REPORT ON INERT MATERIALS IN FERTILIZERS*

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A committee to study the problem of fillers and related materials in fertilizers was appointed by the Association of American Fertilizer Control Officials early in 1947.

This committee proposed that a nation-wide survey be conducted on the filler content of commercial fertilizer mixtures. To implement this proposal it appointed a subcommittee[4] to develop methods which could be used in such a survey to indicate the filler content of fertilizers. At the same time the committee authorized a statement of policy which read in part as follows:

"... If the results of this survey warrant, the Association of Official Agricultural Chemists will be requested to appoint a referee to study this problem. Furthermore, it is the aim and desire of this committee to work in full cooperation with the Association of Official Agricultural Chemists and will present to them the results of our findings for their consideration."

From its studies the subcommittee devised methods for the determination of (a) calcium carbonate equivalent of carbonate carbon, (b) ash in acid-insoluble matter (acid-insoluble matter minus loss on ignition) and ash-free acid-insoluble organic matter (loss on ignition of acid-insoluble matter), and (c) ash-free water-insoluble organic matter. It submitted these methods to several State fertilizer control laboratories for evaluation as to their applicability in the proposed survey. Specially prepared mixtures also were made available to aid in the evaluation.

At this juncture the Association of Official Agricultural Chemists was requested to appoint, and appointed, an Associate Referee to study the proposed methods and others of possible use in the determination of filler.

This report presents the methods proposed by the above-mentioned sub-committee and the results obtained by the several collaborators in their evaluation tests.

METHODS

I. CARBONATE CARBON OR CALCIUM CARBONATE EQUIVALENT

APPARATUS

The apparatus consists of a Knorr alkalimeter (250-ml flask), the guard tube of which is filled with Ascarite (sodium hydroxide asbestos absorbent mixture) and the

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condenser outlet connected with a CO_2 absorption train. The train consists of a Schwartz drying tube containing Ag_2SO_4 saturated H_2SO_4 (1+1) to a depth of ca ½-inch above the bend, a Bowen potash bulb or Schmitz drying tube containing the same soln, a second Schwartz tube filled with Anhydrone (anhydrous magnesium perchlorate), two Stetser and Norton absorption bulbs, the inlet two-thirds of which are filled with Ascarite and the outlet third with Anhydrone for absorbing the evolved CO_2, a third Schwartz tube similarly filled with Ascarite and Anhydrone, a Drechsel gas washing bottle containing conc H_2SO_4 to a depth of 1–2 cm, a protecting trap, and an aspirating bottle or suction source.

DETERMINATION

Aspirate a slow stream of air thru the assembled apparatus until the two Stetser and Norton bulbs have reached a constant weight. Close off the train by turning the necessary stoppers, remove the dry alkalimeter flask, and place 5 g of fertilizer therein, replace the flask, fill the dropping funnel with 50 ml HCl (1+4). Slowly add the acid to the sample while aspirating. After the reaction has substantially subsided, gently heat the flask until the soln boils, and continue boiling for 2–3 min. Discontinue heating and continue aspirating the system for 20 min. Close off the system, remove the two Stetser and Norton bulbs, and hang in a balance case until cool enough to weigh (usually 20–30 min.). Before weighing, the bulbs should be wiped with a dry lint-free cloth using a standardized procedure. Weighings should be made against a tare, packed in the same manner as the absorption bulbs. The increase in weight of the absorption bulbs is due to CO_2. Calculate and report the result as $CaCO_3$ equivalent.

II. ASH IN ACID-INSOLUBLE MATTER AND ASH-FREE ACID-INSOLUBLE ORGANIC MATTER

DETERMINATION

Weigh and place 5.0 g of fertilizer in a 250-ml beaker. Add 25 ml of hot H_2O (98–100°C.), swirl contents of beaker, decant and filter the liquid (11 cm Whatman No. 40, or equivalent rapid filtering paper), transfer residue in the beaker to the filter with a stream of hot H_2O from a wash bottle, and wash 4–5 times. Transfer the washed residue from the filter paper to the beaker by unfolding the paper and directing a stream of HCl (1+1) upon it from a wash bottle. Add HCl (1+1) to give a total volume of 100–125 ml. Cover the beaker with a watch-glass and boil for 15–20 min. Filter thru a tared Gooch crucible with an acid-washed asbestos mat and perforated porcelain plate to hold the mat in place. Wash the insoluble residue 6 times with hot H_2O, dry crucible 1 hr. at 125°C, cool in a desiccator, and weigh. The increase in weight represents the total acid-insoluble matter. Ignite crucible and contents 1 hr at 600°C. in a muffle furnace, cool, and reweigh. Report the loss on ignition as ash-free acid-insoluble organic matter and the net increase in weight of the crucible as ash in acid-insoluble matter.

III. ASH-FREE WATER-INSOLUBLE ORGANIC MATTER

REAGENTS

(a) *$CaCl_2$ soln, sp. gr. 1.40.* Prepare a saturated $CaCl_2$ soln by dissolving $CaCl_2 \cdot 2H_2O$ in hot water. The quantity of the salt required depends on its purity, but usually two pounds per liter of H_2O is sufficient to produce the desired soln, sp. gr. 1.40 at 25°C. Check the sp. gr. with an hydrometer, and if necessary heat the soln with an excess of the salt until a soln of the proper density is obtained. Decant the soln into a suitable bottle for storage. Add 4.0 ml conc HCl (sp. gr. 1.19) per liter of soln, and thoroly mix. Thoroly mix the stock soln each time before withdrawing a portion for use.

(b) *Ammonium carbonate.*

DETERMINATION

Measure 50 ml of the acidified stock soln into a 100-ml tall-form beaker. Place 5.0 g of the fertilizer in a dry beaker, add ca 0.05 g ammonium carbonate, and mix thoroly with a dry stirring rod. Transfer the dry mixture with the use of a camel's-hair brush to calcium chloride soln. Stir carefully but vigorously until sample is thoroly wet. (Proper grounding of the grinding mill during preparation of the sample has been found largely to overcome the difficulty of wetting some samples). Carefully wash and remove the stirring rod, and rinse down the sides of the beaker with a jet of the $CaCl_2$ soln from a wash bottle. Set the beaker aside for 10 to 30 minutes to permit the organic matter to collect on the surface of the soln. Remove the organic matter by skimming the surface of the liquid, using a tube connected to a source of vacuum thru a wide-mouth bottle (4–8 oz.) that traps the organic matter and any $CaCl_2$ soln carried with it. Care should be taken to remove as little $CaCl_2$ soln with the organic matter as possible. Rinse any organic matter remaining in the collecting tube into the bottle. Swab the sides of the beaker at the surface of the soln with a small wet piece of filter paper and rinse any adhering organic matter into the bottle. Transfer the collected organic matter to a previously tared Gooch crucible with asbestos mat, wash thoroly with hot water to remove all $CaCl_2$. Drench the residue in the crucible once or twice with HCl (1+49) to destroy any adhering carbonate, wash with H_2O, dry 1 hr. at 125°C., and weigh. Ignite 1 hr. at 600°C. and reweigh. Report the loss on ignition as ash-free water-insoluble organic matter.

COLLABORATORS

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(3) Department of Chemistry, New Jersey Agricultural Experiment Station, New Brunswick, N. J.

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SAMPLES

The formulas of five mixtures which were submitted to collaborators for evaluation of the methods are given in Table 1. The principal sources of carbonate carbon in Sample 1 were Cal-Nitro and selectively calcined dolomite, whereas the principal source in the other four samples was dolomite. Three samples (1, 2, and 5) were formulated with a single source of insoluble organic material and the others (3 and 4) with two and three sources, respectively. Only Samples 1 and 3 were formulated without the use of sand as a make-weight material.

RESULTS OBTAINED BY COLLABORATORS

The results reported by the collaborators are presented in Tables 2 to 5, inclusive. Collaborator No. 3 was not equipped for the determination of carbonate carbon. Collaborator No. 2 compared the acid solubility in HCl (1+4) in addition to using the suggested concentration of 1+1. Collaborator No. 1 found it desirable to reduce the sample size from 5 g to 1 g in conducting the flotation separation of organic material. Collaborator No. 2 also varied the procedure for collecting the floated material by overflowing it from a small beaker.

TABLE 1.—*Formulas of mixtures submitted to collaborators*

SAMPLE NUMBER	1	2	3	4	5
GRADE	8-16-16	6-8-6	3-9-6	3-9-6	3-12-6
Material	Pounds per ton				
Nitrogen solution 2A, 40.6% N	—	—	—	—	142
Ammonium sulfate, 20.8% N	205	420	—	150	21
Ammonium nitrate, 32.5% N	100	100	—	—	—
Uramon, 42.0% N	—	—	100	—	—
Cal-Nitro, 20.5% N	260	—	—	—	—
Sodium nitrate, 16.0% N	—	—	—	95	—
Garbage tankage, 2.4% N	—	80	—	—	—
Cocoa shell meal, 2.4% N	100	—	—	—	—
Cotton seed meal, 5.7% N	—	—	300	100	—
Processed tankage, 8.5% N	—	—	—	60	—
Fish scrap, 6.1% N	—	—	—	50	—
Peanut hull meal, 1.5% N	—	—	100	—	—
Hyper-humus, 1.0% N	—	—	—	—	100
Ammo-Phos, 10.8% N, 47.0% P_2O_5	280	—	—	—	—
Superphosphate, 20.0% P_2O_5	—	800	900	900	1232
Double superphosphate, 46.0% P_2O_5	420	—	—	—	—
Potassium chloride, 62.4% K_2O	515	195	95	80	205
Potassium sulfate, 48.0% K_2O	—	—	100	150	—
Sulfate of Potash-Mg, 27.0% K_2O	—	—	50	—	—
Calcined dolomite, 25.9% MgO	120	—	—	—	—
Dolomite, 20.1% MgO	—	300	355	215	100
Sand	—	105	—	200	200
Total	2000	2000	2000	2000	2000

TABLE 2.—*Calcium carbonate equivalent of carbonate carbon*

COLLABORATOR	SAMPLE NUMBER									
	1		2		3		4		5	
	per cent	*lbs/ton*	*per cent*	*lbs/ton*	*per cent*	*lbs/ton*	*per cent*	*lbs/ton*	*per cent*	*lbs/ton*
1	9.60	192	14.70	294	18.48	370	11.06	221	5.50	110
	9.52	190	15.20	304	18.80	376	11.22	224	5.50	110
	—	—	15.10	302	18.30	366	—	—	—	—
Average	9.56	191	15.00	300	18.49	370	11.14	223	5.50	110
2	8.91	178	15.19	304	18.94	379	11.04	221	5.50	110
	8.91	178	—	—	—	—	—	—	—	—
Average	8.91	178	15.19	304	18.94	3.79	11.04	221	5.50	110
4	9.60	192	15.40	308	18.92	378	11.05	221	5.07	101
	9.69	194	15.31	306	18.74	375	10.98	220	5.59	112
Average	9.65	193	15.36	307	18.83	377	11.02	220	5.33	107

TABLE 3.—*Ash in acid-insoluble matter (acid-insoluble matter minus loss on ignition)*

COLLABORATOR	SAMPLE NUMBER									
	1		2		3		4		5	
	per cent	*lbs/ton*	*per cent*	*lbs/ton*	*per cent*	*lbs/ton*	*per cent*	*lbs/ton*	*per cent*	*lbs/ton*
1	1.59	32	7.95	159	4.27	85	13.76	275	14.16	283
	1.67	33	8.04	161	4.35	87	13.81	276	13.50	270
	—	—	—	—	—	—	—	—	13.89	278
Average	1.63	33	8.00	160	4.31	86	13.79	276	13.85	277
2	1.44	29	8.73	175	4.45	89	13.92	278	14.60	292
	1.66	33	8.61	172	3.47	89	13.61	272	14.37	287
Average	1.55	31	8.67	173	4.46	89	13.77	275	14.49	290
	1.67[a]	33[a]	8.88[a]	178[a]	4.56[a]	91[a]	13.74[a]	275[a]	14.58[a]	292[a]
3	1.68	34	9.07	181	4.49	90	13.67	273	14.11	282
4	1.31	26	8.71	174	4.54	91	13.88	278	14.10	282
	1.69	34	8.60	172	4.59	92	13.65	273	14.30	286
	1.70	34	8.56	171	4.46	89	—	—	—	—
Average	1.57	31	8.62	172	4.53	91	13.77	275	14.20	284

[a] HCl (1+4) substituted for HCl (1+1).

TABLE 4.—*Ash-free acid-insoluble organic matter*

COLLABORATOR	SAMPLE NUMBER									
	1		2		3		4		5	
	per cent	*lbs/ton*	*per cent*	*lbs/ton*	*per cent*	*lbs/ton*	*per cent*	*lbs/ton*	*per cent*	*lbs/ton*
1	1.78	36	1.38	28	4.68	94	2.14	43	2.78	56
	1.81	36	1.37	27	4.68	94	2.16	43	2.65	53
	—	—	—	—	—	—	—	—	2.76	55
Average	1.80	36	1.38	28	4.68	94	2.15	43	2.73	55
2	1.68	34	1.31	26	4.60	92	2.15	43	2.68	54
	1.77	35	1.33	27	4.63	93	2.21	44	2.86	57
Average	1.73	35	1.32	26	4.62	92	2.18	44	2.77	55
	2.18[a]	44[a]	1.44[a]	29[a]	5.60[a]	112[a]	2.67[a]	53[a]	3.04[a]	61[a]
3	1.82	36	1.32	26	4.71	94	2.27	45	2.79	56
4	2.08	42	1.22	24	4.44	89	2.19	44	2.77	55
	1.71	34	1.16	23	4.40	88	2.24	45	2.69	54
	1.67	33	1.20	24	4.64	93	—	—	—	—
Average	1.82	36	1.19	24	4.49	90	2.22	44	2.73	55

[a] HCl (1+4) substituted for HCl (1+1).

TABLE 5.—*Ash-free water-insoluble organic matter*
(Flotation separation)

COLLABORATOR	SAMPLE NUMBER									
	1		2		3		4		5	
	per cent	*lbs/ ton*	*per cent*	*lbs/ ton*	*per cent*	*lbs/ ton*	*per cent*	*lbs/ ton*	*per cent*	*lbs/ ton*
1[a]	3.66	73	.98	20	9.24	185	7.90	158	4.88	98
	3.67	73	1.07	21	9.65	193	7.77	155	4.26	85
Average	3.67	73	1.03	21	9.45	189	7.84	157	4.57	91
2	6.88	138	3.07	61	12.05	241	9.41	188	2.96	59
	3.01	60	.83	17	6.71	134	4.98	100	3.59	72
Average	4.95	99	1.95	39	9.38	188	7.20	144	3.28	66
	3.33[b]	67[b]	1.27[b]	25[b]	10.75[b]	215[b]	5.97[b]	119[b]	3.45[b]	69[b]
3	2.53	51	1.23	25	9.93	199	7.65	153	2.92	58
4	3.48	70	1.90	38	11.95	239	6.90	138	3.92	78
	3.26	65	2.00	40	11.95	239	7.00	140	3.86	77
Average	3.37	67	1.95	39	11.95	239	6.95	139	3.89	78

[a] Except for sample Number 3, a 1-g rather than 5-g sample was used.
[b] 50-ml beaker used and overflowed into 400-ml beaker.

COMMENTS OF COLLABORATORS

Carbonate carbon or calcium carbonate equivalent

Florida Agricultural Department.—The CO_2 method is about perfect. I checked with control on four samples and a rerun on the other gave perfect check on my first run.

Bureau of Plant Industry, Soils, and Agricultural Engineering.—Reproducible results were readily obtained by the procedure as outlined.

Ash in acid-insoluble matter and ash-free acid-insoluble organic matter

Florida Agricultural Department.—The acid-insoluble ash and organic matter checked well. I made a third run using HCl (1+4) instead of HCl (1+1). The ash by this modification checked with the (1+1) but organic matter ran a little higher. The reason for using HCl (1+4) instead of (1+1) is to get away from breathing strong fumes of HCl. I believe the same thing can be accomplished by diluting the HCl (1+1) digestion when taken off the hot plate before filtering and washing. This is an empirical method. Just what is acid-insoluble organic matter? There is not a great deal of difference in results by HCl (1+1) and HCl (1+4). The latter is not objectionable to work with.

New Jersey Agricultural Experiment Station.—With careful and proper manipulation, concordant results can be obtained with the methods outlined.

Ash-free water-insoluble organic matter

California Department of Agriculture.—A 1-gram sample of four of the mixtures was used instead of a 5-gram sample as specified in the method, because a 5-gram sample of some of the materials gave suspensions which appeared too viscous to accomplish the desired result.

Florida Agricultural Department.—The water-insoluble method is satisfactory to

the point where the organic matter is skimmed off the calcium chloride flotation. At this point I had trouble skimming with suction. I tried overflowing it from a 50-ml. beaker placed inside a larger beaker. This worked better than skimming. The next trouble was filtering and washing. Three of the samples worked fairly well. The other two took 30 hours to complete. The final results do not check. I doubt the value of this determination even when the process is worked out.

New Jersey Agricultural Experiment Station.—Erratic results were obtained by the method as outlined due to the fact that not all the organic matter came to the surface in the 10 to 30 minutes specified in the procedure.

Bureau of Plant Industry, Soils, and Agricultural Engineering.—Non-ammoniated tobacco grades seemed to be the most difficult samples to handle by the flotation method, because more extraneous material (probably dolomite and superphosphate) is carried along with the organic material. The ignition step might be eliminated if the floated material is drenched with hot HCl (1+50) about twice while in the filter crucible. This acid treatment successfully gets rid of 80–90 percent of the extraneous material. The error then in the organic matter floated would not be excessive, and the weight of the sample prior to the ignition step probably would more nearly indicate the organic matter included in the formula than the ash-free water-insoluble matter, or loss on ignition.

DISCUSSION OF RESULTS

Carbonate carbon or calcium carbonate equivalent.—With the exception of the results for one sample the values reported by the collaborators were in good agreement, and no difficulty was reported in making the determination. Probably the method should be evaluated using individual materials, and mixtures formulated from materials of known carbonate content.

Ash in acid-insoluble matter and ash-free acid-insoluble organic matter.—The values reported by the various collaborators were in good agreement, but probably the ash obtained should receive an acid treatment to more nearly insure a more representative value of the silica content. The significance of the ash-free acid-insoluble organic matter has been questioned. This value is the ignition loss obtained on ashing the total acid-insoluble material, and presumably represents difficulty decomposable organic matter of little or no immediate fertilizer value. Additional study should be given to a method for determining acid-insoluble ash.

Ash-free water-insoluble organic matter.—The erratic results obtained and considerable difficulty experienced by the collaborators in making this determination indicate the need for an improved procedure for direct separation of organic material from mixtures. Possibly reducing the sample size, washing the floated material with dilute acid, and omitting the ignition step would accomplish the objective. A comparison, however, should be made between such a revised procedure and the possibility that the ignition loss of the water-insoluble portion of the original sample would provide a satisfactory measure of the organic matter.

RECOMMENDATION

It is recommended that the work be continued.

CONTRIBUTED PAPERS

A STUDY OF CLARIFICATION METHODS IN THE DETERMINATION OF SUGARS IN WHITE POTATOES

USE OF ION-EXCHANGE RESINS

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In the preparation of potato chips (6, 16, 17, 20) and dehydrated white potatoes (5, 12), the reducing-sugar content of the potato is a major factor in the formation of objectionable color. Research on potato processing at this laboratory has therefore made it necessary to determine sugars on a large number of samples. A rapid, accurate method of analysis for sugars would facilitate this work.

The authors have found that each type of plant material must be investigated to determine the most satisfactory procedure for estimation of sugar content. This conclusion is in agreement with that of the Referee, Elroy J. Miller, and co-workers (18).

By comparison of sugar values obtained by different methods of analysis, it was concluded that non-sugar reducing substances introduced significant errors in some of the methods. Therefore, the emphasis of the investigation was directed towards the removal of these substances, hereinafter referred to as clarification.

The substitution of ion-exchange resins for neutral lead acetate and carbon treatments, for the removal of non-sugar reducing materials from potato extracts, resulted in a major improvement in the clarification procedure.

Three methods were used to determine sugars in clarified extracts from raw potatoes, and four methods were used for extracts from dehydrated potatoes (ca. 6–8 per cent moisture). The methods used and the results obtained are presented in this report.

PROCEDURES

(*1*) *Extraction of sugars:* The raw potatoes were quickly peeled, sliced, chopped finely, and added to sufficient boiling 95 per cent ethyl alcohol (freshly redistilled), to give with the water in the potatoes an approximate alcohol concentration of 80 per cent. Calcium carbonate (1–2 g) was added and the mixture was heated 1 hour, with frequent stirring, on a steam bath. The supernatant liquor was decanted through a folded filter paper. The

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residue was covered with 80 per cent alcohol and reheated for 0.5 hour and the liquid was filtered as before. The residue was transferred to a Waring Blendor cup[2] and sufficient 80 per cent alcohol was added to make a slurry. After blending for about 5 minutes, the mixture was transferred to a Soxhlet extractor and the extraction process was allowed to continue for 0.5 hour. The extract was removed, replaced with fresh 80 per cent alcohol, and the extraction continued for 4–5 hours. All of the extracts were combined and stored in the dark until used.

The dehydrated potatoes were ground to pass a 40–mesh screen. The extraction process was similar to that used for fresh potatoes, with the exception that the blending step was omitted and 80 per cent alcohol was used throughout the procedure.

The complete extraction of the sugars was not demonstrated, but for the purpose of comparison of methods of analysis on each extract, this was not necessary.

(*2*) *Clarification of the potato extracts:* The extract was evaporated on a steam bath to remove the alcohol (1), and the residual water solution was diluted to a definite volume. Three methods were used to clarify aliquots of this water solution: (a) An excess of saturated neutral lead acetate solution was added to the water solution, the lead precipitate was removed by filtration, and the excess lead was removed with disodium phosphate solution. (b) An aliquot of the solution obtained in (a) was treated at room temperature with carbon to remove all color (5 mg. of carbon per ml. of solution for 10 minutes). (c) The water solution was passed through a set of ion-exchange resin columns. For brevity, these three methods of clarification will be referred to as the lead, lead-carbon, and resin treatments.

The ion-exchange columns used in this study have been illustrated and described elsewhere (19). One tube contained 15 g. of the cation-exchange resin, Amberlite IR-100 H-AG,[2] and the other tube contained 20 g. of the acid-binding resin, Amberlite IR-4 B-AG. The columns were backwashed with distilled water for 1–2 hours just before they were used. Two 30-ml. aliquots of the water solution were passed slowly through the IR-100 H-AG, and then through the IR-4 B-AG and discarded, to eliminate the dilution error. Then, 100–125 ml. of the water solution to be used for sugar analysis were passed through the columns at the rate of approximately 3 ml. per minute. The amount of solution passed through the columns represented about 70 g. of raw, or 30 g. of dehydrated, potatoes. Although the capacity of the columns was not determined, no breakthrough was observed. Pure solutions of known concentrations of dextrose, levulose, and sucrose were passed through the resin columns in the same manner as the potato solutions with no change in sugar concentration.

[2] The mention of manufacturers and commercial products does not imply that they are endorsed or recommended by the Department of Agriculture over others not mentioned.

Since the insoluble material in the water solution from dehydrated potatoes interfered with the effectiveness of the resins, the procedure was modified by adding 2 g. of Celite Analytical Filter-Aid to the water solution and filtering with suction through a Büchner funnel containing a filter paper covered with a thin mat of Celite. Experiments with pure solutions of dextrose, levulose, and sucrose showed that the filter aid did not adsorb any perceptible amounts of these sugars. This is in agreement with Waldron *et al.* (18).

(*3*) *Inversion:* The clarified solutions were adjusted to a *p*H of 4.8–5.1 with 10 per cent acetic acid solution, and total sugars were determined after treatment of the solutions with invertase (Difco Laboratories, Detroit, Mich.) overnight at room temperature. The extracts were hydrolyzed prior to carbon treatment, to prevent sucrose adsorption (4). It was necessary to add benzoic acid to the untreated and resin-treated solutions before hydrolysis, to prevent microbiological growth which lowered the sugar values.

(*4*) *Analysis of the clarified extracts:* Sugars were determined on the clarified solutions by the following methods: (a) A procedure that will be referred to as the macro method (2), whereby 50–150 mg. of reducing sugars are reacted with cupric salts, the cuprous oxide is filtered off and dissolved in nitric acid, and the amount of copper is determined by thiosulfate titration. This method requires about 50 times the amount of sugar necessary for the micro methods, and it was included primarily as a reference method (9). (b) A procedure that will be referred to as the A.O.A.C. Somogyi method (3, 14), whereby 1–2 mg. of reducing sugars are reacted with cupric salts in the presence of potassium iodide and potassium iodate, and the iodine liberated on acidification is determined by thiosulfate titration. (c) A procedure that will be referred to as the Somogyi modified method (15), which is procedure (b) as improved by Somogyi. (d) A procedure that will be referred to as the Hassid method (7, 8), whereby 1–3 mg. of reducing sugars are reacted with alkaline ferricyanide, and the ferrocyanide formed is titrated in acid solution with ceric sulfate, using the indicator Setopalin C. The sugar values given in the tables are the averages of closely agreeing duplicates.

DISCUSSION OF RESULTS

A previous investigation with different commercially available decolorizing carbons showed that their sugar adsorptive properties varied (4). Four of these carbons, which did not absorb measurable quantities of levulose or dextrose, were selected for use in the clarification procedure on two raw potato samples, one of high and one of low reducing-sugar content. The data are given in Table 1. Any apparent differences in reducing-sugar content resulting from the carbon treatment were due to the removal of non-sugar reducing materials. All of the carbon-treated solu-

tions were water clear, but the sugar values obtained by the Hassid method were higher than those obtained by the macro method, and these differences were greater, proportionately, in the potato of low reducing-sugar content. Throughout this investigation, it has been observed that the removal of color does not indicate the complete removal of non-sugar reducing substances. With raw potatoes, the macro method gave essentially the same sugar values, with or without lead-carbon treatment, irrespective of the type of carbon used. Since the lead-carbon treatment was

TABLE 1.—*Effect of decolorizing carbons on the determination of reducing sugars in raw white potatoes*[1]

SAMPLE NO.	METHOD OF ANALYSIS	PER CENT REDUCING SUGARS[2]				
		NO CARBON TREATMENT	DECOLORIZING CARBON			
			G60[3]	KB[3]	B&A[4]	325[5]
A	Macro (2)	0.195	0.192	0.186	0.197	0.199
	Hassid (7, 8)	0.274	0.224	0.232	0.242	0.256
B	Macro (2)	1.07	1.05	1.05	1.05	1.05
	Hassid (7, 8)	1.10	1.10	1.08	1.11	1.11

[1] Prior to the carbon treatment, the potato extracts (after the removal of alcohol) were treated with excess neutral lead acetate solution and the excess lead was removed with disodium phosphate, as described under Procedures, clarification step (a).
[2] Calculated as dextrose.
[3] Darco Corp., New York, N. Y.
[4] Baker & Adamson Code No. 1551, General Chemical Co., New York, N. Y.
[5] Animal Charcoal 325 Mesh, Consolidated Chem. Ind. Inc., San Francisco, Calif.

not satisfactory with the Hassid method, it was necessary to find a different method of clarification.

Partridge and Westall (10) used ion-exchange resins for the removal of "bases and cations" and "acids" from extracts of biological materials in their procedure for the identification of sugars by chromatography. Putnam *et al.* (11) found ion-exchange resins useful for removing minerals and organic and amino acids from solutions in the preparation of radioactive carbon-labeled sugars. Serbia (13) found that a mixture of ion-exchange resins added to cane molasses would remove the non-sugar reducing materials that interfered with the Lane-Eynon method of analysis for sugars. The authors tested ion-exchange resins, in glass columns, for the removal of non-sugar reducing materials from white potato extracts. This method of clarification was compared with the lead and with the lead-carbon procedures (see (2)) on extracts prepared from raw and dehydrated potatoes (Tables 2 and 3).

When solutions prepared from raw potatoes were clarified by resin treatment, all of the methods gave, within experimental error, the same sugar values. These values were in marked contrast to those obtained by

TABLE 2.—*Percentages of reducing and total sugars in extracts of raw white potatoes*[1] *as determined by different methods of clarification and evaluation*

SAMPLE NO.	METHOD OF CLARIFICATION[2]	METHODS USED TO EVALUATE SUGARS					
		REDUCING SUGARS[3]			TOTAL SUGARS[3]		
		A.O.A.C.		HASSID (7, 8)	A.O.A.C.		HASSID (7, 8)
		MACRO (2)	SOMOGYI (3, 14)		MACRO (2)	SOMOGYI (3, 14)	
1	Resin	0.48	0.50	0.47	1.39	1.41	1.41
	Lead	0.49	0.51	0.55	1.47	1.49	1.53
	Lead-carbon	0.50	0.52	0.53	1.45	1.51	1.51
2	Resin	0.29	0.31	0.32	0.69	0.73	0.71
	Lead	0.31	0.35	0.39	0.70	0.78	0.79
	Lead-carbon	0.32	0.36	0.36	0.70	0.74	0.77
3	Resin	0.26	0.27	0.28	0.92	0.91	0.92
	Lead	0.28	0.29	0.35	0.93	0.95	0.98
	Lead-carbon	0.27	0.29	0.32	0.93	0.95	0.97
4	Resin	0.61	0.59	0.62	1.30	1.31	1.31
	Lead	0.61	0.67	0.67	1.30	1.37	1.36
	Lead-carbon	0.61	0.65	0.64	1.29	1.35	1.33
5*	Resin	0.04	0.05	0.07	0.25	0.24	0.26
	Lead	0.05	0.07	0.14	0.26	0.29	0.33
	Lead-carbon	0.04	0.07	0.11	0.26	0.28	0.31
6	Resin	0.38	0.37	0.40	0.82	0.82	0.84
	Lead	0.40	0.43	0.50	0.85	0.88	0.94
	Lead-carbon	0.42	0.45	0.48	0.85	0.89	0.93
	No treatment	0.39	0.40	0.50			
7*	Resin	0.12	0.12	0.13	0.37	0.36	0.37
	Lead	0.15	0.16	0.20	0.37	0.37	0.45
	Lead-carbon	0.15	0.16	0.19	0.38	0.41	0.42
	No treatment	0.11	—	0.21			
8	Resin	0.26	0.28	0.26	0.48	0.49	0.46
	Lead	0.28	0.30	0.35	0.48	0.55	0.56
	Lead-carbon	0.27	0.31	0.32	0.49	0.53	0.54
	No treatment	0.27	0.32	0.34			
9	Resin				0.64	0.66	0.63
	Lead				0.62	0.70	0.72
	Lead-carbon				0.63	0.69	0.69
	No treatment				0.64	0.69	0.73
10	Resin				0.32	0.34	0.34
	Lead				0.33	0.39	0.42
	Lead-carbon				0.34	0.39	0.38
	No treatment				0.33	0.40	0.44
11	Resin				0.41	0.42	0.42
	Lead				0.42	0.48	0.51
	Lead-carbon				0.43	0.47	0.47
	No treatment				0.41	0.49	0.53

[1] The raw potatoes were purchased at various times over a period of six months at local vegetable markets. It was impossible to ascertain the potato variety or the storage history of each purchase.

[2] Resin = water-solution of potato extract passed through the ion-exchange columns.
Lead = water-solution of potato extract treated with excess neutral lead acetate solution and the excess lead removed with disodium phosphate.
Lead-carbon = same as "Lead" treatment, plus additional 10-minute treatment with Baker & Adamson Code No. 1551 decolorizing carbon (5 mg. carbon per ml. of solution).
No treatment = No chemical treatment of the water-solution of the potato extract.

[3] Calculated as dextrose.

* The reducing-sugar content of Samples No. 5 and No. 7 was below the minimum amount specified for the A.O.A.C. macro method.

TABLE 3.—*Percentages of total sugars in extracts of dehydrated white potatoes as determined by different methods of clarification and evaluation*

SAMPLE NO.	DESCRIPTION OF SAMPLE	METHOD OF CLARIFICATION[1]	METHODS USED TO EVALUATE SUGARS[2]			
			A.O.A.C.		SOMOYGI MODIFIED (15)	HASSID (7),(8)
			MACRO (2)	SOMOGYI (3),(14)		
12	Maine Green Mountain stored 3 yr. @ 34°F. in nitrogen	Resin	1.57	1.49	—	1.60
		Lead	1.62	1.68	—	1.89
		Lead-carbon	1.62	1.66	—	1.78
		No treatment	1.61	1.63	—	1.85
13	Maine Katahdin stored 3 yr. @ 34°F. in nitrogen	Resin	0.51	0.52	—	0.52
		Lead	0.54	0.62	—	0.82
		Lead-carbon	0.55	0.60	—	0.77
		No treatment	0.50	0.56	—	0.79
14	California Russet stored 4 mo. @ 75°F. in air	Resin	1.00	1.06	—	1.03
		Lead	1.09	1.12	—	1.40
		Lead-carbon	1.09	1.10	—	1.31
		No treatment	0.99	1.21	—	1.34
15	Idaho Russet stored 4 yr. @ 34°F. in air	Resin	1.59	1.53	1.63	1.54
		Lead	1.72	1.83	1.84	1.99
		Lead-carbon	1.72	1.73	1.84	1.88
		No treatment	1.72	1.66	1.83	2.04
16	Idaho Russet treated with 200 p.p.m. SO_2 before dehydration. Stored 3 yr. @ 34°F. in nitrogen	Resin	0.97	0.93	0.94	0.99
		Lead	1.01	1.08	1.07	1.16
		Lead-carbon	1.02	1.06	1.07	1.10
		No treatment	1.02	1.07	1.09	1.20
17	Oregon Netted Gem treated with 400 p.p.m. SO_2 before dehydration. Stored 4 yr. @ 75°F. in nitrogen	Resin	1.10	1.02	1.11	1.09
		Lead	1.27	1.40	1.39	1.58
		Lead-carbon	1.29	1.38	1.35	1.50
		No treatment	1.31	1.46	1.41	1.62
18	Oregon Netted Gem stored 4 yr. @ 75°F. in nitrogen	Resin	0.93	0.91	0.93	0.92
		Lead	1.14	1.19	1.19	1.43
		Lead-carbon	1.14	1.18	1.18	1.33
		No treatment	1.06	1.22	1.19	1.42
19	Variety unknown, commercially dehydrated. Stored 4 yr. @ 34°F. in air	Resin	2.81	2.67	2.85	2.73
		Lead	3.12	3.23	3.26	3.33
		Lead-carbon	3.15	3.27	3.23	3.25
		No treatment	3.13	3.12	3.25	3.29

[1] See Footnote No. 2, Table 2.
[2] Calculated as dextrose.

the different methods when lead or lead-carbon clarification was used. For example, in sample No. 8, when lead clarification was used, the reducing-sugar value obtained by the Hassid method was 30 per cent higher than by the macro method; when carbon treatment was included, the

value was 18 per cent higher. It is well demonstrated throughout Table 2 that, under the conditions of procedure, lead or lead-carbon clarification did not adequately remove the non-sugar reducing materials that affected the sugar values obtained by the Hassid method. The fact that the macro method gave the same values, regardless of the method of clarification, suggested the analysis of the untreated potato extract. The results showed (Table 2) that no clarification was necessary in the analysis for sugars in the raw potato by this method. The specificity of the A.O.A.C. macro method recommends it when sufficient sugar is available for the analysis.

