CONTRIBUTED PAPERS

DECOMPOSITION OF CALCIUM METAPHOSPHATE FOR DETERMINATION OF TOTAL P_2O_5*

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The preparation and properties of vitreous calcium metaphosphate are described in several publications (1, 2, 3, 7, 9). Calcium metaphosphate differs markedly from other phosphates used as fertilizers. The individual glassy particles, unless finely ground, present only a limited surface for attack by solvents. When dissolved, the phosphate must be hydrated to the ortho form before it can be estimated by the usual volumetric or gravimetric procedures for fertilizers.

Several investigators have recognized significant errors that can occur in the analysis of metaphosphate unless certain precautions are taken. MacIntire, Hardin, and Oldham (7) recommended in 1937 that solutions of metaphosphate in water or in dilute acid be boiled with nitric acid to convert phosphates to the ortho form before precipitation with ammonium molybdate. Hoffman and Lundell (4) reported in the same year that digestion for 30 minutes in the hydrochloric-nitric acid mixture specified in A.O.A.C. (8) method **2.9 (b)** was adequate for decomposition and hydration of calcium metaphosphate. Hoffman and Lundell preferred a nitric-hydrofluoric acid decomposition, however, to eliminate gelatinous silica which slowed the subsequent filtration.

When TVA began experimental work on fused phosphates, the products from small-scale tests often contained partially fused materials that did not decompose completely in acid digestions. As these materials could not be detected readily in advance of the analysis, decomposition with an alkaline flux became general practice for phosphatic materials that had been fused or calcined during manufacture. This procedure was retained for the evaluation of metaphosphate, even after homogeneous products were being manufactured.

When calcium metaphosphate came into wider use and began to appear in mixed fertilizers, however, its behavior under the conditions of the A.O.A.C. methods for decomposition of analytical samples of fertilizers became an immediate question. This paper describes a study of the applicability of A.O.A.C. and other procedures in the decomposition of calcium metaphosphate alone and in mixed fertilizers.

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MATERIALS

Five samples of calcium metaphosphate from the demonstration plant at Wilson Dam, Alabama, were used. The P_2O_5/CaO mole ratio in the product tapped from the furnace normally varies from 0.95 to 1.00. The ratio in the minus 10-mesh product that is shipped at present is somewhat lower because of the addition of from 3 to 5 per cent of ground limestone as a conditioner. Chemical compositions of the samples are given in Table 1.

TABLE 1.—*Composition of calcium metaphosphate*

SAMPLE	PERCENTAGE COMPOSITION					P_2O_5/CaO MOLE RATIO
	P_2O_5[a]	CaO	SiO_2	Fe_2O_3	Al_2O_3	
A[b]	63.5	26.6	5.2	1.1	1.5	0.94
B	64.6	25.1	6.3	1.2	1.5	1.02
C	64.8	26.3	5.7	1.1	1.3	0.96
D	63.7	23.4	8.0	1.5	2.2	1.08
E	63.2	27.0	6.0	1.2	1.5	0.92

[a] Determined after digestion of sample for 15 minutes according to A.O.A.C. method **2.9 (b)**.
[b] Sample had been conditioned with limestone and contained 0.9 per cent CO_2.

Six samples of mixed fertilizers containing various amounts of calcium metaphosphate were furnished by K. D. Jacob, Associate Referee on Phosphoric Acid. Jacob, Armiger, Caro, and Hoffman (6) have reported details concerning these samples. The phosphatic constituents are shown in Table 2.

TABLE 2.—*Partial formulas of mixed fertilizers containing calcium metaphosphate*

SAMPLE NUMBER[a]	GRADE	POUNDS OF PHOSPHATE PER SHORT TON OF FERTILIZER	
		METAPHOSPHATE	SUPERPHOSPHATE
2829	0-16-16	125	1212.5
2830	0-20-10	172.5	1467.5
2831	0-20-10	300	1100
2832	0-20-20	665	0
2833	4-16-8	130	1200
2834	0-20-20	645	0

[a] Numbers are those reported by Jacob and co-workers (6).

APPLICATION OF OFFICIAL METHODS TO CALCIUM METAPHOSPHATE

The A.O.A.C. (8) specifies four methods for the decomposition of analytical samples of fertilizers. Two of the procedures, **2.9 (a)** and **2.9 (b)**, are intended for use where the amount of organic matter is small. In method **2.9 (a)**, a 2-gram sample is dissolved in 30 ml of nitric acid and

3 to 5 ml of hydrochloric acid, and the resultant solution is boiled until organic matter is destroyed. In method **2.9 (b)**, a 2-gram sample is dissolved in a mixture of 15 to 30 ml of hydrochloric acid and 3 to 10 ml of nitric acid. The other two procedures, **2.9 (c)** and **2.9 (d)**, are intended for use where the amount of organic matter is large.

The conditioned metaphosphate, sample A in Table 1, was crushed to pass a 20-mesh sieve, and portions were ground further to pass 35- and 80-mesh sieves. Alkalimetric determinations of total P_2O_5 on samples of

TABLE 3.—*Efficiency of A.O.A.C. methods 2.9 (a) and 2.9 (b) in the decomposition of conditioned calcium metaphosphate*[a]

FINENESS, MESH	DIGESTION TIME, MINUTES	P_2O_5, PER CENT	
		METHOD 2.9 (a)	METHOD 2.9 (b)
−20	5	54.2	61.8
−20	10	59.8	63.5
−20	15	61.6	63.5
−35	5	54.8	61.2
−35	10	61.8	63.5
−35	15	63.0	63.5
−80	5	59.2	61.1
−80	10	62.8	63.5
−80	15	63.1	63.5

[a] Sample A, Table 1.

the three mesh sizes, each decomposed by fusion with sodium hydroxide and subsequent fuming with perchloric acid, gave identical results of 63.3 per cent P_2O_5.

The three mesh sizes of the conditioned metaphosphate were digested in the reagents specified in methods **2.9 (a)** and **2.9 (b)**. The digestions were made for periods of 5, 10, and 15 minutes, as digestion time was recognized as an important factor in the decomposition of metaphosphate. The P_2O_5 content of the resultant solutions was determined alkalimetrically. The results, Table 3, show that decomposition was more complete with method **2.9 (b)** than with method **2.9 (a)**.

The comparison of the two methods was extended to include two other samples of metaphosphate, B and C in Table 1, that had been ground to pass a 20-mesh screen. Sample B contained 66 per cent of minus 20- plus 35-mesh material, whereas sample C was a typical preparation that contained a high proportion of fines. Portions were digested for 5, 10, and 15 minutes according to methods **2.9 (a)** and **2.9 (b)**. Two aliquots were taken from each solution. In the first aliquot, the P_2O_5 was precipitated immediately; the second aliquot was fumed gently with perchloric

acid to ensure hydration of the phosphate to the ortho form before the molybdate solution was added. The data in Table 4 show that more P_2O_5 was dissolved and more phosphate was hydrated to the ortho form with method **2.9 (b)** than with method **2.9 (a)**, and that 10-minute digestions were adequate for method **2.9 (b)**.

