid.*, **13**, 77 (1919).
(8) SZENT-GYÖRGYI, A., *ibid.*, **22**, 1387 (1928).
(9) WAUGH, W. A., and KING, C. G., *J. Biol. Chem.*, **97**, 325 (1932).
(10) SVIRBELY, J. L., and SZENT-GYÖRGYI, A., *Biochem. J.*, **26**, 865 (1932).
(11) HAWORTH, W. V., and SZENT-GYÖRGYI, A., *Nature*, **131**, 24 (1933).
(12) HERBERT, R. W., HIRST, E. L., PERCIVAL, E. G. V., REYNOLDS, R. J. W., and SMITH, F., *J. Chem. Soc. (London)*, **1270, 1419, 1564** (1933).
(13) SCHREINER, O., and SULLIVAN, M. X., *Botan. Gaz.*, **51**, 121, 273 (1911).
(14) NELSON, E. K., and BROWNE, C. A., *J. Am. Chem. Soc.*, **51**, 830 (1929).
(15) WINTER, H., *Z. Rübenzucker Ind.*, **44**, 1049 (1894).
(16) Cited by PELLIGOT, E., *Ann.*, **30**, 76 (1839).
(17) VON EULER, H., and MARTIUS, E., *ibid.*, **505**, 73 (1933).
(18) HILL, R., *Proc. Roy. Soc. London*, **B. 167**, 205 (1930).
(19) ALBERT, A., and GLEDHILL, W. S., *Biochem. J.*, **41**, 529 (1947).
(20) MCFARLANE, W. D., *ibid.*, **30**, 1473 (1936).
(21) MILLER, M. C., *Food Research*, **12**, 343 (1947).
(22) REHBERG, P. B., and LINDBERG, A. L., *Acta Med. Scand.*, **128**, Supplement 196, 674 (1947).
(23) KERTESZ, Z. I., *J. Biol. Chem.*, **104**, 485 (1934).
(24) SNELL, F. D., and SNELL, C. T., *Colorimetric Methods of Analysis*, 3d Ed., D. Van Nostrand Co., New York, 1949, p. 317.
(25) DIXON, K. C., and HARRISON, K., *Biochem. J.*, **26**, 465 (1942).
(26) UGAMI, H., *J. Agr. Chem. Soc. Japan*, **10**, 727 (1934).

SOME OBSERVATIONS ON THE COLORIMETRIC DETERMINATION OF VANILLIN

By DUANE T. ENGLIS, JAMES W. MILES, and LOUIS A. WOLLERMAN
(Department of Chemistry and Chemical Engineering and Department of Food Technology, University of Illinois, Urbana, Ill.)

The Folin-Denis method (5) for the colorimetric determination of vanillin in flavoring extracts, which involves treatment with a phosphotungstate-phosphomolybdate reagent in acid solution followed by the development of the color upon addition of sodium carbonate, was official for a number of years. Snell (8) called attention to the fact that the troublesome precipitate which formed on addition of saturated sodium carbonate could be lessened by substitution of a 20 per cent solution of the reagent. Wilson (9) adopted the recommendation of Snell, and simplified the procedure by use of a color filter and wedge photometer (in place of a Duboscq instrument) for evaluation of the color.

More recently Ensminger (2) has given additional attention to the Wilson procedure and has made further slight modifications in the quantity of sample taken, the amount of lead clarification reagent used, and the degree of dilution employed. Very satisfactory results were obtained in collaborative studies (3, 4) and the revised procedure was made official (6).

A few years ago an effort was made in this laboratory (7) to find some material which would develop the color and not give rise to the formation of a precipitate, and several other basic reagents were tried in place of sodium carbonate. When sodium cyanide was used, a deep blue color was obtained and no precipitate formed, but the color was very unstable. Sodium borate gave a very slow development of the color, as did trisodium phosphate. In the latter case a period of about twenty-four hours at room temperature was required for the reaction. By heating in a boiling water bath, maximum color was produced in 10–15 minutes. However, when the procedure was applied to genuine and imitation extracts, higher vanillin values were indicated than those found by the early Wilson method. It was subsequently discovered that under the new conditions the reagent was strongly reduced by levulose, although not by dextrose (1). The high results for vanillin were attributed to the presence of small amounts of invert sugar in the extracts.

It has been postulated by earlier workers that the white precipitate which forms on addition of sodium carbonate in the Folin-Denis procedure is sodium phosphate, but a consideration of the solubilities of the various sodium salts of phosphoric acid makes this assumption seem questionable. Although the precipitate may form whether lead acetate has been used as

a clarification reagent or not, it was the belief of the present authors that an additional quantity of precipitate might result from the formation of lead carbonate or phosphate when lead reagent has been used. This belief made it seem probable that sodium hexametaphosphate might exercise some sequestering action and lessen the precipitation tendency. To investigate this possibility, four solutions were prepared containing 1, 5, 10, and 20 per cent of sodium hexametaphosphate. A five ml portion of each of these solutions was added to vanillin solutions just before the addition of the Folin-Denis reagent, and was followed with 10 ml of 20 per cent sodium carbonate. Each mixture was finally made up to a volume of 100 ml with distilled water. A heavy precipitate was observed in the flask containing the least sodium metaphosphate, a slight precipitate in the next lowest, and no precipitate in the two containing the largest amounts of the metaphosphate.

Because 5 ml of the 10 per cent sodium metaphosphate appeared adequate to prevent precipitation in the Wilson procedure, this quantity was adopted for use in later experiments. Standard curves for vanillin were prepared following the early Wilson procedure (9) and also for the modified technique involving the use of metaphosphate. The curves obtained were very similar, but the modified procedure gave absorbance values slightly higher than those observed for the Wilson method. A few analyses of vanilla extracts gave results which were in good agreement for each procedure, but in general, they were slightly higher by the metaphosphate modification. The work was temporarily discontinued but in view of renewed interest in the colorimetric procedure additional work of this nature was undertaken.

EXPERIMENTAL

Although Wilson had found that 10 ml of 20 per cent sodium carbonate was adequate for the development of the color when 5 ml of the acid Folin-Denis reagent was employed, there is no statement as to whether a still further reduction of the amount of the carbonate might be advantageous. This point was the subject of some preliminary tests. Following the general photometric method (6), a 5 ml portion of standard vanillin (0.1 mg per ml) was introduced into each of several 100 ml flasks and 5 ml portions of the Folin-Denis reagent were added to each. After the solutions had stood 5 minutes, the quantities of 20 per cent sodium carbonate and water indicated in Table 1 were added; the solutions were mixed thoroughly, and were examined after 10 mintues with a Lumetron Model 400-A photoelectric colorimeter equipped with a red filter which transmitted a wave band with mean wavelength at 650 mμ. Water was employed as the comparison solution, and when precipitates were present the solutions were filtered before examination. The results are given in Table 1.

TABLE 1.—*Effect of quantity of sodium carbonate upon precipitation tendency and absorbance values in the Folin-Denis procedure*

FLASK[a]	WATER	20% SODIUM CARBONATE	ABSORBANCE ×10	TURBIDITY
	ml	*ml*		
1	5	5	3.28	Clear
2	4	6	(3.65?)	Clear
3	3	7	3.28	Turbid
4	2	8	2.92	Turbid
5	1	9	2.61	Turbid
6	0	10	2.65	Turbid

[a] 5 ml of standard vanillin solution (0.1 mg per ml) plus 5 ml of Folin-Denis reagent were used in each flask.

Because of freedom from immediate precipitation, an advantage in further reduction of the amount of sodium carbonate is shown, since flasks 1 and 2, containing respectively 1 and 1.2 g of sodium carbonate per 100 ml at the final dilution, were clear. All others contained precipitates, the amount increasing slightly with increase of the reagent. Even after filtering, flasks 3 to 6 gave a slight cloudiness. On long standing, flasks 1 and 2 also began to be cloudy.