When the comparison of procedures was made on dehydrated potatoes, only total sugars were determined, because more material was required than was suitable for handling to provide sufficient reducing sugars for all the methods. It should be noted, however, that the non-sugar reducing materials would be a much larger portion of the values obtained for reducing sugars than of those for total sugars (see example given later in this report).

All of the methods gave comparable sugar values for dehydrated potatoes when resin treatment was used (Table 3). The micro methods gave much higher sugar values for the lead and lead-carbon-treated samples than for the resin-treated samples. For example, in sample No. 17 (Table 3), the Somogyi modified method gave values 25 per cent and 21 per cent higher for the lead and lead-carbon treatments, respectively, and the Hassid method gave values 45 per cent and 37 per cent higher. Additional analyses were made on this sample with other decolorizing carbons (Darco KB and Darco G60) in the lead-carbon clarification procedure. Nevertheless, sugar values were consistently high in comparison with resin treatment.[3] The magnitude of this error is much greater in the determination of the reducing sugars, since the same non-sugar reducing materials are included as in the determination of total sugars. For example, the reducing-sugar values obtained for sample No. 17 by the Hassid method were 0.42 per cent when resin treatment was used, and 0.93 per cent with lead-carbon treatment. In this same experiment, the A.O.A.C. Somogyi method gave 0.42 per cent, and the Somogyi modified method gave 0.38 per cent, reducing sugars with resin treatment, against 0.55 per cent and 0.65 per cent, respectively, with lead-carbon treatment.

The data obtained on the dehydrated potato by the macro method were of considerable interest, since additional reducing materials appeared to have been formed during the dehydration of the potato. This was suggested by the fact that the sugar values were, in general, lower with resin treatment than with the other methods of clarification. For raw potatoes, the sugar values determined by the macro method were the same, with or without clarification.

[3] If the quantity of carbon is materially increased, adsorption of dextrose and levulose can be expected (4).

CONCLUSIONS

No clarification step was necessary when the A.O.A.C. macro method was used for the analysis of sugars in raw potatoes.

The removal of color was not necessarily an indication of the complete removal of non-sugar reducing materials.

Non-sugar reducing materials in raw potatoes which interfered with the micro methods of analysis, and in dehydrated potatoes which interfered with both macro and micro methods, were not completely removed by neutral lead acetate or decolorizing carbon.

When ion-exchange resins were used to remove non-sugar reducing materials, concordant results were obtained by all of the methods studied. Furthermore, the sugar values were lower than those obtained with other methods of clarification. (Experiments with pure solutions of dextrose, levulose, and sucrose, in concentrations comparable to those used in this investigation, showed that the resins did not adsorb these sugars.)

The greater accuracy of the sugar values obtained by the use of ion-exchange resins in this study warrants the investigation of their use in sugar determinations on other biological materials. Such studies are in progress at this laboratory.

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STABILIZATION OF STANDARD CAROTENE SOLUTIONS

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In the final measurement of the color of a carotene solution, in the determination of provitamin A pigments, it is necessary to use some apparatus such as an optical or a photoelectric colorimeter or a spectrophotometer. Very frequently it is necessary to recalibrate or check the electrical instruments and, in the case of optical colorimeters, a standard solution is required for comparison with the unknown solution.

A Coleman Model 11 Universal spectrophotometer is used for colorimetry in the author's laboratory. In order to establish a standard curve, crystalline carotene obtained from General Biochemicals, Inc., Chagrin Falls, Ohio, is further purified by precipitation with methanol (1). This purified carotene is used to make up standard solutions of varying concentrations. Transmittance readings from these solutions are then plotted on graph paper as a colorimetric curve. Petroleum ether or Skellysolve B is used as a solvent.

Pure carotene solutions deteriorate rapidly especially when exposed to daylight at room temperature. In fact, at the end of three days a standard carotene solution initially containing 100 micrograms per 100 ml. stored under the above conditions showed an 8 per cent loss of its potency; at the end of one week's storage, a 14 per cent loss; and after 12 weeks, bleaching and oxidation of the carotene had progressed to such an extent that there was only a negligible quantity of the original pigment left in the solution.

Therefore, because of the labile character of carotene, standard solutions cannot be stored for future use, but must be made up fresh as needed. Furthermore, crystalline carotene deteriorates quite rapidly after the sealed ampules, in which it is contained, are broken open.

Obviously a means of stabilizing standard carotene solutions would be desirable from the standpoint of both time and cost economy. Although a dilute solution of potassium dichromate has been used fairly successfully as a standard comparison solution in optical colorimeters, its use as a standard in photoelectric colorimeters is not recommended. The maximum light absorption for a potassium dichromate solution is at a much lower wave length (390 millimicrons) than for a carotene solution. Therefore,

the most ideal and practical method would be to employ a stabilized carotene solution for establishment of a colorimetric curve, as well as for a reference and comparison standard.

Several reports have been made on the antioxidant effect of tocopherols. Buxton (2) used mixed tocopherols as antioxidants for Vitamin A in fish liver oils. Hickman, *et al.*, (4) showed that Vitamin E (tocopherols) helped prevent the destruction of Vitamin A and carotene in the digestive tract. Quackenbush, *et al.*, (5) stated that tocopherols retard the oxidation of carotene.

In the author's laboratory it has been found that mixed tocopherols (alpha, beta, and gamma) are good antioxidants, if used in proportionally larger quantities, for stabilization of Vitamin A and carotene in solutions.

TABLE 1.—*Solutions stored in daylight (not direct sunlight) at room temperature (20°C.)*

MICROGRAMS OF CAROTENE PER 100 ML.	PER CENT REMAINING								
	START	3 DAYS	1 WEEK	3 WEEKS	5 WEEKS	7 WEEKS	9 WEEKS	12 WEEKS	AFTER CHROMATOGRAPHING
100 no Tocoph.	100	92	86	64	40	28	16	10	Negligible
200 no Tocoph.	100	95	90	77	70	66	50	42	30
100+.1 mg. Tocoph.	100	100	96	95	90	80	68	60	55
200+.1 mg. Tocoph.	100	100	98	96	95	90	85	79	76
100+.5 mg. Tocoph.	100	100	100	96	94	90	88	80	75
200+.5 mg. Tocoph.	100	100	100	100	97	94	90	88	87
100+1 mg. Tocoph.	100	100	100	100	100	100	100	100	97
200+1 mg. Tocoph.	100	100	100	100	100	100	100	100	98
100+5 mg. Tocoph.	100	100	100	100	100	100	100	100	100
200+5 mg. Tocoph.	100	100	100	100	100	100	100	100	95

Hence it was deemed necessary to investigate the possibility of tocopherols protecting carotene solutions, under various conditions, for at least 12 weeks. Because of the fact that carotene is sensitive to oxidation, and light, identical sets of solutions were stored in daylight (not direct sunlight) at room temperature (20°C.), in the dark at room temperature, and in the refrigerator.

In the preparation of the solutions, the tocopherols used were derived from a concentrate of mixed tocopherols produced by Distillation Products, Inc., which is claimed to contain 220 mg. of mixed tocopherols per gram. Our assay by the method of Devlin and Mattill (3), which is a modification of the older Emmerie and Engel method, showed 230 mg. mixed tocopherols per gram.

The solutions were stored for 12 weeks, and at regular intervals the color of each was measured in the spectrophotometer at 440 millimicron wave length. At the end of the 12-week period the solutions were chro-

TABLE 2.—*Solutions stored in dark at room temperature (20°C.)*

MICROGRAMS OF CAROTENE PER 100 ML.	PER CENT REMAINING								
	START	3 DAYS	1 WEEK	3 WEEKS	5 WEEKS	7 WEEKS	9 WEEKS	12 WEEKS	AFTER CHROMATOGRAPHING
100 no Tocoph.	100	100	99	92	84	80	70	68	51
200 no Tocoph.	100	100	100	95	90	84	75	74	69
100 + .1 mg. Tocoph.	100	100	100	100	92	90	88	85	79
200 + .1 mg. Tocoph.	100	100	100	100	95	94	94	94	90
100 + .5 mg. Tocoph.	100	100	100	100	100	100	99	98	92
200 + .5 mg. Tocoph.	100	100	100	100	100	100	100	99	95
100 + 1 mg. Tocoph.	100	100	100	100	100	100	100	100	99
200 + 1 mg. Tocoph.	100	100	100	100	100	100	100	100	100
100 + 5 mg. Tocoph.	100	100	100	100	100	100	100	100	100
200 + 5 mg. Tocoph.	100	100	100	100	100	100	100	100	98

matographed by the Wall-Kelley chromatographic procedure (6) to remove oxidation, and other degradation, products.

In Tables 1, 2, and 3 are given results of storage of 100 ml. solutions containing either 100 or 200 micrograms of carotene each, plus varying amounts of tocopherols.

SUMMARY AND CONCLUSIONS

By incorporating small quantities of mixed tocopherols or pure alpha tocopherol in petroleum ether solutions of purified carotene, an antioxidant effect is attained by which the carotene is preserved from deterioration for as long as 12 weeks. Such carotene solutions therefore, can be used as colorimetric standards over such a period. Without the use of tocoph-

TABLE 3.—*Solutions stored in refrigerator*

MICROGRAMS OF CAROTENE PER 100 ML.	PER CENT REMAINING								
	START	3 DAYS	1 WEEK	3 WEEKS	5 WEEKS	7 WEEKS	9 WEEKS	12 WEEKS	AFTER CHROMATOGRAPHING
100 no Tocoph.	100	100	100	98	96	90	88	87	84
200 no Tocoph.	100	100	100	99	97	95	95	92	88
100 + .1 mg. Tocoph.	100	100	100	100	100	98	97	95	94
200 + .1 mg. Tocoph.	100	100	100	100	100	99	98	97	94
100 + .5 mg. Tocoph.	100	100	100	100	100	100	100	99	96
200 + .5 mg. Tocoph.	100	100	100	100	100	100	100	98	96
100 + 1 mg. Tocoph.	100	100	100	100	100	100	100	100	100
200 + 1 mg. Tocoph.	100	100	100	100	100	100	100	100	100
100 + 5 mg. Tocoph.	100	100	100	100	100	100	100	100	100
200 + 5 mg. Tocoph.	100	100	100	100	100	100	100	100	100

erols in these solutions, destruction of the carotene is so rapid that they are no longer reliable after two or three days.

In order to stabilize standard carotene solutions it is necessary to add from 10 to 50 times as much tocopherols as there is carotene present. The use of more than 5 mg. of tocopherols per 100 ml. of petroleum ether may produce a measurable color which would be spuriously read as carotene.

The destruction of carotene in higher potency solutions is not as rapid as in dilute solutions, possibly due to an antioxidant effect by the carotene itself.

The conditions of storage of the stabilized carotene reference standards are not too consequential, although it is no doubt desirable to protect them from light as much as possible, and from heat.

Recovery of carotene from a solution which has been passed through a chromatographic column is not always 100 per cent, due to losses by manipulation, incomplete elution, oxidation, etc. However, in solutions of carotene which have been stored for long periods of time, and destruction of the provitamin A pigment is evident, the magnesia adsorption column removes degradation products.

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REFRACTIVE INDICES OF LACTOSE SOLUTIONS

By F. W. ZERBAN and JAMES MARTIN (New York Sugar Trade Laboratory, New York, N. Y.)

Emma J. McDonald (1) has recently published five-place tables of the refractive indices of lactose hydrate solutions at 20° and 25°C., for concentrations up to 16 per cent, based on weights in vacuo; also four-place tables for concentrations from 16 to 36 per cent at 25°; and for the entire concentration range at 15°. Similar measurements to the fifth decimal place were made in this laboratory during the spring of 1947, at 20°, upon solutions containing up to 32 per cent of lactose hydrate, based on weights in air. A Bausch and Lomb Precision Refractometer was used in this work and the same procedure was followed as previously described for measurements upon dextrose and invert sugar solutions (2). The lactose was prepared by recrystallizing the commercial product, by

the method given in National Bureau of Standards Circular C-440, p. 467, until the specific conductance of a 5-per cent solution did not decrease further. This required six recrystallizations. The purified sugar was first dried in air, and then in a thin layer at 50°C. in an air oven. The residual moisture in the hydrate was determined by heating for two hours at 100°C. at atmospheric pressure. It was found to equal 0.038 per cent, and this was corrected for in weighing out the sugar for each experiment. All solutions were allowed to stand for 24 hours to complete mutarotation. The results of the measurements upon 30 solutions are given in the second column of Table 1.

TABLE 1.—*Refractive indices of lactose hydrate solutions; observed data and values calculated from Equation 1*

CONCN. OF LACTOSE HYDRATE, WEIGHTS IN AIR, PER CENT	nD^{20} OBSERVED	nD^{20} CALCD. BY EQUATION 1	DEVIATION FROM FORMULA	SQUARE OF RESIDUALS $\times 10^{-10}$
1.004	1.33437	1.33439	−0.00002	4
1.973	1.33580	1.33577	+0.00003	9
3.002	1.33721	1.33723	−0.00002	4
4.024	1.33869	1.33869	0.00000	0
5.095	1.34020	1.34025	−0.00005	25
6.045	1.34166	1.34164	+0.00002	4
7.107	1.34323	1.34321	+0.00002	4
7.558	1.34389	1.34388	+0.00001	1
9.286	1.34646	1.34647	−0.00001	1
10.095	1.34771	1.34769	+0.00002	4
10.962	1.34901	1.34901	0.00000	0
11.666	1.35007	1.35003	−0.00001	1
13.349	1.35267	1.35268	−0.00001	1
14.370	1.35427	1.35426	+0.00001	1
15.192	1.35554	1.35555	−0.00001	1
16.288	1.35726	1.35728	−0.00002	4
17.360	1.35897	1.35895	+0.00002	4
17.620	1.35938	1.35939	−0.00001	1
17.709	1.35953	1.35953	0.00000	0
18.426	1.36071	1.36068	+0.00003	9
20.048	1.36332	1.36329	+0.00003	9
20.483	1.36394	1.36400	−0.00006	36
20.856	1.36464	1.36461	+0.00003	9
22.602	1.36747	1.36747	0.00000	0
23.675	1.36925	1.36925	0.00000	0
25.593	1.37255	1.37247	+0.00008	64
25.818	1.37275	1.37285	−0.00010	100
27.795	1.37624	1.37624	0.00000	0
30.207	1.38046	1.38044	+0.00002	4
32.076	1.38380	1.38378	+0.00002	4
				304

From these data the following equation was computed by the method of averages:

$$n_D^{20}, \text{ lactose hydrate} = 1.33299 + 0.0013887\ p + 0.0000076668\ p^2 - 0.00000012179\ p^3 + 0.0000000022419\ p^4 \quad (1)$$

The deviations from this formula are very small below the saturation point, and exceed 0.00005 only at a few concentrations in the supersaturation range above 20 per cent, where it is difficult to prevent incipient crystallization. For the same reason attempts to prepare solutions above about 32 per cent concentration were unsuccessful.

The sum of the squares of the residuals for the 30 experiments equals 304×10^{-10}, which is not as good as the figure 190×10^{-10}, found for our dextrose equation, based on 23 experiments; but much better than the figure 1550×10^{-10} for Saunders's levulose equation, based on the data of Jackson and Mathews, comprising 32 experiments (1). The respective variances for these three series of experiments are thus: 10.48×10^{-10}, 8.64×10^{-10} and 50.0×10^{-10}, respectively. The respective standard errors of estimate are then: 0.000032, 0.000029, and 0.000071.

Refractive indices of lactose hydrate solutions at 20° had previously been reported also by Tolman and Smith (3), and by Rothenfusser and Kotschopoulos (4). Table 2 shows a comparison of the various data, all based on weights in air. McDonald's concentration figures have been converted to this basis by calculation, and her refractive index values for concentrations above 16 per cent have been interpolated between those for 25° and 15°.

It is noted that the authors' figures are a few units of the fifth decimal place lower than McDonald's up to a concentration of 12 per cent lactose hydrate, and one or two units higher between 14 and 16 per cent. They check exactly to the fourth decimal place for concentrations from 17 to 32 per cent. On the whole, the agreement between the two series of results is very satisfactory, considering that the measurements were made by different observers using different instruments, and that McDonald computed quadratic equations by the method of least squares, while the authors used a quartic equation derived by the method of averages. The results of Rothenfusser and Kotschopoulos are slightly lower than those of the authors up to a concentration of 4 per cent, and above that become increasingly lower. The data of Tolman and Smith check, within about one unit of the fourth decimal place, with those of McDonald and of the authors.

Previous literature also gives figures for the refractive indices of lactose solutions, measured at 17.5°C. by Stolle (5), at 18.5°C. by Golse (6), and at 25°C. by Pulvermacher (7). These are compared in Table 3 with the values computed from McDonald's results. To find $n_D^{17.5}$ based on her values, the average between her figures for n_D^{20} and n_D^{15} was taken. The $n_D^{18.5}$ had to be computed by linear interpolation between n_D^{15} and n_D^{25},

TABLE 2.—*Comparison of various data for the refractive indices of lactose hydrate solutions at 20°C.*

CONCN. OF LACTOSE HYDRATE, WEIGHTS IN AIR, PER CENT	MCDONALD	MARTIN EQUATION 1	ROTHENFUSSER AND KOTSCHOFOULOS	TOLMAN AND SMITH
0.0	1.33299	1.33299	1.33300	1.3330
0.5	1.33370	1.33369	1.33368	
1.0	1.33440	1.33439	1.33439	1.3343
1.5	1.33511	1.33510	1.33507	
2.0	1.33583	1.33580	1.33578	1.3357
2.5	1.33654	1.33652	1.33649	
3.0	1.33726	1.33722	1.33720	
3.5	1.33798	1.33795	1.33791	
4.0	1.33871	1.33866	1.33862	
4.5	1.33943	1.33939	1.33930	
5.0	1.34016	1.34011	1.33999	
5.13	1.34035	1.34030		1.3402
5.5	1.34089	1.34084	1.34071	
6.0	1.34162	1.34158	1.34145	
7.0	1.34310	1.34305		
8.0	1.34458	1.34454		
9.0	1.34606	1.34604		
10.0	1.34757	1.34754		
10.13	1.34776	1.34774		1.3477
11.0	1.34908	1.34907		
12.0	1.35061	1.35059		
13.0	1.35214	1.35214		
14.0	1.35368	1.35369		
15.0	1.35523	1.35525		
15.13	1.35544	1.35546		1.3555
16.0	1.35680	1.35682		
17.0	1.3584	1.35840		
18.0	1.3600	1.36000		
19.0	1.3616	1.36160		
20.0	1.3632	1.36322		
21.0	1.36485	1.36484		
22.0	1.3665	1.36648		
23.0	1.36815	1.36813		
24.0	1.3698	1.36980		
25.0	1.3715	1.37147		
26.0	1.3732	1.37316		
27.0	1.3749	1.37487		
28.0	1.3766	1.37659		
29.0	1.37835	1.37833		
30.0	1.3801	1.38008		
31.0	1.38185	1.38185		
32.0	1.3836	1.38364		
34.0	1.3872			
36.0	1.3908			

because the values for 20° were not determined by her for concentrations above 16 per cent lactose. Pulvermacher's original results are for anhydrous lactose, and his concentrations were first converted into corresponding concentrations of lactose hydrate. McDonald's values for n_D^{25} were calculated by her equation 5 for concentrations up to 16 per cent lactose, and by her equation 6 for concentrations above that figure.

TABLE 3.—*Comparison of refractive indices of lactose hydrate solutions at 17.5, 18.5, and 25°*

CONCN. OF LACTOSE HYDRATE	$n_D^{17.5}$		CONCN. OF LACTOSE HYDRATE	$n_D^{18.5}$		CONCN. OF LACTOSE HYDRATE	n_D^{25}	
	STOLLE	MCD.		GOLSE	MCD.		PULV.	MCD.
per cent			*per cent*			*per cent*		
1.0007	1.33473	1.3346	4.65	1.3400	1.3397	1.35	1.3350	1.33441
1.9950	1.33588	1.3360	7.56	1.3433	1.3440	2.93	1.3380	1.33667
3.9489	1.33873	1.3388	14.35	1.3551	1.3544	6.10	1.3423	1.34127
7.7931	1.34448	1.3445	18.47	1.3604	1.3609	12.27	1.3517	1.35047
			22.51	1.3670	1.3675	17.96	1.3605	1.3594
			26.97	1.3746	1.3750	24.61	1.3716	1.3703

Table 3 shows satisfactory agreement between Stolle's and McDonald's values, but those of Golse are from 0.0007 lower to 0.0007 higher than McDonald's, and are evidently unreliable. Pulvermacher's figures are from 0.0006 to 0.0013 higher throughout, the discrepancies averaging 0.0011. But Pulvermacher's results for n_D^{25} of sucrose also average 0.0009 higher than those internationally accepted, and there must have been a systematic error in his measurements.

SUMMARY

The refractive indices of lactose hydrate solutions, containing up to 32 per cent of this sugar, have been determined at 20°C. to the fifth decimal place with a Bausch and Lomb Precision Refractometer. The results agree satisfactorily with those reported by McDonald, given by her to five decimal places in the range up to 16 per cent concentration, and interpolated from her figures for n_D^{25} and n_D^{15} at concentrations between 16 and 36 per cent. Results obtained by previous authors are compared with the more recent ones and critically discussed.

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DETERMINATION OF GLUCOSE, GALACTOSE, AND RHAMNOSE IN MIXTURES*

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In a study of the flavonol glycosides of buckwheat and other plants, it was desirable to determine the carbohydrates in their hydrolysates, both qualitatively and quantitatively. Chemical methods for the separation of glucose, rhamnose, and galactose (6) were not suitable because they were tedious, quantitatively inaccurate, and not successful in identifying sugars present in trace amounts. Polarimetric methods (3) for analyses of the possible combinations were not applicable because of interfering substance resulting from the hydrolysis, and also because of the limited quantities of most of the materials to be analyzed. A combination of methods—namely, filter paper chromatography (5), copper reduction (4), and yeast fermentation (1, 2, 7, 8)—was finally employed, with complete success. Although the procedure to be presented deals exclusively with mixtures of glucose, rhamnose, and galactose, the principles may be applied in analyses of mixtures of other sugars.

METHODS

Hydrolysis.—Several methods of hydrolysis were employed. However, boiling under reflux for 2 hours in 2.5 per cent sulfuric acid, or for 1 hour in 5 per cent sulfuric acid, gave the best recoveries of quercetin and sugars from rutin. Before analysis, the sulfuric acid was neutralized with sodium hydroxide.

Copper Reduction.—The sugar analyses were made by Schoorl's method (4).

Paper Chromatography.—The method of Partridge (5) was used. The solvent employed was the mixture of 40 per cent butanol, 10 per cent ethanol, and 50 per cent water, described in the original paper.

Fermentation.—A culture of the yeast *Saccharomyces bayanus* N.R.R.L.‡ No. 966) was used for fermenting glucose and one of *Saccharomyces carlsbergensis* (N.R.R.L. No. 379) was employed for fermenting glucose and galactose. The methods of fermentation used for the two yeasts were similar to those employed by Wise and Appling (8) and by Auernheimer *et al.* (2) except that the fermentation time was lengthened to 48 hours and the flasks were not shaken continuously.

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TABLE 1.—*Analyses of solutions of single sugars and synthetic mixtures of sugars by copper reduction and selective fermentation*[a]

SUGAR	SINGLE SUGARS			MIXTURES OF TWO SUGARS			MIXTURE OF THREE SUGARS
	GLUCOSE	GALACTOSE	RHAMNOSE	GLUCOSE+GALACTOSE	GLUCOSE+RHAMNOSE	GALACTOSE+RHAMNOSE	GLUCOSE+GALACTOSE+RHAMNOSE
Before Fermentation							
Mg. Sugar/aliq.[b]	55.20	54.76	50.00	*22.08+21.91*	*22.08+20.00*	*21.91+20.00*	*16.56+16.43+15.00*
Ml. Thio. Found	16.65	14.94	14.33	12.90	11.91	12.55	14.35
Ml. Thio. Calc.	16.69	15.11	14.33	13.13	12.17	12.84	14.39
% Recovery	99.8	98.9	100.0	98.3	97.9	97.8	99.7
After Fermentation with Yeast N.R.R.L. No. 966 to Remove Glucose Only							
Mg. Sugar/aliq.[b]	16.56	16.43	15.00	*16.55+16.43*	*16.55+15.00*	*16.43+15.00*	*9.94+9.86+9.00*
Ml. Thio. Found	0.00	4.79	4.68	4.70	4.50	9.00	5.60
Ml. Thio. Calc.	0.00	4.69	4.49	4.70	4.48	9.19	5.66
% Recovery	—	102.1	104.2	100.0	100.2	98.0	98.9
After Fermentation with Yeast N.R.R.L. No. 379 to Remove Glucose and/or Galactose							
Mg. Sugar/aliq.[b]	16.56	16.43	15.00	*16.55+16.43*	*16.55+15.00*	*16.43+15.00*	*9.94+9.86+9.00*
Ml. Thio. Found	0.00	0.00	4.60	0.00	4.33	4.38	2.68
Ml. Thio. Calc.	0.00	0.00	4.49	0.00	4.48	4.48	2.74
% Recovery	—	—	102.5	—	96.7	97.8	98.0

[a] Duplicate stock solutions of the single sugars were made to volume. Aliquots of these solutions were mixed to give the sugar mixtures. All analyses were then made in duplicate.

[b] Mg. sugar/aliquot = weight of the anhydrous sugar used in the final copper reduction; equivalent to the calculated ml. of thiosulfate.

PROCEDURE

Prepare a filter paper chromatogram of the solution to be analyzed. Determine the identity of the sugars present from the position of the spots. Dilute an aliquot of the original solution in the same manner as was done with the fermented samples. Using Schoorl's method, analyze an aliquot of this solution for total reducing sugars. Record the volume of 0.1 *N* thiosulfate required. If the chromatogram indicates that rhamnose is absent, but that either glucose or galactose or a mixture of these two is present, follow fermentation procedure A described below. If rhamnose is present in combination with either or both of the two sugars, follow procedure B. If the chromatogram indicates only rhamnose, fermentation is not necessary.

Fermentation Procedure A.—Ferment an aliquot of the solution with yeast N.R.R.L. No. 966 to remove glucose. Determine reducing materials on an aliquot of the fermented solution by Schoorl's method. Record the volume of 0.1 *N* thiosulfate. If the volume is zero, only glucose is present in the original solution. Calculate the weight of glucose present in the original solution by means of Schoorl's table from the volume of 0.1 *N* thiosulfate used before fermentation. If titration is required, the volume of 0.1 *N* thiosulfate is then equivalent to the galactose. From this volume, calculate the weight of glucose present in the original solution. If the two volumes are equal, only galactose is present in the original sample. Calculate the weight of galactose from either titration. If the two volumes are not equal, subtract the volume used by the solution after fermentation from the volume required for the total reducing sugars, to obtain the volume of 0.1 *N* thiosulfate equivalent to the glucose. Calculate the weight of glucose present in the original solution.

Fermentation Procedure B.—Ferment one aliquot of the original solution with yeast N.R.R.L. No. 966, and another with N.R.R.L. No. 379. The volume of 0.1 *N* thiosulfate after fermentation with yeast No. 379 is equivalent to the rhamnose in the aliquot taken for copper reduction, since both the glucose and/or galactose would be destroyed. Calculate the weight of rhamnose present in the original solution. The volume of 0.1 *N* thiosulfate after fermentation with yeast No. 966 is equivalent to the rhamnose and/or galactose, since only glucose would be destroyed. The difference between the volumes of 0.1 *N* thiosulfate required after fermentation with yeast No. 966 and No. 379 is equivalent to the galactose present. Calculate the weight of galactose present in the original solution. Subtract the volume of 0.1 *N* thiosulfate required after fermentation with yeast N.R.R.L. No. 966 from the volume of 0.1 *N* thiosulfate used in the aliquot analyzed before fermentation to obtain the volume of 0.1 *N* thiosulfate equivalent to the glucose. Calculate the weight of glucose in the original solution.

RESULTS

Table 1 shows the results obtained by using the above-described procedure with pure sugars, and with the several possible combinations of these sugars. To simplify the presentation of the data, the values are reported as ml. of 0.1 *N* thiosulfate rather than as mg. of sugar, since it is impossible to evaluate recoveries for any one step of the over-all procedure without incorporating any errors present in all the other steps. The experimental titres for a given weight of sugar are compared with the calculated titres. Percentage recovery is the ratio of ml. of thiosulfate found to the calculated volume of thiosulfate. Examples of an application of the method to the analysis of rutin are given in Table 2.

TABLE 2.—*Recoveries of Quercetin and Sugars from Rutin*

WEIGHT OF RUTIN	H_2SO_4		TIME OF HYDROLYSIS	RECOVERY OF THEORETICAL		
	CONC.	VOL.		QUERCETIN[a]	RHAMNOSE	GLUCOSE
g.	*per cent*	*ml.*	*hrs.*	*per cent*	*per cent*	*per cent*
0.5000	2.5	25	2	100.3	95.5	96.0
.5000	5.0	25	1	100.6	95.2	95.8

[a] Quercetin was filtered on Gooch crucible after hydrolysis, washed with water, dried to constant weight at 110°C. and weighed.

DISCUSSION

As indicated by the data, synthetic mixtures of glucose, galactose, and rhamnose can be analyzed, with recoveries of 98 to 104 per cent; hydrolysates of a flavonol glycoside can be analyzed, with recoveries of approximately 96 per cent. The value 96 per cent is probably low because of destruction of sugar during the hydrolysis. By use of the qualitative filter paper chromatogram, the amount of fermentation and chemical work required is reduced to the minimum. Application of the technique to other mixtures is being investigated.

SUMMARY

A method is presented for the analyses of mixtures of glucose, galactose and rhamnose in hydrolysates of flavonol glycosides. The sugar determinations are made by Schoorl's copper reduction method before and after fermentation by two yeasts capable of selective destruction of glucose and of glucose and galactose, respectively. Filter paper chromatography is used for qualitatively identifying the sugars.

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STUDIES ON COAL-TAR COLORS, VI*

D&C REDS NOS. 14, 15, 16, AND 31

By KENNETH A. FREEMAN and CHARLES STEIN (Division of Cosmetics, Food and Drug Administration, Federal Security Agency, Washington 25, D. C.)

This paper represents the sixth in a series of papers (1, 2, 3, 4, 5) describing the preparation of purified samples of certifiable colors and the optical properties of solutions of those colors.

In this study the preparation, analysis, and optical properties in various solutions of 1-(2-carboxyphenylazo)-2-naphthol, the sodium, barium and calcium salts of which are certifiable as D&C Reds Nos. 14, 15, and 16 (6), respectively, and 1-phenylazo-3-carboxy-2-naphthol, the calcium salt of which is certifiable as D&C Red No. 31 (6), are described.

EXPERIMENTAL

Preparation of D&C Red No. 14.—Anthranilic acid and β-naphthol were recrystallized twice from water and benzene, respectively. Ten grams of anthranilic acid were dissolved in 300 ml. of water containing 20 ml. of conc. hydrochloric acid and the solution cooled to 5°C.; 5.5 grams of sodium nitrite previously dissolved in water was then added, and the mixture kept at 5°C. for one hour, stirring constantly. The excess nitrite was then destroyed with an excess of sulfamic acid. The solution of the resulting diazonium compound was added to a cold solution (5°C.) of 5 grams of sodium hydroxide, 15 grams of sodium carbonate, and 10.7 grams of β-naphthol in 500 ml. of water. The mixture was stirred continously for one hour at a temperature of 5°C. and then was allowed to warm to room temperature. The product was collected in a Büchner funnel, and washed with two 50 ml. portions of water followed by two 50 ml. portions of ethyl alcohol and, finally, by several small portions of ether. The dye was dried thoroughly on a steam bath; then pulverized in a mortar and dried overnight at 135°C. Yield—18 grams.

Preparation of 1-(2-carboxyphenylazo)-2-naphthol-(color acid of D&C Red No. 14).—Ten grams of the D&C Red No. 14 prepared above was suspended in 100 ml. of (1+5) HCl and the suspension warmed on a steam bath for 30 minutes. After cooling, the product was collected in a Büchner funnel and then recrystallized three times from glacial acetic acid.

Analysis:
Calculated: N, 9.59%
Found: N, 9.60%

Preparation of D&C Red No. 15.—A saturated solution of the sodium

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salt in boiling water was prepared and filtered, the filtrate heated to boiling and the barium salt precipitated by the addition of a slight excess of 10 per cent barium chloride solution. The product was collected in a Büchner funnel and washed with three 50 ml. portions of hot water, then with two 50 ml. portions of 95 per cent alcohol and, finally, with several small portions of ether. The material was dried on a steam bath, pulverized, and then dried for several hours at 135°C.