TABLE 4.—*Comparison of A.O.A.C. methods 2.9 (a) and 2.9 (b) in the decomposition of minus 20-mesh calcium metaphosphate*

SAMPLE	DIGESTION TIME, MINUTES	P_2O_5, PER CENT			
		METHOD 2.9 (a)		METHOD 2.9 (b)	
		IMMEDIATE PPTN.	AFTER $HClO_4$	IMMEDIATE PPTN.	AFTER $HClO_4$
B	5	43.3	50.5	64.0	64.2
	10	55.2	57.2	64.8	64.8
	15	61.7	62.6	64.7	64.8
C	5	58.5	61.8	64.6	64.8
	10	60.1	62.4	64.8	65.2
	15	63.8	64.0	64.8	65.0

Additional tests with samples A and B, ground to pass a 20-mesh screen, showed that after 60-minute digestions by method **2.9 (a)** less P_2O_5 was dissolved than after 10-minute digestions by method **2.9 (b)**. Analysis of the residues from the digestions showed that the low P_2O_5 values with method **2.9 (a)** were attributable to incomplete decomposition.

Calcium metaphosphate conditioned with ground limestone probably would be placed in the category of dry mixtures that tend to segregate. In this event it would be ground to pass a 35-mesh sieve before analysis. Also, mixed fertilizers containing calcium metaphosphate presumably would be crushed to pass a 35-mesh screen. Portions of the five samples in Table 1 therefore were ground to this fineness and were decomposed according to the four official methods. Each sample was digested for 15 minutes. The results are given in Table 5.

TABLE 5.—*Comparison of four official methods in the decomposition of minus 35-mesh calcium metaphosphate*

DIGESTION METHOD	PERCENTAGE P_2O_5				
	SAMPLE A	SAMPLE B	SAMPLE C	SAMPLE D	SAMPLE E
2.9 (a)	63.0	64.4	64.8	63.4	63.1
2.9 (b)	63.5	64.6	64.8	63.7	63.2
2.9 (c)	63.6	64.5	64.6	63.2	63.3
2.9 (d)	64.4	65.4	65.6	64.0	64.1

Fifteen-minute digestions by methods **2.9 (a)** and **2.9 (b)** gave practically the same results. In other tests of method **2.9 (a)**, lower results were obtained in 12-minute digestions; a minimum digestion period of 15 minutes is necessary, therefore, if method **2.9 (a)** is used.

Good reproducibility was obtained in replicate determinations with method **2.9 (c)**. This method should be adequate for the decomposition of mixed fertilizers containing metaphosphate and high percentages of organic matter.

High results were obtained consistently when method **2.9 (d)** was used, although all precipitations of ammonium molybdiphosphate were made in the recommended temperature range of 25° to 30°C. (5). That the high results by method **2.9 (d)** were directly attributable to the presence of sulfuric acid was demonstrated through experiment. A sample of metaphosphate was digested according to method **2.9 (b)**, and several aliquots were taken for analysis. To these aliquots was added 0.1 to 1 ml of phosphate-free sulfuric acid in 0.1-ml increments. Progressively higher results were obtained with increased additions of sulfuric acid.

APPLICATION OF OTHER METHODS TO CALCIUM METAPHOSPHATE

A few tests were made in which the minus 35-mesh samples were digested with nitric acid, with hydrochloric acid, and with nitric and perchloric acids in sequence. The results are given in Table 6 along with results by method **2.9 (b)**.

TABLE 6.—*Comparison of method 2.9 (b) with other digestion methods in the decomposition of minus 35-mesh calcium metaphosphate*

DIGESTION METHOD	PERCENTAGE P_2O_5				
	SAMPLE A	SAMPLE B	SAMPLE C	SAMPLE D	SAMPLE E
HCl—15 min.	63.1	64.3	64.6	63.1	63.1
HNO_3—15 min.	60.9	63.7	63.2	62.7	62.6
HNO_3 —10 min. $HClO_4$— 8 min.	63.2	64.3	64.5	63.3	63.2
2.9 (b)—15 min.	63.5	64.6	64.8	63.7	63.2

The superiority of hydrochloric acid over nitric acid (Table 6) is consistent with the finding that method **2.9 (b)**, in which the reagent contains a preponderance of hydrochloric acid, is superior to method **2.9 (a)** in the decomposition of metaphosphate.

Perchloric acid is a good solvent for metaphosphate, and it leaves the acid-insoluble residue in a readily filterable form. Ordinarily a preliminary digestion with nitric acid is not used on thoroughly oxidized materials, such as metaphosphate. Nitric acid was used in the present tests for consistency with other tests on a mixed fertilizer that contained organic matter.

DECOMPOSITION OF MIXED FERTILIZERS CONTAINING CALCIUM METAPHOSPHATE

The six mixed fertilizers that contained calcium metaphosphate (Table 2) were treated for 15 minutes according to methods **2.9 (a)** and **2.9 (b)**. The results in Table 7 represent averages of multiple analyses.

TABLE 7.—*Comparison of A.O.A.C. methods 2.9 (a) and 2.9 (b) in the decomposition of mixed fertilizers*

SAMPLE NUMBER	GRADE	PERCENTAGE P_2O_5	
		METHOD **2.9 (a)**	METHOD **2.9 (b)**
2829	0-16-16	16.8	16.8
2830	0-20-10	21.5	21.5
2831	0-20-10	21.4	21.5
2832	0-20-20	18.6	18.6
2833	4-16-8	16.3	16.3
2834	0-20-20	20.1	20.6

There was little difference in the results obtained by the two methods, an exception being sample 2834, which contained flax shive as a filler. Metaphosphate was the only phosphatic constituent of this sample. The discrepancy could not be attributed to particle size, because this mixed fertilizer, as were the others, was finely ground, with approximately 95 per cent passing a 35-mesh screen. The insoluble residue from a digestion by method **2.9 (a)** was found to contain 0.4 per cent P_2O_5. This sample was heated to dryness with magnesium nitrate solution and ignited according to method **2.9 (c)**, and then was digested with hydrochloric acid. The result by method **2.9 (b)**, 20.6 per cent P_2O_5, was confirmed.

In other tests the samples were digested successively with nitric acid and perchloric acid. The results agreed closely with those obtained by **2.9 (b)**.

CONCLUSIONS

This study has shown that minus 20-mesh calcium metaphosphate can be digested by A.O.A.C. method **2.9 (b)** with assurance of solution and hydration of the phosphate within a reasonable period of time, whereas the same cannot be said for method **2.9 (a)**. Finer grinding permits use of method **2.9 (a)**, provided the period of digestion is of sufficient length. In general, however, method **2.9 (b)** is faster and more dependable than method **2.9 (a)**.