No disadvantage results from the use of the lesser amount of sodium carbonate; in fact, slightly higher color values are usually observed. Slightly lower color values in the higher concentrations might be assigned to greater fading or loss by adsorption on the paper in the filtration process. The inconvenience of filtration along with possible influence of the paper makes an elimination of the filtration step very desirable.

These general observations concerning precipitate formation directed attention to the previous work (7) in which sodium hexametaphosphate had offered promise of relieving certain difficulties. Parallel tests were made in which samples of standard vanillin were put through the clarification operations with lead acetate solution and then filtered, and aliquots of the filtrates were treated with Folin-Denis reagent. In one series the reagents and procedure were exactly as prescribed in the recent paper (6). In a second series, operations were identical except that 5 ml of 10 per cent sodium hexametaphosphate was added just before the Folin-Denis reagent. The absorbances were then measured with the Lumetron instrument.

In the first series the solutions were filtered as prescribed. No filtration was necessary in the modified method. The results are given in Table 2 and from these data working curves were drawn. It was again noted that the modified method gave slightly higher absorbances throughout the range. This might be assigned to a slight difference in the action of the reagent because of the presence of the metaphosphate or to a slight loss in color in the filtration operation of the first method. However, each

TABLE 2.—*Absorbances of standard vanillin solutions by modified Folin-Denis photometric method*

STANDARD VANILLIN	ABSORBANCE×10	
	OFFICIAL PROCEDURE	HEXAMETAPHOSPHATE MODIFICATION
(*1 ml=1 mg*)		
2.5	0.97	1.14
5.0	1.64	1.84
7.5	2.43	2.53
12.5	3.61	—
20.0	4.85	5.05

working curve should be satisfactory for an analysis by the respective procedure.

A word of caution concerning sodium metaphosphate might be added. This product may vary in composition and properties, and should be given a preliminary test for satisfactory performance. The salt hydrolyzes slowly on standing so that it may eventually lose its sequestering properties; however, a stock solution was used over a period of several weeks without loss of its ability to prevent precipitation.

To determine whether or not the basicity of the final solution would be altered by the use of the lead clarifying reagent and the presence of the metaphosphate, a blank run was made. A two ml portion of lead reagent was added to 80 ml of water and diluted to volume in a 250 ml flask. The solution was allowed to stand a short time and then was filtered, and three 10 ml portions of the filtrate were placed into 100 ml flasks. These were handled as follows:

	Sample 1	*Sample 2*	*Sample 3*
Ml lead filtrate	10	10	10
Ml 10% sodium metaphosphate	5	—	—
Ml Folin-Denis reagent	5	5	5
	(allowed to stand 5 minutes)		
20% Na_2CO_3	10	10	5
	(allowed to stand 10 minutes and diluted to 100 ml; *p*H observed with a *p*H meter)		
*p*H	9.8	9.7	9
Condition	clear	quite turbid	slightly turbid

It is definite that when the amount of sodium carbonate is kept constant the metaphosphate has only a very slight effect on the basicity of the final solution. Addition of only half the usual quantity of carbonate lowers the *p*H slightly but markedly diminishes the precipitation tendency when metaphosphate is absent. After 24 hours, Sample 1 was still clear, but both Samples 2 and 3 had moderately heavy precipitates. Since the

metaphosphate is effective at the higher carbonate concentration, this quantity can be retained for the procedure and the method will be less subject to difficulties in case the acid concentration of the Folin-Denis reagent varies slightly.

Some suggestions might be made as to possible changes in details of the official procedure (6). Since 2 ml is a rather small volume of extract to measure accurately, it may be desirable to increase this volume to 5 ml and dilute to 500 ml instead of 250 ml. Furthermore, if vanillin tends to be carried down by the lead precipitate when present in concentrated solution, one could dilute more nearly to final volume 250 ml (rather than 80 ml) before the lead reagent is introduced.

It is known that vanillic acid has a stronger reducing action on the Folin-Denis reagent than the vanillin from which it is derived. Information as to whether or not it is removed by the lead reagent does not seem to be available. If it is not, this substance along with levulose may tend to give high results by the modified Folin-Denis methods.

Work in which results by the colorimetric and other procedures will be compared is in progress.

SUMMARY

Reduction in the amount of sodium carbonate beyond the quantity specified in the recently recommended modified Folin-Denis method lessens the tendency toward formation of an objectionable precipitate without any disadvantage in regard to color development.

The precipitation tendency can be entirely eliminated by introduction of a small amount of sodium hexametaphosphate when the usual quantity of sodium carbonate is employed. No significant change in final *p*H results from introduction of the metaphosphate. Elimination of the precipitate and necessity for the subsequent filtration step enables an analyst to regulate the time intervals for the various operations of the more or less empirical procedures with greater exactness. These factors contribute a great deal to the convenience and precision of the process.

REFERENCES

(1) ENGLIS, D. T., and MILES, J. W., *Anal. Chem.*, **21**, 583 (1949).
(2) ENSMINGER, L. G., *This Journal*, **34**, 330 (1951).
(3) ———, *ibid.*, **35**, 271 (1952).
(4) ———, *ibid.*, **36**, 679 (1953).
(5) FOLIN, O., and DENIS, W., *Ind. Eng. Chem.*, **4**, 670 (1912).
(6) *Report of Subcommittee D*, and *Changes in Methods of Analysis, This Journal*, **36**, 63 (1953); *ibid.*, **35**, 77 (1952); *ibid.*, **34**, 72 (1951).
(7) MILES, J. W., *Determination of Vanillin in Flavoring Extracts.* M.S. Thesis, University of Illinois, (1947).
(8) SNELL, F. P., and SNELL, CORNELIA T., *Colorimetric Methods of Analysis*, Vol. II, D. Van Nostrand, New York, 1937, p. 87.
(9) WILSON, J. B., *This Journal*, **25**, 155 (1942).

ANALYSIS OF NAIL LACQUERS*

By S. H. NEWBURGER (Division of Cosmetics, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.)

Several years ago the author attempted to develop a scheme of analysis for nail lacquers[1] but was handicapped by inability to identify all of the component materials. At best, inadequate chemical tests were used to guess the composition of the lacquer. Recently, infrared and ultraviolet spectrophotometry supplied the key necessary for the identification of the complex materials found in nail lacquers.

This investigation concerns the non-volatile ingredients commonly found in colorless nail lacquers, *viz.*, nitrocellulose, *n*-butyl phthalate, and aryl sulfonamide-formaldehyde resin. It is possible to separate the nitrocellulose from the plasticizer and resin by benzene precipitation; the butyl phthalate is separated from the resin by a petroleum ether extraction from 80 per cent methanol. The materials are then identified by their infrared and ultraviolet spectra.

EXPERIMENTAL

A number of mixtures containing nitrocellulose, *n*-butyl phthalate, and aryl sulfonamide-formaldehyde resin, as well as the solvents toluene and *n*-butyl acetate, were analyzed by the proposed procedure.

The following materials were used in experimental samples: *n*-Butyl phthalate (b.p. 203–206°C., 20 mm); nitrocellulose (approximately ½ sec.); aryl sulfonamide-formaldehyde resin;[2] toluene (b.p. 110–111°C.); and *n*-butyl acetate (b.p. 123–126°C.).

The analytical results are presented in Tables 1 and 2. Both tables refer to the same set of experiments. Table 1 gives the data for the separation of the nitrocellulose from the resin and plasticizer; Table 2 details the end results of the analyses. For brevity the following symbols are used in the tables: NC for nitrocellulose; BP for *n*-butyl phthalate; and ASF for aryl sulfonamide-formaldehyde resin.