Analysis:
Calculated: Ba 19.1%
Found: Ba 18.9%
Titration with standard $TiCl_3$ soln: 99.7%

Preparation of the sodium salt of 1-phenylazo-3-carboxy-2-naphthol.—Aniline was purified by distillation. A commercial sample of 3-hydroxy-2-naphthoic acid was recrystallized three times from hot 50 per cent ethyl alcohol. This material melted at 221°C.; the reported value is 216°C. (7). Recrystallization of a small portion of the material from 95 per cent ethyl alcohol did not alter the melting point.

Ten grams of aniline was dissolved in 300 ml. of water containing 25 ml. of conc. hydrochloric acid and the solution cooled to 5°C. While stirring mechanically, 50. ml of a cold (5°C.) 16 per cent sodium nitrite solution was added and the solution held at 5°C. for one hour; then the excess nitrous acid was destroyed with sulfamic acid. The solution of the resulting diazonium compound was added to a cold (5°C.) solution of 20.5 grams of 3-hydroxy-2-naphthoic acid, 15 grams of sodium hydroxide and 15 grams of sodium carbonate, dissolved in 500 ml. of water. The mixture was stirred continuously for 30 minutes at 5°C., and then allowed to warm to room temperature. The product was collected on a Büchner funnel, and the material on the filter was washed successively with about 250 ml. of ca 0.1 *N* sodium hydroxide, about 200 ml. of distilled water, three 50 ml. portions of ethyl alcohol, and two 25 ml. portions of ether.

Preparation of 1-phenylazo-3-carboxy-2-naphthol.—The product obtained above was converted to the color acid and recrystallized three times from glacial acetic acid in the manner described for the preparation of 1-(2- carboxyphenylazo)-2-naphthol. The product crystallized in long, dark, red needles, m.p. 233°C. The melting point given in the Colour Index (8) is 232°C.

Analysis:
Calculated: N, 9.58%
Found: N, 9.60%

Five grams of the purified color acid was dissolved in about 300 ml. of hot ethyl alcohol and added slowly with rapid stirring to 500 ml. of hot water containing 10 grams of calcium acetate. After all of the color acid solution had been added, the resulting suspension was cooled to room

temperature and filtered on a Büchner funnel. The product remaining on the filter was resuspended in 500 ml. of distilled water, filtered on a Büchner funnel, and washed on the filter with three 50 ml. portions of ethyl alcohol and two 25 ml. portions of ether. The product was dried on a steam bath, pulverized in a mortar and dried at 135°C.

Analysis:
Calculated: N, 9.0 % Ca, 6.44%
Found: N, 9.01% Ca, 6.39%

SPECTROPHOTOMETRIC DATA

All spectrophotometric measurements were made with a General Electric recording spectrophotometer utilizing a wave length band of 8 mμ. Matched 1 cm. pyrex cells were used, and all solutions were allowed to come to room temperature before being made to volume. The *p*H values of the solutions were measured with a glass electrode *p*H meter in the range of *p*H 1.0 to 11.0, and the other values estimated. (Since the solvent in all cases was 50% alcohol it is to be understood that the term *p*H, as used here, is the apparent value.)

(*a*) *1-(2-carboxyphenylazo)-2-naphthol:*

(1) A 13.80 mg. sample of the purified color, weighed on a semimicro balance, was dissolved in exactly 500 ml. of 95% alcohol. Fifty ml. aliquots of this solution were diluted to 100 ml. with aqueous solutions of acid, alkali, and buffers, and the *p*H values of the solutions determined. The spectrophotometric data obtained from these solutions are shown in Figure 1.

(2) A 14.78 mg. sample of the purified color was dissolved in exactly 500 ml. of 95% alcohol. Aliquot portions of this solution were then diluted with water containing about 50 grams of ammonium acetate per liter, and the absorption spectra determined. The results are shown in Table 1 and Figure 2.

(*b*) *1-phenylazo-3-carboxy-2-naphthol:*

(1) Solutions containing 17.70 mg. of the purified color per liter were prepared as described under *a*(1). The spectrophotometric data obtained from these solutions are shown in Figure 3.

(2) Solutions of the purified color were prepared as described under *a*(2), except that the final dilutions were made with water containing 10 ml. of conc. hydrochloric acid per liter, and the absorption spectra determined. The results are shown in Table 2 and Figure 4.

DISCUSSION

The absorption curve of 1-(2-carboxyphenylazo)-2-naphthol in (1+1) alcohol-water solution is little changed with varying *p*H values between *p*H 1 and 13. At *p*H values between 7.0 and ca 12.0 there is practically no change in the curve. In this *p*H range the wave length of maximum ab-

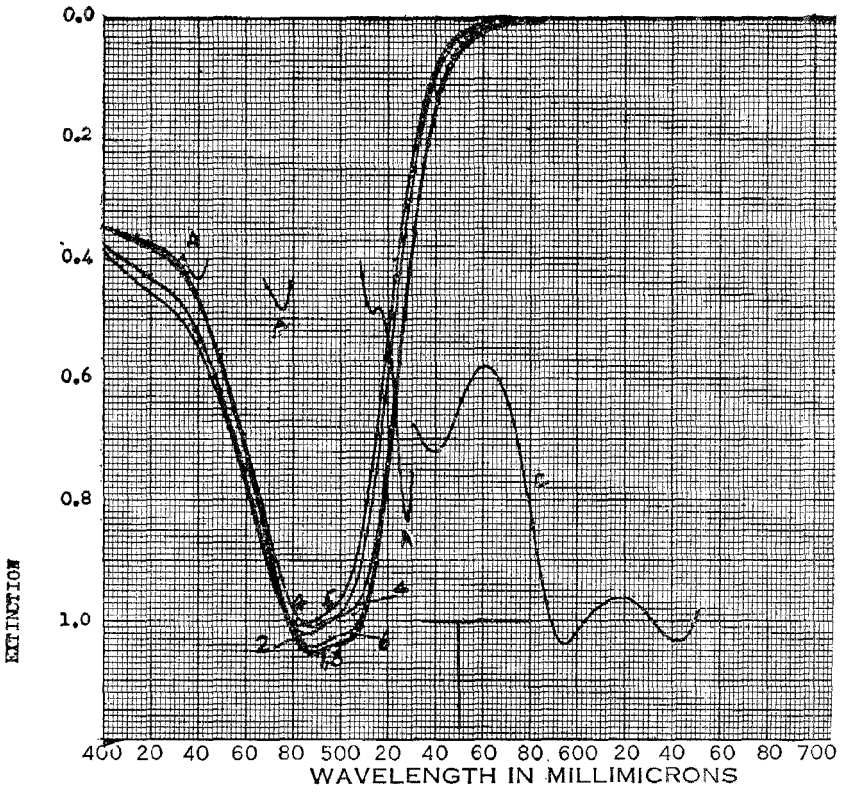

FIG. 1.—Absorption curves of D&C Red No. 14 in (1+1) alcohol-water solution at various *p*H levels (conc. 13.80 mg. per liter).

Curve 1—*p*H 7.1
Curve 2—*p*H 4.7
Curve 3—*p*H 8.2
Curve 4—*p*H 13.0
Curve 5—*p*H 1.2
Curve 6—*p*H 12.3

*p*H for curves 4 and 6 calculated, others measured with *p*H meter.

A = Corning Didymium Glass 512, 6.0 mm. (Absorption peaks at 400.4, 441.4, 477.1, 529.0, and 684.8 mμ).

C = Signal Lunar White Glass-II-6946236.

sorption is 488 mμ ± 2 mμ. Solutions of this color at concentrations between 3.70 and 14.78 mg./liter were found to follow Beer's law (with a maximum deviation of 0.8% and an average deviation of 0.5%). The average extinction per milligram per liter in (1+1) alcohol-water solution at a *p*H between 7.0 and 12.0 and at 488 mμ was found to be 0.0770. This figure is based on the results of four determinations. Solutions stored at room temperature for 24 hours gave curves identical with those of freshly prepared solutions.

The absorption curve of 1-phenylazo-3-carboxy-2-naphthol in (1+1) alcohol-water solution changes in shape, wave length of maximum ab-

TABLE 1.—*Extinction values of purified 1-(2-carboxyphenylazo)-2-naphthol dissolved in 50% alcohol*

CURVE NO.	CONCENTRATION MG./LITER	$E_{433\,m\mu}$	$\frac{E_{433\,m\mu}}{\text{CONCENTRATION}}$
1	14.78	1.133	0.0767
2	11.09	0.849	0.0766
3	7.39	0.568	0.0769
4	3.70	0.287	0.0776
Average			0.0770

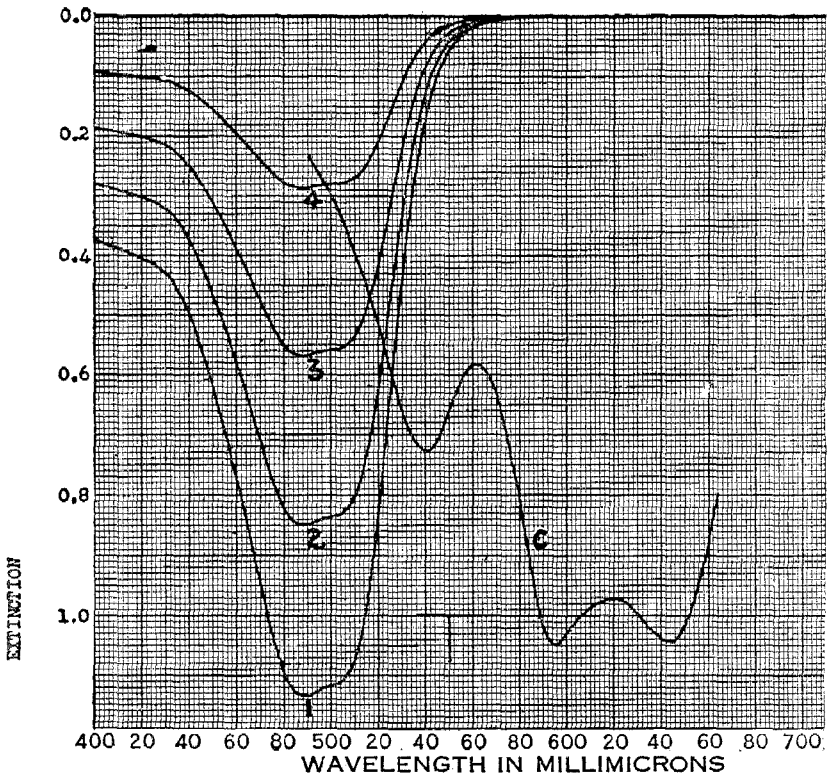

FIG. 2.—Absorption curves of D&C Red No. 14 in (1+1) alcohol-water solution buffered with ammonium acetate.

Curve 1—14.78 mg. per liter
Curve 2—11.09 mg. per liter
Curve 3— 7.39 mg. per liter
Curve 4— 3.70 mg. per liter
Cells—1 cm.

C = Signal Lunar White Glass-H-6946236.

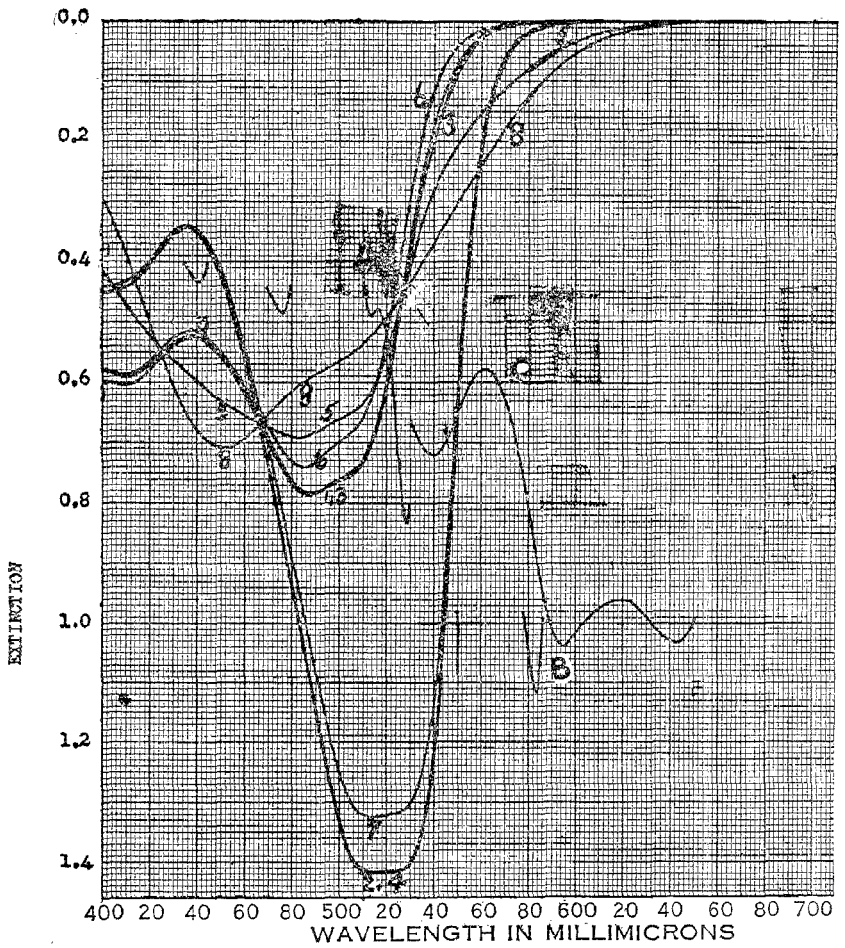

FIG. 3.—Absorption curves of D&C Red No. 31 in (1+1) alcohol-water solution at various *p*H levels (conc. 17.70 mg. per liter).

Curve 1—*p*H 6.7	Curve 5—*p*H 13.0
Curve 2—*p*H 3.9	Curve 6—*p*H 8.9
Curve 3—*p*H 6.9	Curve 7—*p*H 4.3
Curve 4—*p*H 1.2	Curve 8—*p*H 14.0

*p*H for curves 5 and 8 calculated, others measured with *p*H meter.

A = Corning Didymium Glass 512, 6.0 mm. (Absorption peaks at 400.4, 441.4, 477.1, 529.0, and 684.8 mμ).

B = Corning Didymium Glass 592, 4.02 mm. (Absorption peak at 583.7 mμ).

C = Signal Lunar White Glass-H-6946236.

sorption, and unit extinction with varying values above *p*H 4.0. At *p*H values between 1.0 and 4.0 the curve is unchanged with variations in *p*H. Solutions of this color should, therefore, be adjusted to *p*H values be-

TABLE 2.—*Extinction values of purified 1-phenylazo-3-carboxy-2-naphthol dissolved in 50% alcohol*

CURVE NO.	CONCENTRATION MG./LITER	$E_{515\ m\mu}$	$\frac{E_{515\ m\mu}}{\text{CONCENTRATION}}$
1	3.31	0.268	0.0810
2	6.62	0.535	0.0808
3	13.23	1.062	0.0803
Average			0.0807

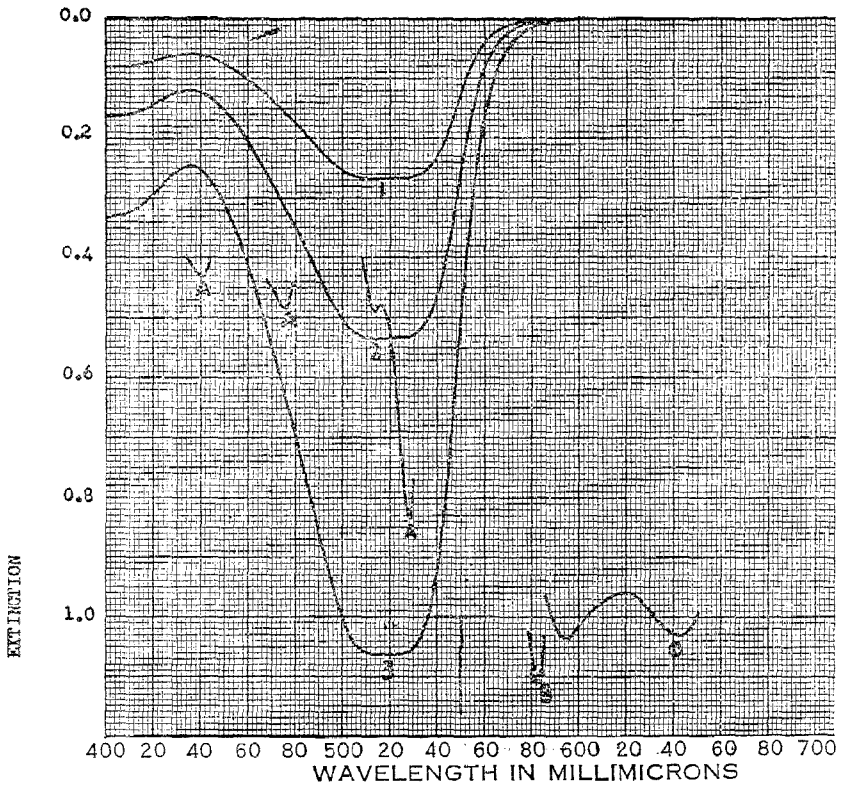

FIG. 4.—Absorption curves of D&C Red No. 31 in (1+1) alcohol-water solution adjusted to *p*H 2.5.

Curve 1— 3.31 mg. per liter
Curve 2— 6.62 mg. per liter
Curve 3—13.23 mg. per liter
Cells—1 cm.

A = Corning Didymium Glass 512, 6.0 mm. (Absorption peaks at 400.4, 441.4, 477.1, 529.0, and 684.8 mμ).
B = Corning Didymium Glass 592, 4.02 mm. (Absorption peak at 583.7 mμ).
C = Signal Lunar White Glass-H-6946236.

tween 1.0 and 4.0 before spectrophotometric measurements are made. The wave length of maximum absorption is 518 mμ ± 6 mμ. An unusual characteristic of curves of this color in acid solution is the broad, flat peak, there being practically no difference in the absorption between 512 and 524 mμ. Solutions of this color at concentrations between 3.31 and 13.23 mg./liter were found to follow Beer's law (with a maximum deviation of 0.5% and an average deviation of 0.3%). The average extinction per milligram per liter in (1+1) alcohol-water solution at a *p*H between 1.0 and 4.0 and at 518 mμ was found to be 0.0807. This figure is based on the results of three determinations. Solutions stored for 24 hours at room temperature gave curves identical with those of freshly prepared solutions.

APPLICATION TO COMMERCIAL SAMPLES

Two commercial samples each of D&C Red No. 15, Barium Lake; and D&C Red No. 31, Calcium Lake, were analyzed spectrophotometrically. The samples were dissolved by refluxing with 250 ml. of 95% alcohol containing one ml. of conc. hydrochloric acid, cooling and diluting to exactly 500 ml. with 95% alcohol. Aliquots of the D&C Red No. 15, Lake, were diluted with equal volumes of water containing 50 grams of sodium acetate per liter. Aliquots of the solutions of D&C Red No. 31 were diluted with water containing 10 ml. of conc. hydrochloric acid per liter. The results of spectrophotometric examination of the resulting solutions are shown in Table 3.

TABLE 3.—*Analysis of commercial samples*

D&C RED NO. 15, BARIUM LAKE			D&C RED NO. 31, CALCIUM LAKE		
	DYE SPECTROPHOTOMETRICALLY	DYE BY TITRATION WITH $TiCl_3$		DYE SPECTROPHOTOMETRICALLY	DYE BY TITRATION WITH $TiCl_3$
	per cent	*per cent*		*per cent*	*per cent*
Sample No. 1	15.5	15.8	Sample No. 1	44.7	44.0
Sample No. 2	84.5	85.3	Sample No. 2	85.5	84.5

SUMMARY

Purified samples of D&C Red No. 14, 15 and their color acid 1-(2-carboxyphenylazo)-2-naphthol, and D&C Red No. 31 and its color acid (1-phenylazo-3-carboxy-2-naphthol), were prepared and studied spectrophotometrically.

The curve obtained from 1-(2-carboxyphenylazo)-2-naphthol in (1+1) alcohol-water solution adjusted to *p*H values between 7.0 and 12.0 is unchanged with changes in *p*H. At *p*H values below 7.0 and above 12.0 the curve changes slightly in regard to wave length of maximum absorption and general shape, but changes considerably in extinction. At *p*H values between 7.0 and 12.0 the wave length of maximum absorption is 488

mμ ± 2 mμ. Beer's law is shown to be applicable to solutions containing 3.7 to 14.8 mg. of dye per liter. The average extinction per mg. per liter is 0.0770 ± 0.006 at 488 mμ.

The curve obtained from 1-phenylazo-3-carboxy-2-naphthol in (1+1) alcohol-water solution adjusted to *p*H values between 1.0 and 4.0 is unchanged with changes in *p*H. At *p*H values above 4.0 the curve changes in regard to wave length of maximum absorption, extinction, and general shape of the curve. At *p*H values between 1.0 and 4.0 the wave length of maximum absorption is 518 mμ ± 6 mμ, there being practically no difference in absorption between 512 and 524 mμ. Beer's law is shown to be applicable to solutions containing 3.31 to 13.23 mg. of dye per liter. The average extinction per mg. per liter is 0.087 ± 0.003 at 518 mμ.

These data have been applied to the determination of "pure color" in commercial samples of D&C Red No. 15, Barium Lake, and to D&C Red No. 31, Calcium Lake.

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STUDIES ON COAL-TAR COLORS, VII*

D&C RED NO. 34

By KENNETH A. FREEMAN and CHARLES GRAICHEN (Division of Cosmetics, Food and Drug Administration, Federal Security Agency, Washington 25, D.C.)

This is the seventh in a series of papers† dealing with the preparation, analysis, and optical properties of solutions of certifiable coal-tar colors. In this paper, the results obtained in the investigation of 4-(1-sulfo-2-

* Presented at the Annual Meeting of the Association of Official Agricultural Chemists, held at Washington, D. C., October 10–12, 1949.
† For references to previous papers see No. VI. *This Journal*, p. 718.

naphthylazo)-3-hydroxy-2-naphthoic acid, the calcium salt of which is certifiable as D&C Red No. 34,[1] are described.

EXPERIMENTAL

Purification of intermediates

Tobias acid (2-naphthylamine-1-sulfonic acid).—A sample of commercial Tobias acid was recrystallized three times from warm (1+1) alcohol-water solution. The temperature was not allowed to exceed 70°C. in order to avoid possible rearrangement of the sulfonic acid group.

Analysis:
Calculated: S, 14.30%
Found: S, 14.34%

3-Hydroxy-2-naphthoic acid.—A sample of the commercial material was recrystallized three times from hot (1+1) alcohol-water solution. The melting point of the purified material (221°C.) was not changed by a fourth recrystallization from hot 95% alcohol.

Preparation of D&C Red No. 34.—Thirty grams of Tobias acid was dissolved in 950 ml. of distilled water containing 7.0 grams of sodium hydroxide. Forty-seven ml. of conc. hydrochloric acid was added slowly, with vigorous stirring, to form a finely divided suspension of the acid. A 10% sodium nitrite solution cooled to below 5°C. was added to the cold suspension (5°C.) and the mixture stirred for one hour at 5°C. The excess nitrite was then destroyed with excess sulfamic acid. The suspension of the resulting diazonium compound was immediately added to a cold (5°C.) solution of 27 grams of 3-hydroxy-2-naphthoic acid, 12.9 grams of sodium hydroxide and 29 grams of sodium carbonate in one liter of distilled water. The mixture was stirred continuously for one hour at a temperature of 5°C. and then was allowed to warm to room temperature. The precipitated product was collected on a Büchner funnel and recrystallized from distilled water. The dye was again collected on the Büchner funnel and washed with three 100-ml portions of ethyl alcohol, followed by several small portions of ether.

A part of the dye was suspended in water containing twice the amount of calcium chloride calculated to give the calcium salt of the dye, and boiled for 30 minutes. The material was filtered while hot and was washed successively with water, alcohol, and ether. The ether was evaporated on a steam bath and the dye pulverized in a mortar.

The dried color absorbs moisture so rapidly from the air that accurate weighing is impractical. The material was therefore spread out on a large watch-glass in a dust-free atmosphere until the moisture content had reached equilibrium. The results of the analyses are shown in Table 1.

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[1] Service and Regulatory Announcements, F.D.C. No. 3, Food and Drug Administration.

There is a difference in water content of 1.66% between the dye dried at atmospheric pressure and at 2 mm. pressure. Since this is equivalent to 0.48 mols of water, it is probable that one-half molecule of water of crystalization remains in the product dried at 135°C. at atmospheric pressure.

SPECTROPHOTOMETRIC DATA

All spectrophotometric measurements were made with a General Electric recording spectrophotometer, utilizing a wave length band of 8 mμ. Matched 1 cm. Pyrex cells were used and all solutions were allowed to come to room temperature before being made to volume.

(a) A 15.17 mg. sample (corrected to the anhydrous color) of the puri-

TABLE 1.—*Analysis of purified D&C Red No. 34*

DETERMINATION	CALCULATED FOR PURE COLOR	FOUND	PURE DYE BY CALCULATION
	per cent	*per cent*	*per cent*
Nitrogen	6.08	5.42	89.1
Sulfur	6.96	6.20	89.1
Calcium	8.70	7.68	88.2
Titration with 0.1 *N* $TiCl_3$	—	—	88.4
Volatile matter at 135°C. and atm. press.	—	8.94	—
Volatile matter at 138°C. and 2 mm. press.	—	10.60	—
Titration of the dried material with 0.1 *N* $TiCl_3$	—	—	99.3*

* This figure is probably low because of the absorption of moisture by the sample while weighing.

fied color was weighed on a semi-micro balance and dissolved by refluxing for 15 minutes in 250 ml. of 95% alochol containing 1 ml. of conc. hydrochloric acid. The solution was cooled to room temperature and diluted to exactly 500 ml. with 95% alcohol. 50 ml. aliquots of this solution were diluted to 100 ml. with aqueous solutions of acid, alkali, or buffers. The *p*H values of these solutions were determined with a glass electrode *p*H meter in the range of *p*H 1.0 to 11.0, and the other values estimated. (Since it is doubtful whether the indicated *p*H values of alcoholic solutions are true values, it is to be understood that the term *p*H, as used here is the apparent value.) The spectrophotometric data obtained from these solutions are shown in Figure 1.

(b) A 17.09 mg. sample (corrected to the anhydrous color) of the purified color was dissolved in exactly 500 ml. of 95% alcohol as described in (a). Aliquot portions of this solution were then diluted with water containing 10 ml. of conc. hydrochloric acid per liter. The spectrophotometric data obtained from these solutions are shown in Table 2 and Figure 2.

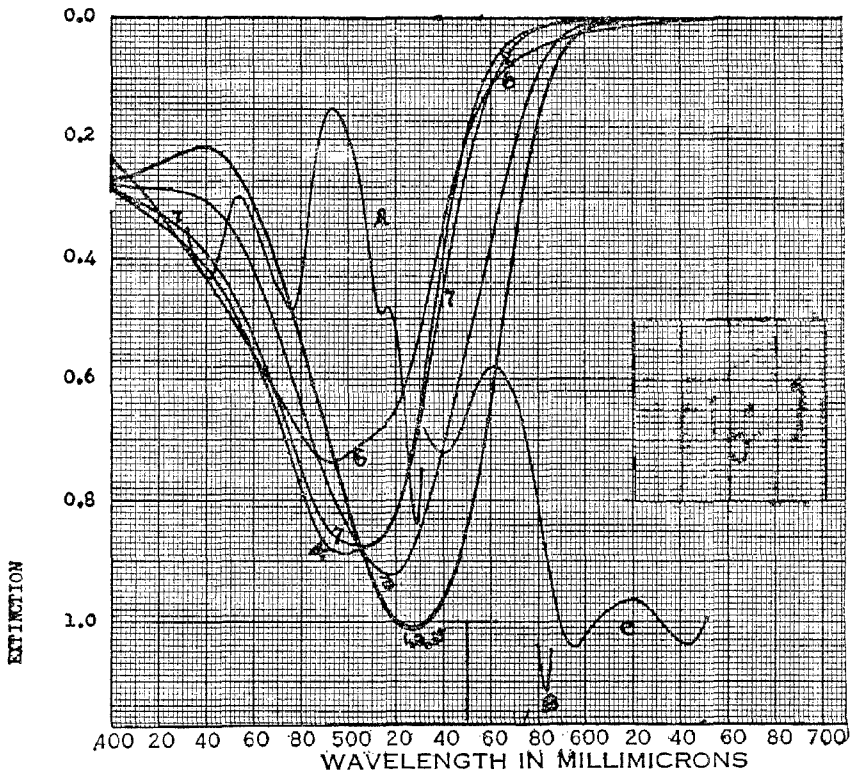

FIG. 1.—Absorption curves of D&C Red No. 34 in alcohol-water solution of various *p*H levels (conc. 15.17 mg. per liter).

Curve 1—*p*H 2.2
Curve 2—*p*H 3.4
Curve 3—*p*H 5.8
Curve 4—*p*H 13.0
Curve 5—*p*H 1.1
Curve 6—*p*H 14.0
Curve 7—*p*H 8.4

A = Corning Didymium Glass 512, 6.0 mm. (Absorption peaks at 400.4, 441.4, 477.1, 529.0, and 684.8 mμ).
B = Corning Didymium Glass 592, 4.02 mm. (Absorption peak at 583.7 mμ).
C = Signal Lunar White Glass-H-6946236.

DISCUSSION

The absorption curve of D&C Red No. 34 in (1+1) alcohol-water solution changes in shape, wave length of maximum absorption, and unit extinction, with varying values above *p*H 3.5. At *p*H values between 1.0 and 3.5 the curve is unchanged with variations in *p*H. Solutions of this color should therefore be adjusted to *p*H values between 1.0 and 3.5 before spectrophotometric measurements are made. The wave length of maximum absorption is 528 mμ ± 2 mμ.

Solutions of this color at concentrations between 6.8 and 17 mg./liter

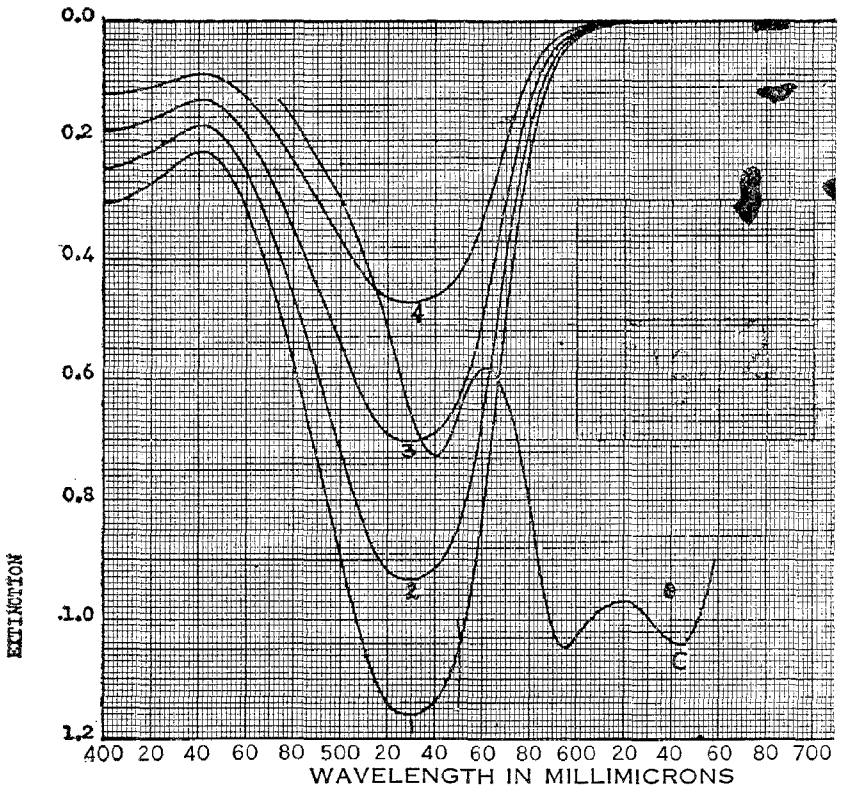

FIG. 2.—Absorption curves of D&C Red No. 34 in (1+1) alcohol-water adjusted to *p*H 2.6.

Curve 1—17.09 mg./liter
Curve 2—13.68 mg./liter
Curve 3—10.25 mg./liter
Curve 4—6.84 mg./liter
Cells—1 cm.

C = Signal Lunar White Glass-H-6946236.

All concentrations calculated as anhydrous colors.

TABLE 2.—*Extinction values of purified D&C Red No. 34 dissolved in 50% alcohol*

CURVE NO.	CONCENTRATION MG/LITER	$E_{528\ m\mu}$	$\frac{E_{528\ m\mu}}{\text{CONCENTRATION}}$
1	17.09	1.160	0.0679
2	13.68	0.933	0.0682
3	10.25	0.703	0.0686
4	6.84	0.471	0.0688
	Average		0.0684

follow Beer's law (with a maximum deviation of 0.7% and an average deviation of 0.5%). The average extinction per milligram per liter in (1+1) alcohol-water solution at a *p*H between 1.0 and 3.5, and at 528 mμ, was found to be 0.0684. This figure is based on the results of four determinations. Solutions stored for 24 hours at room temperature gave curves identical with those of freshly prepared solutions.

APPLICATION TO COMMERCIAL SAMPLES

Two commercial samples of D&C Red No. 34, Calcium Lake, were analyzed spectrophotometrically following the procedure described for the standard sample. The data are shown in Table 3.