Method **2.9 (c)** is suitable for the decomposition of 35-mesh calcium metaphosphate and should be adequate for the decomposition of mixed fertilizers containing metaphosphate and high percentages of organic matter.

The large amount of sulfuric acid specified in method **2.9 (d)** leads to high results for P_2O_5.

Other procedures suitable for the decomposition of calcium metaphosphate are digestions with hydrochloric acid alone or with nitric and perchloric acids in sequence.

ACKNOWLEDGMENT

B. B. Luff contributed to this study through the performance of some of the chemical analyses.

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WATER-INSOLUBLE FATTY ACIDS AND BUTYRIC ACID IN BUTTER MANUFACTURED BY THE "CONTINUOUS" PROCESS*

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During the past few years the Food and Drug Administration has carried on a rather extensive investigation into chemical methods applicable to butter designed to indicate the relative fitness of the cream from which the butter was churned. As a result of this investigation, water-insoluble fatty acids (WIA) and butyric acid appear to be satisfactory indices of the condition of the cream (1, 2, 3, 4). All data supporting these observations have been obtained on butter churned in the conventional barrel-type churn. A major technical development in the manufacture of butter during the past few years has been the introduction of the "continuous" process, so called to distinguish it from the one employing the conventional churn. It has been thought desirable to extend the investigation to butter produced by the "continuous" process in order

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TABLE 1.—*Effect of neutralization of oil on WIA and butyric acid*

SAMPLE NO.	CLASSES OF CREAM IN CHURN			COMMERCIAL CLASSIFICATION OF CREAM IN VAT	SEPARATED OIL AFTER VACREATION AND PASTEURIZATION BUT BEFORE NEUTRALIZATION				SEPARATED OIL AFTER NEUT. AND ADJUSTMENT TO 80 PER CENT FAT		BUTTER "CONTINUOUS" PROCESS		
	1	2	3		SERUM ACIDITY	FAT	WIA	BUTYRIC	WIA	BUTYRIC	WIA	BUTYRIC	MOLD
	per cent	*per cent*	*per cent*		*per cent as lactic*	*per cent*	*mg/100 g fat*	*mg/100 g fat*	*mg/100 g fat*	*mg/100 g fat*	*mg/100 g fat*	*mg/100 g fat*	*per cent*
1				1	0.41	90.5	293	3.1	290	2.5	300	2.9	0
2	79	13	8	1	0.79	87.5	295	5.2	294	4.8	300	5.1	0
3				1	0.52	87.4	293	2.6	294	2.0	267	2.3	0
4				1	0.63	87.4	335	3.6	327	4.0	281	4.9	0
5				2	1.00	87.6	375	2.9	352	3.6	363	3.3	0
6				1	0.67	85.3	323	5.1	312	5.5	317	5.5	0
7				1	0.64	87.7	309	6.6	302	7.1	309	6.0	0
8	54	39	7	2	0.80	87.5	430	6.8	434	7.6	441	7.3	0

to determine what effect that process might have on the WIA and butyric acid content of the butter.

In the continuous process, the cream is heated to about 50°C. in a centrifugal heater equipped with agitators to break the fat emulsion and is then separated in a high speed separator to a fat content of 82 to 92 per cent. This fat concentrate is then passed through a vacuum pasteurizer. In the plant where the work was done the vacuum pasteurizer used was called the "Vacreator." It consists of three chambers. In the first, the material is flash pasteurized by being quickly heated to about 90°C.; in the second, it is subjected to a combined vacuum and steam treatment and the temperature drops to about 75°C.; in the third or high vacuum chamber, the temperature is further reduced to about 43°C. The complete "vacreation" treatment takes about 5 seconds. The pasteurized material is collected in a standardization vat where it is neutralized and adjusted to 80 per cent fat by the addition of water (and salt, if salted butter is being made). The material is held at a temperature of about 43°C. under continuous agitation in order to prevent fat crystallization and to keep the contents of the vat homogeneous. It is then pumped to the chilling machine, which consists of two jacketed horizontal tubes equipped with scraper blades and agitators for working the butter. The material is cooled in the first tube to about 10°C. and in the second tube to about 4°C. It then passes from the second tube through the "texturator," where further chilling and working takes place. This is an artificially chilled cylinder, approximately 1×4 feet in dimensions with constricted, funnel-shaped ends. The butter is forced continuously into one end and expands to fill the interior. It is then extruded through a constricted rectangular orifice in the form of a "bar" which is cut into blocks. These may go directly to the printing machine or they may be held in storage for future printing.

In the barrel-type churning process the cream is first neutralized and then pasteurized, while in the "continuous" process the cream is separated to a fat content of between 82 and 92 per cent, the oil is then pasteurized, collected in a standardization vat and neutralized. Since in the latter process most of the water phase has been removed, very little water-soluble acid (lactic, acetic etc.) remains to be neutralized.

Under these conditions, the effect of neutralization on WIA and butyric acid was studied in a series of experimental, commercial scale, churnings. The oil concentrates were sampled after pasteurization but before neutralization and adjustment to 80 per cent fat. They were again sampled after the neutralization and adjustment. Finally, the finished butters were sampled. In two cases (Table 1, Samples No. 2 and 8) each can of cream comprising the churning was graded according to the scheme employed in previous studies (2, 3). This allowed calculation of the percentage of each grade of cream in the churning. The cream in the

TABLE 2.—*Effect of various steps in "continuous process" on WIA and butyric acid*

SAMPLE NO.	CLASSES OF CREAM IN CHURN				COMMERCIAL CLASSIFICATION OF CREAM IN VAT	VAT CREAM			SEPARATED OIL BEFORE VACREATION AND PASTEURIZATION		SEPARATED OIL AFTER VACREATION AND PASTEURIZATION BUT BEFORE NEUTRALIZATION				BUTTER "CONTINUOUS" PROCESS			BUTTER CHURNED IN BARREL-TYPE CHURN		
	0	1	2	3		ACIDITY AS LACTIC	WIA	BUTYRIC	WIA	BUTYRIC	SERUM ACIDITY	FAT	WIA	BUTYRIC	WIA	BUTYRIC	MOLD	WIA	BUTYRIC	MOLD
	per cent	*per cent*	*per cent*	*per cent*		*per cent*	*mg/100 g fat*	*mg/100 g fat*	*mg/100 g fat*	*mg/100 g fat*	*per cent as lactic*	*per cent*	*mg/100 g fat*	*mg/100 g fat*	*mg/100 g fat*	*mg/100 g fat*	*per cent*	*mg/100 g fat*	*mg/100 g fat*	*per cent*
A		61	30	9	1	0.44	314	9.6	186	2.6	0.50	86.3	178	2.8	190	3.0	0	220	1.2	48
B					1	0.65	277	10.6	228	4.0	0.60	90.5	226	3.7	200	4.1	3	193	0.0	70
C					1	0.28	251	0.0	220	0.0	0.32	90.4	205	0.0	224	0.0	0	*		
D					1	0.58	347	9.0	240	3.1	0.60	89.0	248	3.1	230	3.4	2	228	0.7	50
E					1	0.55	231	6.7	207	2.2	0.56	91.4	203	2.5	206	2.3	0	*		
F	4	77	18	1	1	0.57	239	7.7	177	3.2	0.36	90.3	172	2.3	175	3.9	2	173	0.0	
G		9	71	20	2	0.87	326	8.1	288	4.2	0.60	90.5	281	5.1	296	5.7	2	263	0.6	
H		84	16		1	0.62	190	12.0	169	3.7	0.70	90.5	198	3.3	178	3.5	0	175	1.1	56
I					1	0.63	298	9.9	216	5.6	0.80	87.0	224	5.9	217	5.9	0	*		
J		8	78	14	2	0.71	424	18.6	346	5.4	0.80	87.9	335	5.0	290	4.5	0	324	0.0	84

* Not churned in Barrel Churn.

other six churnings was commercially classified on a vat basis. In all cases the normal routine of the creamery was not changed.