The infrared spectra of the nitrocellulose, resin, and plasticizer are presented in Figs. 1, 2, and 3.

The ultraviolet spectra for butyl phthalate and aryl sulfonamide-formaldehyde resin are given in Figs. 4 and 5. The phthalate at 276 mμ and the resin at 269 mμ both obey Beer's law to within ±1% in the concentration range 100–300 mg/l in alcohol.

---

* Presented at the Sixty-eighth Annual Meeting of the Association of Official Agricultural Chemists, held Oct. 11–13, 1954, at Washington, D. C.

[1] NEWBURGER, S. H., *Assoc. Food and Drug Officials U. S.*, XV, No. 2, 42 (April 1951).

[2] Sold under the trade name of "Santolite M-H-P" by the Monsanto Chemical Company, St. Louis, Mo.

TABLE 1.—*Separation of nitrocellulose from resin and plasticizer*

EXPERIMENT NO.	SAMPLE COMPOSITION[a]		RECOVERY IN GRAMS					
			RESIN AND PLASTICIZER			NITROCELLULOSE		
			1ST FRACTION	2ND FRACTION	TOTAL	PPTED	RESIDUE ON FILTER PAPER	TOTAL
		grams						
1	BP	0.191	0.365	0.048	0.413	0.588	0.018	0.606
	ASF	0.247						
		0.438						
	NC	0.595						
2	BP	0.285	0.454	0.056	0.510	0.485	0.038	0.523
	ASF	0.251						
		0.536						
	NC	0.509						
3	BP	0.252	0.389	0.048	0.437	0.539	0.026	0.565
	ASF	0.210						
		0.462						
	NC	0.552						
4	BP	——	0.266	0.046	0.312	0.608	0.022	0.630
	ASF	0.328						
		0.328						
	NC	0.611						
5	BP	0.251	0.224	0.026	0.250	0.612	0.018	0.630
	ASF	——						
		0.251						
	NC	0.642						
6	BP	——	0.003	0.002	0.005	0.668	0.013	0.681
	ASF	——						
	NC	0.697						

[a] Each sample also contained 2 ml toluene and 2 ml *n*-butyl acetate.

One of the commercial colorless nail lacquers contained tricresyl phosphate and a phthalate as the plasticizers. A sample containing the organic phosphate was prepared and analyzed according to the proposed procedure. The phosphate was found in the petroleum ether fraction where butyl phthalate is extracted. The tricresyl phosphate used was a technical grade containing 80 per cent of the *para* and 20 per cent of the *meta* isomers. For the analytical data see Table 3. The initials TCP were used to designate the tricresyl phosphate.

TABLE 2.—*Analysis of colorless nail lacquers*

EXPERIMENT NO.	SAMPLE COMPOSITION[a]		RECOVERY	
		grams	*grams*	*per cent*
1	NC	0.595	0.606	102
	BP	0.191	0.190	99.5
	ASF	0.247	0.214	86.6
2	NC	0.509	0.523	103
	BP	0.285	0.281	98.6
	ASF	0.251	0.212	84.5
3	NC	0.552	0.565	102
	BP	0.252	0.244	96.8
	ASF	0.210	0.180	85.7
4	NC	0.611	0.630	103
	ASF	0.328	0.286	87.2
5	NC	0.642	0.630	98.1
	BP	0.251	0.240	95.6
6	NC	0.697	0.681	97.7

[a] Each sample contained 2 ml toluene and 2 ml *n*-butyl acetate.

The infrared spectrum of the organic phosphate is shown in Fig. 6 and the ultraviolet spectrum in Fig. 7. In alcohol solution at 265 mμ it obeys Beer's law to within ±1% in the concentration range 100 to 300 mg/l.

Figure 8 shows the infrared spectrum of the plasticizer fraction obtained from the commercial nail lacquer containing tricresyl phosphate.

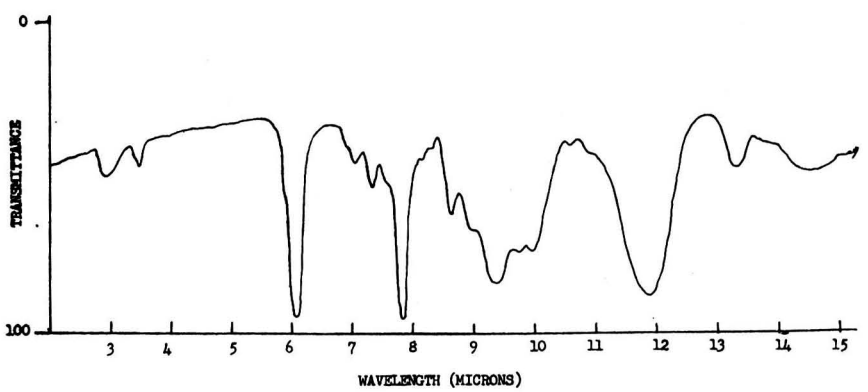


FIG. 1.—Nitrocellulose. Thin film on NaCl crystal.

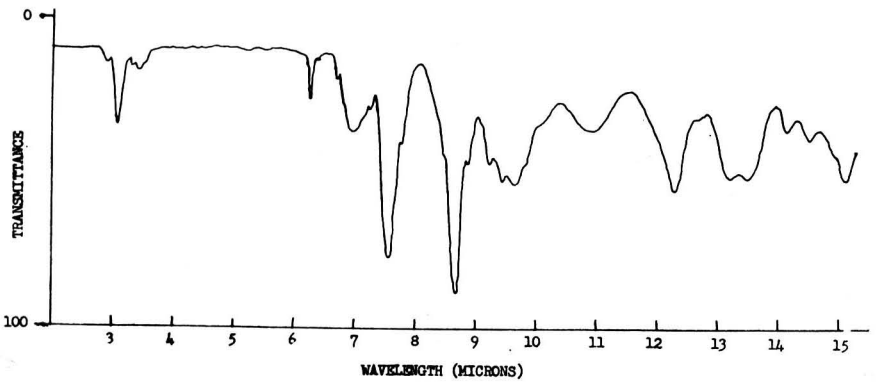


FIG. 2.—Aryl sulfonamide-formaldehyde resin. Thin film on NaCl crystal.

PROCEDURE

(See Chart 1, *Diagramatic Scheme of Colorless Nail Lacquer Analysis*)

A.—Dil. a 4–6 g sample with 5 ml acetone, add 20 ml benzene, and pour slowly with stirring into a 400 ml beaker contg 150 ml hot benzene. Rinse sample container with 5 ml acetone and add rinsings to the benzene. Evap. benzene on the steam bath under gentle jet of air to ca 80 ml, dil. with 90 ml benzene, and cool to room temp. Pour mixt. into centrifuge tube, rinse pptn beaker with 10 ml benzene, and add rinsing to centrifuge tube. Reserve pptn beaker and any ppt that adhered to the sides. After centrifuging, decant supernatant liquid into a 250 ml beaker labeled 1 and set it aside.

B.—Dissolve the residue in the pptn beaker and centrifuge tube (*A*) with 15, 10, and 10 ml portions of acetone, and pour the combined acetone solns into a 100 ml beaker. Evap. the acetone on steam bath, redissolve residue in 10 ml acetone, add 20 ml benzene, and repeat the pptn and centrifuging procedure described in *A*. Decant the supernatant liquid into a beaker labeled 2. Reserve the pptn beaker and centrifuge tube contg the residues.

C.—Filter the decanted liquids in beakers 1 (*A*) and 2 (*B*) thru the same 12.5 cm S&S No. 597 filter paper into 2 tared beakers labeled I and II. Reserve the filter paper and beakers 1 and 2.