TABLE 3.—*Analysis of commercial samples of D&C Red No. 34, Calcium Lake*

	DYE SPECTROPHOTOMETRICALLY	DYE BY TITRATION WITH $TiCl_3$
	per cent	*per cent*
Sample No. 1	60.9	61.5
Sample No. 2	69.5	70.5

SUMMARY

Purified D&C Red No. 34 was prepared and studied spectrophotometrically. The curve obtained from the color in (1+1) alcohol-water solution, adjusted to *p*II values between 1.0 and 3.5, is unchanged with changes in *p*H. At *p*H values above 3.5 the curve changes in regard to wave length of maximum absorption, extinction, and general shape. At *p*H values between 1.0 and 3.5 the wave length of maximum absorption is 528 mμ+2 mμ. Beer's law is shown to be applicable to the solutions containing 6.84 to 17.09 mg. of dye per liter. The average extinction per mg. per liter is 0.0684 $\pm$ 0.004 at 528 mμ. These data have been applied to the determination of "pure color" in commercial samples of D&C Red No. 34, Calcium Lake.

WATER-INSOLUBLE FATTY ACIDS AND BUTYRIC ACID IN CREAM AND BUTTER*

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INTRODUCTION

Spoilage resulting from improper practices in the production of cream intended for use in the manufacture of butter has long been a problem,

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† W. B. White, Chief.

not only for the manufacturer, but also for the officials charged with the enforcement of food laws. One of the corrective steps taken by industry has been the inauguration of widespread cream improvement programs. While considerable improvement has resulted from these programs, decomposed cream, unfit for human consumption, is still being used in the manufacture of butter.

The time required for a given raw cream to become decomposed will depend chiefly on the temperature at which it is held unrefrigerated but decomposition is progressive in every case. The chemical break-down which occurs during these progressive changes seems to center mainly in three cream constituents. Lactose is degraded into smaller molecules such as lactic acid, acetic acid, and sometimes (particularly in the later stages) propionic acid and butyric acid. Protein is hydrolyzed to polypeptides, and to some extent to the individual amino acids. Fat is hydrolyzed to its constituent fatty acids and glycerol; and the unesterified water-insoluble fatty acids (WIA)[1] can be determined. In any particular case any one of these types of chemical changes may or may not occur; *i.e.*, cream may decompose into other organoleptically disagreeable substances without a significant increase in WIA or butyric acid.

STUDIES ON PROGRESSIVE DECOMPOSITION

Progressive decomposition studies similar to those reported in a previous paper,[2] were conducted on a series of creams produced on farms in the vicinity of Cincinnati during the summer of 1947. It was originally planned to use the cream from one producer only; but owing to the fact that most of the producers in the neighborhood of Cincinnati are small ones, it was not possible to obtain from any single source the 10 gallons of cream required for the experiment. Arrangements were therefore made with several producers along one route to hold the cream separated from combined morning and evening milkings. These creams were then collected on the following morning and composited in a 10 gallon cream can.

On receipt at the laboratory it was found that the creams had developed some acidity (0.25 to 0.37 per cent titrable acidity as lactic). Obviously authentic cream with no developed acidity would have been preferable as the starting material for progressive decomposition studies. Although the creams were not under observation from the beginning, and souring had started when the experiments were begun, valuable information was obtained by allowing these creams to progressively decompose. They were well mixed and about 18 pounds were removed from the can. After samples for chemical analysis were taken the balance of the 18 pound portion was pasteurized at 150°F for 30 minutes, cooled, and churned in an electrically driven churn of 12 gallon capacity. The remainder of the cream in each 10 gallon can was allowed to stand on the receiving floor of the creamery

[1] *This Journal*, 30, 575 (1947).
[2] *This Journal*, 31, 750 (1948).

for periods up to 11 days. The creams were stirred daily, and samples were taken for analysis at three intervals several days apart. At each sampling a portion was neutralized, pasteurized, and churned. Data obtained on the first cream studied are given in Table 1.

TABLE 1.—*Changes in cream during progressive decomposition*
Temperature range 65–91°F. Relative humidity 73–99%

DETERMINATIONS	CREAM				BUTTER			
	AGE IN DAYS				AGE IN DAYS			
	1	4	8	11	1	4	8	11
Titrable Acidity % as Lactic	0.25	0.76	1.59	1.62	—	—	—	—
Butyric Acid[1] mg/100 g Fat	None	16.4	18.7	12.8	None	2.4	3.0	6.6
Propionic Acid mg/100 g Fat	None	None	None	5.4	None	None	None	1.5
Fat, Per Cent	31.0	31.0	31.0	31.0	87.5	83.9	84.9	82.7
Acidity of Fat ml 0.1 *N*/100 g	—	—	—	—	13	11	16	11
Lactic Acid, Per Cent	0.222	0.358	0.977	0.727	—	—	—	—
Lactose, Per Cent	1.33	0.85	0.00	0.00	—	—	—	—
WIA mg/100 g Fat	234	657	934	1052	225	659	952	1084
WIA Buttermilk mg/100 g	—	—	—	—	14	29	32	27
Odor and Flavor	Normal	Moldy	Moldy Cheesy	Cheesy Putrid	Rancid taste after 4 days at 40°F.	Decided objectionable flavor	Cheesy	Cheesy Putrid

[1] *This Journal*, 28, 644 (1945).

The titrable acidities increased rapidly, but there was no increase in lactic acid after 8 days; the lactose had by then completely disappeared from the cream. Some butyric acid was found in the cream after 4, and also after 8 and 11 days, and some of this acid carried over into the butter. Propionic acid was found in the cream after 11 days of holding. It has been shown in a previous report[2] that the presence of determinable quantities of butyric acid (or propionic acid) in butter is associated with the presence of some decomposed cream in the vat mixture from which the butter was churned. Progressively larger quantities of WIA were found in the cream and butter after the 4, 8, and 11 days of holding. Two of the producers used mechanical separators and the other four, water separators.

In like manner a second composite can of cream was obtained from a different group of producers. The analytical data are given in Table 2.

Lactose did not completely disappear from this cream even after holding 9 days. An increase in WIA was found in the sample taken on the 6th day, at which time butyric and propionic acids had also appeared in the cream. Four of the seven producers used mechanical separators, and the other three, water separators.

TABLE 2.—*Changes in cream during progressive decomposition*
Temperature range 54–92°F. Relative humidity 68–100%

DETERMINATIONS	CREAM				BUTTER			
	AGE IN DAYS				AGE IN DAYS			
	1	3	6	9	1	3	6	9
Titrable Acidity % as Lactic	0.30	0.70	1.20	1.80	—	—	—	—
Butyric Acid[1] mg/100 g Fat	Trace	None	10.4	—	Trace	—	Trace	8.5
Propionic Acid mg/100 g Fat	Trace	Trace	7.4	—	Trace	—	Trace	None
Fat, Per Cent	37.0	36.0	36.6	37.2	83.7	87.6	85.0	87.3
Acidity of Fat ml 0.1 *N*/100 g	—	—	—	—	6	5	6	9
Lactic Acid, Per Cent	0.185	0.466	0.940	1.078	—	—	—	—
Lactose, Per Cent	2.43	1.99	1.24	0.41	—	—	—	—
WIA mg/100 g Fat	75	68	314	758	153	135	312	714
WIA Buttermilk mg/100 g	—	—	—	—	20	26	24	35
Odor and Flavor	Normal	Normal	Moldy Slight Cheesy	Moldy Cheesy Rancid	Normal	Normal	Slight Cheesy	Cheesy

[1] *This Journal*, **28**, 644 (1945).

A third cream similarly obtained from a still different group of producers yielded, on a study of its progressive decomposition, the analytical data in Table 3.

At the end of 4 days holding, it was found that the cream had undergone considerable deep-seated chemical break-down, as is evidenced from the quantity of WIA found, and also by the appearance of butyric and propionic acids. The cream was not held beyond the 7th day, since its condition had so deteriorated that, it appeared nothing could be gained by carrying the experiment further. It is interesting to note that each of

the producers who furnished the cream for this experiment used water separators.

A progressive decomposition study was also conducted on a cream, freshly separated from about 100 gallons of sweet milk obtained from a single producer. The data are given in Table 4.

WIA in the butter (made as before in the churn of 12-gallon capacity) from this cream, at the start of the experiment, is well within the range

TABLE 3.—*Changes in cream during progressive decomposition*
Temperature range 60–101°F. Relative humidity 82–100%

DETERMINATIONS	CREAM			BUTTER		
	AGE IN DAYS			AGE IN DAYS		
	1	4	7	1	4	7
Titrable Acidity % as Lactic	0.37	0.96	1.38	—	—	—
Butyric Acid[1] mg/100 g Fat	None	26.0	103.6	—	2.8	2.8
Propionic Acid mg/100 g Fat	None	11.1	43.3	—	2.1	2.1
Fat, Per Cent	36.0	33.8	29.0	85.5	78.7	84.2
Acidity of Fat ml 0.1 *N*/100 g	—	—	—	11	26	15
Lactic Acid, Per Cent	0.253	0.466	0.680	—	—	—
Lactose, Per Cent	1.09	0.54	None	—	—	—
WIA mg/100 g Fat	237	1037	1711	235	970	1170
WIA Buttermilk mg/100 g	—	—	—	17	45	336
Odor and Flavor	Normal	Moldy	Moldy	Distinct objectionable taste	Cheesy Rancid	Putrid Rancid

[1] *This Journal*, 28, 644 (1945).

found for sweet cream butter[3] and it did not increase until after 8 days of holding the cream. The initial lactose closely approximated that normally found in machine-separated sweet cream. As the cream became more and more sour, the lactose never completely disappeared, as was the case in two of the other progressive decomposition experiments on multiple-source cream described above, where the initial quantity of lactose was smaller. Furthermore the quantity of lactic acid found in the original cream corresponded to that usually found in sweet cream (0 to 3

[3] *This Journal*, 31, 739 (1948).

milligrams per 100 grams). Butyric and propionic acids did not appear, other than in traces, in the cream until after 8 days.

STUDIES ON CREAM OF INDIVIDUAL PRODUCERS

Arrangements were made with each of six of the producers on one of the cream routes to furnish cream separated from the morning and evening milking of the preceding day. The analysis of these samples is given in Table 5.

TABLE 4.—*Changes in cream during progressive decomposition*
Temperature range 71–95°F. Relative humidity 86–100%

DETERMINATIONS	CREAM				BUTTER			
	AGE IN DAYS				AGE IN DAYS			
	1	3	8	11	1	3	8	11
Titrable Acidity % as Lactic	0.12	0.66	1.09	1.54	—	—	—	—
Butyric Acid[1] mg/100 g Fat	None	Trace	39.0	22.1	None	Trace	None	2.4
Propionic Acid mg/100 g Fat	None	Trace	36.8	12.3	None	Trace	None	None
Fat, Per Cent	28.0	27.0	26.0	27.0	84.5	81.9	69.4	75.7
Acidity of Fat ml 0.1 *N*/100 g	—	—	—	—	7	18	19	16
Lactic Acid, Per Cent	0.003	0.309	0.635	0.966	—	—	—	—
Lactose, Per Cent	2.75	2.35	1.63	0.86	—	—	—	—
WIA mg/100 g Fat	176	251	684	762	158	219	700	740
WIA Buttermilk mg/100 g	—	—	—	—	20	24	38	74
Odor and Flavor	Normal	Normal	Slight Cheesy Slight Rancid	Cheesy Putrid	Normal	Normal	Slight Cheesy	Cheesy Rancid

[1] *This Journal*, 28, 644 (1945).

There is a wide variation in the percentage of lactose in these creams. Samples 1, 4, 5, and 6, in which the cream was separated in a water separator, contained the lower quantities of lactose and, with one exception, contained higher quantities of WIA; samples 1, 4, and 6 contained somewhat more of these acids than has been found in authentic undecomposed cream.

In order to compare water and mechanically-separated cream the farm of producer No. 1 was revisited and arrangements made to obtain the

TABLE 5.—*Analysis of cream from individual producers*

PRODUCER NUMBER	TITRABLE ACIDITY	FAT	LACTOSE	WIA	SEPARATOR USED
	Per cent Lactic	*per cent*	*per cent*	*Mg/100 g Fat*	
1	0.11	44	0.36	234	Water
2	0.35	47	2.40	74	Mechanical
3	0.30	51	2.43	176	Mechanical
4	0.43	38	0.50	366	Water
5	0.45	38	0.88	163	Water
6	0.42	35	1.28	246	Water

evening milk as soon as possible after milking. A small hand separator was taken along and the cream was separated immediately after milking. Analysis was started immediately in order to stop any bacterial action, and then finished in the laboratory. The data are given in Table 6. This table also gives data obtained 4 weeks earlier on cream from the same farm which had been separated in a water separator. The samples were taken from the combined creams of the morning and evening milking in each instance.

The data are rather striking. Titrable acidities are the same. Lactose in the mechanically separated cream is normal for this type of separation, while the small quantity found in the cream obtained from the water separator indicates that most of the lactose was lost because of its solubility in the serum. WIA in the mechanically separated cream is well below the maximum found for sweet cream.

In a further study of cream from these producers, arrangements were made with the centralizer receiving these producers' creams to set aside the normal weekly deliveries made by each. These creams were not mixed with creams from other producers. Thus there was, in each delivery, cream from 1 to 7 days old. After samples of the cream were taken for analysis the balance was neutralized, pasteurized, and churned as before. Analyses of the creams and butters are given in Table 7.

All these creams were cheesy in taste and odor, demonstrating that a material quantity of decomposed cream was present. This is confirmed

TABLE 6.—*Comparative analysis of creams, water and mechanically separated*

	WATER SEPARATED	MECHANICALLY SEPARATED
Titrable Acidity (per cent as Lactic)	0.11	0.10
Fat (per cent)	44.0	56.0
Lactose (per cent)	0.36	2.13
WIA (Mg/100 g fat)	234	82

TABLE 7.—*Analysis of weekly delivery of cream from individual producers*

PRODUCER NO.	CREAM					BUTTER				
	TITRABLE ACIDITY	BUTYRIC[1] ACID	PROPIONIC ACID	FAT	WIA	FAT	ACIDITY OF FAT	BUTYRIC[1] ACID	WIA	WIA IN BUTTERMILK
	Per cent as Lactic	*mg/100 g*	*mg/100 g*	*per cent*	*mg/100 g Fat*	*per cent*	*ml 0.1 N/100 g*	*mg/100 g*	*mg/100 g Fat*	*mg/100 g*
1	0.55	44.6	10.8	39.0	1440	83.4	33	6.5	1361	112
2	0.88	43.9	17.6	33.0	1229	80.1	25	4.5	1097	129
3	1.41	—	—	53.0	475	84.6	5	2.6	410	209
4	1.09	195.0	47.4	35.0	1399	—	—	—	—	—
5	1.00	81.9	31.7	38.0	1615	82.1	20	9.0	1288	256
6	1.43	61.0	17.8	35.0	1556	82.9	24	3.5	1517	89

[1] *This Journal*, **28**, 644 (1945).

TABLE 8.—*Creams and butters churned therefrom obtained from individual producers*

SAMPLE NO.	CREAM					BUTTER					BUTTER MILK	COMMENT
	TITRABLE ACIDITY	FAT	BUTYRIC[1] ACID	PROPIONIC[1] ACID	WIA	WIA	ACIDITY OF FAT	BUTYRIC[1] ACID	PROPIONIC[1] ACID	FAT	WIA	
	per cent as lactic	*per cent*	*mg/100 g fat*	*mg/100 g fat*	*mg/100 g fat*	*mg/100 g fat*	*ml 0.1 N per 100 g*	*mg/100 g fat*	*mg/100 g fat*	*per cent*	*mg/100 g*	
1	0.93	34.0	11.6	3.6	206	213	6	Trace	None	84.0	23	Sour cream.
2	1.37	32.0	7.9	None	355	317	12	Trace	None	85.0	22	Cream had disagreeable taste; not identified.
3	0.62	24.6	—	—	45	97	4	None	None	70.1	20	Sour cream.
4	0.90	26.0	7.3	8.1	281	257	8	None	None	78.2	27	Sour cream.
5	1.01	26.2	29.7	11.6	447	330	9	None	None	79.3	37	Cream slightly cheesy. Butter slightly cheesy
6	0.71	35.0	None	None	—	159	5	None	None	84.0	23	Sour cream.
7	1.52	42.4	22.4	5.0	802	857	13	5.4	None	83.6	55	Cream slightly cheesy. Butter slightly cheesy.
8	0.88	40.0	10.3	6.8	501	518	10	2.4	Trace	84.0	22	Slightly cheesy. Fruity odor. Entire surface of can covered with heavy corrugated mold layer.
9	0.59	39.0	12.2	None	404	419	12	None	None	82.8	34	Cream after pasteurization had a slight cheesy odor. Butter had disagreeable taste.
10	0.72	43.0	None	None	130	137	8	None	None	83.8	29	Sour cream.
11	1.44	34.0	144.4	9.0	1342	693	13	10.8	3.1	70.9	605	Cream was cheesy and putrid.
12	1.36	25.2	None	None	159	130	9	None	None	85.7	19	Cream after pasteurization had a slight yeasty odor.
13	1.39	22.0	Trace	Trace	82	126	7	None	None	85.8	21	Sour cream.
14	2.12	36.0	50.1	10.9	2402	1435	20	2.5	None	82.6	463	Cream was cheesy and putrid.
15	2.46	12.0	171.6	88.4	5983	918	9	3.0	2.3	85.3	487	Cream was cheesy and putrid.
16	0.62	45.0	28.2	None	405	1810	46	28.6	None	82.4	61	Cream slightly cheesy. Butter had disagreeable taste.
17	0.94	42.0	—	—	242	269	7	None	None	84.2	30	Sour cream.
18	0.73	32.0	19.8	6.6	697	663	10	None	None	84.0	46	Cream slightly cheesy. Butter slightly cheesy and had disagreeable taste.
19	0.99	32.0	None	None	330	337	7	None	None	85.5	8	Butter slightly cheesy and had disagreeable taste.
20	1.02	38.0	None	None	2418	1388	29	None	None	80.4	525	Cream slightly cheesy.
21	0.89	24.0	51.5	14.5	245	252	11	6.4	None	86.0	24	Sour cream.
22	0.65	56.0	18.7	5.4	654	767	27	6.4	None	73.9	34	Butter slightly cheesy and had disagreeable taste.

[1] *This Journal*, 28, 644 (1945).

TABLE 9.—*Analysis of survey samples of butter*[1] *(1945)*

CHICAGO				ST. LOUIS				NEW ORLEANS			CINCINNATI		
SAMPLE NO.	WIA	BUTYRIC[2]	MOLD	SAMPLE NO.	WIA	BUTYRIC[2]	MOLD	SAMPLE NO.	WIA	MOLD	SAMPLE NO.	WIA	MOLD
	mg/100 g Fat	*mg/100 g Fat*			*mg/100 g Fat*	*mg/100 g Fat*			*mg/100 g Fat*			*mg/100 g Fat*	
1	29	—	2	1	81	—	0	1	21	6	1	98	0
2	39	—	18	2	99	—	0	2	29	8	2	104	0
3	45	—	16	3	106	—	0	3	54	10	3	116	6
4	51	—	2	4	124	—	6	4	63	10	4	150	44
5	53	—	2	5	129	—	8	5	65	6	5	150	92
6	54	—	0	6	138	—	0	6	73	0	6	181	0
7	56	—	0	7	146	—	16	7	94	0	7	197	45
8	63	—	24	8	151	—	4	8	104	8	8	210	75
9	66	—	0	9	154	—	79	9	108	8	9	249	97
10	66	—	0	10	160	—	44	10	119	26	10	254	0
11	75	—	0	11	161	—	0	11	134	14	11	266	91
12	89	—	0	12	161	—	0	12	149	18	12	266	92
13	101	—	0	13	165	—	48	13	190	54	13	269	15
14	109	—	0	14	171	—	0	14	218	62	14	298	89
15	114	—	42	15	174	—	60	15	223	18	15	314	70
16	116	—	52	16	191	—	0	16	226	22	16	330	93
17	118	—	0	17	198	—	87	17	244	26	17	425	93
18	119	—	0	18	203	—	85	18	269	54	18	441	66
19	119	—	4	19	211	—	56	19	278	32	19	453	93
20	121	—	2	20	226	—	74	20	280	10	20	465	90
21	133	—	40	21	235	—	56	21	285	34	21	598	83
22	133	—	29	22	244	—	84	22	288	30	22	649	82
23	136	—	34	23	251	—	40	23	294	42	23	2063	96
24	141	—	0	24	264	—	50	24	299	42	24	2341	82

TABLE 9—(*continued*)

CHICAGO				ST. LOUIS				NEW ORLEANS			CINCINNATI		
SAMPLE NO.	WIA	BUTYRIC[2]	MOLD	SAMPLE NO.	WIA	BUTYRIC[2]	MOLD	SAMPLE NO.	WIA	MOLD	SAMPLE NO.	WIA	MOLD
	mg/100 g Fat	*mg/100 g Fat*			*mg/100g Fat*	*mg/100 g Fat*			*mg/100 g Fat*			*mg/100 g Fat*	
25	143	—	28	25	278	—	62	25	331	51			
26	145	—	21	26	285	—	44	26	325	28			
27	151	—	2	27	293	—	50	27	335	38			
28	168	—	2	28	296	—	42	28	340	30			
29	174	—	4	29	304	—	44	29	343	18			
30	181	—	2	30	320	—	52	30	359	58			
31	195	—	35	31	333	—	70	31	386	38			
32	215	—	29	32	363	—	91	32	414	72			
33	238	3	88	33	386	—	78	33	450	22			
34	243	3	82	34	394	—	81	34	456	44			
35	464	11	22	35	414	—	72	35	456	22			
36	501	23	58	36	541	9	22	36	505	20			
37	599	13	26	37	589	—	64	37	519	56			
38	733	11	24	38	593	15	28	38	534	32			
39	969	19	44	39	600	16	44	39	730	52			
40	1050	28	64	40	621	—	80	40	766	42			
				41	753	—	100	41	1278	50			
				42	964	20	42						

[1] Arranged by WIA content.
[2] The revised distillation method was used, *This Journal*, **25**, 176 (1942).

TABLE 10.—*Analysis of survey samples of butter*[1] *(1948)*

CINCINNATI				ST. LOUIS				CHICAGO			
SAMPLE NO.	WIA	BUTYRIC[2]	MOLD	SAMPLE NO.	WIA	BUTYRIC[2]	MOLD	SAMPLE NO.	WIA	BUTYRIC[2]	MOLD
	mg/100 g fat	*mg/100 g fat*			*mg/100 g fat*	*mg/100 g fat*			*mg/100 g fat*	*mg/100 g fat*	
1	121	Trace	2	1	163	Trace	6	1	102	Trace	4
2	219	None	38	2	182	Trace	26	2	103	Trace	6
3	276	None	54	3	201	Trace	6	3	111	Trace	2
4	276	Trace	94	4	204		43	4	124	Trace	8
5	276	Trace	40	5	211		64	5	131	Trace	0
6	289	Trace	44	6	221	None	18	6	131	Trace	2
7	290	Trace	42	7	228	None	24	7	173	Trace	4
8	297	Trace	66	8	231	Trace	4	8	203	.28	0
9	298	Trace	75	9	255	Trace	22	9	215	Trace	14
10	300	Trace	68	10	257	Trace	53	10	217	3.5	30
11	311	None	30	11	257	None	34	11	231	20.0	8
12	312	Trace	76	12	257	2.2	26	12	239	3.1	12
				13	263	None	28				
13	314	Trace	78	14	263		59	13	247	2.3	40
14	318	2.3	74					14	249	2.6	0
15	322	None	68	15	264	None	18	15	255	Trace	24
16	322	None	30	16	269	Trace	28	16	270	3.8	12
17	322	None	88	17	270	Trace	42	17	281	3.8	42
18	322	Trace	88	18	271	Trace	32	18	286	2.5	28
19	337	2.0	82	19	282		50	19	305	Trace	28
20	337	Trace	90	20	286	None	54	20	308	None	24
21	331	Trace	48	21	288		35	21	313	Trace	44
22	339	Trace	56	22	294		48	22	326	Trace	52
23	347	Trace	96	23	297	Trace	38	23	326	3.1	18
24	367	Trace	84	24	297	Trace	32	24	326	Trace	30
				25	302	Trace	32				
25	377	Trace	86	26	304	Trace	60	25	335	4.1	26
26	396	Trace	16	27	312	None	46	26	339	Trace	36
27	405	2.0	60	28	312		52	27	342	Trace	26
28	405	None	50					28	349	4.9	28
29	408	Trace	46	29	312	3.0	20	29	347	2.8	32
30	420	Trace	82	30	313	Trace	52	30	367	Trace	24
31	422	None	32	31	324	Trace	80	31	393	7.0	40

TABLE 10—(*continued*)

CINCINNATI				ST. LOUIS				CHICAGO			
SAMPLE NO.	WIA	BUTYRIC[2]	MOLD	SAMPLE NO.	WIA	BUTYRIC[2]	MOLD	SAMPLE NO.	WIA	BUTYRIC[2]	MOLD
	mg/100 g fat	*mg/100 g fat*			*mg/100 g fat*	*mg/100 g fat*			*mg/100 g fat*	*mg/100 g fat*	
32	425	Trace	88	32	326	5.5	19	32	399	3.3	38
33	426	Trace	82	33	331	Trace	66	33	300	Trace	54
34	432	Trace	84	34	333	None	54	34	405	Trace	44
35	450	2.0	50	35	335	None	10	35	416	3.6	44
36	470	None	88	36	337		30	36	417	2.5	36
				37	343	Trace	44	37	441	3.1	36
37	473	Trace	100	38	354		47	38	443	Trace	40
38	490	Trace	84	39	354	Trace	28	39	453	3.8	48
39	493	2.9	100	40	354		52	40	452	3.9	74
40	495	Trace	100	41	354	Trace	55	41	454	Trace	38
41	503	2.5	94	42	355	None	70	42	459	4.1	86
42	507	2.1	80					43	469	3.1	56
43	527	Trace	72	43	366	Trace	92	44	469	38.4	46
44	551	2.1	92	44	375	Trace	46	45	472	5.4	18
45	553	4.0	94	45	380	None	52	46	476	6.1	66
46	556	6.9	98	46	382	None	33	47	493	4.4	60
47	583	Trace	100	47	384	2.0	36	48	501	Trace	58
48	587	2.8	82	48	384	None	62				
				49	391	Trace	60	49	501	9.6	56
49	587	Trace	82	50	403		60	50	555	3.6	44
50	590	Trace	68	51	406	Trace	30	51	658	8.6	64
51	591	2.2	80	52	408	None	32	52	721	9.0	68
52	602	None	80	53	412		40	53	998	12.8	66
53	655	6.9	96	54	414	None	62	54	3299	7.9	78
54	663	5.5	100								
55	664	2.8	98	55	418	Trace	47				
56	779	6.0	66	56	424	Trace	34				
				57	433		32				
				58	445		68				
				59	476	Trace	95				
				60	480	5.5	34				
				61	486	Trace	62				
				62	500	Trace	34				
				63	508		48				
				64	700	6.6	40				

[1] Arranged by WIA content.
[2] *This Journal*, **28**, 644 (1945).

by the chemical analysis, which shows large quantities of WIA and also substantial amounts of butyric and propionic acids.

During the summer of 1947, a number of random samples of cream were obtained from centralizer-type creameries located in Cincinnati. These samples were said to represent the normal production and delivery made by an individual producer, and in each case the cream was unmixed with other producers' cream. The majority of the samples were delivered to the centralizer by means of route pick-ups. At the time this investigation was under way the usual time for route pick-up was every seven days. Thus, it is obvious that such seven-day cream, in most cases, contained cream from one, all the way up to seven, days old. Unless such cream had been properly refrigerated by the producer some decomposition was likely before delivery to the centralizer. The daily addition of newly separated cream to the can prior to the time of pick-up would naturally dilute the products of decomposition produced in older cream in the can. No information was available as to the sanitary conditions under which these creams were produced, nor as to whether the cream was hand skimmed or separated in a mechanical or water separator.

These creams were neutralized, pasteurized at 150°F for 30 minutes, cooled, and churned. The analytical data, together with comments, are given in Table 8. The results show that in a number of the creams decomposition actually did occur.

It is obvious that the 50 or so individual cans of cream comprising a commercial churn of butter will vary in their WIA content, depending upon the history, and therefore the condition, of the cream. Analysis of numerous cans of cream that were actually used in commercial churns has shown a variation in WIA of from less than 50 mg./100 g. of fat up to 6000 mg.

COMMERCIAL BUTTERS

As a part of this investigation two surveys of commercial butters were conducted. During the summer of 1945 a number of butters were purchased in the open market in Chicago, St. Louis, New Orleans, and Cincinnati, and examined for their WIA content. In each case mold count was also made, and in some cases butyric acid was determined. The data on the samples collected are given in Table 9.

In 1948, samples of butter were collected at creameries in the vicinity of Chicago, St. Louis, and Cincinnati, representing commercial grades of #1 and #2 butter. WIA, mold, and butyric acid were, in most cases, determined on each sample. Data obtained are given in Table 10.

The data in Tables 9 and 10 show that several of the samples contained large quantities of WIA, the amount in some cases even being very much in excess of that found in the authentic churns of butter churned from vat cream containing varying quantities of decomposed cream (1). Also substantial amounts of butyric acid were found in a number of the samples.

SUMMARY

These progressive decomposition experiments show that, as cream ages and decomposes, the fat may break down forming water-insoluble acids (WIA): in some cases in quantities far in excess of those normally present in sweet cream. Also butyric acid, and sometimes propionic acid, are often found in the samples of cream containing the larger quantities of WIA. It is suggested that the use of water separators might be one of the contributing factors causing early decomposition of cream. Data are presented on the determination of WIA in 321 samples of commercial butters. It has been shown that some of these samples contained materially larger quantities of WIA than were found even in authentic churns of butter known to have been churned from vats of cream which contained varying quantities of decomposed cream. Butyric acid was found in some of the butters containing the larger quantities of WIA.

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RAPID DETERMINATION OF SULFUR DIOXIDE IN WINE*

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A review of the literature indicates extensive work on methods for determining the sulfur dioxide content in beverages and foods. Reports by L. V. Taylor on sulfur dioxide determinations in wine and beer (1, 2, 3), reviews by Nichols and Reed (4) and Monier-Williams (5) evaluate some of the methods in use. Volumetric, gravimetric, and titrimetric methods have been generally employed in determining sulfur dioxide in wine. No attempt is made in this paper to compare the relative merits of the above-mentioned methods nor to draw a comparison between them and the method outlined below.

The experimental work on wine samples reported herewith indicates some rather interesting facts, but is not based on a sufficient number of analyses and does not cover a range of conditions suitable for drawing

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definite conclusions. Possible oxidations and reductions during distillation were considered, but the scope of such side reactions is not discussed. Mention only is made of the fact that addition of oxalic acid or sodium arsenite to the solutions before distillation seems to make for higher and more consistent sulfur dioxide recoveries.

In wines, sulfur dioxide, when used as a preservative, occurs both in the free and combined forms. In bottled beverages the content of sulfur dioxide is not constant but decreases with time, even when the containers are tightly sealed (6, 7) In the chemical examination of wines both the total and the free sulfur dioxide concentration is desired.

Upon distillation, the free sulfur dioxide passes into the distillate and is only partially absorbed, unless a suitable absorbing medium is used. The absorption medium used in the following procedures is a dilute solution of lead acetate. The salt, lead sulphite, which is formed by interaction of sulfur dioxide and lead acetate, is highly insoluble and forms a colloidal

TABLE 1.—*Spectral transmittance through lead sulphite suspension**

WAVE LENGTH mμ	TRANSMITTANCE
	per cent
400	32.0
450	36.8
500	41.7
550	46.6
600	51.3
650	56.2
700	61.0

* Sulfur dioxide content about 25 mg./liter.

suspension. At concentrations of 100 mg./liter or less (calculated as sulfur dioxide) the stability of the colloid is sufficient to permit photometric measurements. The optical density of the suspension is also a limiting factor at about this same concentration. After photometric measurements have been made, the same distillate may be used in iodimetric titrations. This test is hereafter referred to as the "Lead Sulphite Method" to distinguish it from other methods mentioned.

A Coleman D. M. Spectrophotometer, Model 10-S, was used to determine a suitable wave length for making photometric measurements on colloidal suspensions of lead sulphite. Table 1 gives the per cent spectral transmission at various wave lengths from 400–700 mμ. Figure 1 shows wave length plotted against per cent transmission. The resulting straight line indicates that the selection of the wave length of light for the transmission measurements is not critical.

From the standpoint of the volatile acids, exclusive of sulfur dioxide, in wine distillates it is immaterial if a portion of sulfur dioxide escapes. The amount absorbed in the distillate gives the correction to be applied to volatile acid figure.

Free sulfur dioxide is readily given off on distillation so that portions of the distillate used in alcohol determination may be utilized in testing for sulfur dioxide. The "alcohol distillate" is selected instead of the "volatile acid distillate" because it has the same volume as that of the wine sample, whereas, upon quantitatively steam distilling the volatile acids, the quantity of the distillate is often several times as great as the original sample. The concentration of acetic acid and ethyl alcohol in wine distillates affects the solubility of lead sulphite, but the error therefrom does not prohibit the use of the "alcohol distillate" in approximating the free sulfur dioxide content.

A series of tests utilizing several fractions of wine distillates show that

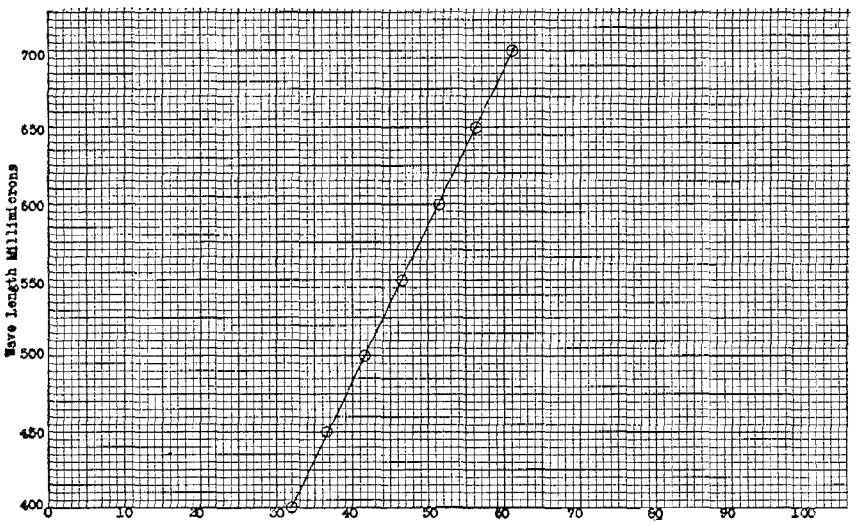

FIG. 1.—Per cent spectral transmittance through lead sulphite suspension. Negative log transmittance Coleman spectrophotometer

free sulfur dioxide is removed early in the distillation. An approximation of the quantity of free sulfur dioxide in distillates may be accomplished very rapidly by the procedure outlined below.