Serum acidity (acidity of the water-curd phase of the separated oil), fat, WIA, and butyric acid were determined in the oil after vacreation and pasteurization; and WIA and butyric acid were determined in the oil after neutralization and adjustment to 80 per cent fat, and in the finished butter. Analytical data, including mold counts on the butters, are given in Table 1.

With the exception of Sample No. 4 there is little difference in the WIA content of the oil (both before and after neutralization) and in the butter. Likewise, butyric acid in the oil before and after neutralization, and in the butter, was essentially the same.

In another study of the effect of the various steps of the process on WIA and butyric acid, the vat cream itself, prior to centrifuging (instead of the pasteurized oil) was made the starting point material. Four samples were taken to represent the process: (1) the vat cream, (2) the separated oil as it leaves the centrifuge (prior to vacreation), (3) the vacreated and pasteurized oil before neutralization, and (4) the finished butter. In the case of Samples A, F, G, H, and J (Table 2), individual cans of cream comprising each churning were classified and the per cent of each class of cream in the churning was computed. In order to obtain comparable information on butter churned in a barrel-type churn in the cases of Samples A, B, D, F, G, H, and J, about 4 gallons of the vat cream were neutralized with the same neutralizer used by the creamery, pasteurized at 65°C. for 30 minutes, cooled and churned in a barrel-type churn of about 12 gallons capacity. Data obtained in this study are given in Table 2.

DISCUSSION

Some WIA is removed during the centrifuging step since the quantity determined in the separated oil before vacreation is in all cases less than that found in the cream. There is very little difference in the WIA content of the oil before and after vacreation and in the finished butter. Neutralization neither decreases nor increases WIA. Comparison of the butter obtained by the "continuous" process and that churned in the barrel-type churn from cream from the same vat shows that both processes give a product containing approximately the same quantity of WIA. Approximately one-third of the butyric acid present in the cream is retained in the oil and apparently none of this acid is lost when the oil is subjected to vacreation, neutralization, and churning. This is in contrast to butter churned in the barrel-type churn, where as the data show, most of the butyric acid is removed in the churning process.

SUMMARY

The effect of each step of the "continuous" process on WIA and butyric acid was studied. A comparison was made of the WIA and butyric acid

content of butter produced by the continuous process with that of butter churned from the same cream in a barrel-type churn. While some WIA and butyric acid are removed in the centrifuging step of the continuous process, vacreation and neutralization have no appreciable effect on either the WIA or butyric acid remaining in the oil. There is little difference in the quantity of WIA found in the butter prepared by the "continuous" process and that churned in the barrel-type churn.

Grateful appreciation is extended to A. F. Ratay, who made the barrel-type churnings, and to T. E. Byers, who assisted in the analysis of some of the samples, both of the Cincinnati District of the Food and Drug Administration; also to Margaret MacLean, of the Division of Food, who analyzed some of the samples, and to F. R. Smith, of the Division of Microbiology, who made the mold counts. Thanks are also due to H. A. Lepper and W. I. Patterson for their suggestions and assistance during the development of this study.

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EFFECT OF EXCESS ALKALI IN THE DETERMINATION OF WATER-INSOLUBLE FATTY ACIDS IN BUTTER*

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In the determination of water-insoluble fatty acids[1] (WIA), normal sodium hydroxide solution is added to an ether solution of the butter until the phenolphthalein indicator shows a *decided* pink color; then an additional 0.5 ml of N NaOH is added.† It has been reported[2] that—"for certain samples, the value obtained is influenced by the amount of alkali used in neutralizing the ether-water solution of the butter."

When the potential significance of WIA in cream and butter was first recognized, it was decided that the logical procedure for isolating these acids from butter was by extraction of their salts into water after neutralization in ether solution. The question of possible saponification of neutral fat by the dilute alkali used was investigated at that time and there was no indication that significant saponification took place. As

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[1] *Methods of Analysis, A.O.A.C.*, 7th Ed. 1950, p. 260.
† The instructions in the method calling for neutralization to a "decided pink color" may be subject to individual interpretation. Experience has shown that this color can be obtained by neutralizing the material to the *first* faint pink and then adding 0.1 ml of the alkali in excess. Thus, the conditions of the method can be met by adding 0.6 ml of the alkali after the appearance of the first faint pink color.
[2] J. of Dairy Sci., **34**, 478 (1951).

the question has now been raised by others, we have again investigated the possibility of saponification at this point in the procedure. This paper presents data obtained in a study of the influence of the amount of alkali on the quantity of WIA determined; specifically, whether or not appreciable saponification of the fatty acid glycerides occurs during the neutralization of the material in the centrifuge bottle (to a decided pink color) or when 0.5 ml of the alkali, in excess of that required to so neutralize it, is added.

Ten commercial butters were used in the first phase of the investigation. These had been authenticated by grading each can of cream comprising each respective churning. The cream came from a rather wide area, and

TABLE 1.—*Effect of varying quantities of excess N NaOH in in the determination of WIA*
(Mg WIA/100 g fat)

SAMPLE NO.	NEUTRALIZED WITH *N* NaOH TO DECIDED PINK COLOR	NEUTRALIZED WITH *N* NaOH TO DECIDED PINK COLOR (0.5 ML ADDED IN EXCESS)	NEUTRALIZED WITH *N* NaOH TO DECIDED PINK COLOR (2 ML ADDED IN EXCESS)
1	127	144	154
2	93	125	129
3	251	248	261
4	437	418	446
5	308	301	298
6	198	179	200
7	347	353	377
8	200	209	218
9	310	325	326
10	159	140	163
Av.	243	244	257

comprised "station," as well as "direct shipper" cream gathered in Kentucky, Illinois, Arkansas, and Missouri. The butters varied widely as regards the quality of the cream from which they were churned; some of them contained no decomposed cream and others contained varying quantities. WIA was determined on each of the 10 butters after the ether-water mixtures were neutralized only to a decided pink color. In other determinations, 2 ml of the alkali (rather than only 0.5 ml) was added in excess of that required for neutralization to a decided pink color. The results are given in Table 1, which also includes the results obtained on the same butters with the official method (middle column).