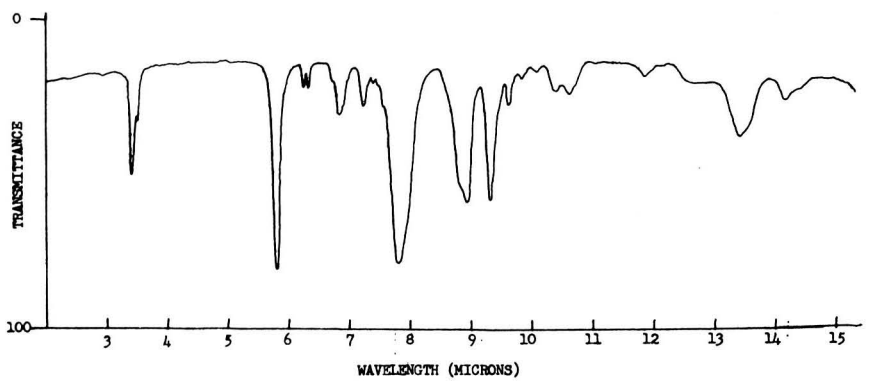


FIG. 3.—*n*-Butyl phthalate. Thin liquid film on NaCl crystal.

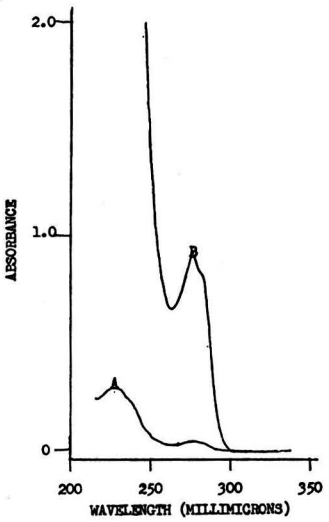


FIG. 4.—*n*-Butyl phthalate. *Curve A:* 10 mg/l in 95% alcohol. *Curve B:* 200 mg/l in 95% alcohol. Cell length, 1 cm.

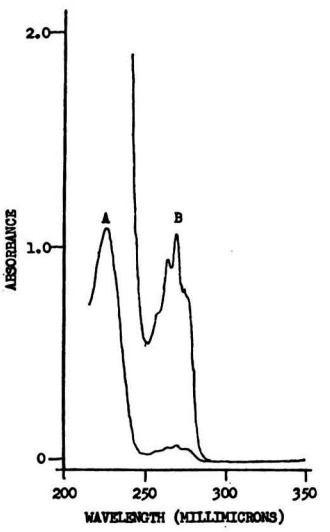


FIG. 5.—Aryl sulfonamide-formaldehyde resin. *Curve A:* 20 mg/l in 95% alcohol. *Curve B:* 300 mg/l in 95% alcohol. Cell length, 1 cm.

TABLE 3.—*Analysis of a colorless nail lacquer containing TCP*

SAMPLE COMPOSITION		RECOVERY	
	gram	*gram*	*per cent*
NC	0.663	0.669	101
TCP	0.324	0.323	99.7
ASF	0.248	0.212	85.5

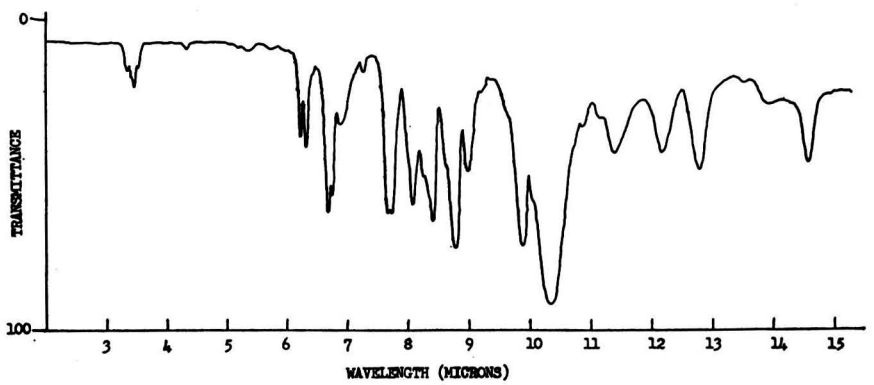


FIG. 6.—Tricresyl phosphate, technical grade (80 per cent *para* and 20 per cent *meta* isomers). Thin liquid film on NaCl crystal.

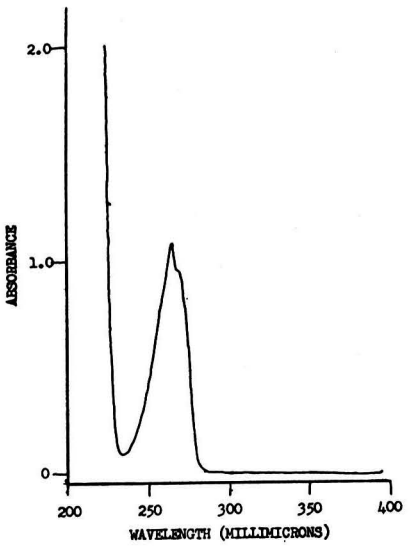


FIG. 7.—Tricresyl phosphate, technical grade (80 per cent *para* and 20 per cent *meta* isomers). 300 mg/l in 95% alcohol. Cell length, 1 cm.

D.—Evap. the filtrates in beakers I and II (*C*) on steam bath under jets of air, and dry residues in an oven at 105°C. for 10 min. Cool, and weigh I and II as combined resin and plasticizer.

E.—Rinse the reserved beakers 1 and 2 (*C*) with two 30 ml portions of hot methyl ethyl ketone and pour rinsings thru the reserved filter paper (*C*) into a tared beaker. Discard the filter paper, evap. the filtrate on the steam bath under a jet of air, dry residue in an oven at 105°C. for 20 min., cool, and weigh as a mixt. consisting essentially of nitrocellulose.

F.—Dissolve the residues in the reserved pptn beaker and centrifuge tube (*B*) in acetone, and transfer the acetone solns to a tared 250 ml beaker. Evap. the acetone on steam bath, redissolve the residue in 5 ml acetone, and add 75 ml (1+2)