METHOD

To 10 ml of distillate from the alcohol determination add 0.5 ml of 5% neutral lead acetate soln. Compare the cloud produced with that of standards containing from 5–100 mg/liter of sulfur dioxide. If the distillate contains quantities greater than 50 mg/liter it should be diluted with distilled water until it approaches this value. The optical density may be obtained by use of a colorimeter or photometer. For photometric use a standard curve may be prepared and used henceforth.

The value obtained cannot be considered the free sulfur dioxide content of the wine, but it is of comparable magnitude to that in the volatile acid distillate and may be applied as a correction in expressing the volatile acids, exclusive of sulfur dioxide.

The above method provides a means of rapidly screening a number of samples to select those which do contain appreciable amounts of sulfur dioxide.

Table 2 shows the sulfur dioxide values obtained, with alcohol distillates by comparison method, photometrically and iodimetrically; and iodimetrically with the volatile acid distillate.

TABLE 2.—*Sulfur dioxide in distillates of commercial wines*

WINE SAMPLES	ALCOHOL DISTILLATE			ACID DISTILLATE, IODIMETRIC
	COMPARISON	PHOTOMETRIC	IODIMETRIC	
Muscadine	0 mg/l	0 mg/l	0 mg/l	0 mg/l
Muscadine	30	30	32	30
Catawba	60	55	58	62
White Bordeaux (imported)	120	138	132	116
Red Bordeaux (Imported)	35	30	32	38
Red Burgundy	5	3	4	4
Blackberry	130	116	115	120

Distillates containing more than 60 mg/l of SO_2 were diluted 50% with water for comparison and photometric tests.

PREPARATION OF COMPARISON STANDARDS AND A STANDARD CURVE

Sulphite solutions are not stable over extended periods of time and must be prepared immediately before use. About 200 mg. of sodium acid sulphite is added to 100 ml. of water acidified with sulfuric acid (3 ml.), and sulfur dioxide is distilled through a reflux condenser into a series of five receivers containing 50 ml. of 1% lead acetate solution. The receivers were changed so that gradations from a faint cloud to a milky suspension were obtained. The volume is each receiver was brought up to 100 ml. with distilled water.

The suspensions were examined with both the Coleman spectrophotometer at wave length 600 mμ and the neutral wedge photometer employing a green filter No. 56. After completion of the optical examination the sulfur dioxide was determined by acidifying with 5 ml. of concentrated hydrochloric acid and titrating with 0.02 *N* iodine solution using starch indicator. Table 3 shows this data. In Figure 2 the concentration of sulfur dioxide is plotted against the negative log of the transmission. In Figure 3 the neutral wedge photometer readings are plotted against the concentration of sulfur dioxide.

Photometric and turbidimetric standards may be prepared from aqueous suspensions of lead phosphate. Either phosphoric acid or monosodium phosphate appear satisfactory. Stock solutions of either are fairly stable and will not have to be freshly prepared each time they are used.

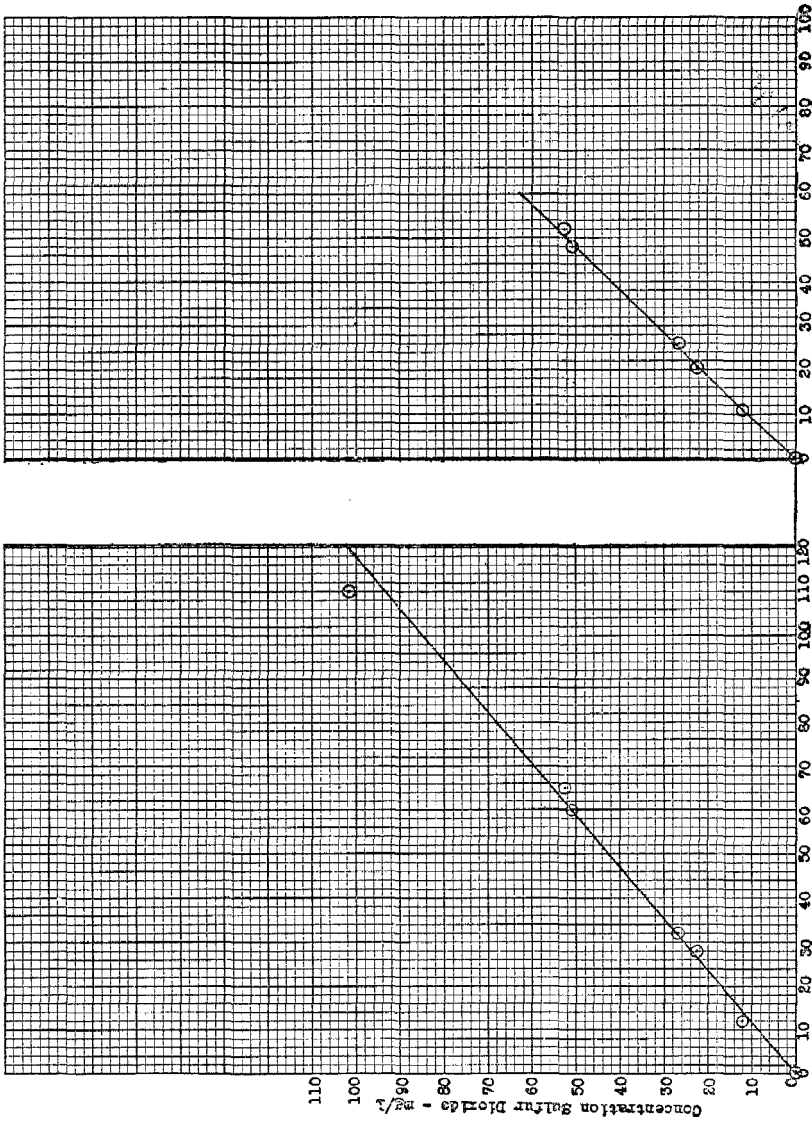

FIG. 2.—Standard curve for SO_2 determination

FIG. 3.—Standard curve for SO_2 determination

TABLE 3.—*Photometric standards for sulfur dioxide*

SAMPLE NO.	NEUTRAL WEDGE PHOTOMETER	COLEMAN SPECTRO-PHOTOMETER TRANSMISSION	NEG. LOG. TRANSMISSION	IODIMETRIC SO_2 mg/l
0	0	100.0%	0	0
1	11.0	75.7	0.12	12.0
2	20.5	52.5	0.28	22.5
3	26.0	47.8	0.32	26.5
4	52.0	22.0	0.65	52.5
5	not readable	7.9	1.10	101.5
5*	48.0	25.1	0.60	(50.8)

* Diluted sample No. 5 one-half with distilled water.

Neutral lead acetate, 5% solution, is added directly to the phosphoric acid or sodium phosphate solution. Turbidity is compared with that of lead sulphite suspensions.

DETERMINATION: TOTAL SULFUR DIOXIDE IN WINE

To 50 ml. of wine sample are added boiling stones and 50 ml. of 5% sulfuric acid. The receiver consists of a graduate containing 50 ml. of 1% neutral lead acetate solution. The end of the condenser or adapter is immersed about two inches below the surface of the liquid in the receiver. About 50 ml. of distillate is collected and the volume made up to 100 ml. with distilled water. After optical examination the suspension is acidified with hydrochloric acid and titrated with 0.02 *N* iodine. A small amount of lead sulphite usually adheres to the adapter or condenser and must be washed into the titration flask with acid. Table 4 shows the results obtained on a number of wines by the "Lead Sulphite Method" using the Coleman Spectrophotometer followed by iodimetric titration and a check employing Monier-Williams method (8), both the titrimetric and gravimetric results being reported.

Distillation of wines in the preceding manner renders the photometric

TABLE 4.—*Total sulfur dioxide in wine distillates*

WINE SAMPLES	MONIER-WILLIAMS		LEAD SULPHITE PHOTOMETRIC	METHOD IODIMETRIC
	TITRIMETRIC	GRAVIMETRIC		
Blackberry	0.0 mg./l.	0.0 mg./l.	0.0 mg./l.	0.0 mg./l.
Blackberry (603.8 mg./l. SO_2)	608.0	600.6	—	602.8
Grape Catawba	12.8	18.0	13.0	17.9
Zinfandel	30.4	32.0	26.0	30.8
Burgundy (White)	132.0	138.0	120.0	140.7
Sauterne	60.4	68.4	60.0	67.6

test for sulfur dioxide considerably less accurate than iodimetric titrations. Tests on a number of wines which had undergone additional acetic fermentation gave sulfur dioxide values as low as one-half that obtained by iodimetric titration or the Monier-Williams determination. This may have been due to the excess acetic acid in the distillate partially solubilizing the lead sulphite. Those same wines when distilled under reflux after addition of oxalic acid or sodium arsenite, gave photometric values closely approximating those by iodimetric titration and the Monier-Williams method. The use of a current of air through the distillation apparatus to aid in driving over sulfur dioxide caused no appreciable variation in the recovery of sulfur dioxide, so it is suggested that a reducing agent added to wine sample may prevent oxidation of the sulfur dioxide by air during distillation.

SUMMARY

A photometric method of determining sulfur dioxide in wine distillate is presented.

Neutral lead acetate solution is shown to be a good absorption medium for sulfur dioxide.

Lead sulphite suspensions may be acidified and the sulfur dioxide content determined by iodimetric titration.

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AN ULTRAVIOLET SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF BENZENE HEXACHLORIDE*

By BERNARD DAVIDOW and GEOFFREY WOODARD (Division of Pharmacology, Food and Drug Administration, Federal Security Agency, Washington 25, D.C.)

Immediately following the introduction of benzene hexachloride as an insecticide, there arose a host of analytical problems related to the determination of this compound and its individual isomers.

* Part of the material in this paper was presented before the American Society of Pharmacology and Experimental Therapeutics, Federation Meetings, Detroit, 1949. The data are taken from the dissertation submitted by Bernard Davidow to the Faculty of the Graduate School, Georgetown University, 1948, in partial fulfillment of the requirements for the degree of Master of Science.

Although analytical methods based on infrared spectrophotometry (1) partition chromatography (2) and on total or hydrolyzable chlorine (3) were available, they were not considered applicable to the determination of benzene hexachloride in biological tissues or spray residues since they lacked either sensitivity or specificity. Therefore, a method more suitable for the specific estimation of the relatively small quantities of benzene hexachloride which might occur in biological tissues or spray residues was sought.

Advantage was taken of the following observations in developing the proposed method. Mitscherlich (4) in 1833 described the formation of trichlorobenzenes from benzene hexachloride by treatment with alkali. Van der Linden (5) studied the products of alkaline hydrolyses of the alpha, beta, and gamma isomers of benzene hexachloride and by a method of mixed melting points estimated that of the mixture of 1, 2, 3-, 1, 3, 5-, and 1, 2, 4-trichlorobenzene formed, approximately 82 per cent was the 1, 2, 4-trichlorobenzene. Furthermore, Conrad-Billroth (6) in 1932 reported that the 1, 2, 4-trichlorobenzene had an absorption spectrum in the ultraviolet region characteristically different from the other trichlorobenzenes.

The method as finally evolved consists of the initial extraction of the insecticide from the material to be examined, the subsequent conversion of the benzene hexachloride with alkali to 1, 2, 4-trichlorobenzene, the purification of this latter compound and its estimation by measurement of its optical density at 286 millimicrons.

METHOD

APPARATUS

(1) Beckman quartz spectrophotometer with ultraviolet attachments and quartz cells with 1 cm. absorption path.
(2) Refluxing apparatus: 125 ml. flasks, condensers (all connections standard taper joints).
(3) Soxhlet apparatus.
(4) Chromatographic tubes, 20 cm. long, 2 cm. O.D.: 30 cm. long, 5 cm. O.D.

REAGENTS

(1) *Benzene hexachloride, gamma isomer.*[1]
(2) *Ether,* ACS.
(3) *Sodium sulfate anhydrous powder.*
(4) *Methanolic potassium hydroxide,* 1.5 *N.*—Dissolve 98 g of potassium hydroxide C.P. in absolute methanol C.P. to make one liter of solution.
(5) *Magnesium oxide activated.* No. 2641, Westvaco Chlorine Products Corp.
(6) *Aluminum oxide anhydrous.*—Merck.
(7) *Celite* No. 545.[2]
(8) *Silica gel activated.*—Mesh size 28–200. Davison Chemical Corporation. Reactivate after use by washing with alcohol, followed by water and then

[1] Lindane, manufactured by Hooker Electrochemical Company.
[2] Manufactured by Johns Manville Company.

heating in oven at 400° for 24 hours. (*Caution:* All organic solvents must be removed before putting in oven.)

(9) *Normal hexane, commercial.*[3]—Purify by passage through silica gel column (500 g silica gel will purify 500 ml hexane).(7) In case the unknown is passed thru an alumina or magnesium oxide column the hexane used for the blank should be similarly purified.

PREPARATION OF STANDARDS

(1) Prepare a standard soln of benzene hexachloride, gamma isomer in absolute methanol to contain 1 mg per ml. To five 125-ml flasks with ground glass condensers add first 0, 1.00, 2.00, 4.00, and 8.00 ml, respectively, of the standard soln.
(2) Reflux with 20 ml of 1.5 *N* methanolic potassium hydroxide for one hour on a steam bath.
(3) Cool, then transfer to a separatory funnel with 25 ml normal hexane and 250 ml of distilled water.
(4) Shake for 2 min., allow the two phases to separate, and discard the aqueous phase.
(5) Wash hexane phase with ten 400-ml portions of distilled water without shaking, by pouring each portion in a slow stream into the soln.
(6) Dry the hexane soln by filtering thru 10–12 g of anhydrous sodium sulfate which has been previously wet with hexane and adjust volume to 25 ml (a 40-mm. chemical funnel is satisfactory for filtration).
(7) Determine optical density of the standards at wave lengths 284, 286, and 290 millimicrons.

DETERMINATION

(1) Extract sample containing between 0.5 and 15 mg of benzene hexachloride with ether (for 10 p.p.m. a 200-g sample is suitable).
   a) *Biological tissue*—Place weighed tissue in a mortar, add sodium sulfate equal to about three times the weight of the tissue. Grind to a coarse dry powder to insure dehydration and complete breaking up of the cells. Transfer to a Soxhlet and carry out extraction for a period corresponding to at least 10 syphonings of the apparatus.
   b) *Dry materials*, such as animal laboratory feed, are ground and extracted directly without dehydration.
(2) Transfer ether soln to flask to be used for hydrolyses and evaporate the ether, using a current of air.
(3) Reflux the ether extract with 20 ml of 1.5 *N* methanolic potassium hydroxide for one hour on a steam bath.
(4) Cool, then transfer to a separatory funnel with 25 ml normal hexane and 250 ml of distilled water.
(5) Shake for 2 min., allow the two phases to separate, and discard the aqueous phase.
(6) Wash hexane phase with ten 400-ml portions of distilled water without shaking, by pouring each portion in a slow stream into the soln.
(6) Dry the hexane soln by filtering thru 10–12 g of anhydrous sodium sulfate which has been previously wet with hexane and adjust volume to 25 ml (a 40-mm chemical funnel is satisfactory for filtration).
(7) (a) *Tissues (except liver)* from rats and dogs require no further purification.

[3] Phillips Petroleum Company.

(b) *Liver*—Purify extracts from liver by passage thru a magnesium oxide column:

Pass the hexane soln from 5 g liver thru a column 3–4 cm long and 2 cm in diameter, containing equal parts of magnesium oxide and celite (packed dry), and wet with hexane before adding the extract. (To decrease the chance of disturbing the surface of the column about 4 g of anhydrous sodium sulfate may be put on top before adding the solvent.)

(c) *Diet*—For commercial laboratory diet pass the hexane soln thru a column of alumina (same size, and packed as in (b)).

Collect 5 ml fractions of eluate in 10 ml glass-stoppered graduates. Transfer the fourth 5-ml portion to the absorption cell.

(8) Determine optical density of the unknown at wave lengths 284, 286, and 290 millimicrons using the hexane (reagent 9) as a blank. Compare with a standard curve prepared from gamma benzene hexachloride. Calculate the concentration of benzene hexachloride in the unknown.

OBSERVATIONS

It was noted that hexane solutions of the nonsaponifiable fractions of laboratory diets, spinach, potatoes, apple wax, and biological tissues, free from benzene hexachloride, had absorption properties in the ultraviolet region which interfered with a quantitative determination of benzene hexachloride unless treated as outlined above. To overcome this difficulty, it was necessary to characterize the spectrum of the components which contribute to the expression of density as the critical wave length (286 millimicrons). Therefore spectral absorption curves were prepared on the nonsaponifiable fraction of these materials and on the products of alkaline hydrolysis of the isomers of benzene hexachloride. (Figures 1 and 2).

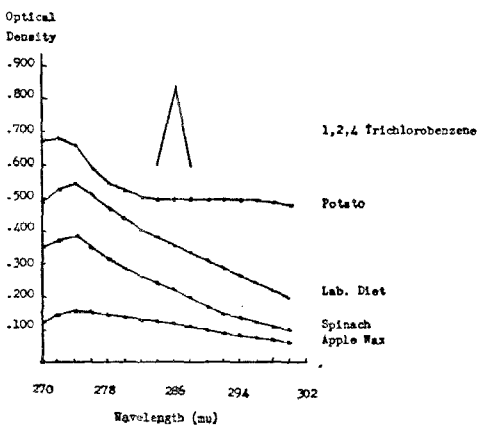

FIG. 1.—Background spectra of Nonsaponifiable fractions.

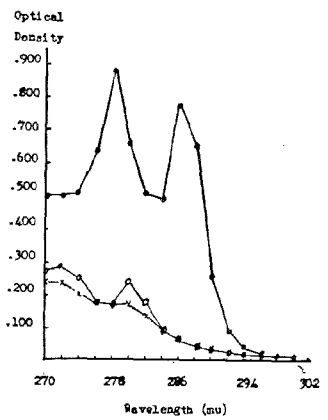

FIG. 2.—Absorption curves of the trichlorobenzenes in hexane

•—1,2,4—Trichlorobenzene 250 μg/ml

○—1,3,5—Trichlorobenzene 250 μg/ml

×—1,2,3—Trichlorobenzene 250 μg/ml

These data reveal that the spectral absorption curves of the control material are linear through the range of 284 to 290 millimicrons. On account of this linearity, resolution of the components contributing to the density at 286 millimicrons may be accomplished by two methods. The first consists of noting the optical densities at wave lengths 284 and 286 millimicrons and applying simultaneous equations for the resolution of a two component color system.

Derivation and Application of a Formula for the Resolution of a Two Component Color System

Legend:

a = ratio of optical density at wave lengths 284/286 due to benzene hexachloride.
b = ratio of optical density at wave lengths 284/286 due to background.
X = optical density due to benzene hexachloride.
Y = optical density due to background.
D = optical density.

From Beers Law.

(1) $D_{284} = X_{284} + Y_{284}$
(2) $D_{286} = X_{286} + Y_{286}$
Using ratios a and b and substituting in equation (1)
(3) $D_{284} = aX_{286} + bY_{286}$
Multiply equation (2) by b
(4) $bD_{286} = bX_{286} + bY_{286}$
Subtract equation (4) from (3)
(5) $D_{284} - bD_{286} = aX_{286} - bX_{286}$
(6) $D_{284} - bD_{286} = X_{286}(a - b)$

$$(7)\quad X_{286} = \frac{D_{284} - bD_{286}}{a - b}$$

A second method which can be applied for the elimination of spectral absorption due to other substances is the base-line method (8). In this case, optical density readings at wave lengths 284, 286, and 290 millimicrons are plotted graphically. A line called the base line is ruled between the points at wave length 284 and 290 millimicrons. The distance between the point at 286 millimicrons for an unknown or standard and the point on the line directly below it is called the base-line density. The base-line density (Fig. 3) is directly proportional to the concentration of benzene hexachloride. This method of analysis can be adapted with slight modification to the determination of benzene hexachloride in many substances. To do so, a spectral absorption curve must be plotted on control samples free of benzene hexachloride, so as to be certain that the background absorption spectrum is linear through the critical wave lengths. Stopcock lubricants may contribute to the optical density in the ultraviolet region, and therefore should be avoided.

With some substances the determination of benzene hexachloride may be complicated by the presence of materials which have a great deal of

absorption in the ultraviolet region and may raise the optical density readings above the optimum range, or may affect the linearity of the spectral absorption curve through the critical wave lengths. Many of these substances, however, can be removed by chromatographing with the proper adsorbent. For example, in the determination of benzene hexachloride in liver tissue or butter, interference was encountered by the presence of vitamin A. The interference has been eliminated in this laboratory by chromatographing the hexane solution containing the vitamin A and the 1, 2, 4-trichlorobenzene through magnesium oxide(9). The vitamin A is strongly held on the adsorbent while the 1, 2, 4-trichlorobenzene is recovered in the hexane eluate. In a similar manner, many plant pigments may be adsorbed on a chromatographic column of alumina.

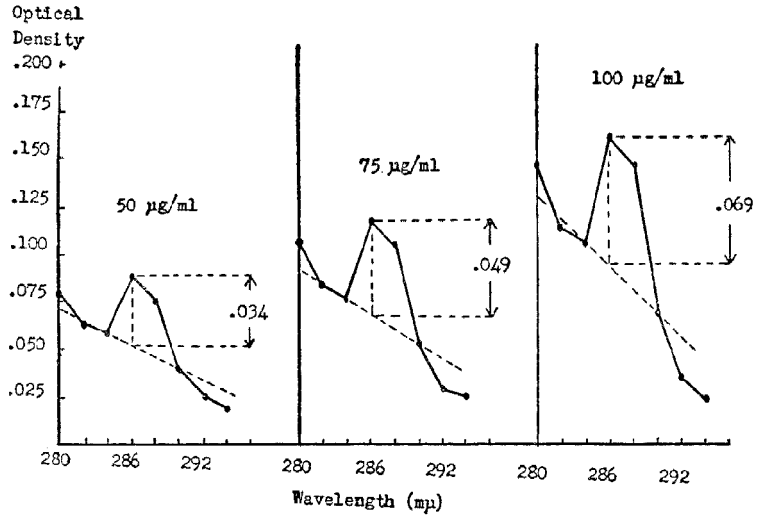

FIG. 3.—Estimation of benzene hexachloride by base-line technique.

The recoveries of benzene hexachloride added to animal laboratory diet and rat fat are illustrated in Tables 1 and 2. The hexane containing the trichlorobenzenes and plant pigments from the laboratory diet was chromatographed through a column of alumina. In the case of rat fat,the hexane solution needed no such purification.

An analysis of the recovery data reveals a standard deviation of 8%. The source of error may be due in part to:

(1) Loss of benzene hexachloride and 1, 2, 4-trichlorobenzene, because of their volatility.

(2) Emulsification of a portion of the 1, 2, 4-trichlorobenzene and its discard with the aqueous wash.

The method as described is of an empirical nature; therefore, the analy-

sis should be performed under the same conditions as used for the preparation of the standard curve. In this manner, as little as 500 micrograms of benzene hexachloride per total sample may be determined in biological tissues, in spray residues on vegetables such as spinach and cabbage, and in animal laboratory diets.

TABLE 1.—*Recovery of benzene hexachloride added to animal laboratory diet*

ISOMER	ADDED	RECOVERED	PER CENT RECOVERED
	p.p.m.	*p.p.m.*	
Alpha	100	100	100
Alpha	100	100	100
Beta	100	106	106
Beta	100	112	112
Gamma	100	92	92
Gamma	100	101	101
Delta	100	101	101
Delta	100	101	101

TABLE 2.—*Recovery of benzene hexachloride added to 1 gram of rat fat*

ISOMER	ADDED MICROGRAMS	RECOVERED MICROGRAMS	PER CENT RECOVERED
Alpha	500	500	100
	1000	1120	112
	2000	1720	86
Beta	1000	1010	101
	1500	1243	83
	2000	1760	88
Gamma	1000	900	90
	1500	1400	93
	2000	1750	88
Delta	1000	1000	100
	1500	1460	97
	2000	2040	102

SUMMARY

A method for the determination of benzene hexachloride has been presented. The method is based upon the conversion of the benzene hexachloride to 1, 2, 4-trichlorobenzene and the estimation of the trichlorobenzene with an ultraviolet spectrophotometer.

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AN ULTRAVIOLET SPECTROPHOTOMETRIC METHOD FOR THE QUANTITATIVE ESTIMATION OF BENZENE HEXACHLORIDE IN MILK

By JOHN P. FRAWLEY and BERNARD DAVIDOW (Division of Pharmacology, Food and Drug Administration, Federal Security Agency, Washington 25, D.C.)

An ultraviolet spectrophotometric procedure for the quantitative estimation of benzene hexachloride has been developed by Davidow and Woodard (1). The method of these workers is based on the dehydrohalogenation of benzene hexachloride to 1,2,4-trichlorobenzene and the estimation of the latter compound by means of an ultraviolet spectrophotometer. The method is applicable to all the isomers.

Recently, when applying the method to milk analyses, several difficulties were encountered which led to (1) a unique procedure for the extraction of fat from milk samples and (2) modifications of the original method to yield greater sensitivity, 0.5 p.p.m. on a 200 ml. sample of milk.

Since the method of Davidow and Woodard (1) is based on the conversion by alkali of the benzene hexachloride to trichlorobenzene, the preferred method of analysis would be one capable of distinguishing the benzene hexachloride from any possible trichlorobenzene present in milk, assuming that some dehydrohalogenation of the insecticide may take place in the animal. In order to detect the presence of 1,2,4-trichlorobenzene in milk, an ultraviolet spectrophotometric examination of a steam distillate of the extracted fat may be used. Thus, any treatment with alkali before or during the extraction, with the possible conversion of benzene hexachloride to trichlorobenzene, was undesirable. If preliminary analysis showed the absence of trichlorobenzene, then any benzene hexachloride can be determined by alkaline conversion to trichlorobenzene, as described below. If, however, the preliminary analysis indicated the presence of trichlorobenzene, the benzene hexachloride content could

be determined by the increase in trichlorobenzene after treatment with alkali.

Since benzene hexachloride is stable in the presence of acid, various methods of treatment with acids were tried in an attempt to minimize the tendency for the milk to emulsify when shaken with an organic solvent. In all cases the protein material in the milk caused sufficient emulsification to require centrifugation in order to separate the solvent layer from the milk. For milk samples of about 200 ml., it was considered preferable to use the procedure described below rather than centrifuge large volumes.

The procedure finally employed consisted in adjusting the *p*H of the milk to approximately 4.6, the isoelectric point of casein. This enabled the precipitated protein and the fat, which was quantitatively occluded on the protein, to be separated from the liquid portion of the milk. Extraction of the fat from the precipitated protein was then carried out in a Soxhlet apparatus. This method of treatment and extraction was found to be equally effective for both regular milk and homogenized milk. It also eliminated centrifugation and offered no strict limitation to the sample size.

Glacial acetic acid was employed and 0.75 ml. per 100 ml. of milk was found to give a *p*H of 4.5. Most rapid precipitation and flocculation were obtained when the milk was first diluted with an equal volume of distilled water and stirred slowly during the addition of the acetic acid.

The precipitate was then collected on a Büchner funnel. The filtrate frequently foamed during filtration, but this was controlled by the addition of an antifoaming agent, *i.e.*, isoamyl alcohol. Extraction of the aqueous filtrate with ether yielded only 0.01 gram of residue per 100 ml. of milk and therefore was not considered necessary for quantitative recoveries.

The casein-fat residue was ground and dried with anhydrous sodium sulfate and extracted with ether in a Soxhlet apparatus. As a general rule 20 grams of sodium sulfate per 100 ml. of milk yield a coarse dry power. However, if the residue has not been adequately dried on the Büchner funnel, a considerably larger quantity of the drying agent is required.

Fat recoveries determined by this precipitation and extraction procedure were compared with the A.O.A.C. Method of Roese and Gottlieb and the results are presented in Table 1. The average recovery was found to be 97 per cent.

After extraction in a Soxhlet apparatus with ether, the ether is evaporated and the fat saponified with 25 ml. of 1.5 *N* methanolic potassium hydroxide per 100 ml. of milk. This quantity is in excess of the amount required for the saponification of average milk samples (3.5 to 4.5% butter fat). By titration of the excess potassium hydroxide, it was found that 25 ml. of 1.5 *N* methanolic potassium hydroxide will saponify approximately

7 grams of butter fat. Therefore, this quantity of alkali is sufficient except in extreme cases where the milk contains more than 7% butter fat.

During saponification, dehydrohalogenation of the benzene hexachloride is affected, resulting chiefly in the formation of 1,2,4-trichlorobenzene (2, 3). The saponified material is then shaken with normal hexane to extract the trichlorobenzene. After washing and chromatographing the hexane solution, it is concentrated to 5 ml. with gentle boiling (caution: without a stream of air.)

The optical density of the concentrated hexane solution is determined at 284, 286, and 290 millimicrons and the quantity of benzene hexachloride

TABLE 1.—*Fat recoveries as compared with A.O.A.C. method*

EXPERIMENTAL METHOD	A.O.A.C. METHOD	RECOVERY
g. fat/100 ml milk	*g. fat/100 ml milk*	*per cent*
3.74*	3.89*	96
5.23	5.52	95
4.17	3.97	105
3.64	4.00	91
3.60*	3.70*	97

* Indicates homogenized milk sample.

is determined by a base-line technique (4). The selection of the wave lengths 284, 286, and 290 millimicrons was made because the base line obtained through this range for control milk samples was found to yield a straight line. Readings made at other wave lengths on the same blank sample indicated a variance from a straight line and would consequently result in slight positive base-line densities on blank samples.

The base-line technique consists in graphically plotting wave length against optical density. The optical density readings at 284, 286, and 290 millimicrons are located on the graph. A straight line, called the base line, is constructed through the points at wave lengths 284 and 290. The distance from the point at 286 millimicrons and the point on the line directly below it, is called the base-line density. The quantity of benzene hexachloride in the unknown can be estimated from a standard curve relating base-line density to concentration of benzene hexachloride.

A mathematical resolution for the determination of the base-line density may also be employed. Using the wave lengths previously indicated, the following formula may be adopted:

A = optical density at 284 millimicrons
B = optical density at 286 millimicrons
C = optical density at 290 millimicrons

$$\text{Base-line density} = B - \left(A - \frac{A-C}{3}\right)$$

Comparison of the base-line density with the standard curve establishes the concentration of benzene hexachloride.

METHOD

Apparatus and reagents are the same as those used in the preceding paper with the addition of a special* Erlenmeyer flask with a 10 ml. tube, graduated at 5 ml., sealed to the side of the flask near the bottom.

PROCEDURE (FOR 200 ML. OF MILK)

(1) Dilute 200 ml of milk with 200 ml of water.
(2) Add 1.5 ml of glacial acetic acid, stirring slowly—allow precipitate to settle for twenty minutes.
(3) Filter the precipitate through an approximately 1 or 2 mm layer of celite on a Büchner funnel (18 cm size is convenient).
(4) Grind precipitate to a coarse dry powder with anhydrous sodium sulfate.
(5) Extract dry powder with ether for 2 hours in a Soxhlet apparatus (a minimum of 20 syphonings of the apparatus).
(6) Transfer ether extract of 125 ml flat-bottom flask (standard taper) and evaporate the ether on a steam bath using a gentle stream of air.
(7) Add 50 ml of 1.5 *N* methanolic potassium hydroxide and reflux for 1 hour.
(8) Cool and transfer quantitatively to a 500 ml separatory funnel with 25 ml of normal hexane and 250 ml of distilled water. Shake for two minutes and allow the two phases to separate.
(9) Discard the aqueous phase and wash the hexane phase without shaking with ten 400 ml portions of distilled water.
(10) Dry the hexane by filtering through anhydrous sodium sulfate which has been previously wet with hexane. Wash the sodium sulfate with three 5 ml portions of hexane.
(11) Pass the hexane solution through a column containing a 1-1 mixture of magnesium oxide and celite, followed by a 15 ml wash with hexane.
(12) Transfer the eluate to the Erlenmeyer flask with the graduated side arm and concentrate the hexane solution to 5 ml. by gentle heating on a steam bath using a 10 cm long air condenser. This volume can be easily determined by running the solution into the graduated side arm. In case this special flask is not available, transfer the evaporated solution to a 10 ml volumetric flask and make to volume. This will decrease the sensitivity of the procedure by one-half.
(13) Determine the optical density of the hexane solution at 284, 286, and 290 millimicrons.
(14) A line called the base line is ruled between the points at wave lengths 284 and 290 millimicrons. The distance between the point at 286 millimicrons and the point on the line directly below it is called the base-line density.
(15) The quantity of benzene hexachloride in the unknown is estimated from a standard curve relating base-line density with concentration of benzene hexachloride.

The performance of the method is illustrated by the following recovery experiments using regular milk and homogenized milk (Table 2). An ether solution of the benzene hexachloride was added to milk and the ether evaporated with a stream of air. The average recovery was found to be 96.1 per cent.

* Use of the flask was suggested by Mr. L. Tufts of Hooker Electrochemical Co., Niagara Falls, N. Y

TABLE 2.—*Recoveries of benzene hexachloride added to control milk*

MG. ADDED TO 200 ML. MILK	MG. RECOVERED		PER CENT RECOVERED	
	REGULAR	HOMOGENIZED	REGULAR	HOMOGENIZED
1.00	0.99	0.97	99	97
0.60	0.58	0.58	97	97
0.40	0.37	0.37	93	93
0.20	0.18	0.19	90	95
0.10	0.10	0.10	100	100

SUMMARY

A method for the determination of benzene hexachloride in milk has been presented. The method is sensitive to 0.1 mg. of benzene hexachloride and, as outlined, is capable of quantitatively estimating 0.5 p.p.m. in milk.