Statistical treatment of the data shows that there is a significant difference in WIA where 2 ml of *N* NaOH are used in excess of that necessary to give a decided pink color. However, there is no significant difference between results obtained with only enough alkali to give a definite pink color, and 0.5 ml in excess of this amount; the average re-

sults differ by only one mg. Even though there is a statistically detectable difference in the results with 2 ml of *N* NaOH (4 times that specified in the method) this difference is so slight that it would have no practical effect on the analytical interpretation.

To further test the possibility that fat may be saponified during neutralization, the ether-fat extracts from the determination on Sample 4 (437 mg WIA; excess alkali avoided) were evaporated. The residual fat was transferred to a centrifuge bottle and 25 ml of H_2O, 50 ml of ether, and one drop of *N* NaOH were added. On shaking the mixture it was found alkaline to phenolphthalein. The procedure for extraction of WIA was then applied to the fat solution. No precipitate was formed when the

TABLE 2.—*Analysis of commercial butters*

SAMPLE NO.	ML *N* NaOH ADDED AFTER NEUTRALIZATION TO DECIDED PINK	WIA MG/100 G FAT	MEAN MOLECULAR WEIGHT
1	0.0	141	250
	0.5	149	259
	1.0	145	247
	2.0	139	253
	*	119	257
2	0.0	337	264
	0.5	371	265
	1.0	372	262
	2.0	375	264
	*	207	255

* Samples neutralized to *faint* pink color.

aqueous residue in the centrifuge bottle was acidified with sulfuric acid. The acids which go to make up the WIA value separate at this point and our experience shows that as little as one mg of WIA will be visible here. Accordingly, in this experiment, less than one mg was liberated. This shows that no detectable saponification occurs during neutralization to a decided pink color.

Again, the ether-fat extracts from the determination of Sample 4 (446 mg WIA; 2 ml of *N* NaOH) were evaporated. The residual fat was treated as just described, with the exception that 2 ml of *N* NaOH were added in excess. Again no precipitate was formed on acidifying the aqueous residue with sulfuric acid. This demonstrates that an excess of even 4 times the quantity of alkali specified in the method failed to cause saponification of neutral fat.

A study was made with a butter churned from sweet cream produced by a single herd. (The cream was separated to 35 per cent fat from 40 gallons of sweet milk, pasteurized for 30 minutes at 155°F., cooled, and churned.) When up to 10 times the quantity of *N* NaOH specified in

the method was used the data show no significant saponification of fat.

Two samples of commercial butter, in which it had been claimed that increased values for WIA were found with increasing quantities of alkali, were next analyzed. The results are given in Table 2.

Low results were obtained in both samples when the material in the centrifuge bottle was neutralized to only a faint pink color. However when the neutralization was carried to a *decided* pink color (0.0 ml of *N* NaOH in Table 2) increased quantities of WIA were found, and results approached those obtained with the addition of either 0.5, 1.0, or 2.0 ml of excess *N* NaOH. The results demonstrate that no saponification of the glycerides of the fatty acids took place due to the action of excess alkali.

A series of 20 butters was used to study further the effect of the addition of excess alkali. In each case one sample was neutralized to a faint pink color, and another to a decided pink color. 0.2 ml and 0.5 ml (official method) of the alkali in excess of that required to neutralize to a decided pink color were added to 2 other samples. The results are given in Table 3.

TABLE 3.—*Effect of varying quantities of N NaOH in the determination of WIA*
(mg WIA/100 g fat)

SAMPLE NO.	NEUTRALIZED WITH *N* NaOH TO FAINT PINK COLOR	NEUTRALIZED WITH *N* NaOH TO DECIDED PINK COLOR	NEUTRALIZED WITH *N* NaOH TO DECIDED PINK COLOR (0.2 ML ADDED IN EXCESS)	NEUTRALIZED WITH *N* NaOH TO DECIDED PINK COLOR (0.5 ML ADDED IN EXCESS*)
1	283	503	539	548
2	510	573	557	592
3	736	786	758	761
4	1090	1069	1085	1090
5	569	547	574	590
6	458	488	495	498
7	471	452	455	466
8	777	793	770	779
9	413	462	482	481
10	404	450	452	452
11	886	874	866	899
12	190	191	190	191
13	469	497	488	505
14	611	627	632	657
15	381	438	435	454
16	971	1001	1023	1024
17	600	634	652	655
18	639	675	660	671
19	502	519	520	527
20	1263	1274	1306	1257
Av.	611	643	647	655

* Official Method: *Methods of Analysis*, 7th Ed., 15.118, p. 260.

When the material in the centrifuge bottle was neutralized to only a faint pink color, lower results were obtained in 17 of the 20 samples. Neutralization to a decided pink color increased the quantity of WIA found. The addition of either 0.2 ml or 0.5 ml of the alkali in excess of that required to neutralize to a decided pink color did not cause a significant further increase in WIA and this indicates that some excess alkali is necessary to assure complete extraction of the fatty acids. Particular attention is called to Sample No. 1 where the addition of the requisite amount of alkali in excess of that required to neutralize to a faint pink color caused an increase in WIA from 283 mg to 548 mg/100 g fat.

In searching for a possible explanation of this difference, the background

TABLE 4.—*WIA in butter churned from cream neutralized with lime*

SAMPLE NO.	WIA SAMPLE NEUTRALIZED WITH *N* NaOH TO FAINT PINK	WIA SAMPLE NEUTRALIZED WITH *N* NaOH TO DECIDED PINK COLOR. (0.5 ML ADDED IN EXCESS
	mg/100 g fat	*mg/100 g fat*
1	283	548
	239	
	290	
12	190	191
A	204	234
B	244	259
C	197	239
D	260	390

of this sample was investigated. The vat cream had been neutralized with lime, as was also the case with Sample No. 12. The vat creams from which the other butters of Table 3 were churned, were neutralized with sodium hydroxide, sodium bicarbonate, sodium carbonate or some combination of these. Sample No. 12 gave the same WIA figure regardless of the amount of NaOH solution used to neutralize the sample.

To study further the effect of lime neutralization upon WIA in the butter, 4 other butters, prepared on a commercial scale, were analyzed. The results in Table 4 show that the WIA value of butters prepared from vat creams neutralized with lime may sometimes be much higher with the 0.5 ml of excess alkali (Official Method) than when the sample is neutralized only to a faint pink color.

In Samples 1 and D, neutralization to a faint pink color failed by a wide margin to return all of the WIA. The loss of WIA on these 2 samples is much greater than on the other 4 samples listed in the table as well as on the 18 samples listed in Table 3 in which some form of sodium neutralizer was used.