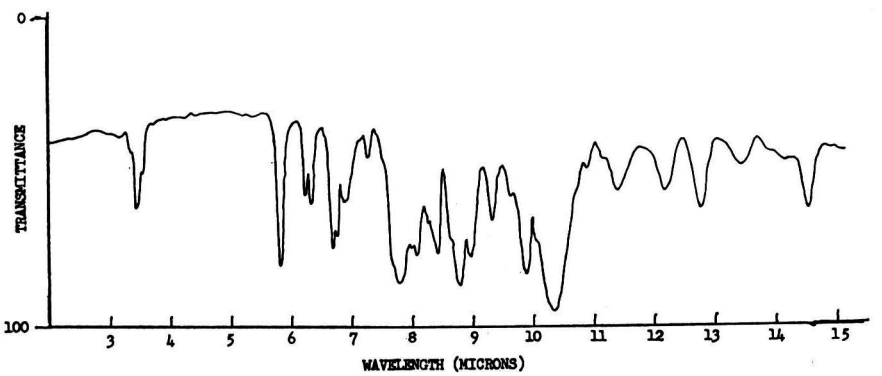
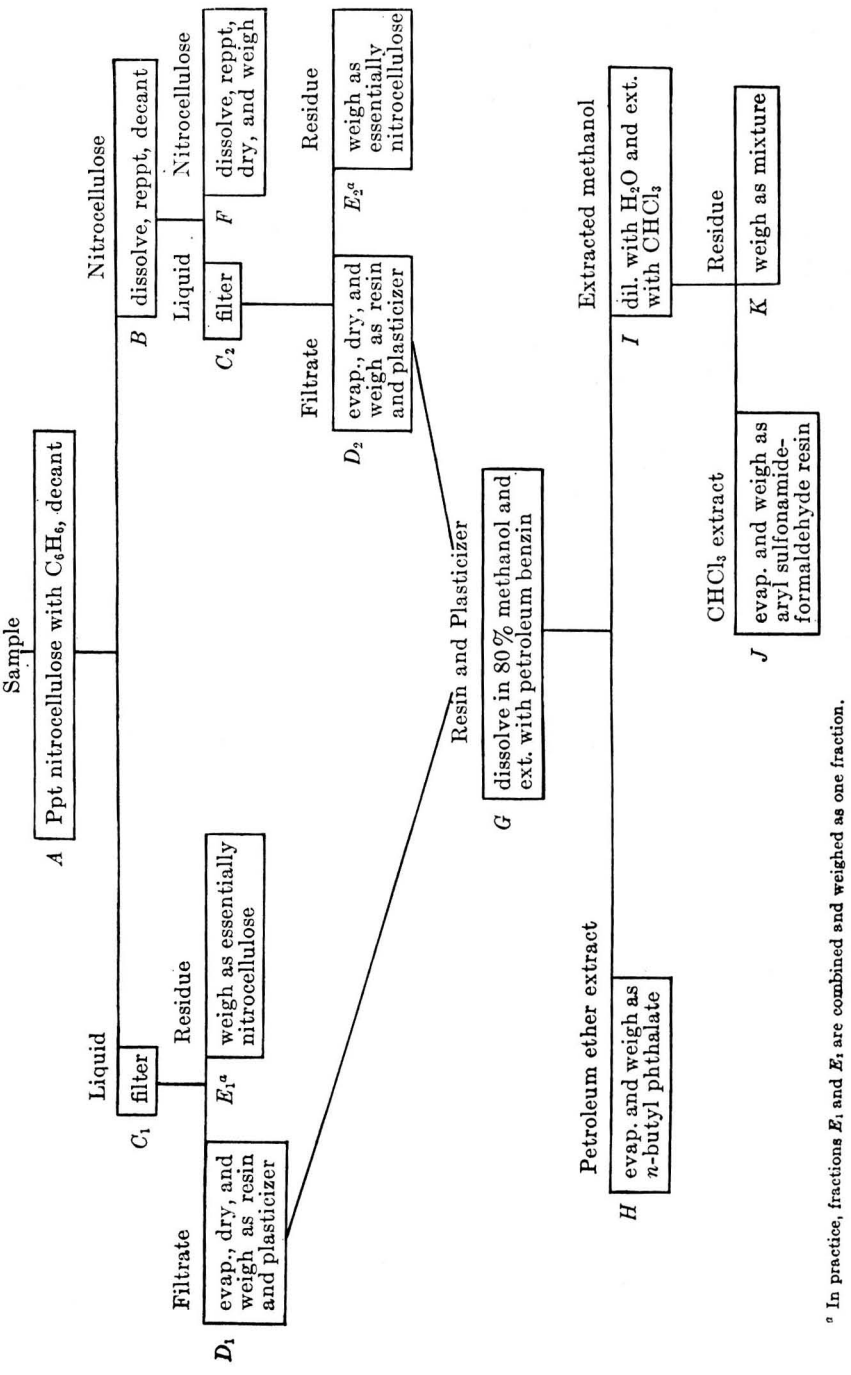


FIG. 8.—Plasticizer fraction from commercial colorless nail lacquer. Thin film on NaCl crystal.

CHART 1.—DIAGRAMATIC SCHEME OF COLORLESS NAIL LACQUER ANALYSIS



[a] In practice, fractions E_1 and E_2 are combined and weighed as one fraction.

alcohol-ether soln followed by 10 ml H_2O. Evap. the solvent on steam bath under a gentle jet of air and dry residue in an oven at 105°C. for 1.5 hrs. Cool, and weigh as nitrocellulose.

G.—Use 32 ml methanol to dissolve and transfer the resin and plasticizer residues in beakers I and II (*D*) into a 250 ml separatory funnel. Add 8 ml H_2O and ext. with four 40 ml portions of petroleum ether. Reserve the extd methanol soln. Set separatory funnels aside for rinsing later.

H.—Filter the combined petroleum ether exts (*G*) thru a 12.5 cm S&S No. 597 filter paper into a tared 250 ml beaker. Follow with an addnl 40 ml petroleum ether wash thru the filter paper. Reserve filter paper, evap. the filtrate on the steam bath under a jet of air, dry residue in an oven at 105°C. for 10 min., cool, and weigh as butyl phthalate.

I.—Transfer the reserved extd methanol soln (*G*) to a 500 ml separatory funnel, add 50 ml $CHCl_3$, dil. with 200 ml H_2O, and acidify with a little HCl. Shake the mixt. well and draw off the $CHCl_3$ layer. Continue the extn with addnl 50, 50, and 30 ml portions of $CHCl_3$. Reserve the extd aq. soln. Set separatory funnel aside for rinsing later.

J.—Filter the combined $CHCl_3$ exts (*I*) thru the reserved filter paper (*H*) and follow with an addnl 40 ml wash $CHCl_3$. Reserve filter paper, evap. filtrate on the steam bath under jet of air, dry residue in an oven at 105°C. for 10 min., cool, and weigh as aryl sulfonamide-formaldehyde resin.

K.—Filter the extd aq. soln (*I*) thru the reserved filter paper (*J*). Discard filtrate. Rinse all the separatory funnels used in the extns (*G*, *I*) with two 30 ml portions of acetone, and filter the acetone solns thru the filter paper into a tared 250 ml beaker. Discard filter paper. Evap. filtrate on steam bath, dry residue in an oven at 105°C. for 10 min., cool, and weigh as a mixt. of resin and nitrocellulose.

Identify the product and isolated fractions by their infrared spectra:

1. *Nail lacquer.*—Prep. sample by coating a salt crystal with a thin film of the lacquer and drying in an oven at 105°C. for 5 min.

2. *n-Butyl phthalate.*—Spread a thin film of the liquid on a salt crystal to obtain the spectrum.

3. *Nitrocellulose.*—Dissolve a little of the material in acetone, pour some of the soln on a salt crystal, allow the acetone to evap. in the air, and dry film on crystal in an oven at 105°C. for 5 min. Allow crystal to cool, and obtain infrared spectrum of film.

4. *Aryl sulfonamide-formaldehyde resin.*—Follow procedure given for nitrocellulose.

Verify the identity of the butyl phthalate and aryl sulfonamide-formaldehyde resin by the ultraviolet spectra of their alcohol solutions.

DISCUSSION

The samples described in Tables 1, 2, and 3 do not contain camphor, an ingredient found in nail lacquers. Its omission permitted a more careful study of the analytical data. In any case camphor would be gradually volatilized in the course of the analysis. Incidentally, the presence of camphor can be detected by its odor in the resin and the plasticizer fraction after the solvents have been evaporated on the steam bath.

The infrared spectrum of the residue on the filter paper (Table 1) indicates that it is essentially nitrocellulose with some admixture of plasticizer and resin. Little error results from assigning this fraction, varying from 13 to 38 mg, to nitrocellulose.

The end results of the nail lacquer analyses are presented in Table 2.

The infrared spectra indicated that the separations were quite clean with little contamination. In the samples containing the three ingredients, the values for nitrocellulose are somewhat high, those for butyl phthalate a little low, while the recoveries for the aryl sulfonamide-formaldehyde resin average about 86 per cent.

The resin loss can be attributed to absorption by the nitrocellulose, extraction with the butyl phthalate, and incomplete extraction from the aqueous solution and the unextractable water-insoluble residue. This unextractable water-insoluble residue was ignored in the calculations, as it is a mixture of resin and nitrocellulose which in only one experiment exceeded 5 mg (in experiment No. 2 it was 14 mg). Actually, the final step *K* in the experimental procedure can be eliminated, as its result is not used. However, it is a check on the amount of unextractable water-insoluble residue.