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THE RELATION BETWEEN FILTH IN WHOLE FIGS AND FILTH RECOVERED FROM FIG PASTE*

By J. T. KELLY and L. I. PUGSLEY (Food and Drugs Laboratory, Dept. of National Health and Welfare, Ottawa)

A method of sampling, examination and classification of the filth in bulk figs has been described by Howard (1). Hodges (2) extended the work of Howard and introduced a gasoline flotation technique for the estimation of filth in bulk figs. He showed that a much better recovery of insects, particularly of whole larvae, was obtained by the gasoline flotation technique than by the macroscopic method described by Howard. This was confirmed by work in progress in this laboratory designed to adopt the flotation method to a quantitative procedure for bulk figs. Hodges (2) showed that the gasoline flotation was also applicable to the estimation of filth in fig paste but thus far it has not been placed on a quantitative basis.

The work presented here was undertaken to determine the relationship between the estimation of filth in bulk figs by the macroscopic method

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and the estimation of filth in fig paste by the gasoline flotation method. The determination of this relationship appeared of interest for the evaluation of the filth content of fig paste in terms of the filth content of bulk figs from which it was produced.

MATERIALS AND METHODS

Six bags or boxes each containing 25 to 30 pounds of Smyrna or Calimyrna figs (1948 crop) were obtained from each of five different lots of bulk figs at the time of importation. A random sample of 200 figs was drawn from each bag of each lot and examined for filth by the method described by Howard (1) using a dissecting microscope (Greenough type). Each type of defect was recorded for each individual fig examined, and after examination the fig was returned with its defect to the bag from which it was drawn. In lot 4 the identity of the original bags was not kept, and the examination was made on a sample of 200 figs drawn from the pooled lot. After examining the five lots of bulk figs they were sent to a firm engaged in processing such products, and fig paste was prepared from them. Each lot was processed separately and three boxes of paste of approximately 60 pounds each were obtained from each.

Samples of six cores or plugs of 40 to 60 g. each were taken by means of a butter trier from each of the boxes of each lot. The individual plugs were placed in separate beakers with about 1000 ml. of water, and the mixture boiled for approximately 30 minutes until the paste was thoroughly dispersed. The mixture was then transferred to a 4000 ml. beaker and about 30 ml. of white, lead-free gasoline was added. This was stirred vigorously and the beaker was filled with cold water and allowed to stand until the gasoline layer rose to the top. The gasoline layer and approximately 2000 ml. of the water were transferred to a Wildman trap flask and the gasoline layer trapped off and filtered by means of a Büchner funnel. The flotation was repeated on the mixture, and the gasoline layer filtered through the same paper. The combined residues from each core were then examined for insects, insect heads and larvae, using a Greenough type microscope at a magnification of 7× to 15×. The number of heads and whole insects or larvae found was expressed as heads per 100 g. of fig paste. Insect fragments, such as legs and wings, were recorded but were disregarded in the results reported here. A second series of six-core samples was taken and examined in like manner, except that 100 g. cores were drawn in order to determine if the above extrapolation procedure was justified.

RESULTS

Table 1 shows the results obtained in the examination of each box of each lot of bulk figs. It is noted that of the five lots the mean per cent defective varied from 25.2 per cent to 5.4 per cent. The number of defectives in the 200 figs examined from each box in each lot showed con-

siderable variations; however, an analysis of variance showed no significant difference between boxes within each lot. The standard deviation for lot 4 is higher than the others because of the smaller number of figs examined in the lot.

TABLE 1.—*Filth content of each box of bulk figs used for paste*

BOX NO.	NO. FIGS. EXAMINED PER BOX	NO. DEFECTIVES FOUND IN				
		LOT 1	LOT 2	LOT 3	LOT 4	LOT 5
1	200	59	48	46	35	13
2	200	61	55	29	—	8
3	200	43	47	46	—	9
4	200	44	52	31	—	10
5	200	55	47	37	—	13
6	200	40	46	40	—	12
Mean %		25.2	24.6	19.1	17.5	5.4
s		1.8	0.7	1.5	2.7	0.7

Table 2 shows the average results obtained in the examination of each box of fig paste made from each lot of the bulk figs described above. The recovery of the insects and insect heads using cores of 40 to 60 g., and cores of 100 g., are shown separately, expressed as heads per 100 g. paste.

TABLE 2.—*Recovery of heads calculated per 100 g. of fig paste*

BOX NO.	NO. PLUGS PER BOX	LOT 1		LOT 2		LOT 3		LOT 4		LOT 5	
		WT. OF PLUG		WT. OF PLUG		WT. OF PLUG		WT. OF PLUG		WT. OF PLUG	
		40–60g.	100g.	40–60g.	100g.	40–60g.	100g.	40–60g.	100g.	40–60g.	100g.
1	6	2.8	3.0	3.2	2.7	1.7	2.8	1.3	2.0	1.5	0.7
2	6	2.2	1.8	4.0	3.0	1.2	2.5	0.9	1.0	0.5	1.2
3	6	5.1	6.1	5.0	5.3	1.2	2.2	2.0	2.2	0.7	1.0
Mean		3.5		3.9		1.9		1.5		0.9	
F		3.70		1.17		1.33		1.39		2.18	

TABLE 3.—*Relation between filth content of bulk figs and fig paste*

LOT NO.	NO. FIGS EXAMINED	MEAN DEFECTIVE	WT. PASTE EXAMINED	MEAN RECOVERY HEADS PER 100 G. PASTE
		per cent	*g.*	
1	1200	25.2	2752	3.5
2	1200	24.6	2777	3.9
3	1200	19.1	2857	1.9
4	200	17.5	2739	1.5
5	1200	5.4	3600	0.9

An analysis of variance showed no significant difference between the two core sizes. The mean of the results obtained for both sizes is shown at the bottom of Table 2. The F value for lot 1 was of borderline significance (F at .01 = 3.699) because of the relatively higher recovery in box 3 of this lot. It is seen that the recovery varied from 3.9 to 0.9 insect heads per 100 g. of paste.

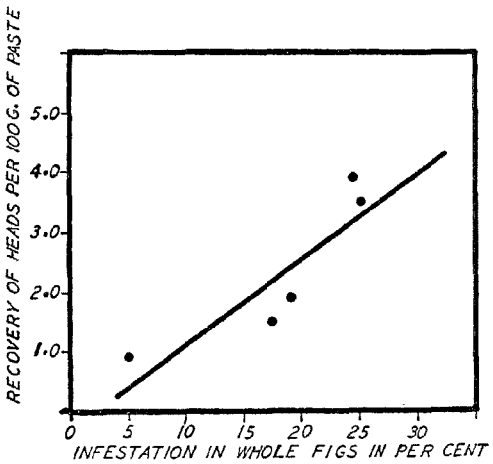

FIG. 1.—The relation between the insect infestation in whole figs in per cent and the recovery of insects and insect heads per 100 grams of fig paste.

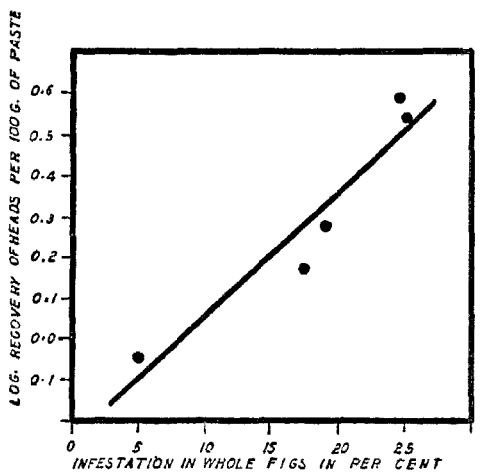

FIG. 2.—The relation between the insect infestation in whole figs in per cent and the logarithmic recovery of insects and insect heads per 100 grams of fig paste.

The mean values obtained for the per cent defective of each lot of bulk figs, in Table 1, and the mean values for the recovery of insects and insect

heads, in Table 2, are combined with the number of whole figs and the weight of paste examined to form Table 3. The data from this table were used to calculate a regression line by the method of least squares, shown in Figure 1. A significant correlation was obtained ($r=0.88$). When the logarithm of the mean recovery per 100 g. of paste was used against the mean per cent defective a highly significant correlation was obtained ($r=0.95$) in Figure 2. On the basis of the latter results it is considered that this is the better correlation to use.

DISCUSSION

The relationship shown in Figures 1 and 2 is deemed to be of considerable value in yielding an estimate of the filth content of fig paste in terms of bulk figs. Growing and handling conditions of this fruit have not advanced sufficiently to produce a product completely free of insects, hence the allowance in commerce of a small amount of filth in this product. In the production of fig paste the insects are broken up and the filth content is more difficult to estimate. Despite the grinding, the recovery of whole insects and heads correlates with the original infestation, and may be used to evaluate the paste in terms of the original figs from which it was made.

SUMMARY

A significant correlation is shown between the amount of insect filth recovered from fig paste and the amount of filth found in the whole figs from which it was made. This correlation provides a quantitative means for assessing filth in fig paste in terms of per cent defective whole figs.

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STEREOISOMERIC ANALYSIS OF BETA-CAROTENE*

By E. M. BICKOFF, M. E. ATKINS, G. F. BAILEY, and FRED STITT
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Although it has been known for several years that the vitamin A potencies of all-trans-β-carotene, neo-β-carotene-B, and neo-β-carotene-U as measured in experiments on rats (5, 6, 9) stand in the approximate ratios of 6:3:2, respectively, there is still no widely used method of analy-

[1] Now out of print.
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[1] One of the Laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.

sis for carotene content in foods or feeds which attempts to determine the relative quantities of the stereoisomers present. All-trans-α-carotene is known to have only one-half the vitamin A activity of all-trans-β-carotene (7). In view of the absence of α-carotene in some commodities, such as alfalfa, broccoli, apricots, and sweet potatoes (8, 12, 16), and the predominance of β-carotene in most foodstuffs containing carotene (3, 13), a method for determining the approximate stereoisomer composition of β-carotene should prove useful in obtaining better estimates of vitamin A potency through carotene analysis.

Slowness in adopting methods for evaluating the neo-β-carotene content of carotene extracts has been due to the difficulty of quantitatively separating the closely related stereoisomers. Polgár and Zechmeister (14) showed that the components of an isomerized β-carotene solution could be resolved on a hydrated-lime column with an acetone-hexane mixture as a developer. However, the neo-B zone remained in contact with that of the all-trans form, making it necessary to extrude the column and separate the bands mechanically. The adsorbed pigments were eluted from each of the separate segments with petroleum ether containing alcohol. Kemmerer and Fraps (12) applied this technique to various plant materials. Their method was included in the AOAC collaborative study on carotene in 1945 (11). The difficulty of separating the bands quantitatively led to conflicting results from different laboratories, and it was recommended that the method be further studied and simplified before adoption.

Since approximately 95 per cent of β-carotene occurs as the all-trans, neo-B, and neo-U isomers in an equilibrium mixture (14), work at this Laboratory has been aimed at developing a comparatively rapid, reliable, liquid chromatogram procedure for separating β-carotene extracts into three fractions, each containing primarily only one of these three isomers. Quantitative chromatographic analysis requires careful control of the significant variables such as adsorbent, developer, adsorbate, size of column, and method of packing. In this paper the results of a study of these variables are reported in which the adsorbate was an iodine-isomerized solution of crystalline β-carotene. As a result of this study a procedure is described for the stereoisomeric analysis of β-carotene extracts by liquid chromatogram procedure into neo-B, all-trans, and neo-U fractions, followed by colorimetric analysis of the fractions. The method is sufficiently simple to make its routine application practical. The application of the method to both fresh and dehydrated alfalfa is described in a subsequent publication (3).

STUDY OF VARIABLES IN CHROMATOGRAPHIC SEPARATION

The present study is an extension of earlier work (2) in which various developers were evaluated for their efficiencies in separating an iodine-isomerized petroleum ether solution of crystalline β-carotene into neo-B,

all-trans, and neo-U fractions on a hydrated lime column. Except where otherwise noted, the size of chromatographic tube, the method of packing the column, the adsorbate solution, and the adsorbent were the same as used previously (2). A 1.5 per cent solution of *p*-cresyl methyl ether in petroleum ether (b.p. 88–99°C.) was used as standard developing solution. For each variable studied, these standard conditions were maintained for the other variables.

Adsorbate: The total amount of carotene added to a column is more important than its concentration in determining the degree of separation of the isomers. However, it is preferable to add the desired quantity in a volume of one ml. or less to produce compact, well-defined bands. Our studies showed that increasing the amount of carotene on the column from 20 to 1000 micrograms gives successively poorer separations. Although very small quantities of carotene readily yield good separation of isomers, the resulting bands are quite pale. Thus it is necessary to choose sufficient carotene to produce bands easily visible on the column. About 100 mmg. total carotene (0.2 ml. 0.536 g./l. solution) was used in studying the other chromatographic variables. This quantity produces bands which are easily visible and clearly separated when the standard developing solution is used (2). In the liquid chromatogram procedure given below, 40 mmg. is recommended.

Developers and solvents: Petroleum ether (b.p. 88–99°C.) was used as solvent for most of this work, with some parallel experiments with isooctane. Lower-boiling solvents were unsatisfactory because of excessive volatility. Isooctane has the advantage of uniformity from batch to batch, while petroleum ether usually produced significantly cleaner separations and is less expensive. Occasional lots of petroleum ether were found to be unsuitable because of unknown impurities.

Since earlier work (2) indicated that certain aromatic-aliphatic ethers were superior to the more common developing agents for separating stereoisomers of β-carotene adsorbed on a hydrated-lime column, approximately forty additional compounds of this type were investigated for their influence on the course of the separation of the stereoisomers. None of these was found to be significantly better than the previously investigated compound *p*-cresyl methyl ether, although a number were effective in producing a separation between neo-B and all-trans-β-carotene on the hydrated lime column, especially anisole, phenetole, anethole, β-chloroethyl phenyl ether, *p*-bromo anisole, *p*-, *m*-, and *o*-cresyl ethyl ether, *n*-butyl phenyl ether, phenyl ether, ethyl anisate, α-naphthyl ethyl ether, *p*-methoxydiphenyl, and hydroquinone diethyl ether.

Adsorbent: An adsorbent may be considered suitable for use in a rapid routine, chromatographic procedure if adsorption columns prepared from it are satisfactory with respect to the following properties: rate of permeation of developing solution, rate of development of the column when a

suitable developer is used, separation of bands in the developed column, and visibility of the bands on the column. Since Shell brand hydrated lime,[2] the adsorbent used in earlier work on separation of β-carotene stereoisomers (2), is no longer commercially available, a survey was made of approximately seventy different samples of available hydrated limes or calcium hydroxides for their suitability for use in this chromatographic

TABLE 1.—*Hydrated lime adsorbents suitable for chromatographic separation of β-carotene stereoisomers*

SOURCE[a]		
Eimer and Amend Co.	New York, N. Y.	Calcium hydroxide, C.P. Grade
Mallinckrodt Chemical Works	St. Louis, Mo.	Calcium hydroxide, U.S.P. Grade
Kraft Chemical Co.	Chicago, Ill.	Hydrated lime
Mississippi Lime Co.	Ste. Genevieve, Mo.	Mississippi Vertical Combination
Green Mountain Lime Corp.	New Haven Junction, Vt.	Hydrated lime
Baker and Adamson	New York, N. Y.	Reagent calcium hydroxide, Code 1523
Consolidated Chemical Industries	Houston, Texas	Pioneer Hydrated Lime
E. & F. King & Co.	Boston, Mass.	Kemikal Hydrated Lime
Wm. H. Scheel	Brooklyn, N. Y.	Hydrated Lime

[a] The mention of these products does not imply that they are endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.

procedure. These materials differed greatly in permeation rates and in separating powers, in general showing no correlation between these properties. Approximately two-thirds of the samples showed such low solvent permeation rates that they were not tested further. Over half of the remaining samples produced little or no separation of the isomer zones, required excessively long times for developing the column, or were too dark in color for satisfactory visibility of the bands on the column. The first five products listed in Table 1 were found to be comparable to Shell brand lime for chromatography of the stereoisomers of β-carotene as

[2] The mention of this product does not imply that it is endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.

judged by the criteria listed above. Since only one sample of each product was tested and no attempt was made to make the survey exhaustive, there are doubtless other commercially available products which are equally satisfactory for this purpose. Likewise there is no assurance that other samples of the products listed will perform satisfactorily in this application.

In efforts to perpare adsorbents with superior properties for chromatographic separations, calcium hydroxide was prepared in this laboratory by precipitation from solutions of calcium salts, by hydrating calcium oxide, and by hydrolysis of calcium carbide. Variations of each of these methods yielded products which could be used to separate the stereoisomers of β-carotene, but most of them were inferior to the best commercially available samples. The most satisfactory laboratory product was obtained by slaking reagent-grade calcium oxide with the stoichiometric quantity of water and drying 15 to 30 minutes at 100°C., but successive small batches showed large differences in adsorptive properties. Calcium hydroxide prepared by hydrolysis of calcium carbide was so dark in color as a result of impurities in the carbide that the chromatograph zones were difficult to see, but was otherwise satisfactory. No work was done with adsorbents other than calcium hydroxide.

It was found that the moisture content of hydrated lime is an important factor influencing its adsorptive powers. When samples of Shell brand lime were conditioned in atmospheres varying in relative humidity from 0 to 91 per cent by exposure for 48 hours over saturated salt solutions *in vacuo*, appreciable differences in efficiency of separating the stereoisomers resulted. The optimum range of relative humidity was found to be 30 to 50 per cent. Storage over a desiccant definitely lowered band separation. Heating 16 hours at 100°C. destroyed the separating power, but it was at least partially restored after conditioning in an atmosphere having a relative humidity of 33 per cent. It is thus desirable to store calcium hydroxide used for chromatography in closed containers and to avoid unnecessary exposure of the material to the atmosphere when it is used.

The dependence of the adsorptive properties of calcium hydroxide on both the manner in which it is formed and its treatment subsequent to formation reflects the fundamental roles of particle size and shape, surface area, and surface condition in determining these properties.

Preparation of column: It is desirable that the time required to develop the chromatogram be kept as low as possible, both for economy of time and to minimize the possibility of re-isomerization of any band during the procedure. The development time is determined by the manner in which the column is prepared as well as by the properties of the adsorbent and developing solution. The tightness of packing the column affects both the development time and the degree of separation of the zones. Tightly packed columns usually gave well defined zones, but required 2 to 3

hours for development. Packing the column uniformly under vacuum with a minimum of tamping resulted in more diffuse zones, but the speed of development was increased so that all three major zones could be collected in from 40 to 60 minutes. Columns packed with mixtures of hydrated lime and diatomaceous earth in ratios of 3:1, 2:1, and 1:1 developed increasingly rapidly (15) but were considered unsatisfactory because of diffuseness and resulting low color intensities of the bands.

Increase in packed length of the column increases the separation of the bands but requires longer development times. Analyses by the procedure described below, using 7.5, 10, and 12 cm. effective column lengths, showed a slight difference in results for the first two, but no difference for the last two. The intermediate column length is accordingly recommended. The method of packing routinely employed is described in the following section.

PROCEDURE FOR STEREOISOMERIC ANALYSIS

The following procedure has been found satisfactory for routine stereoisomeric analyses of β-carotene solutions. Twenty-four samples can be analyzed by two experienced operators in an eight-hour day.

Adsorbent: It is recommended that the hydrated lime or calcium hydroxide employed meet the following performance characteristics when an iodine-isomerized petroleum ether solution of crystalline β-carotene is analyzed by this procedure: (1) V_c, the rate of flow of developing solvent through the column when a state of constant flow has been reached, should be at least 2 mm. per minute; (2) the time required to develop the column to the point where the neo-U zone is beginning to leave the column should not exceed 90 minutes; (3) there should be no obvious overlapping of the neo-B and all-trans bands in the developed column. These specifications are rather arbitrary and are intended only as a guide in deciding whether a particular product is suitable for routine analytical use. The products listed in Table 1 easily met these requirements. Columns prepared with the most satisfactory adsorbents clearly separate the neo-B and all-trans bands by at least 5 mm., have flow rates (V_c) of at least 5 mm./min., and require only 30 minutes for the development time mentioned.

Preparation of chromatographic column: The chromatographic tube is 9 mm. inside diameter and 28 cm. in length, with the upper end flared to a diameter of 2.5 cm. to form a convenient funnel for the addition of the adsorbent. The tube is constricted at the lower end and sealed to a capillary tube having a bore of 1 mm. and length of 16 cm. The column is packed to a height of 10 cm., while the pressure is reduced at the lower end to 100 to 200 mm. Hg by means of an aspirator. The adsorbent is added in 5 to 10 separate portions with gentle tapping of the side of the column to obtain uniform packing.

Development of the column: The preparation of the β-carotene extract or

solution to be used for stereoisomeric analysis, as well as the analytical procedure, should be carried out in a darkened room. Two to four foot candles of light intensity have been found adequate for observation of the progress of chromatographic development and cause no detectable isomerization during the time required for the analyses (3, 4).

A sufficient quantity of carotene solution is added to the dry column to give about 40 micrograms of carotene. The chromatogram is developed with a solution of 1.5 per cent para-cresyl methyl ether in petroleum ether (b.p. 88–99°C.). By means of a Fisher Filtrator,[2] or equivalent, maintained at an absolute pressure of 100 to 200 mm. Hg., the eluate is collected directly in 25-ml. volumetric flasks. The bands are eluted from the column in the following order: neo-β-carotene B, all-trans-β-carotene, neo-β-carotene U. The neo-B fraction contains all the eluate leaving the column before the leading boundary of the all-trans zone reaches the bottom of the column. Further eluate is collected as the all-trans fraction until the leading boundary of the neo-U zone reaches the column outlet. The elution of the neo-U fraction is accelerated by addition to the column of 15 ml. of 5 per cent acetone in petroleum ether when the collection of the all-trans fraction is nearly completed. If colored oxidation or reaction products of β-carotene are present, they remain firmly adsorbed near the top of the column.

Colorimetric analyses: As soon as each fraction is collected, the solution in the flask is made up to volume with petroleum ether and the optical density is determined at once by an Evelyn colorimeter with a 440 mμ filter. If D_b, D_t and D_u are the optical densities of the neo-B, all-trans, and neo-U fractions, respectively, the isomer contents of the three solutions are then given by the equations

$$T = KD_t \qquad (1)$$
$$B = 0.78\ KD_b \qquad (2)$$
$$U = 0.95\ KD_u \qquad (3)$$

where B, T, U are conveniently expressed in micrograms of isomer present in the fraction. The value of K is determined from measurement of the optical densities of petroleum ether solutions of crystalline (all-trans) β-carotene with the colorimeter and filter to be used for stereoisomeric analyses. The factors 0.78 and 0.95 appearing in the above equations were determined from measurements on solutions of the pure crystalline neo-B, all-trans, and neo-U isomers, the preparation of which was recently described (4, 10). These calibrations have not been checked with other types of colorimeters.

STEREOISOMERIC ANALYSIS OF AN ISOMERIZED BETA-CAROTENE SOLUTION

Table 2 shows the results of eleven replicate stereoisomeric analyses of a solution of crystalline β-carotene isomerized by iodine in the presence of

ordinary laboratory illumination. These analyses were run on the same day by the above procedure. The apparent concentration of the isomerized solution was determined colorimetrically and 43.7 mmg. were placed on the column for each analysis. In a separate experiment it was found that 1.7 mmg. of the 43.7 mmg. apparent carotene content were accounted for by oxidation or reaction products which were readily separated from the β-carotene on a magnesium oxide column (1). When this is taken into consideration, it is seen from the first column of the table that the recovery

TABLE 2.—*Stereoisomer composition of iodine-isomerized β-carotene solution*

TOTAL CAROTENE RECOVERED	STEREOISOMER COMPOSITION		
	NEO-B	ALL-TRANS	NEO-U
	per cent	*per cent*	*per cent*
42.0	33.3	44.1	22.6
42.4	32.9	44.0	23.1
41.7	32.9	43.8	23.3
42.0	33.2	43.7	23.1
41.6	33.0	44.2	22.8
41.6	31.1	45.1	23.8
41.8	32.4	44.6	23.0
42.4	32.6	43.7	23.7
41.6	33.6	43.6	22.8
42.2	32.8	43.9	23.3
41.6	32.6	44.5	22.9
Average 41.9	32.8	44.1	23.1
σ 0.39	0.65	0.46	0.37

σ =standard deviation from the mean.

of β-carotene isomers from the column was complete within experimental error. The standard deviations shown in the table indicate that the reproducibility of the procedure under favorable circumstances is probably greater than its accuracy, in view of the instability of the neo-β-carotenes. It should also be borne in mind that the fractions as collected in this procedure contain small amounts of the less stable and hence less abundant stereoisomers of β-carotene (14).

The stereoisomer composition of Table 2 can be compared with the chromatographic analysis on hydrated lime of a similar solution by Polgár and Zechmeister (14). They extruded the developed column, carved out the bands, eluted the stereoisomers from the separate zones, and determined the amount of each isomer colorimetrically. Their results are given in Table 3. The first row shows the isomer composition as they reported it, assuming the same extinction coefficient for all the isomers. The second row shows the composition found when the lower extinction coefficients

TABLE 3.—*Stereoisomer composition of iodine-isomerized β-carotene solution (41)*

	NEO-B	ALL-TRANS	NEO-U	NEO-E	LABILE ISOMER
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Uncorrected[a]	25	48	22	3	2
Corrected[b]	30	44	21	3	2

[a] As given in reference (*14*), assuming equal extinction coefficients for all isomers.
[b] Corrected for differences in extinction coefficients of all-trans, neo-B, and neo-U isomers in accordance with Equations *1*, *2*, and *3*.

of the neo-B and neo-U isomers are taken into account in accordance with the above equations. Since the neo-E and labile isomers are included in the neo-B fraction in our procedure, the results in Tables 2 and 3 are in good agreement.

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SUMMARY

The variables affecting the chromatographic separation of the stereoisomers of β-carotene on hydrated-lime columns have been studied. As a result of this study, a liquid chromatogram procedure has been developed for stereoisomeric analysis of β-carotene extracts. Three fractions consisting primarily of neo-β-carotene B, all-trans-β-carotene, and neo-β-carotene U are collected separately and analyzed colorimetrically. The method was applied to the analysis of an iodine-isomerized solution of β-carotene. It should be applicable to the stereoisomer analysis of β-carotene extracts from any source.

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DETERMINATION OF BETA-CAROTENE STEREOISOMERS IN ALFALFA*

BY E. M. BICKOFF and C. R. THOMPSON (Western Regional Research Laboratory,† Albany, California)

Alfalfa meal is bought largely for its β-carotene content and consequent vitamin A potency. The purified carotene fraction of alfalfa meal may be considered to be primarily a mixture of all-trans-β-carotene and its partially cis isomers, since it contains no significant quantity of α-carotene (7, 11). Two of these cis isomers (neo-β-carotene B and neo-β-carotene U) may constitute as much as half of the purified "total carotene" fraction and have considerably lower nutritional value than the all-trans form (4, 5, 6, 9). It is important, therefore, that the relative amounts of these isomers be known.

A convenient chromatographic method for separating β-carotene extracts into three fractions consisting primarily of neo-β-carotene B, all-trans-β-carotene and neo-β-carotene U is described in the preceding paper (3). The application of this method to alfalfa is reported herein and a procedure is outlined for the determination of these stereoisomers of β-carotene in fresh and dehydrated alfalfa.

Influence of light on analytical results: Zechmeister and Polgár showed (12) that all carotenoids tested were photo-labile and stated that the rate of steric change is dependent on the initial configuration. In order to determine if ordinary laboratory illumination would influence the reproducibility of results, a series of analyses were performed on the same meal sample on different days. Between analyses, the meal was stored in the dark under refrigeration. In all cases the "total carotene" fractions were prepared by rehydrating the meal, extracting quantitatively with acetone, transferring to petroleum ether, and passing through a magnesia column (micron brand No. 2642)[1] to remove chlorophyll and xanthophyll prior to separation of the stereoisomers on hydrated lime (Method I). The analytical results on the same meal with uncontrolled light conditions varied widely. In order to ascertain whether this variation was caused by light during the analysis, a meal sample was analyzed both under ordinary light conditions (150–300 foot candles) and under greatly reduced light intensity. Analyses performed under the reduced light (2–4 foot candles)

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yielded higher values for neo-B (Table 1). When the extraction was carried out under reduced light but the chromatography was carried out under ordinary laboratory light conditions, the neo-B value again was lower. In another experiment, the extract was irradiated under an incandescent lamp, followed by chromatography under reduced light. This mixture also yielded lower values for neo-B, in accord with observations that cis isomers

TABLE 1.—*Effect of light on isomerization of carotene in extracts from dehydrated alfalfa meal*

LIGHT CONDITIONS DURING ANALYSIS		STEREOISOMER COMPOSITION		
EXTRACTION IN	CHROMATOGRAPHY	NEO-β-CAROTENE B	ALL-TRANS-β-CAROTENE	NEO-β-CAROTENE U
		per cent	*per cent*	*per cent*
Daylight	Daylight	19.0	61.5	19.5
		18.0	62.4	19.5
Daylight	Daylight[1]	5.7	71.1	23.2
		8.1	72.5	19.3
Reduced light	Daylight	29.1	52.7	18.1
Reduced light	Reduced light	40.0	44.8	15.2
		42.5	43.4	14.1
Darkness[2]	Darkness[2]	41.5	43.1	15.4
		42.2	43.8	14.0

[1] 40-minute irradiation of extract at 100 f.c. before chromatography.
[2] All apparatus covered with dark paper or cloth.

are much more photosensitive than the all-trans form (12). In one experiment the entire analysis was performed in almost complete darkness, with equipment for extraction and chromatography covered with dark paper or cloth. The total isomer contents found for the various experiment of Table 1 were the same. The analytical results agree with those obtained under reduced light (Table 1), indicating that a light intensity of 2–4 foot candles causes no detectable isomerization during the time required for the analysis. Under these conditions, the light intensity, is, however, sufficient to permit adequate observation of the progress of separation of the zones on the column. These experiments demonstrate that light has a very pronounced effect on the isomerization reaction in the total extract prior to chromatography and on the separated isomers after chromatography.

Relation of quantity of carotene extracted to isomer composition: Since light has such a marked influence on the analytical results, it is desirable to keep the manipulations prior to chromatography at a minimum. Elimination of the need for quantitative extraction of the total carotene would greatly simplify the manipulations, since the meal need then only

be soaked in a petroleum ether solution in the dark for a few minutes. An experiment was set up to determine whether partial extraction of the carotene from the meal would result in preferential extraction of any of the isomers. It was found that varying the extraction time over intervals of 15 minutes to 25 hours gave a constant isomer composition with varying amounts of carotene extracted (Table 2). Accordingly, it is not necessary to extract quantitatively the carotene from the meal to determine the isomer composition. Soaking the meal in petroleum ether for 15 minutes gives an extract containing sufficient carotene for stereoisomeric analysis.

TABLE 2.—*Relation of stereoisomer composition to quantity of carotene extracted from alfalfa meal*[1]

EXTRACTION PERIOD	STEREOISOMER COMPOSITION			PROPORTION OF TOTAL CAROTENE EXTRACTED[2]
	NEO-β-CAROTENE B	ALL-TRANS-β-CAROTENE	NEO-β-CAROTENE U	
(hours)	*per cent*	*per cent*	*per cent*	*per cent*
¼	39.8	43.0	17.2	47
1	37.2	46.0	16.8	52
4	38.5	44.7	16.8	68
25	41.5	43.2	15.3	84

[1] Cold extraction in dark with petroleum ether.
[2] Total carotene was determined by the method of Bailey, Atkins, and Bickoff (1).

Effect of xanthophylls and chlorophylls on separation of β-carotene isomers: Another experiment was designed to determine whether petroleum-ether extract obtained by soaking the meal for 15 minutes in the dark could be chromatographed directly on hydrated lime, omitting the preliminary purification to separate the "total carotene" fraction from the chlorophylls and xanthophylls. Several meal samples were analyzed by this rapid, partial extraction procedure (Method II). The same samples were also analyzed by the procedure described above for the "total carotene" fractions (Method I). Similar results were obtained by the two procedures (Table 3).

Fresh whole alfalfa: Eight samples of fresh alfalfa obtained from various localities were analyzed for stereoisomer content by the method given in the procedure for fresh plant materials. Small but significant amounts of neo-B and neo-U were found in all the samples studied (Table 4). Considerable precautions were taken to prevent isomerization between the time the samples were cut and the time they were analyzed. Most samples were frozen immediately after cutting and kept frozen until analyzed. Fresh samples cut from a plot adjacent to the laboratory and analyzed immediately without freezing had the same isomer content as comparable samples taken from the same plot and frozen prior to analysis (Table 4). Several experiments were carried out in darkness, with all equipment

TABLE 3.—*Comparison of two methods of preparing β-carotene extracts for stereoisomeric analysis*

MEAL	METHOD[1]	STEREOISOMER COMPOSITION		
		NEO-β-CAROTENE B	ALL-TRANS-β-CAROTENE	NEO-β-CAROTENE U
		per cent	*per cent*	*per cent*
Dehydrated No. 1	I	37.1	41.3	21.6
		38.7	40.3	21.0
	II	37.6	39.8	22.6
		37.5	38.9	23.6
Dehydrated No. 2	I	32.1	56.0	11.9
		33.5	55.3	11.1
		33.7	55.2	11.0
	II	31.6	55.3	13.0
		32.0	54.2	13.7
		33.2	53.8	12.9
Sun-cured	I	12.1	76.6	11.3
		12.4	76.5	11.0
	II	13.3	72.2	14.5
		13.0	72.0	15.1

[1] Method I—Quantitative extraction with acetone, followed by preliminary purification on magnesia column prior to chromatography on hydrated lime.
Method II—Partial extraction with petroleum ether, followed by chromatography on hydrated lime.

covered with black paper or cloth. In all cases, neo-U as well as neo-B was found. A sample of commercially obtained crystalline β-carotene prepared from alfalfa contained less cis isomers than the fresh alfalfa. This is to be

TABLE 4.—*Stereoisomer composition of "total carotene" fraction of fresh whole alfalfa*

LOCATION OF FIELD	CONDITION OF SAMPLE	NEO-β-CAROTENE B	STEREOISOMER COMPOSITION	
			ALL-TRANS-β-CAROTENE	NEO-β-CAROTENE U
Albany, Calif.	as cut	6.3	84.8	9.0
Albany, Calif.	as cut*	7.1	84.8	8.0
Albany, Calif.	frozen	7.6	83.4	9.0
Albany, Calif.	frozen	7.0	84.7	8.1
Firebaugh, Calif.	frozen	3.0	89.1	7.9
Kerman, Calif.	frozen	4.0	87.6	8.4
Ryer Island, Calif.	frozen	4.6	90.0	5.4
Vorden, Calif.	frozen	8.1	84.6	7.3

* Analyzed in darkness.

expected, since it has been shown that all-trans-β-carotene will crystallize first from a solution containing a mixture of stereoisomers of β-carotene (2).