DISCUSSION

It is recognized that a definite alkalinity is necessary for the extraction of fatty acids from fat. It is a well known fact that soaps in water solution have a tendency to hydrolyze. This hydrolysis can be suppressed through the addition of excess alkali. In the determination of WIA it was found that an excess of 0.5 ml of *N* NaOH over and above that required for neutralization to a decided pink was sufficient to suppress the hydrolysis of the sodium salts of the fatty acids and thus make for more complete extraction of the acids from the ether phase into the water phase. This is demonstrated by the results given in Tables 2 and 3.

SUMMARY

In the determination of water-insoluble fatty acids (WIA) in butters, the addition of 0.5 ml of *N* NaOH in excess of that required to neutralize the sample to a decided pink color of phenolphthalein (the conditions specified in the method) does not cause any significant increase in WIA. It has been shown that some excess alkali is necessary in order to assure complete extraction of the fatty acids.

DETERMINATION OF RESORCINOL IN HAIR DYES*

By S. H. Newburger and J. H. Jones (Division of Cosmetics, Food and Drug Administration, Federal Security Agency, Washington, D. C.)

Shupe[1] has described a method for the determination of resorcinol in hair dyes. Essentially, the method consists of a preliminary extraction of oils and fatty acids with chloroform from an acid aqueous solution of the sample; the resorcinol is then extracted with ether. The ether is evaporated; the extracted material is dried in a desiccator and weighed as resorcinol. In our experience, this procedure, when applied to hair dyes now on the market, usually yields an unidentifiable tarry material. The introduction of sulfated surface active agents into hair dyes has also complicated the analysis because of the emulsion formed when extractions with chloroform are made.

We have found that the resorcinol can be extracted by Shupe's procedure and identified and determined quantitatively by ultraviolet spectrophotometry. When sulfated surface active agents are present a preliminary acid hydrolysis eliminates emulsification difficulties.

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* Presented at the Annual Meeting of the Association of Official Agricultural Chemists, held at Washington, D. C., October 1–3, 1951.

[1] Shupe, I. S., *This Journal*, 24, 871 (1941).

METHOD

APPARATUS

A spectrophotometer capable of isolating a wave band of 5 mμ or less in the region 220–330 mμ. (A Cary Recording Spectrophotometer Model 11 and a Beckman Model DU were used in this study.)

REAGENTS

Ethyl ether, c.p.

Chloroform, c.p.

Standard resorcinol soln (40 mg per liter in 0.1 N HCl).—Dissolve 100 mg of resorcinol in 250 ml of 0.1 *N* HCl. Dilute 10 ml of the soln to 100 ml with 0.1 *N* HCl. [The resorcinol used was a white crystalline powder (m.p. 109–110°C.)]

ISOLATION OF RESORCINOL

(a) *If a sulfated surface active agent is present.*—Transfer 5 ml of sample to a 50 ml round-bottom flask, add 2 ml of HCl and a few carborundum chips, connect to a water-cooled condenser, and reflux for one-half hour. Cool and transfer to 125 ml separatory funnel with the aid of 25 ml of chloroform and 8 ml of water. Proceed as directed in (b), beginning with "... extract with three 25 ml portions of chloroform."

(b) *If a sulfated surface active agent is absent.*—Acidify 5 ml of sample with HCl, dilute to ca 15 ml with water, and extract with three 25 ml portions of chloroform. Combine the chloroform extracts, wash with 5 ml of water and in turn wash the water with 5 ml of chloroform. Discard all chloroform extracts and add the 5 ml of wash water to the remaining aqueous fraction.

Extract the acid aqueous soln with five 25 ml portions of water-washed ethyl ether. Combine the ether extracts in a 250 ml beaker, add 25 ml of water containing a few drops of HCl, and heat on steam bath until no odor of ether remains. Cool the remaining aqueous soln to room temp, filter thru a cotton plug into a 100 ml volumetric flask, dilute to mark with 0.1 *N* HCl and mix.

SPECTROPHOTOMETRIC DETERMINATION

Dilute 10 ml of the soln containing the extracted resorcinol to 100 ml with 0.1 *N* HCl. Determine the absorbancy in 1 cm cells of the unknown soln and the standard resorcinol soln at 273 mμ, against a blank of 0.1 *N* HCl.

$$\text{Per cent resorcinol} = \frac{A_{sple}}{A_{std}} \times C \times \frac{1}{50} \times \frac{1}{\text{Sp. gr. of sple}},$$ where C is the concentration of the standard in mg/liter.

(The recommended dilution will give a suitable absorbancy reading if the 5 ml sample contains 30–60 mg of resorcinol. If the absorbancy reading is too high or too low for accurate results, make a dilution of the extract which will give an absorbancy reading between 0.5 and 1.0.)

Determine the absorbancy of the unknown solution at a sufficient number of points in the region 220–300 mμ to enable the plotting of an absorbancy vs. wavelength curve.

EXPERIMENTAL

Figure 1 shows the ultraviolet absorption curve of resorcinol in 0.1 *N* HCl. The ultraviolet absorption curve of resorcinol in $NH_4OH(1+99)$ is shown in Figure 2. Solutions of resorcinol in 0.1 *N* HCl were found to follow Beer's law to within ±0.5 per cent.

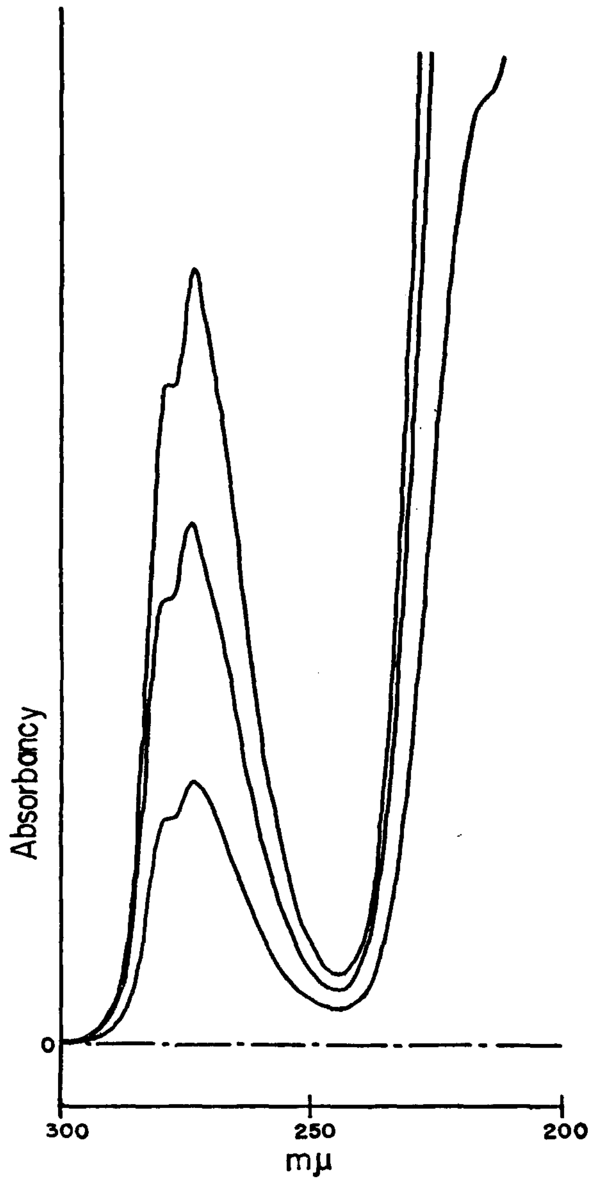

FIG. 1.—Absorption curves of resorcinol (25, 50, and 75 mgs per liter) in 0.1 N HCl.