It is worth noting that since the absorbance of alcohol solutions of the resin and the plasticizer follows Beer's law, it is possible to determine these fractions by ultraviolet spectrophotometry.

When tricresyl phosphate is used with butyl phthalate as the plasticizer, the two substances will be isolated together. The infrared spectra of these two materials are such that each can be identified in the presence of the other (see Figs. 3, 6, and 8). If it is necessary to determine the amount of each compound, the ultraviolet spectra (Figs. 4 and 7) are sufficiently different to apply the methods available for the analysis of two-component mixtures.

One of the advantages of spectrophotometric identification is that it facilitates the detection of materials that might not be suspected as present. This was certainly the case with the tricresyl phosphate. Occasions may arise, however, where a chemical confirmation test may be desirable.[1]

Experience has shown that the proposed procedure can be applied to the colored opaque nail lacquers which differ from the colorless type in containing inorganic and organic pigments. The pigments are precipitated with the nitrocellulose. An investigation is now under way to quantitatively separate the nitrocellulose from the pigments.

Other problems that remain are analytical methods for camphor and the various volatile solvents.

SUMMARY

An analytical procedure has been developed for the colorless nail lacquers that contain, as their non-volatile constituents, nitrocellulose, *n*-butyl phthalate, and aryl sulfonamide-formaldehyde resin. Infrared and ultraviolet spectrophotometry are used for identifying the nail lacquer constituents.

The identification and determination of tricresyl phosphate in nail lacquers was investigated.

Some of the remaining problems of nail lacquer analysis are discussed.

NOTES

RESPONSE OF GOLDFISH TO SEVERAL CHLORINATED INSECTICIDES

By BERNARD DAVIDOW and GEORGE SCHWARTZMAN (Divisions of Pharmacology and Food, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.)

Using the technique reported by Davidow and Sabatino (1), a study was made of the response of goldfish to microquantities of four additional chlorinated insecticides. The insecticides were endrin, isodrin, strobane, and TDE. The tests consisted of placing two goldfish in a series of solutions containing increasing quantities of the listed insecticides. A positive reaction was indicated by the onset of convulsions (extremely rapid and eccentric swimming). Loss of equilibrium and death occurred at higher doses.

The response of goldfish to isodrin and strobane is presented in Table 1. It will be noted that the delay in onset of symptoms ranged from 25 to 120 minutes and showed little relationship to concentration of insecticide.

TABLE 1.—*Response of goldfish to isodrin and strobane*

CONCENTRATION	ONSET OF CONVULSIONS	
	ISODRIN	STROBANE
mmg/250 ml	*minutes*	
200	30	40
100	40	46
50	25	40
25	66	120
10	Neg.	92
5	Neg.	Neg.

In the case of TDE, concentrations up to 6 mg per 250 ml of distilled water failed to produce any toxic effect. At a concentration of 7 mg per 250 ml water, one fish died in approximately three hours but without the characteristic train of symptoms.

In contrast, endrin produced a definite time pattern of response which varied

TABLE 2.—*Determination of endrin by two methods of bioassay*

SAMPLE	ENDRIN BY FLY ASSAY	ENDRIN BY FISH ASSAY
	mmg	*mmg*
A_1	25	25
B_1	25	14
A_2	5	5
B_2	5	5

[a] Solution became cloudy when extract of sample was added to water. The low value may be due to excessive wax in sample.

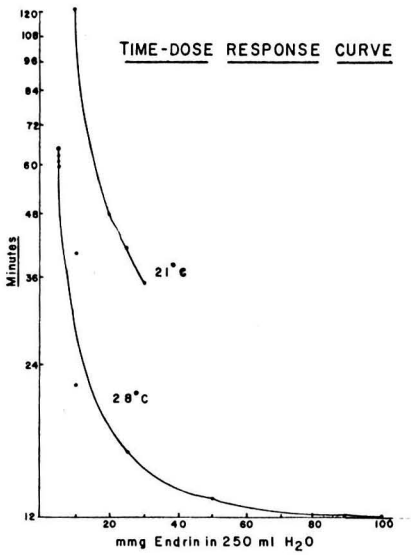


FIG. 1.—Response curve for endrin at two temperatures.

with the concentration. It was also noted that the temperature of the water as well as the concentration influenced the time of onset of symptoms. The time pattern of convulsions as modified by temperature is presented in Fig. 1. The lowering of the water temperature decreased the sensitivity and increased the time necessary to produce the characteristic response of the fish. Therefore, standards and unknowns must be run at the same time if this method is to be used quantitatively.

This technique compares favorably with the fly assay method (2) for estimating the quantity of endrin residue extracted from plant material. Aliquots of the residue solutions were selected to give a response which could be plotted on a standard dose-response curve. The findings of this assay are compared with results obtained with the same samples by the fly assay method (Table 2). Goldfish appear to be a suitable test animal for the bioassay of endrin.

REFERENCES

(1) DAVIDOW, B., and SABATINO, F. J., *This Journal*, **37**, 902 (1954).
(2) LAUG, E. P., *J. Pharmacol. Exptl. Therap.*, **86**, 324 (1946).

APPARATUS FOR THE DETERMINATION OF CARBAMIC ACID DERIVATIVES*

By R. PAYFER (Laboratory Service, Plant Products Division, Department of Agriculture, Ottawa, Ontario, Canada)

Free carbamic acid is unknown, but its derivatives are used as pesticides and weed killers. They are either salts such as ferbam (ferric dithiocarbamate), thiram

* Presented at the Sixty-eighth Annual Meeting o fthe Association of Official Agricultural Chemists. Oct. 11–13, 1954, at Washington, D. C.

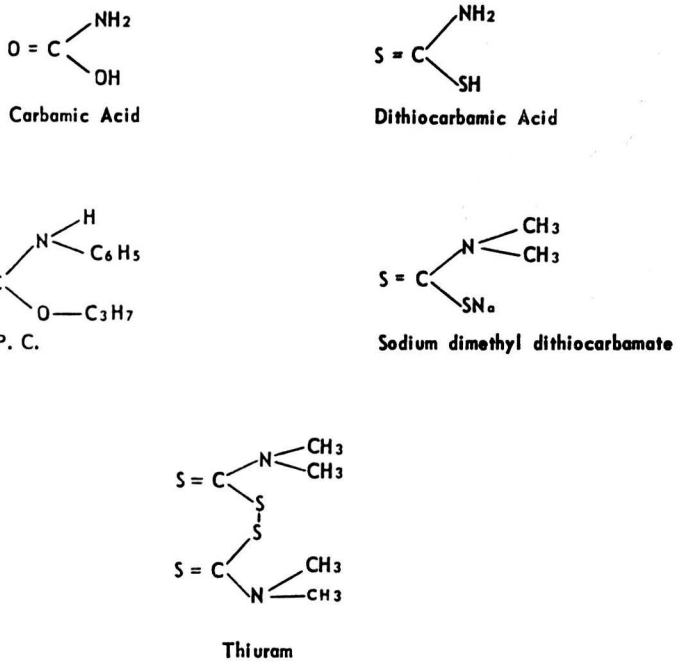


FIG. 1.—Carbamic acid derivatives.

(tetraalkyl thiuram disulfide), or esters, known as urethanes, of which I.P.C. (*o*-isopropyl N-phenyl carbamate) is an example (Fig. 1).

Callan and Stratford (1), Clarke, *et al.*, (2), Rosenthal, *et al.*, (3), and Lowen (4), among others, have studied the dithiocarbamic compounds, while Gard (5) and Shaw (6) have studied the ester known as I.P.C. The former analyzed the dithiocarbamates by means of a carbon disulfide determination and the latter analyzed the ester by means of acid hydrolysis and measurement of evolved carbon dioxide.