PREPARATION OF CAROTENE EXTRACTS FROM ALFALFA FOR STEREOISOMERIC ANALYSIS

Dehydrated or sun-cured meal: About 10 g. of meal are soaked in 25 ml. of petroleum ether (b.p. 88° to 99°C.) for 15 minutes in the dark. After filtering, the extract is ready for stereoisomeric analysis as described in the preceding paper (3). For good quality dehydrated meals 1 ml. extract will contain about 40 micrograms of carotene.

Fresh plant material: About 20 g. of fresh or frozen alfalfa is ground in a Waring Blendor[2] with 200 ml. of acetone for ½ minute. The extract is then filtered rapidly through a coarse filter and added to 25 ml. of petroleum ether in a 500 ml. separatory funnel. The acetone is removed with about 5 separate washes with water and the extract dried over sodium sulfate. After drying it is ready for stereoisomeric analysis.

DISCUSSION OF RESULTS

In accordance with previous work (8) the results reported herein show small but significant amounts of neo-B and neo-U in all samples of fresh alfalfa examined. The amount of cis isomers found in dehydrated meal is much greater than that found in the fresh tissues and demonstrates that much of the stereoisomers present in the meal must have been formed during the dehydration process. The meal samples described in this paper were obtained directly from the dehydrators, with minimum opportunity for change in isomer content.

Some dehydrated meals contained a slightly higher apparent neo-B content than the 33 per cent found in an iodine-catalyzed equilibrium mixture at room temperature (3). This may be due to the formation of higher amounts of the less abundant isomers at the high temperatures required for dehydration. Polgár and Zechmeister (10) found larger amounts of members of the neo-β-carotene A, B, C . . . group to be present in mixtures of the stereoisomers obtained by melting crystals of all-trans-β-carotene than in the equilibrium mixture at room temperature. In our work with alfalfa meal, we have obtained chromatographic evidence of the existence of members of this group other than neo-B. These are included in the neo-B fraction in our analytical procedure.

The results on sun-cured meal and fresh alfalfa (Tables 3 and 4) show that sun-curing produces some cis-isomers at the expense of all-trans. The total carotene content of the sun-cured meal is considerably lower than that of the dehydrated meals.

Nutritional implications: Neo-β-carotene B has 53 per cent of the

[2] The mention of these products does not imply that they are endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.

growth-promoting activity of all-trans-β-carotene in rats (5) or chicks (6), and neo-β-carotene U is variously reported to have 25 per cent (9) or 38 per cent (4) of the activity of all-trans-β-carotene when fed to rats. Most estimates of the vitamin A equivalence of carotene in dehydrated meal are based on the assumption that all the β-carotene is in the all-trans form. Data in this paper show that as much as 60 per cent of the carotene may exist in partially cis forms. If the corrections mentioned above are applied to a commercial meal containing 38 per cent neo-B, 41 per cent all-trans, and 21 per cent neo-U, the calculated nutritional value will be 30 per cent lower than found by a "total carotene" analysis, which assumes an isomer composition of 100 per cent all-trans-β-carotene.

SUMMARY

The liquid chromatogram procedure has been applied to the stereoisomeric analysis of β-carotene extracts of alfalfa into three fractions consisting primarliy of neo-β-carotene B, neo-β-carotene U, and all-trans-β-carotene. The presence of light during extraction and analysis will cause isomerization of the pigments. In reduced light this source of error is minimized.

Neo-β-carotene B and neo-β-carotene U may constitute as much as one-half of the β-carotene content of dehydrated alfalfa meal. If no correction is made for them, analyses which yield only "total carotene" values will indicate vitamin A potencies as much as 30 per cent higher than those in which due allowance is made for the reduced nutritional value of these stereoisomers.

ACKNOWLEDGMENT

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DETERMINATION OF DDT IN SOILS

By Louis Koblitsky and R. D. Chisholm (U. S. Department of Agriculture, Agricultural Research Administration, Bureau of Entomology and Plant Quarantine, Moorestown, N. J.)[1]

In one of the treating procedures authorized as a basis for certification of plants to be shipped from areas regulated by the Japanese beetle quarantine, DDT is applied at the rate of 25 pounds per acre and cultivated into the soil to a depth of 3 inches (1). Owing to cultural practices, retreatment is often necessary when analyses of soil samples taken from treated areas indicate a loss in DDT content. The method described was developed for the purpose of simplifying the work of annual adjustment of nursery soils to this prescribed level. The program involved the sampling of a large number of plots of various soil types and provided only a short period of time for analysis.

METHOD OF ANALYSIS

The samples are composites of 50 borings, each 2 inches in diameter and 3 inches in depth (471.24 cubic inches (V)[2]) and taken at locations rather evenly spaced throughout areas of 20,000 square feet or less. Sampling is best accomplished when the soil is moist.

PREPARATION OF SAMPLE

Brush the composite sample thru a 4-mesh screen, mix thoroly, and weigh (W_3).[2] Pour the sample into a can of such dimensions that the soil is at least 8 inches deep. Subsample by means of a thin-walled steel tube 1 inch in diam., taking seven cores, one from the center and one two-thirds the distance from the center to the rim on each of six radii equally spaced around the can. Weigh the subsample (W_2),[2] expose in shallow trays until nearly air-dry, reweigh (W_1),[2] and store in air-tight jars pending analysis. If duplicate subsamples are required, shake the can to close the holes left by the subsampler and take another subsample in the same way.

SPECIAL APPARATUS

(1) *Extraction jars.*—Quart Mason jars fitted with glass or plastic-lined lids.

(2) *Shaking machine.*—A machine that rotates the jars end over end about 25 times per minute.

(3) *Electrometric titrimeter.*

(4) *Electrode system:*

(a) Silver electrode.—Solder insulated copper wire to one end of a 2-inch length of 10-gauge pure silver wire. Seal into a 6-inch length of glass tubing with De Khotinsky cement so that $\frac{3}{4}$ inch of the silver wire is exposed. Connect the other end of the copper wire to the electrometric titrimeter. Clean this electrode occasionally by gently rubbing the silver surface with fine emery cloth.

(b) Mercury-mercurous sulfate electrode (Fig. 1).—Pour enough electrolytic mercury (A) into a wide-mouth 4-ounce jar (B) to make a layer 3 to 5 mm deep, and

[1] Grateful acknowledgment is made to S. R. Dutky, for his suggestions on the mercury-mercurous sulfate electrode, and to L. B. Parker for the preparation of the photograph.

[2] Term used in formula for calculating results.

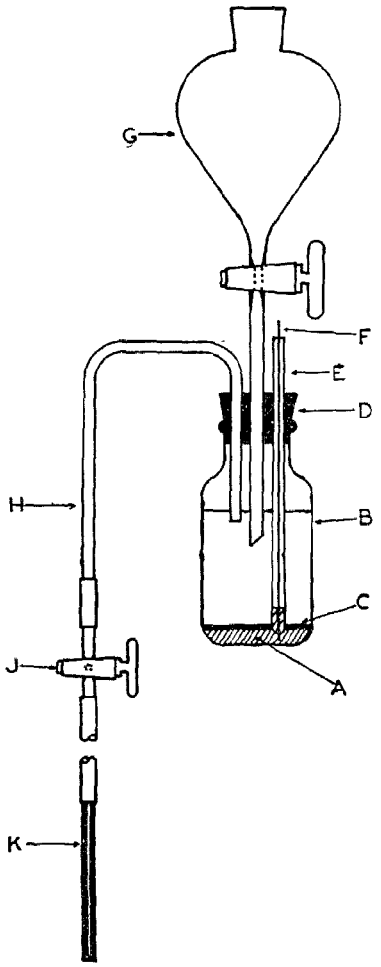

FIG. 1.—Mercury-mercurous sulfate electrode.

REAGENTS

Benzene.—Chlorine and thiophene free.
Isopropanol.—99%.
Metallic sodium.—A.C.S. grade.
Hydrogen peroxide.—30% reagent grade.
Bromthymol blue.—0.4% soln.
Sulfuric acid.—A.C.S. grade.
Celite.—C.P. analytical filter aid.
Silver nitrate soln (0.025 *N*).
Potassium thiocyanate soln (0.025 *N*).

then add a paste (*C*) made from a small amount of mercurous sulfate and 0.1 *N* sulfuric acid. Allow the paste to settle and fill the jar three-quarters full with the acid. Close the jar with a three-hole rubber stopper (*D*). Thru one hole pass a glass tube (*E*) with a sealed-in platinum wire tip until contact with the mercury layer is made. Join the other end of the platinum wire to a copper wire (*F*) by means of a mercury seal. Connect the other end of the copper wire to the electrometric titrimeter. Thru another hole pass the stem of a separatory funnel (*G*) until its tip is below the surface of the acid in the jar. Thru the third hole pass one end of a glass siphon tube (*H*) until the end in the jar is below the level of the acid. To the other end connect a stopcock (*J*). Apply narrow bands of lubricant to the ends of the stopcock plug to provide for a continuous acid film at all times. Connect a capillary tube (*K*) 6 inches long to the other end of the stopcock. Mount the electrode above the soln to be titrated, partially fill the separatory funnel (*G*) with 0.1 *N* sulfuric acid, and fill the siphon (*H*). Flush the capillary tube (*K*) by opening stopcock *J* for a few seconds and insert into the solution to a depth of about 2 inches.

DETERMINATION

Weigh 300 g of the subsample and pipette 300 ml of extracting solvent (benzene 2 plus isopropanol 1) into an extraction jar, seal, and place on the shaking machine. Remove after ½ hour of agitation and filter into a suction flask thru a Büchner funnel, using gentle suction. Transfer a 200-ml. aliquant into a 500-ml Erlenmeyer flask and evaporate almost to dryness on a water bath kept close to 90°C. Add 25 ml of isopropanol and 2.5 g of metallic sodium cut into small pieces. Reflux for at least ½ hour on a hot plate, shaking occasionally. Eliminate the undecomposed sodium by cautiously adding thru the condenser 20 ml of dilute (1 +1) isopropanol. Boil for a few minutes, add 25 ml of water, cool, add 5 ml of hydrogen peroxide 1 ml at a time, and boil for 15 minutes. Disconnect the flask from the condenser, cool, add a few drops of bromthymol blue, neutralize with dilute (1 +2) sulfuric acid, add 5 ml of the acid in excess, and cool. Add an excess of 0.025 *N* silver nitrate, coagulate the precipitated silver chloride, add about 1 g of celite, and filter thru a fast quantitative paper into a 400-ml beaker. Wash flask, paper, and precipitate thoroly with small portions of water, keeping the filtrate and washings between 250 and 300 ml. Titrate the excess silver nitrate in the filtrate with 0.025 *N* potassium thiocyanate. Use an electrometric titrimeter equipped with silver and mercury-mercurous sulfate electrodes. Between titrations wash the electrodes with water and flush the capillary tip. Conduct a reagent blank and, whenever possible, soil blanks at the same time and in the same manner.

CALCULATION OF RESULTS

Subtract the quantity of silver nitrate found in the filtrate from that originally added. The difference (corrected for the blank) will be that required to combine with the chlorine liberated from the DDT. The weight of chlorine times 2 will give the amount of DDT in the aliquant titrated (*U*).[2] Calculate the DDT as pounds per 3-inch acre by the following formula, rounding to the nearest ½ pound:

$$\text{Pounds per 3-inch acre} = \frac{U \times W_1 \times W_3 \times 41{,}486}{S \times W_2 \times V}$$

Where U = Weight of DDT in aliquant titrated (grams).

S = Weight of nearly air-dry subsample represented by aliquant (grams).

W_1 = Weight of nearly air-dry subsample (grams).

W_2 = Weight of subsample taken from screened composite sample (grams).

W_3 = Weight of screened composite (grams).

V = Volume of composite = Top area × depth of boring × number of borings (cubic inches).

41,486 = Factor to convert grams per cubic inch to pounds per 3-inch acre.

DISCUSSION

The method is based on the determination of total organic chlorine as described by Umhoefer (2). Though not specific for DDT, his method has been adapted to the determination of DDT in agricultural sprays, spray residues, and various foods (3, 4, 5, 6). The colorimetric method of Schechter *et al.* (7) was found to be too time-consuming for routine work. The color bodies extracted from soils interfered with the method of Stiff and Castillo (8).

It is conceivable that chlorine-bearing decomposition products of DDT may accumulate in the soil from year to year and eventually vitiate results by the present method, but there is no evidence at this time that such is the case. Final judgment must be based upon critical studies of the ultimate fate of DDT in various types of soil, using more specific chemical (or biological) methods.

Laboratory-prepared mixtures of DDT and a sandy loam, a clay soil, and a muck soil were used in developing the method. These soils were selected as being representative of soils that might be encountered in nurseries. It was found that the recovery of DDT from these mixtures was influenced by such variables as the extracting solvent, the moisture content, and the type of soil.

The recovery of DDT from these mixtures was best when they were extracted for 30 minutes with a mixture of 2 parts by volume of benzene and 1 part of 99 per cent isopropanol. Several investigators (3, 5, 9) have used benzene as the solvent to remove DDT from spray residues. However, when this extractant was used, the recoveries from a sandy-loam mixture prepared at the rate of 50 pounds of DDT per 3-inch acre were 49, 41, 35.5, 36.5, and 34.5 pounds when analyzed immediately, 1 week, 2, 5, and 7 months after preparation, respectively. From a sandy-loam mixture prepared at the rate of 25 pounds of DDT per 3-inch acre, analyzed 7 months after preparation with petroleum ether, acetone, ethanol, or a mixture of benzene and acetone as the extractant, the recoveries were all about 17 pounds. With benzene and isopropanol, 2+1 parts by volume, as the extractant, close to theoretical recoveries were obtained immediately after preparation and periodically over a period of 2 years.

Benzene and isopropanol mixed 2+1 parts by volume (2.2+1 parts by weight) approximates very closely the azeotropic mixture of these compounds. That mixture, 2+1 parts by weight, has a boiling point of 71.9°C., which is considerably lower than the boiling points of its constituents.

The possibility of decomposing the DDT by overheating while the solvent is evaporating, as suggested by Fleck (4) and others (5, 10), is thus reduced. When a soil extract to which DDT had been added was evaporated in a water bath kept close to 90° C., the recovery of DDT was 99.6 per cent. A similar recovery was obtained when the residue was allowed to remain in the water bath 1 hour after evaporation of the solvent was complete.

Electrometric titration is used because color retained in the treated extract from most soils obscures the end point of a visual titration.

The silver and mercury-mercurous sulfate electrodes are easily prepared and maintained. The silver, or indicating, electrode is not attacked by the solution acidified with sulfuric acid. In the reference electrode the concentration of the mercurous ions in contact with the mercury is kept constant by covering the mercury with a suspension of slightly soluble mercurous sulfate in dilute sulfuric acid, thus providing a common anion in both the salt and the solution (11).

Back titration with potassium thiocyanate has been found to be more sensitive and consequently preferable to direct titration with silver nitrate for the analysis of miscellaneous soils. One-tenth ml. of 0.025 *N* potassium thiocyanate gives a potential change near the equivalence point of about 35 millivolts, as compared with about 10 millivolts by direct titration with silver nitrate of the same normality. Thus a sharp end point is obtained more rapidly by back titration. However, this procedure is a little longer than the direct procedure. Since certain soils contain a considerable amount of extractable material, both procedures involve filtration. Direct titration in the presence of this material may result in the fouling of the silver electrode. The additional time required for precipitation in the back-titration procedure is partly compensated for by the time saved in titrating.

The average recoveries of DDT, rounded to the nearest ½ pound, from laboratory-prepared mixtures of DDT with several soils of variable moisture content are shown in Table 1.

TABLE 1.—*Average recovery of DDT, in pounds per 3-inch acre, from several soil mixtures prepared at the rate of 25 pounds of DDT per 3-inch acre*

CONDITION OF SOIL MIXTURE WHEN ANALYZED	SANDY LOAM		CLAY SOIL		MUCK SOIL	
Air-dry	22	(10)*	24	(6)	26	(5)
Nearly air-dry	24	(30)	23	(10)	23.5	(16)
Just below maximum water-holding capacity	21	(2)	19	(2)	21	(2)

* Figures in parentheses are the number of determinations made.

With air-dry soils the recoveries were a little higher in the clay and muck soils and lower in the sandy loam than in the nearly air-dry soils. With the wet soils the recoveries were considerably lower. The variation between the various soils was least when the soils were nearly air-dry.

These recoveries were obtained by subtracting soil and reagent blanks from the total DDT determined. Since in many cases it is impossible to secure untreated soils for blanks, the results were recalculated by subtracting the reagent blank only. The soil blanks were 1, 1, and 3.5 pounds for the sandy loam, clay soil, and muck soils, respectively. The reagent blank was 1.5 pounds. The recalculated recoveries from the nearly air-dry soils were 25, 24, and 27 pounds DDT per 3-inch acre for the respective sandy, clay, and muck soil-DDT mixtures.

A total of 84 samples taken from nursery plots treated with DDT were analyzed. The differences between analyses on duplicate subsamples from the same composite averaged 1 pound per 3-inch acre, and ranged between 0 and 2.5 pounds.

This method has also been adapted to the determination of DDT in spray and dust residues on apples, peaches, potatoes, elm twigs, foliage, paper toweling, and glass plates.

SUMMARY

A method for the determination of DDT in soil is given. The average recoveries from sandy-loam, clay-soil, and muck-soil mixtures prepared at the rate of 25 pounds DDT per 3-inch acre were 24, 23, and 23.5 pounds, respectively. The recoveries from the same mixtures were 25, 24, and 27 pounds when no soil blanks were subtracted, since in many cases it is inpossible to secure untreated soils. The variation between soils was least when analysis was made when the soils were nearly air-dry. The differences between recoveries from duplicate subsamples averaged 1 pound per 3-inch acre and ranged between 0 and 2.5 pounds. The method has been successfully used for the determination of DDT in spray and dust residues. The description of a convenient dip-type electrode is given.

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MICROSCOPIC IDENTIFICATION OF SUCCINIC ACID AS BARIUM SUCCINATE

By WILLIAM V. EISENBERG (Division of Microbiology)[1] and FRED HILLIG (Division of Food,[2] Food and Drug Administration, Federal Security Agency, Washington, D. C.)

In a paper[3] on the determination and identification of lactic and succinic acids in foods, the method for succinic acid there given directs that, after the final titration with barium hydroxide, the aqueous layer be evaporated

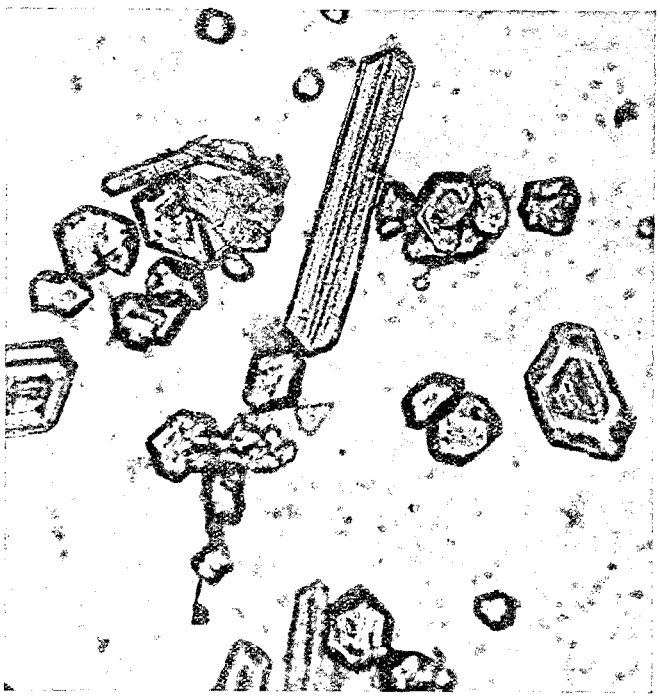

FIG. 1.—Barium succinate crystallized from water.

until crystals appear and that these be compared with crystals of pure barium succinate similarly prepared. However, the microscopic-crystallographic constants of barium succinate crystals were not given at that time.

These measurements have been made on crystals obtained from decomposed eggs, and were found to be identical in every respect with those of pure barium succinate. In both cases the aqueous solution of barium succinate was evaporated almost to dryness and allowed to stand until the

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[2] W. B. White, Chief.
[3] Clayborn and Patterson, *This Journal*, **31**, 134 (1948).

crystals formed. The crystals were then removed to a microscopic slide, dried, and examined under the polarizing microscope by the immersion method.

MICROSCOPIC-CRYSTALLOGRAPHIC PROPERTIES OF BARIUM SUCCINATE

Barium succinate crystallizes from water as bipyramids and prisms of the tetragonal system, having a tabular and long prismatic habit (see Fig. 1). In parallel polarized light (crossed nicol prisms) the crystals exhibit parallel and symmetrical extinction. Elongated prisms exhibit extinction parallel to the long *c* axis and positive elongation. In convergent polarized light a positive uniaxial interference figure slightly inclined to the optic axis is observed on tabular bipyramidal crystals and on crushed particles which do not extinguish sharply. The principal refractive indices are $n_\omega = 1.580$ and $n_\epsilon = 1.633$, both ± 0.002. The index of the ϵ ray may be measured on the long axis of the elongated prisms and on crushed particles showing maximum birefringence.

DETERMINATION OF MONOFLUOROACETIC ACID IN FOODS AND BIOLOGICAL MATERIALS*

By L. L. Ramsey and P. A. Clifford (Division of Food,† Food and Drug Administration, Federal Security Agency, Washington 25, D. C.)

Sodium monofluoroacetate, also known as 1080, is finding increasingly widespread use as a rodenticide. Because of its extreme toxicity to all warm-blooded species, the use of 1080 by commercial exterminators in food warehouses and manufacturing plants presents problems of grave concern to health and regulatory officials. Hence, in regulatory and pharmacological work there is urgent need for a method for determining micro amounts of monofluoroacetic acid in foods and biological materials.

The method proposed here for the determination of monofluoroacetic acid is based upon the fluorine content of an isolated fraction containing only organic acids. The essential steps of the method are:

(1) Sample preparation, which includes a protein separation step in case of protein-containing materials and may include a hot aqueous extraction or an enzymatic digestion in certain instances,

(2) Ether extraction, followed by removal of all acids from the ether with aqueous alkali,

(3) Separation of fluoroacetate from inorganic fluorine compounds by partition chromatography, employing a silicic acid column with 0.5 *N*

* Presented in part at the meeting of the Washington Section of the American Chemical Society May 12, 1949. Essentials of the method in mimeographed form have been distributed to regulatory officials and other interested persons.

† W. B. White, Chief.

sulfuric acid as the immobile solvent and chloroform containing 10 per cent by volume of tertiary amyl alcohol or *n*-butyl alcohol as the mobile solvent, and

(4) Ignition of the fluoroacetic acid with lime to convert the fluorine to inorganic fluoride, which is then determined by an established technique (1).

The method is highly specific for monofluoroacetic acid. Although inorganic fluorides or fluosilicates are readily extracted from an acid solution with ether, a 1000-fold excess of mineral fluoride does not materially affect the analysis. The critical step in the procedure is the chromatography, in which the separation of monofluoroacetic acid from inorganic acids containing fluorine is 100% complete. Since the percolate from the chromatographic column contains only organic acids, only fluorine-containing organic acids are measured. No such acids are known to exist naturally in foods or biological tissue,[1] but the method would measure the fluorine of difluoroacetic and trifluoroacetic acids, if present. Apparently these acids do not occur in commercial 1080 to any appreciable extent, as indicated by a total fluorine analysis.

Sensitivity of the procedure is governed by that of the micro-method for the determination of fluorine. With careful evaluation and stability of the reagent and apparatus blank (about 5 micrograms F), as little as 10 micrograms F can be determined with good precision. This quantity of organic F represents about 41 micrograms of monofluoroacetic acid. With a 200-gram sample containing 41 micrograms of fluoroacetic acid in the aliquot taken for titration, this permits a quantitative determination at a level of about 0.2 p.p.m.

A colorimetric microanalytical method for acetate and fluoroacetate, based upon the lanthanum nitrate-iodine qualitative test for the acetate ion, has been proposed by Hutchens and Kass (2). Besides lacking specificity their method failed to recover fluoroacetate added to grain and tissue; furthermore, they were unable to recover the fluorine of the added 1080.

Our findings by the method presented here do not confirm those of Hutchens and Kass and do not support their suggestion that the fluoroacetate is firmly bound or converted to another substance. We have found that fluoroacetic acid added as the potassium salt to biological tissue and a wide variety of foods can be recovered essentially quantitatively. However, in order to obtain reproducible quantitative recoveries of added fluoroacetate we did find it necessary to subject flour samples to a pancreatic digestion, when the fluoroacetate had been added in aqueous solution and the flour air dried for a period of several weeks prior to analysis. Furthermore, it has been found that a pancreatic digestion,

[1] A South African plant, Gifblaar, *Dichopetalum Cymosum*, however, has been found to contain monofluoroacetic acid. (*Onderstepoort Journal of Veterinary Science and Animal Industry*, **20**, 67 (1944).)

or even a pre-cooking of the sample in boiling water, will improve the recoveries of 1080 from poisoned rats.

METHOD

APPARATUS

(1) Chromatographic tubes, 18 mm O.D. ×250 mm long, prepared from Pyrex tubing.

(2) Suitable pressure source, such as compressed air or a cylinder of nitrogen or carbon dioxide; and a means of keeping the pressure constant, such as a column of mercury or a diaphragm type pressure regulator.

(3) Mixer of the Waring Blendor type.

(4) Fluorine apparatus listed in *Methods of Analysis, A.O.A.C.*, Sixth Ed., sec. 29.24.

REAGENTS

(1) *Silicic acid.*[2]

(2) *Mobile solvent.* Add 100 ml of tertiary amyl alcohol[3] or *n*-butyl alcohol to 900 ml of U.S.P. chloroform and mix.

(3) *Sulfuric acid solns*: 1 +1, ca 0.1 *N* and ca 0.5 *N*.

(4) *Sodium hydroxide solns*, ca 1.0 *N* and ca 0.1 *N*.

(5) *Phenolphthalein soln*, 1%.

(6) *Trisodium phoshate soln*, saturated.

(7) *Pancreatin, U.S.P.*

(8) *Ether, A.C.S.*

(9) *Phosphotungstic acid*, 20% *W/V*.

(10) Fluorine reagents listed in *Methods of Analysis, A.O.A.C.*, Sixth Ed., sec. 29.25.

PROCEDURE

(1) *Preparation of sample.*—This will vary with different types of materials. Sugar can merely be dissolved in water, acidified with sulfuric acid, and extracted directly. A simple water wash may be adequate to prove contamination of certain other foods.

(a) *Sugar.*—Dissolve 100 g of sugar in sufficient water to give a volume of ca 350 ml.

(b) *Flour.*—Place 100 g of flour in the mixer, add 400 ml of water and 5 g of pancreatin, and comminute ca 2 minutes. Adjust to *p*H 7–8, using the saturated soln of Na_3PO_4 and a suitable indicator paper.[4] Transfer the comminuted material to a tared 1-liter Erlenmeyer flask, washing the mixer three times with 25 ml portions of water. Incubate the mixture at 35–40°C. for at least 3 hours. Add 5 ml of (1 +1) H_2SO_4 and swirl. Add 20 ml of 20% *W/V* phosphotungstic acid and swirl again. Make up to 750 g with water, stopper, and shake vigorously ca 2 minutes. Filter thru a Büchner funnel (16 cm size is convenient) with suction or thru a fluted filter. Use at least 375 g aliquot of filtrate. (Since the specific gravity of the filtrate is very close to 1, measuring out the aliquot in a graduated cylinder is satisfactory.) If only small continuous extractors (less than 400 ml capacity) are available, neutralize the filtrate aliquot to phenolphthalein with strong NaOH and finally adjust the soln just to the alkaline color of the indicator with the 0.5 *N* H_2SO_4 and *N* NaOH. Concentrate the soln to a volume of ca 100 ml by boiling. If the soln bumps or spatters while boiling, place on a steam bath; a current of air will hasten the evaporation.

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[2] Mallinckrodt's Analytical Reagent Grade precipitated powder was used in this work.
[3] Sharples Chemicals, Inc. Technical Product was used.
[4] Fisher Alkacid test paper is satisfactory.

(c) *Wheat.*—Grind finely in a suitable mill such as a Wiley mill. Proceed as for flour.

(d) *Corn meal.*—Proceed as for flour in (b) above, except omit the pancreatic digestion.

(e) *Corn.*—Grind and proceed as for corn meal.

(f) *Peanuts.*—Grind finely (like peanut butter) and proceed as for cornmeal, except use 100 ml of the 20% phosphotungstic acid per 100 g sample. It may be necessary to refilter thru a folded filter to remove oil.

(g) *Cheese.*—Proceed as for cornmeal, except use 40 ml of phosphotungstic acid for the 100 g sample.

(h) Other foods such as chili peppers, cacao beans, etc., can be treated in a manner similar to the above.

(i) *Biological tissue.*—If the material is tough or fibrous, grind it twice thru a food chopper. (Soft tissues, *e.g.* brain and liver, need not be ground.) Place 100 g of the ground tissue in an 800 ml beaker, add ca 300 ml of water, cover with a watchglass, and cook by boiling gently ca one-half hour. Transfer the material to the mixer, rinsing the beaker with two 25 ml portions of water, and comminute thoroly (ca 2 minutes). Transfer the comminuted material to a tared liter Erlenmeyer flask, rinsing the mixer with two 25 ml portions of water. Add 5 ml of the 1 + 1 H_2SO_4 and mix. Add sufficient 20% phosphotungstic acid (usually 50–75 ml) to precipitate all the proteins, then water to make the weight 600 g. Shake vigorously for ca 2 minutes and filter thru a fluted filter paper, or with suction thru a Büchner funnel. If the material does not filter rapidly, return the mixture to the flask, add ca 10 ml more of the phosphotungstic acid, shake vigorously, and refilter.

Alternative procedure to cooking the tissue: Place 100 g of ground tissue in the mixer, add 300 ml water and 15 g of pancreatin, and comminute thoroly (ca 2 minutes). Adjust the *p*H to ca 8 with the saturated Na_3PO_4 soln using a suitable indicator paper. Transfer the comminuted material from the mixer to a tared liter Erlenmeyer flask, washing the mixer with two 25 ml portions of water; and incubate ca 3 hours at 35–40°C. Continue by precipitating the proteins and making to volume as above.

(2) *Ether extraction.*—Transfer the soln in the case of sugar or a weight-aliquot of the protein-free filtrate (in the case of protein-containing materials) to a large continuous extractor (200 ml size described in *Methods of Analysis, A.O.A.C.*, **26.45 (b)**; 1500 ml extractors of this type have been used successfully. An extra coarse fritted filter tip[5] on the bottom end of the inner tube aids in getting smaller droplets of extracting solvent). For each 50 grams of soln, add 1 ml of H_2SO_4 (1 + 1). Extract with ether until all fluoroacetic acid has been extracted, as determined by a preliminary experiment (usually 3–4 hours with a 400 ml extractor). Transfer the ether extract to a separatory funnel of appropriate size.

To the extraction flask add ca 20 ml of water, two drops of 1% phenolphthalein indicator soln, and sufficient 1.0 *N* NaOH from a buret to give the strong alkaline color of the indicator after swirling. Pour the rinse soln into the separatory funnel and add more alkali until the alkaline color of the indicator persists in the aqueous phase after vigorous shaking. Record the volume of alkali required. Draw off the aqueous layer into a 100-ml beaker and wash the ether with two 10-ml portions of water, rinsing the extraction flask each time with the water before pouring it into the separatory funnel. Add the washings to the beaker. Carefully adjust the alkalinity of the extract just to the alkaline color of phenolphthalein with 0.1 *N* H_2SO_4 and NaOH solns. Evaporate the neutralized extract to dryness on the steam bath.

[5] Ace Glass Inc. Cat. No. 8600, porosity A is suitable.

(A current of air will hasten the evaporation.) If during the evaporation the alkaline color of the indicator should disappear, add just sufficient 0.1 *N* NaOH to give the alkaline color again. Do not continue heating after the residue is apparently dry. (One can continue with a residue which is slightly moist.)

(3) *Chromatography.*—To 5 g of silicic acid in a mortar add the maximum amount of 0.5 *N* H_2SO_4 that it will hold without becoming "sticky" (ca 50–80% of its weight). Mix well with the pestle, then add ca 35 ml of the mobile solvent and work up into a slurry. (This should be smooth; if the silica agglomerates in the solvent, too much 0.5 *N* H_2SO_4 was used.) Place a small cotton plug in the bottom of a chromatographic tube and pour in slurry tilting the tube slightly to avoid air bubbles. Allow the silicic acid to pack down under 2–10 pounds pressure applied thru a gas pressure regulator. When excess solvent has drained thru (column firm and viscous enough to resist pouring when tipped), the column is ready for use. (In this step, care must be exercised to avoid cracking or drying out of the gel; this is caused by leaving the pressure on after the column has packed down and all the solvent has sunk into the gel. However, if a column does crack, the determination need not necessarily be lost even though the sample has been added to the column. Rejuvenate the column by removing the Na_2SO_4, adding ca 10 ml of the mobile solvent to the gel, and stirring it thoroly with a long glass rod. Allow the column to repack under pressure, return the Na_2SO_4 to the column, fill the tube up with solvent, and then collect sufficient percolate from this point, to obtain all the fluoroacetic acid when combined with that which had passed thru prior to the cracking, if any.)