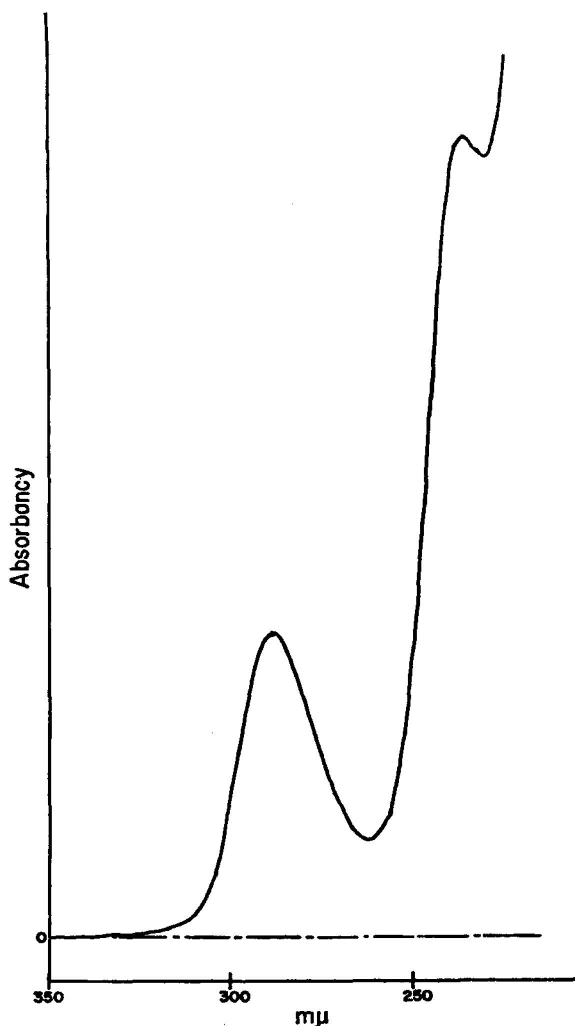

FIG. 2.—Absorption curve of resorcinol (25 mg per liter) in NH_4OH (1+99).

The composition of the control hair dye samples used in this investigation is given in Table 1. Varying amounts of resorcinol were added to 5 ml portions of the control samples and the preparations were then analyzed by the proposed procedures. The results tabulated in Table 2 were obtained with the Cary Recording Spectrophotometer; substantially the same results were obtained with a Beckman Model DU spectrophotometer.

TABLE 1.—*Composition of control hair dye samples*

FORMULA	1	2	3	4
	per cent	*per cent*	*per cent*	*per cent*
95% Alcohol	10	10	10	10
Oleic Acid	10	10	10	10
Ammonia	2	2	2	2
Na_2SO_3	0.2	0.2	0.2	0.2
Triton X-100	—	5	—	—
Tween 20	—	—	5	—
Sodium lauryl sulfate	—	—	—	5
p-Phenylenediamine	2	2	2	2
p-Aminophenol	1	1	1	1
Water	74.8	69.8	69.8	69.8
Total	100	100	100	100

TABLE 2.—*Recoveries of resorcinol*

FORMULA	RESORCINOL ADDED	RESORCINOL RECOVERED	
	mg	*mg*	*per cent*
1	25	24.8	99
	50	49	98
	150	143	95
2	50	47.5	95
3	50	47	94
4	50	47.5	95
	50	48	96
		Average	96

DISCUSSION

The average recovery of the seven determinations was 96%, the greatest deviations from this value being +3% and −2%. The inclusion of the surface active agents Triton X-100, Tween 20, and sodium lauryl sulfate made little difference in the recoveries. Other polyhydric phenols, and other materials which are extractable by ether from acid aqueous solution and absorb in the ultraviolet, will interfere with the proposed determination.

The complete ultraviolet curve of the unknown is plotted to establish the identity of the extracted material as resorcinol. Any doubt as to the identity of the extracted material can be resolved by obtaining its ultraviolet spectrum in dilute NH_4OH (see Fig. 2).

CONCLUSION

Resorcinol in hair dyes can be satisfactorily identified and determined by ultraviolet spectrophotometry.

The method should be applicable to other preparations containing resorcinol.

ETHYLENEDIAMINE TETRA-ACETIC ACID AS AN AID IN THE ANALYSIS OF CERTAIN COAL-TAR COLOR LAKES*

By NATHAN ETTELSTEIN (Division of Cosmetics, Food and Drug Administration, Federal Security Agency, Washington 25, D. C.)

Spectrophotometric methods for the determination of pure dye in samples of coal-tar colors are generally accurate (1) and are usually more rapid and convenient than chemical methods. However, they are applicable only when a clear and homogeneous solution of the color in an appropriate solvent can be made. The calcium, barium, and strontium salts and lakes of some of the certifiable coal-tar colors are practically insoluble in the solvents usually employed in spectrophotometry, and special techniques must be used to prepare satisfactory solutions.

It is well known that ethylenediamine tetra-acetic acid is a powerful chelating agent for many metals. As expected, it was found that this compound does, under proper conditions, form soluble complex chelates with the calcium, barium, or strontium combined with certain acid dyes. Results of this investigation also show that the common substrata for lakes of these dyes, with the exception of titanium dioxide and talc, are brought into aqueous solution by ethylenediamine tetra-acetic acid. When samples of these lakes are boiled with alcohol and water containing ethylenediamine tetra-acetic acid, solutions satisfactory for spectrophotometric examination are obtained.

PROCEDURE

REAGENTS

10% Ethylenediamine tetra-acetic acid soln.—Dissolve 25 g of ethylenediamine tetra-acetic acid in 165 ml of 10% NaOH solution and dilute to 250 ml with water.

Dilute ethanol (1+1).—Mix equal volumes of 95% ethanol and water.

PREPARATION OF SOLUTIONS

Place an accurately weighed sample of ca 100 mg of the color in a 250 ml beaker Add 7 ml of 10% ethylenediamine tetra-acetic acid soln, 3 ml of 10% NaOH soln, and 15 ml of water. Cover the beaker with a watch glass and bring the mixture to a gentle boil on a hot plate. Stir until the entire sample is wetted, and boil for about two minutes longer. Remove the beaker from the hot plate, stir the contents for 30–60 seconds, add 25 ml of 95% ethanol, and 100–150 ml of (1+1) ethanol, and

* Presented at the Annual Meeting of the Association of Official Agricultural Chemists, held at Washington, D. C., October 1–3, 1951.

mix thoroly. Replace the covered beaker on the hot plate and heat just below the boiling point until all the color is in solution. Allow the soln to cool to room temp. and transfer to a 250 ml volumetric flask. Rinse the beaker with several small portions of (1+1) ethanol, add the rinsings to the flask, and dilute the soln to volume with (1+1) ethanol. (If talc or titanium dioxide is present, filter the soln thru a fine porosity sintered glass filter before it is transferred to the volumetric flask.)