About five years ago, the author built an apparatus for the determination of ferbam, thiuram, etc. (Fig. 2). The absorbing towers are made from 50 ml washing bottles connected by standard ball joints. The 4-bulb Allihn condenser is fitted with a standard taper joint to receive the distilling head. The stem of the top funnel (*G*) extends down through the apparatus to within $\frac{1}{8}$–$\frac{1}{4}$ inch from the bottom of flask (*A*). It fits into the distilling head by means of a standard thermometer ground joint and is provided with a side outlet connecting by means of a ball joint to the CO_2 scrubber (*H*). The scrubber (similar to a Meyer sulfur bulb) is made from $\frac{3}{8}$ inch inside diameter tubing and has 10 blown bulbs and funnel.

It was later found that the apparatus served equally well for the determination of I.P.C. by the procedure of Gard (5).

Determination of I.P.C.—Absorption bottles *B* and *C* are not used, and 25, 15, and 10 ml portions of 0.1 *N* NaOH are placed in bottles *D*, *E*, and *F*, respectively. The phosphoric-sulfuric acid mixture is boiled and cooled before use; the blank is thereby lowered. Air is passed very slowly.

Determination of dithiocarbamate.—The CO_2 scrubber is unnecessary. Bottle *B* contains 25 ml of water, bottle *C* contains 10 ml of 10% lead acetate, and bottles

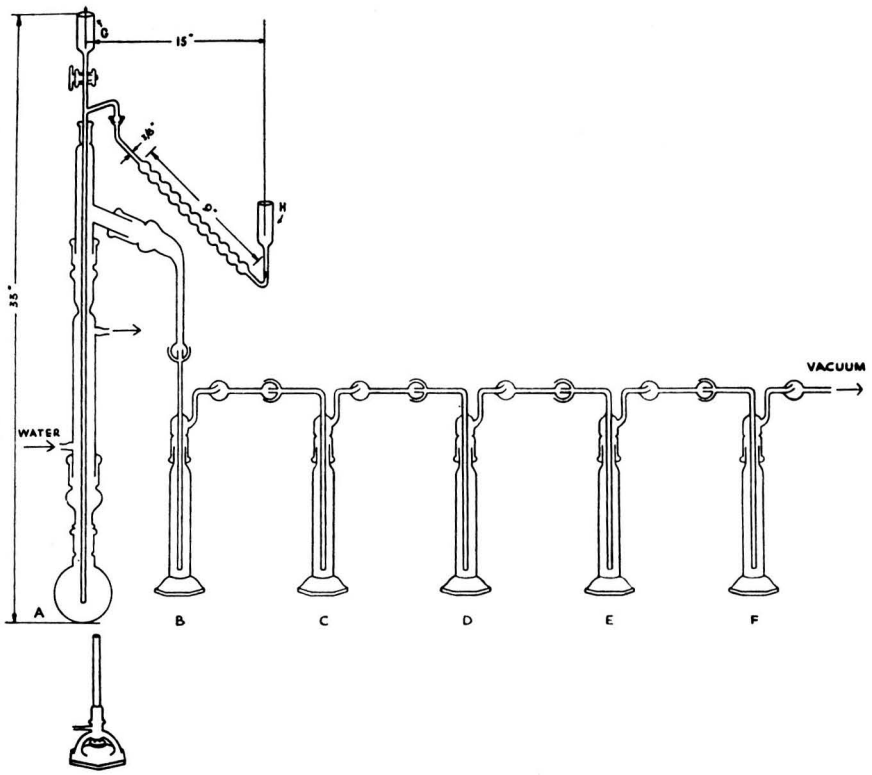


FIG. 2.—Apparatus for determination of carbamic acid derivatives.

D, *E*, and *F* contain 25, 15, and 10 ml of freshly prepared 2 *N* alcoholic potash solution, respectively.

REFERENCES

(1) CALLAN, T., and STRAFFORD, N., *J. Soc. Chem. Ind.*, **43**, 1–8T (1924).
(2) CLARKE, D. G., BAUM, H., STANLEY, E. L., and HESTER, W. F., *Anal. Chem.*, **23**, 1842 (1951).
(3) ROSENTHAL, I., CARLSEN, R. L., and STANLEY, E. L., *This Journal*, **36**, 1170 (1953).
(4) LOWEN, W. K., *Anal. Chem.*, **23**, 1846 (1951).
(5) GARD, L. N., *ibid.*, **23**, 1685 (1951).
(6) SHAW, R. L., *This Journal*, **36**, 381 (1953).

BOOK REVIEWS

Organic Syntheses, Collective Volume III. Edited by E. C. HORNING. John Wiley and Sons, Inc., New York, 1955. x+890 pp. Index, bibl. Price $15.00.

Over thirty years of use has made *Organic Syntheses* the "bible" of the chemist who specializes in the preparation of organic compounds. The editors of this annual compilation of selected and tested synthetic procedures have striven toward and achieved a well-indexed, comprehensive, and detailed manual.

The addition of Collective Volume III, which covers the third decade of annual issues, allows ready and confident reference to an even greater range of procedures. The present collective volume details over three hundred synthetic procedures and includes many revisions and notations added since the original appearance of these procedures in Volumes 20 through 29. In addition, seven new procedures are included which are not covered in the annual Volumes 20 through 29. A new index has been added which allows easy reference to procedures for the preparation of such special reagents as Claisen's alkali, ozone, powdered sodium, etc

The entire *Organic Syntheses* series should be instantly available to every organic chemist.

LEE S. HARROW

Organic Syntheses, Vol. 34. WILLIAM S. JOHNSON, Editor-in-Chief. John Wiley & Sons, Inc., New York, 1954. vi+121 pp. Illus, index. Price $3.50.

In keeping with the tradition established by previous volumes of this series the present volume provides reliable directions for the laboratory preparation of a number of organic compounds. The outstanding virtue of the preparations given is that each has been checked by independent workers in somewhat the same manner that methods of analysis are studied collaboratively before adoption by the A.O.A.C. In this way unreliable procedures are weeded out and do not appear. This book is a valuable addition to any chemical library.

Methods are given for preparing the following compounds: 2-*p*-Acetylphenylhydroquinone; azelanitrile; β-(*o*-carboxyphenyl)propionic acid; cetylmalonic ester; 2-chloro-1,1,2-trifluoroethyl ethyl ether; cycloheptanone; di-*tert*-butyl malonate; 3,4-dihydro-2-methoxy-4-methyl-2H-pyran; 9,10-dihydrophenanthrene; *p,p'*-dinitrobibenzyl; 1,4-dinitrobutane; dimethylfurazan; diphenylacetylene; diphenyl succinate; ethoxyacetylene; ethylchlorofluoroacetate; ethyl enanthylsuccinate; ethyl β,β-pentamethyleneglycidate; hemimellitene; *o*-methylbenzyl alcohol; 2-methylbenzyldimethylamine; N-methyl-1,2-diphenylethylamine and hydrochloride; methylisourea hydrochloride; 3-methyl-1,5-pentanediol; 3-methylthiophene; phenanthrenequinone; 1-phenylpiperidine; *o*-phthalaldehyde; sodium β-styrenesulfonate and β-styrenesulfonyl chloride; tetralin hydroperoxide; *p*-toluenesulfinyl chloride; *p*-tolylsulfonylmethylnitrosamide; and *o*-xylylene dibromide.

K. A. FREEMAN

Organic Reactions, Vol. VIII. ROGER ADAMS, Editor-in-Chief. John Wiley and Sons, Inc., New York, 1954. viii+437 pp. Index. Price $12.00.