To the dry or slightly moist residue in the 100-ml beaker add sufficient 1+1 H_2SO_4 (ca 18 *N*), usually 0.5–1.0 ml, to give an excess of ca 0.25 ml of (1+1) H_2SO_4 over the amount calculated as being required to convert all the salts to the free acid, based upon the amount of *N* NaOH required to neutralize the acid extracted by the ether. Wet the salts *thoroly* with the acid, using a small narrow blade spatula (steel or monel metal) to loosen the salts from the glass and using a glass tamping rod (stirring rod flattened on one end) to break up the solid particles and mix the resulting slurry. Add anhydrous granular Na_2SO_4, 5–10 g usually being sufficient, to take up the excess liquid. Stir well with the tamping rod, breaking up any lumps that may form. Add 10 ml of the mobile solvent, stir thoroly, and decant the solvent carefully onto the column.

Place a graduated cylinder under the column to catch the percolate and apply pressure. When all the solvent has sunk into the gel, release the pressure. Add 5 ml of the mobile solvent to the beaker and again stir thoroly. Decant the solvent carefully onto the column and with the aid of a narrow blade spatula, transfer the bulk of the material in the beaker, mostly Na_2SO_4, to the column. Renew the pressure. When the solvent has passed ca halfway thru the Na_2SO_4, release the pressure. Rinse out the beaker with another 5 ml portion of solvent and transfer to the column. After this washing has sunk ca half-way into the sodium sulfate, fill the tube with the mobile solvent and complete the collection, under pressure, of sufficient percolate, dropwise (3–4 ml per minute is a convenient rate), to obtain all the fluoroacetic acid as determined by a preliminary experiment. (With several batches of silicic acid this amount of percolate was found to be 50 ml.)

Transfer the percolate to a 125 ml separatory funnel, add ca 20 ml of water and sufficient 1.0 *N* NaOH to give the alkaline color of phenolphthalein (present in percolate; no additional phenolphthalein required) in the aqueous phase, after vigorous shaking. Draw off the aqueous layer into a 125-ml Erlenmeyer flask and return the solvent layer to the separatory funnel. Wash the solvent twice with 10 ml portions of water and add the washings to the Erlenmeyer flask. Aerate the soln with a current of air to remove traces of chloroform. (If excess chloroform is not removed, excessive chloride may complicate the F distillation in step 4.)

(4) *Determination of fluorine.*—Transfer the aqueous extract to a platinum dish with a little water, mix with ca 20 ml of the lime suspension (*Methods of Analysis, A.O.A.C.*, **29.25,**) evaporate to *dryness*, and ash briefly (15–20 minutes) at 600°C. (A little carbon in the ash will not interfere in the determination.) Proceed as directed under **29.26 (a), 29.27, 29.28.** (Use of the 100-ml size Nessler tubes is preferable.) Convert F results to fluoroacetic acid or to sodium monofluoroacetate (1080) as desired and correct for the aliquot taken, if any, in the extraction step. The volume occupied by the insoluble solids is ignored.

RESULTS AND DISCUSSION

Analysis of commercial sodium monofluroacetate (1 lot) for fluorine gave 91 and 94% (duplicate determinations) of the theoretical value. This material contained 0.9% chlorine.

Since the fluorine content of commercial 1080 was somewhat low and an appreciable amount of chlorine was present, the product was purified for the experimental work. The commercial 1080 was dissolved in water, acidified with an equivalent amount of sulfuric acid, extracted in a large continuous extractor with ether, and the ether distilled off. The fluoroacetic acid was distilled and refractionated, the fraction boiling at 167–167.5°C. being taken. This fraction was dissolved in alcohol, neutralized with alcoholic potassium hydroxide, and the precipitated potassium salt recrystallized from alcohol. Fluorine in the potassium salt was found to be 16.1 and 16.2% (duplicate determinations); the theoretical value is 16.36%. This salt was deemed to be pure enough for our purpose; in fact, it was considered 100% pure in calculating the recovery experiments. The pure potassium salt as well as commercial 1080 was found to be appreciably hygroscopic when the atmospheric humidity was above a certain undetermined level.

Step two of the method directs that the aqueous extract containing the fluoroacetic acid be neutralized just to the phenolphthalein end point and evaporated to apparent dryness and that heating is not to be continued after this point. Prolonged heating after apparent dryness has been reached always leads to low results, regardless of the *p*H at which the solution was adjusted prior to the evaporation. If the *p*H is too high, there appears to be some hydrolysis and if the *p*H is too low, the loss appears to be due to volatilization of the acid. Table 1 shows the effect on fluoroacetic acid recovery by evaporating its solution at various *p*H levels and drying the residue for various periods of time at 100°C. in preparation for the chromatography step. In these experiments, solutions of potassium fluoroacetate equivalent to 1 mg of fluoroacetic acid were used. It will be noted from the table that evaporating a solution of the pure potassium monofluoroacetate in the presence of ca 0.7 g of Na_2SO_4, and heating the residue overnight, resulted in a low recovery, 70%.

An experiment was conducted to determine the ease of hydrolysis of fluoroacetic acid. One milligram of fluoroacetic acid in the form of its potassium salt was added to 25 ml of *N* NaOH and placed on a steam bath

TABLE 1.—*Effect of pH and heat on recoveries of monofluoroacetic acid*

pH	PERIOD OF HEATING	RECOVERY
		per cent
1. Alkaline color of phenolphthalein	Just to dryness	96, 98
2. Alkaline color of methyl red	About 5 min. after apparent dryness	90
3. Alkaline color of bromcresol green	About 5 min. after apparent dryness	81
4. *p*H7 (borax-phosphate buffer)	Overnight—about 16 hours	66
5. *p*H8 (borax-phosphate buffer)	Overnight—about 16 hours	56
6. Alkaline color of phenolphthalein	Overnight—about 16 hours	77, 79
7. Soln containing 0.7 g of Na_2SO_4 added to potassium fluoroacetate	Overnight—about 16 hours	70
8. Fluoroacetic acid neutralized with ammonium hydroxide	About 5 min. after dryness	48

for two hours, a procedure which will completely hydrolyze monochloroacetic acid (3). Fluoroacetic acid was determined in the hydrolysate by the proposed method; 84% was found, compared, with controls of 95–98% (Table 2).

TABLE 2.—*Recovery of monofluoroacetic acid added as the potassium salt to aqueous solution*

ADDED	FOUND
mg	*per cent*
1.00	98.4
1.00	97.5
1.00	95.5
0+270 mg NaF*	No fluorine found
0.50+270 mg NaF	82

* Quantity of inorganic fluorine in 270 mg NaF is 1000 times the quantity of organic fluorine in 0.5 mg potassium monofluoroacetate.

The somewhat low recovery of fluoroacetic acid (82%) in the presence of a huge excess of inorganic fluoride (Table 2) is believed to be due to incomplete extraction of the fluoroacetic acid from the acidified salts in

the beaker with mobile solvent; at this stage the inorganic fluorine is largely in the form of fluosilicate, which is somewhat insoluble in the 1+1 sulfuric acid.

An experiment was performed to determine the threshold (break-thru) volume, if any, for inorganic fluorides. Fifty mg of inorganic fluorine, one-half in the form of sodium fluoride (55.3 mg) and the other half in the form of potassium fluosilicate (48.3 mg), were placed in a 100 ml beaker, 0.5 ml H_2SO_4 (1+1) added, the material extracted with mobile solvent and chromatographed in the usual way. Fractions of the percolate were tested for fluorine. No trace of fluorine was found in 300 ml of percolate; at this point the collection of fractions was discontinued. Therefore, the chromatographic separation of inorganic fluorine from organic fluorine is complete and there is a wide margin of safety in collecting the fluoroacetic acid fraction.

Measured amounts of fluoroacetic acid as the potassium salt, in the range 0.2–10 p.p.m., were added to various foods and to biological tissues. From foods such as peanuts and cacao beans, which were only washed (not comminuted), recoveries varied from 35 to 70%. Recoveries after comminution of various samples usually fell in the range 90–100%, but in the case of chili peppers, the recovery was markedly lower, about 80% (Table 3). In general, the results indicate that the method is suitable for regulatory problems connected with this compound.

Recovery experiments on flour to which potassium monofluoroacetate was added led to some interesting but not completely explained results. In the early work on flour an enzymatic digestion step was not employed. Recoveries of added fluoroacetic acid in the range 1–10 p.p.m. were 90–100%; however, in all these experiments the analysis was performed within two or three days after the addition of the solution of potassium monofluoroacetate. Subsequently, a collaborative study of the method by several analysts in our laboratories was deemed desirable. Two series of 100 g flour samples were prepared for the study. To each of the samples in one series there was added one ml from a stock solution of potassium monofluoroacetate containing 176 micrograms of fluoroacetic acid per ml and to each sample in the other series 5 ml from the same stock solution, giving a concentration in each series of 1.76 and 8.80 p.p.m. of fluoroacetic acid, respectively. Recoveries obtained by one analyst at the lower level, 1.76 p.p.m., were 97 and 119%, and by a second analyst, 100%. Since the amount of fluorine being determined was rather small, these results are regarded as satisfactory. On the other hand recoveries at the higher level, 8.8 p.p.m., were erratic. Results obtained by one analyst on 9 identical samples were 64, 69, 72, 75, 78, 83, 83, 92, and 95%; by a second analyst on one sample of the same series, 81%; and by a third analyst on one sample of the same series, 72%. However, when the "aged" samples were partially digested with pancreatin before precipitation of proteins with

TABLE 3.—*Recovery of monofluoroacetic acid added as the potassium salt to biological tissue and a wide variety of foods*

PRODUCT	ADDED	RECOVERY
	p.p.m.	*per cent*
Rat Tissue, skinned and disemboweled carcass ground	10	90
	0	None found
Meat and Bone Scrap	10	94, 98
	5	95
	1	96
	0	None found
Sugar	10	90, 92
	1	88, 89
	0.2	86
Flour	10	93, 97
	1	86, 105
	0.2	94
Wheat	10	95
	1	98
Corn	10	97
	1	94
Cheese	10	92
	1	97
Peanuts	10	93
	1	88
Chili Peppers	10	78
	5	81
Cacao Beans	1	92

phosphotungstic acid, the recoveries were normal: 91, 92, and 92% on the same series at the higher level, 8.80 p.p.m.

An experiment was performed to determine, independently of the method presented here, whether the organic fluorine compound extracted from foods to which potassium monofluoroacetate had been added was actually fluoroacetate. To 100 g samples each of flour and hamburger 20 mg of potassium monofluoroacetate was added. The samples were deproteinized, a 60% aliquot extracted with ether, and the ether extracted with aqueous alkali, in accordance with the method. The aqueous alkaline extract of the ether in the case of hamburger gave a positive indigo test (4); but in the case of flour, a negative test. However, upon repeating the

experiment with flour the quantitative method was followed through the chromatography step, in which instance the alkaline (Na_2Co_3) extract of the percolate gave a positive indigo test. This qualitative test[5] is highly specific for organic acids containing a reactive alpha halogen atom and indicates that the organic fluorine compound extracted is, in fact, unchanged fluoroacetate.

SUMMARY

A microanalytical method is described for the determination of monofluoroacetic acid in foods and biological materials; it is based upon the total fluorine content of an isolated organic acid fraction. Partition chromatography is employed to effect complete separation of fluoroacetate from inorganic acids containing fluorine. The method is highly specific, a quantitative determination being readily made in the presence of a 1000-fold excess of inorganic fluorine. Essentially quantitative results can be obtained on foods and biological materials have a concentration of monofluoroacetic acid as low as 0.2 p.p.m.

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PROTECTION OF RIBOFLAVIN FROM DESTRUCTIVE LIGHT RAYS DURING ANALYSIS*

By R. T. OTTES and FLOYD ROBERTS (State Laboratories Department, Bismarck, North Dakota)

Various investigators have reported the sensitivity of riboflavin to light. All procedures for assay warn that the samples must be protected from light during the course of analysis.

In arranging our laboratory for vitamin analysis we desired to provide proper lighting conditions for riboflavin determinations and still have adequate illumination so that the facilities of the same room could be used for other vitamin determinations which would be in progress simultaneously. We felt that by some means it should be possible to filter out the light rays which are most destructive to riboflavin and yet have ample illumination for other work.

A sample of amber transparent shade, the type commonly used in department store display windows for filtering out ultraviolet rays, was obtained from The Transhade Company, 49 West 27th St., New York, N. Y. The light filtering characteristics of this shade were determined by

[5] We wish to thank John B. Wilson, Division of Food, for performing this test.
* Presented at the Annual Meeting of The Association of Official Agricultural Chemists, held at Washington, October 10–12, 1949.

checking its spectral absorption. The results are shown in Figure 1. To determine the actual effect of light passing through this shade on riboflavin, preliminary tests were made on a prepared solution containing one p.p.m. of riboflavin in distilled water. One portion of the solution in a small volumetric flask of clear glass was enclosed in a common buff-colored corrugated paper box, having an opening of approximately 3×5 inches cut into its side, and which was covered by the amber shade. The box was placed in a south window so that the entering rays of light would fall directly on the flask through the amber shade. For comparison a

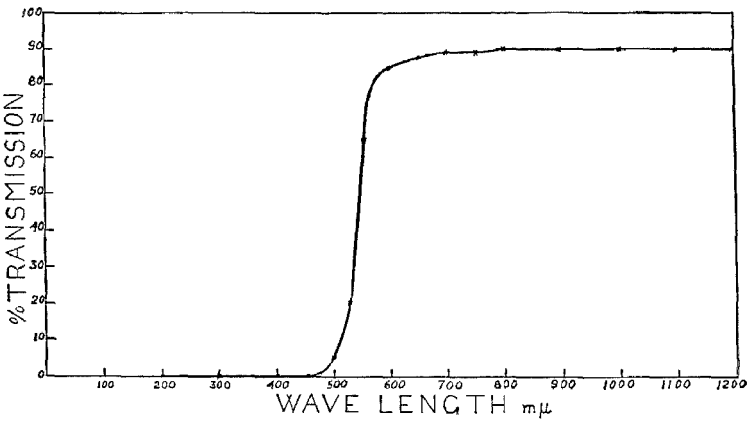

FIG. 1.—Spectral Absorption of Amber Shade

second portion of the solution in a volumetric flask of clear glass and a third portion in a flask of amber glass were also placed in this same window. After exposure to the light of bright sunny days for two hours, three hours, and five days, the amount of riboflavin remaining undestroyed in each flask was determined by placing a portion of the solution from each

TABLE 1.—*Loss of riboflavin in solution placed in direct light of window facing south*

	CONCENTRATION IN p.p.m.				% LOSS		
	INITIAL	2 HRS.	3 HRS.	5 DAYS	2 HRS.	3 HRS.	5 DAYS
Clear Glass	.98	.21	.20	.11	78.6	79.6	88.8
Amber Glass	.96	.75	.67	.17	21.9	30.2	82.3
Amber Shade	1.00	.98	.97	.84	2.0	3.0	16.0

flask directly into fluorometer cells. Fluorescent readings were taken on these cells in a Model DU Beckman spectrophotometer equipped with a fluorescent attachment. These results are given in Table 1.

TABLE 2.—*Loss of riboflavin in solutions placed in diffused light at laboratory bench*

pH		CONCENTRATION IN p.p.m.							PER CENT LOSS					
		INITIAL	1 DAY	2 DAYS	3 DAYS	4 DAYS	5 DAYS	7 DAYS	1 DAY	2 DAYS	3 DAYS	4 DAYS	5 DAYS	7 DAYS
2.2	Control	2.09	2.09	2.09	2.09	2.09	2.09	2.09	0	0	0	0	0	0
	With Shade	1.97	1.97	1.97	1.97	1.97	1.97	1.97	0	0	0	0	0	0
	Without Shade	1.97	1.54	.22	.22	.11	.11	.11	21.8	88.8	88.8	94.4	94.4	94.4
4.0	Control	2.09	2.09	2.09	2.09	2.09	2.09	2.09	0	0	0	0	0	0
	With Shade	1.97	1.97	1.97	1.97	1.97	1.97	1.97	0	0	0	0	0	0
	Without Shade	1.87	.55	.38	.33	.33	.33	.33	70.6	79.7	82.5	82.5	82.5	82.5
6.0	Control	1.98	1.98	1.98	1.98	1.98	1.98	1.98	0	0	0	0	0	0
	With Shade	1.98	1.98	1.98	1.98	1.98	1.98	1.98	0	0	0	0	0	0
	Without Shade	1.97	.44	.33	.33	.33	.33	.22	77.7	83.2	83.2	83.2	85.8	88.8
8.0	Control	2.04	2.04	2.04	2.04	2.04	2.04	2.04	0	0	0	0	0	0
	With Shade	1.87	1.87	1.87	1.87	1.82	1.82	1.76	0	0	0	2.7	2.7	5.9
	Without Shade	1.98	.55	.44	.44	.44	.33	.33	72.2	77.8	77.8	77.8	83.3	83.3

In view of the remarkable possibilities of this shade as shown in the preliminary tests, sheets of the material were procured large enough to cover the window, 40 by 70 inches in size, in the room which was chosen for vitamin assays. A sheet was placed permanently over the upper half of the window. For the lower half the sheet was mounted in a frame so that it could be removed at any time to provide normal light for other work when riboflavin was not involved.

Having adapted the amber shade to the window, additional tests were made with the shade covering the window. The method employed in making the tests was essentially the same as used by Conner and Straub.[1]

TABLE 3.—*Effect of added artificial light on riboflavin stability*

	% LOSS		
*p*H	2 HRS.	5 HRS.	7 HRS.
2.2	0	4.6	4.6
4.0	0	6.8	6.8
6.0	0	4.8	4.8
8.0	0	4.8	7.4

Solutions of pure riboflavin (2 p.p.m.) were prepared in phosphate-citric acid buffers ranging in *p*H values from 2.2 to 8.0. These solutions were kept in clear glass bottles with screw caps. One set of solutions was exposed to diffused light on the work bench, approximately 6 feet from the window, for varying periods of time with the shade covering the window. Another set was used as a control and was kept in a dark cupboard. A third set was tested simultaneously with these, but was placed approximately 6 feet from the window of the same size in an adjacent room, but with no filter over this window. The amount of riboflavin destruction was determined fluorometrically by placing a 1 ml. aliquot of the test solution in 10 ml. of buffer solution of *p*H 6.0. The results of this work are shown in Table 2.

In order to determine the effect that the use of artificial lights in the laboratory might have on the determination, the above procedure was repeated with the amber shade on the window and with added artificial illumination from two 150-watt ceiling lights under white glass globes. The results of this test are shown in Table 3. Except for possible differences in the intensity and quality of the daylight illumination resulting from the fact that determinations were made on different dates, these results were obtained under the same conditions as were the results in Table 2.

The final tests were actual analyses of an enriched flour and enriched bread. The procedure used was the fluorometric procedure taken from

[1] Conner and Straub, J. Ind. Eng. Chem., Vol. 13, 386, 1941.

Cereal Chemistry, Vol. 22, pp. 455–456. When the amber shade was used, the flour analyzed 2.52 p.p.m. of riboflavin, while with the shade removed, the same sample showed 1.97 p.p.m. of riboflavin, or a 21.8% loss. The bread gave results of 1.83 p.p.m. of riboflavin and 1.06 p.p.m. of riboflavin, respectively, or a 42.1% loss.

SUMMARY

The results of this investigation show the value of a light filter such as the amber transparent sun shade over laboratory windows in minimizing the destruction of riboflavin by light rays during the course of assay according to the method found in Cereal Chemistry, Vol. 22, pp. 455–456. Furthermore, such a filter is found to be more effective in preventing destruction of this vitamin than the amber glassware usually provided for this purpose. The quality of the filtered daylight and its intensity is such as to permit normal laboratory work. The use of added artificial light results in appreciable destruction of riboflavin.

A NEW STANDARD FOR VITAMIN D*

By E. M. NELSON (Food and Drug Administration, Federal Security Agency, Washington, D. C.)

The World Health Organization has adopted pure vitamin D_3 as the International Standard for vitamin D in place of the irradiated ergosterol preparation that has been in effect since 1931. For use in this country the U.S.P. Vitamin Advisory Board has recommended the adoption of a similar vitamin D_3 standard that will replace the U.S.P. Reference Cod Liver Oil. This change in the U.S.P. standard will affect the value of the chick unit for vitamin D and it is deemed important to have those concerned with the feeding of poultry, mixing of poultry feeds, and the labeling of vitamin D-containing products for poultry, fully informed of this fact before the new standard becomes effective. It is not possible to give a date when the new standard will become effective, but it may become official before the next A.O.A.C. meeting. The new standard will be announced by the *U. S. Pharmacopoeia* at least six months before it becomes official.

At the First International Conference on Vitamin Standardization, sponsored by the Health Organization of the League of Nations, and held in London in 1931, a special preparation of Irradiated Ergosterol in Oil was adopted as the standard for vitamin D, and the unit for that vitamin was defined in terms of a given weight of that solution. At a Second International Conference on Vitamin Standardization in 1934 the standard previously adopted was retained. It was also decided that in the event that the standard solution needed to be replaced, calciferol or vitamin D_2

* Presented at the Annual Meeting of the Association of Official Agricultural Chemists, held at Washington, D. C., October 10–12, 1949.

should be used in making the new standard solution on the basis that .025 microgram of calciferol was equal to 1 unit of vitamin D. However, except for one interval of six months the Irradiated Ergosterol Solution has been issued as the standard.

Since the International Standard was not available in sufficient quantities to meet the needs of the United States, the *U. S. Pharmacopoeia* adopted a specific lot of cod liver oil as a standard for vitamin D. This oil was carefully assayed against the International Standard and 1 U.S.P. unit was defined as being equal to 1 International unit on the basis of rat assays. Subsequently this so-called U.S.P. Reference Cod Liver Oil became the standard for the assay for vitamin D products for poultry because the A.O.A.C. chick assay, which was adopted in 1935, specified its use for determining the potency of such products in A.O.A.C. chick units.

Other lots of U.S.P. Reference Cod Liver Oil were prepared later for use in the same manner as the original U.S.P. Reference Cod Liver Oil. Each lot was assayed against the International Standard for vitamin D by the rat assay and against the previously used Reference Cod Liver Oil by the chick assay before adoption.

It has long been recognized that vitamin D_3 is preferable to D_2 as a standard, because the physiological effect is more uniform in different species of animals. However, vitamin D_3 has not been available in sufficient quantities and in a sufficient degree of purity to serve as a standard until recent years. Two years ago the British Accessory Food Factors Committee initiated an international collaborative study to establish the suitability of vitamin D_3 as an International Standard and to establish its potency in terms of the International Standard. More than 25 grams of pure D_3 obtained from five firms, including two from the United States, was pooled and recrystallized. In the coolaborative study, including both chick and rat assays, the potency of this material was compared with that of the International Standard, of pure vitamin D_2, and of the British Standards Institute's vitamin D_3 standard. In the United States and Canada, under the sponsorship of the Vitamin Advisory Board of the United States Pharmacopoeia, these studies were expanded to include the U.S.P. Reference Cod Liver Oil. Without revealing the identity of this oil, two samples of it were used and instructions were issued for assay of them on the basis that one sample contained 115 and the other 96 units of vitamin D per gram. In all, 54 assays, 29 using rats and 25 using chicks, were conducted by 30 laboratories. Eighteen laboratories in the United States and Canada submitted 13 rat and 16 chick assays. The results of these studies and also chemical and physical studies show that vitamin D_3 is suitable as a standard for vitamin D and that it has a potency very close to 40 million units per gram.

At a meeting of a Committee on Fat-Soluble vitamins, in London in April 1949, it was recommended to the Expert Committee on Biological

Standardization of the World Health Organization that vitamin D_3 be adopted as the International Standard for Vitamin D, and the unit be defined as the vitamin D activity of .025 microgram of vitamin D_3. This report was adopted by the Expert Committee on Biological Standardization. At a meeting of the Vitamin Advisory Board of the United States Pharmacopoeia, in June 1949, it was recommended that vitamin D be adopted as the U.S.P. standard for this vitamin.

If we now use vitamin D_3 as a basis for evaluating the U.S.P. Reference Cod Liver Oil the results of the collaborative studies show that a product assayed against the Reference Oil with rats and found to contain 100 U.S.P. units per gram, will contain 93.4 units when assayed against the new International Standard; and a product found to contain 100 A.O.A.C. chick units per gram when assayed against the present standard will contain 75 units per gram when assayed against the new standard. In other words, four A.O.A.C. chick units are equal to three "International Chick units."

Since supplies of U.S.P. Reference Cod Liver Oil Standard will probably be exhausted in less than a year it became necessary to revise the A.O.A.C. method so as to specify the use of the new U.S.P. Vitamin D_3 Standard. At the same time the name of the unit was changed from the "A.O.A.C. Chick Unit" to the "International Chick Unit."

The adoption of vitamin D_3 as a standard will put the chick unit on a permanent basis because it will be based on a definite weight of a pure and readily reproducible material. It will make for uniformity of labeling of products in international commerce and uniformity in the recording of results in scientific publications in various countries.

NOTE ON CAROTENE PAPER BY DERBY AND DEWITT

By MONROE E. WALL and EDWARD G. KELLEY
(Eastern Regional Research Laboratory,[1] Philadelphia 18, Pennsylvania)

In a recent communication (1), Derby and DeWitt compared a modified Association of Official Agricultural Chemists procedure (2) with a procedure attributed to Wall and Kelley (3) for the extraction of carotene from *dehydrated* alfalfa. The Association of Official Agricultural Chemists method involves extraction by refluxing with 30% acetone in petroleum ether, and the method attributed to Wall and Kelley utilizes cold extraction with ethanol-petroleum ether in the Waring Blendor. The experimental conclusions of Derby and DeWitt are undoubtedly correct, but we feel that their naming of the various extraction procedures is based on an incorrect interpretation of the literature.

In 1941, Moore and Ely (4) presented the method for extraction of carotene with ethanol-petroleum ether in the Waring Blendor. They applied their procedure to both fresh and dehydrated plant material.

In 1943, Wall and Kelley published their carotene procedure (3). Two extraction methods were presented. *Dehydrated* material was extracted by refluxing with 30% acetone in petroleum ether, and fresh tissue was extracted by the Moore and Ely technique (4). They did not apply the Waring Blendor extraction to *dehydrated* products.

It is obvious, therefore, that the modified Association of Official Agricultural Chemists procedure is essentially the same as the Wall and Kelley method for dehydrated products, whereas the procedure attributed to Wall and Kelley is actually the method of Moore and Ely.

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[1] One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.

CORRECTIONS IN AUGUST JOURNAL

Page 686. Determination of Gamma-Benzene Hexachloride in Insecticide Products, under "*Preparation of Sample* (a)," line 2, change "150–200 mg" to read ".75–1.0 g."

BOOK REVIEWS

The Chemistry and Technology of Enzymes. By HENRY TAUBER. John Wiley & Sons, Inc., New York, 1949, 550 pages. Price $7.50.

This volume, which is an expansion of the author's previous book *Enzyme Technology*, fills an important need, since it not only covers technology of enzymes but also a description of enzymes and their action. A knowledge of enzymes is certainly helpful in their application.

In Part I, "The Chemistry of Enzymes," the chapters are determined by the various changes brought about by enzymes. For example in chapter II, "Esterases," various lipase, liver esterase, tannase, sulfatase, lecithinase, chlorophyllase, various phosphatases, phytase, and cholinesterase are all treated descriptively. References rather than details of the methods of enzyme assay are given.

In Part II, "The Technology of Enzymes," various processes involving enzymes are discussed. This part has no equal in its comprehensive treatment of practical problems involving the use and control of enzyme action. For an example: in the chapter on enzymes in food industries, such things as meat tenderizing, blanching to destroy enzymes in dehydrated and frozen vegetables, enzyme tests for adequacy of blanching, and the role of proteinase and amylase in bread making are considered. Chapters on such relatively recent developments as antibiotic production, and the microbiological assay of vitamins and amino acids, are included, the latter being particularly noteworthy.

Certain errors, mostly of typographical nature, occur throughout the book. Such errors, however, are almost inevitable in such a comprehensive undertaking as this. Little interrelationship between enzymes is given, *e.g.*, the Krebs citric acid cycle is not included. However, in spite of these shortcomings the book is to be recommended particularly to those interested in the use and control of enzyme actions.

EUGENE F. JANSEN

Laboratory Manual Methods of Analysis of Milk and Its Products. 2nd Edition. Published by the Milk Industries Foundation, Washington, D. C., 1949. 627 pages. Price $15.00.

This is the second edition of the manual, the first of which appeared in 1933. It is a compilation of methods by a committee of members of the Foundation who are actively engaged, or directly concerned, in industry with the control of quality of dairy products. The methods represent best practice in the industry on the chemical and bacteriological methods. The manual, however, includes other procedures to meet the needs of production as well as distribution.

The first part is devoted to an illustrated description of the organization of a milk control laboratory, and a schedule of routine laboratory procedures suggested for control of quality in a market milk distribution plant. This is followed by three parts dealing with bacteriological, chemical, and physical control methods, and a part giving miscellaneous and special tests of dairy products. The greater portion of the text is allotted to these five parts.

The committee drew liberally from the methods of the American Public Health Association and the Association of Official Agricultural Chemists and supplemented these by many procedures from the general literature. No determination the dairy analyst is called upon to make seems to have been overlooked, from the composition, quality and cleanliness of the raw material to the composition, condition, quality and acceptability of the finished dairy products.

To assist the producer and distributor, methods are also included for composition and effectiveness of washing and sterilizing solutions, testing of brine, determin-

ing the strength of milk bottles, and testing of tin plate. The closely allied ice cream industry is provided methods for examining and evaluating gelatin, flavors, sugars, cacao products, and fruits. The sewage disposal problem which, under some conditions, seriously confronts segments of the industry, is recognized by the inclusion of directions for determination of biological oxygen demand.

The book closes with a section on preparation of media, reagents, and standard solutions; numerous conversion and engineering tables; ice cream manufacturing techniques; legal standards; and tables of the properties of dairy and related products.

HENRY A. LEPPER

Biochemical Preparations. Vol. I. HERBERT E. CARTER, Editor-in-Chief. xi+76 pages. John Wiley & Sons, Inc., 440 Fourth Ave., New York 16, N. Y., 1949. Price $2.50.

In the ordinary duties of the regulatory chemist, seldom does the necessity arise for preparing an unusual biochemical compound or reagent which cannot be obtained from a commercial source. When such an occasion does occur, however, it is very helpful to have a source in which one can find a reliable procedure for whatever reagent must be made. Even if this first volume of the series does not contain the specific biochemical preparation in which one is interested at the moment, a similar preparation may be described from which valuable information may be obtained.

The procedure for each preparation in this book has been subjected to limited collaborative study in much the same way as are the official methods of analysis of the A.O.A.C. Thus one can have confidence in the directions. To illustrate the types of synthesis or isolation to be found in this book there may be mentioned: adenosine diphosphate, L-serine and the special reagents used in its isolation from silk fibroin, lycopene, and lysozyme. A total of 17 preparations are described. In addition, references are given for the preparation, as described in Organic Syntheses, of 58 other compounds of biochemical interest. The style of presentation resembles in major aspects that of the well-established Organic Syntheses (28 volumes); for example, each title and structural formula (where a definite formula can be written) is followed by: I. Principle; II. Starting material; III. Procedure; IV. Properties and Purity of Product; V. Methods of Preparation, and a résumé including references to methods other than the one described in detail.

W. I. PATTERSON

Diagnostic Techniques for Soils and Crops. Edited by Herminie Broedel Kitchen. xxiii+308 pages. American Potash Institute, Washington, D. C. 1948. Price $2.00.

Diagnostic Techniques for Soils and Crops is a book that should be welcomed by many readers. In addition to its use by soil and plant scientists the book should be useful to County Agricultural agents, Extension workers, Farm Bureau leaders, and many others. Parts of it may, however, be too technical for lay readers.

This volume gives an excellent review of the diagnostic techniques that it is designed to cover. Authors from nine institutions wrote the book. They represent State Institutions, the United States Department of Agriculture, and private industry. Each author is an authority in his field and has done his job splendidly.

The titles of chapters describe the subject matter so well that they are quoted here, some of them with a slightly critical remark, not intended to impart discredit to the high quality of the chapters:

"Chemical Methods for Assessing Soil Fertility" might well include a reference to the original Veitch Method for determination of soil acidity published in 1902, to-

gether with a somewhat more precise statement of the distinctive characteristics of the method.

"Correlation of Soil Tests with Crop Response to Added Fertilizers and with Fertilizer Requirement" tends to emphasize the views of one institution in subject matter and in literature cited to a greater extent than seems desirable in a publication of this kind.

"Operation of a State Soil-Testing Service Laboratory" probably understates the necessity of extensive Statewide knowledge on the part of the operator of the soils of the area and their response to soil-management practices.

"Visual Symptoms of Malnutrition in Plants" would be improved by inclusion of another page or two of colored plates showing additional plants and additional element deficiencies.

Other titles are: "Historical Introduction"; "Operation of an Industrial Service Laboratory for Analyzing Soil and Plant Samples"; "Plant-Tissue Tests as a Tool in Agronomic Research"; "Plant Analysis—Methods and Interpretation of Results"; "Biological Methods of Determining Nutrients in Soils."

A total of more than 500 references are cited. The reviewer believes that this book is destined to be very useful to many persons. He is indeed happy to have a copy for frequent reference.

M. S. ANDERSON

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