This volume extends the high tradition established by the first volume and continued with each succeeding one. The same general easy style and readability is preserved.

As in the other volumes each chapter of the book is devoted to a specific topic. The topics are presented in a thorough and highly readable fashion. Perhaps the

outstanding feature of the treatment of each topic is the presentation of detailed examples which illustrate the subject discussed better than a mere prose description. This feature is not often found in "reviews."

The book is recommended as a "must" addition to any chemical library.

The chapters of Vol. VIII are: (1) Catalytic Hydrogenation of Esters to Alcohols; (2) The Synthesis of Ketones from Acid Halides and Organometallic Compounds of Magnesium, Zinc, and Cadmium; (3) The Acylation of Ketones to Form β-Diketones or β-Keto Aldehydes; (4) The Sommelet Reaction; (5) The Synthesis of Aldehydes from Carboxylic Acids; (6) The Metalation Reaction with Organolithium Compounds; (7) β-Lactones; (8) The Reaction of Diazomethane and Its Derivatives with Aldehydes and Ketones.

KENNETH A. FREEMAN

Organic Chemistry. By LEWIS F. HATCH. McGraw-Hill Book Co., Inc., New York, 1955. vii+324 pp. Index, illus. Price $4.50.

Organic Chemistry is a textbook offered by Professor Hatch of the University of Texas to the students who (in the words of the author) "though not primarily interested in organic chemistry, must understand organic chemistry because of its close alliance to their major fields or interest."

The reviewer does not quarrel so much with the particular book under consideration, since it is in fact a good book of its type, but with the whole philosophy of survey texts. Professor Hatch's book is as informative as other survey textbooks, and points up the student's interest by use of many problems at the end of each chapter. The general description of the petroleum industry and of medicinals and pharmaceuticals are comprehensive to the degree that an adequate balance between the chemistry of the materials involved and the layman's side of the issue has been achieved, although certain glaring exceptions exist. However, the reviewer is compelled to point out that much of the information is readily available in many standard encyclopedias.

Criticism can be leveled at the extreme exhibited in the balance of material between technical and nontechnical information. Assuming for the moment that survey texts in elementary organic chemistry are useful entities, it seems incongruous that the present survey should include a table of physical properties of various acid derivatives (p. 148), a picture of the Monsanto plant at Texas City, Texas (p. 149), a picture of workmen inspecting textile dyeing machinery (p. 187), and the details of indigo white synthesis (p. 188). This contrast is too great. Does the author show the physical properties of the acid derivatives to illustrate the relationship between chemical structure and physical properties, or does he intend to illustrate the wealth of information available in chemistry handbooks?

The reviewer feels that the book under review is of little use to those who, in the course of their work, must come into contact with organic chemistry. A much higher level of information is necessary for these people to comprehend the nature of the material that they must consider.

LEE S. HARROW

Electrometric pH Determinations: Theory and Practice. By ROGER G. BATES. John Wiley and Sons, Inc., New York, 1954. xiii+331 pp. Illus., index. Price $7.50.

As the author states in the preface to this book, "The acute observer is likely to be appalled at the widespread misunderstanding of the principles of *p*H determinations." If all chemists and technicians who made, or use, *p*H measurements in their work were required to read this book, the misunderstanding would virtually disappear.

The volume offers an authoritative survey of almost every aspect of *p*H determinations. The chapters on the theoretical aspects of the subject are a comprehensive and complete presentation of the complex subject matter involved. These chapters may be rather "heavy going" for the average chemist but a thorough study of them is worth the effort involved.

The book also includes several chapters on the practical aspects of *p*H measurement. These chapters contain much much information of concrete value to the chemist or technician who wishes to make *p*H measurements.

JOHN H. JONES

Two Ears of Corn by Way of the Chemical Kettle. By CLAIRE LEAVITT MACDOWELL. The Pequot Press, Stonington, Conn., 1954. xii+252 pp. Illus., index. Price $5.00.

This biography of Charles H. MacDowell, who guided the destinies of the Armour Fertilizer Works from its earliest days to his retirement in 1932, will be welcomed by students of the American fertilizer industry. Mrs. MacDowell has chronicled in easily readable style his connection with many notable events in the industry's history, including the German potash controversy in the earlier years of the century, the introduction of the mechanical superphosphate den, and the development of potash production from Utah alunite during World War I. The problems of fertilizer production and supply with which Mr. MacDowell was confronted during the war as director of the Chemicals Division, War Industries Board, are recounted.

A listing of Mr. MacDowell's published papers and addresses, some 75 in number—dealing chiefly with the technical and economic phases of the fertilizer industry —concludes the volume.

It should be mentioned that this is one of the very few published biographies of outstanding figures in the American fertilizer industry. In fact, it may be unique in this respect.

K. D. JACOB

Advances in Agronomy. Vols. 5 and 6. Edited by A. G. NORMAN. Academic Press Inc., New York, 1953 and 1954. x+422 pp. and xi+383 pp., resp. Illus., index to Vols. 1–6, incl. Prices $8.80 and $8.00, resp.

These two volumes are in the nature of progress reports. They form continuations of a series of which volumes 4 and 5 were reviewed in *This Journal*, **36**, 503 (1953), and **37**, 218 (1954), respectively. The high standard of their predecessors is maintained. They impress us with the breadth and the vigorous development of the field of agronomy, and the diverse interests and accomplishments of those who can be called agronomists or who work in ancillary fields. It is the function of this series to review progress in basic research in soil and crop science and developments in agronomic practice. The central theme of all the volumes is soil-crop relationships, yet in the selection of material the choice is not restricted to papers dealing with the conventional subdivisions of soil and crop science, but is guided by considerations of usefulness to the professional agronomist.

Since Volume 5 has already been reviewed in *This Journal*, further comments on it are unnecessary.

Volume 6 follows the general pattern of its predecessors. It is noteworthy that each volume increases in practical usefulness. In view of the attention now centered in the provision of aid and advice in the improvement of agricultural practices in less developed countries, some of the material therein is particularly pertinent. Its contents, nine papers in all, deal with such subjects as "Progress in Range Improvement," by E. H. McIlvain and D. A. Savage of the U. S. Department of Agriculture; "Requirement and Availability of Soil Water," by O. J. Kelley, also of the U. S.

Department of Agriculture; "A Pattern for International Cooperation in Agriculture," by J. G. Harrar, Rockefeller Foundation; "New Concepts of Management of Corn Belt Soils," S. W. Melsted, University of Illinois; "Flax-seed Improvement," by J. O. Culbertson and others: "Technological Aspects of Trace Element Usage," by A. A. Nikitin, Tennessee Corporation Research Laboratories; "Crop Adaptation and Climate," by C. P. Wilsie and R. H. Shaw of Iowa State College; "Potassium in Plant Nutrition," by Kirk Lawton and R. L. Cook, Michigan State College; and "Sorghum Improvement," by J. R. Quinby of Texas Agricultural Experiment Station and J. H. Martin of the U. S. Department of Agriculture. The article by Harrar, outstanding in treatment, recounts the principles which have been followed by the Rockefeller Foundation in their highly successful programs in Central America. On the domestic scene, Savage and McIlvain review the great changes that have been brought about in range improvement as a result of the application of basic agronomic principles to range problems. Wilsie and Shaw take a broad viewpoint and discuss the adaptation of crops to environment and the influence of climatic factors on problems of crop production. Their examples are mostly taken from North American experience, but the information summarized is international in application.

Through advances in our knowledge of soils and soil management, and crops and crop husbandry, food production in much of the New World and in most other countries where scientific agriculture is practiced keeps in step with the requirements of ever-expanding populations. This is the achievement of professional agronomists, and the accomplishment by which their science may be judged.

J. S. WADE