DETERMINATION

Determine the pure dye content of the sample soln as directed in *Official Methods of Analysis, A.O.A.C.*, Seventh Edition (1950), Sec. **34.29–32**.

DISCUSSION

The procedure described above has been applied to a number of coal-tar color lakes prepared with all of the usual substrata. In all cases, clear solutions resulted unless talc or titanium dioxide was present. Ethanol (1+1) is an ideal solvent because it dissolves the dye liberated by the chelating agent as well as the metallic chelates and organic substrata such as rosin, calcium rosinate, and aluminum benzoate.

The absorbancy of solutions of D&C Red No. 31 in (1+1) ethanol containing from one to three grams of ethylenediamine tetra-acetic acid per liter followed Beer's law and ethylenediamine tetra-acetic acid present had no apparent effect on the absorbancy of the solutions.

In order to test the applicability of the procedure, a number of lakes were analyzed for pure dye content by the method described, and by titration with standard titanium trichloride solution (2). The results are shown in Table 1. In most cases, the values obtained by titration with titanium trichloride were slightly lower than those obtained by the spectrophotometric procedure. The author believes that the low results are probably due to partial decomposition of the dyes by the concentrated sulfuric acid used as a solvent in the titration procedure.

A few investigations have shown the method to be applicable to aluminum and zirconium lakes. Since these lakes are dissolved readily by refluxing for a short time in dilute acid or alkali, a procedure no less convenient than the treatment with ethylenediamine tetra-acetic acid, the procedure was not applied to many of these. However, the ethylenediamine tetra-acetic acid would be useful in analysis of aluminum lakes extended on a substratum containing calcium, barium, or strontium.

This procedure cannot be used for dissolving lakes preparatory to titration of the color with titanium trichloride. Trivalent titanium forms a chelate with ethylenediamine tetra-acetic acid, and the complex will not serve as a reducing agent.

SUMMARY

Ethylenediamine tetra-acetic acid in (1+1) ethanol has been shown to be an effective reagent for dissolving calcium, barium, and strontium salts of certain certifiable dyes and their lakes. The solutions are suitable for

TABLE 1.—*Pure dye determined spectrophotometrically and by titration*

COLOR	SAMPLE NO.	SUBSTRATUM*	PURE DYE	
			PROPOSED METHOD	TITRATION
			per cent	*per cent*
D&C Red No. 7	A	BS+T	13.7	13.0
	B	AO	30.0	31.0
D&C Red No. 8	A	BS+R	81.5	80.4
	B	T	23.9	23.4
D&C Red No. 9	A	GW	38.5	38.4
	B	BS	92.8	92.2
	C	GW	40.8	40.2
	D	None	96.7	97.0
D&C Red No. 10	A	GW	82.1	79.2
	B	BS+T	20.7	20.5
	C	AO	93.1	90.7
D&C Red No. 11	A	BS+T	11.7	12.2
	B	BS	82.1	83.2
	C	R	57.9	58.0
	D	BS+CR	48.7	47.2
D&C Red No. 12	A	BS	61.2	60.4
	B	BS+R	61.4	60.1
	C	BS	89.9	88.6
D&C Red No. 13	A	BS	50.6	50.1
	B	BS+R	43.0	42.3
D&C Red No. 31	A	GW	44.6	44.9
	B	AO	49.4	48.0
D&C Red No. 34	A	TO+CR	38.0	38.0

* AO—Aluminum oxide, BS—Barium sulfate, GW—Gloss white, CR—Calcium rosinate, R—Rosin, T—Talc, TO—Titanium dioxide.

spectrophotometric determination of the amount of color present. Typical results obtained by the proposed procedure are given.

REFERENCES

(1) JONES, J. H., *This Journal*, **33**, 401 (1950).
(2) *Official Methods of Analysis, A.O.A.C., 7th Ed.* (1950), Sec. **34.21 (f)**.
(3) *Ibid.*, Sec. **34.29–32**.

PAPER CHROMATOGRAPHY OF FLUORESCEIN AND THE HALOGENATED FLUORESCEINS*

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

Paper chromatography is a well-established method for the separation of mixtures and has been applied to a great many materials. Recent reviews (1, 2) list several hundred references on this subject.

Paper chromatography seems to be ideally suited for the separation of colored materials. There are several references to the separation of coal-tar colors by this technique, but as far as the author knows, the fluorescein colors have not been studied as a group. This study of the application of paper chromatography to the fluorescein colors had two objectives: 1. an aid in the identification of the fluorescein colors present in food, drugs, and cosmetics, and 2. an aid in the determination of the composition of samples of the fluorescein colors submitted for certification.

EXPERIMENTAL

In the course of this work, several solvent systems were tested. The two found to be the most satisfactory are: 1. NH_4OH (1+99), saturated with isoamyl alcohol and 2. a mixture of 200 ml *n*-butyl alcohol, 88 ml distilled water, 2 ml concentrated NH_4OH, and 40 ml ethyl alcohol.

Solvent 1 is the most satisfactory of the two. With it, the R_F factors for the various fluorescein colors differed considerably; in general, the R_F factor decreased with increasing molecular weight. In some cases, the spots spread a little more than is desirable. With solvent 2 the range of R_F factors was much less and in general they were found to be in reverse order from those for solvent 1.

The colors were spotted from dilute ammonia solution. An appropriate amount of a fluorescein color, if pure, is a small drop of a solution containing ½ mg of the color per ml. When samples were to be examined for traces of colored material, one drop of a solution containing 2 to 4 mg of the color per ml was used.

The chromatograms were developed in the usual manner by spotting on one corner with a solution of the material to be examined. The dried spot was developed in one direction with one of the solvents, and the paper was dried. The chromatogram was then further developed at right angles to the original with the other solvent. Ascending solvent flow was used. Results were more satisfactory when solvent 1 was used for the initial development; the somewhat diffuse spots obtained became smaller and more clearly delineated upon subsequent development with solvent 2.

In most of the work a 23×23 cm square of No. 1 Whatman filter paper

* Presented at the Annual Meeting of the Association of Official Agricultural Chemists, held at Washington, D. C., October 1–3, 1951.

TABLE 1.—*Comparison of purified samples with commercial samples*

COLOR	PURITY	INTENSITY OF SPOT	APPROXIMATE SOLVENT 1	RF FACTOR: SOLVENT 2
Fluorescein	99+%	Major	0.85	0.18
Dibromofluorescein	99+%	Major	0.58	0.26
		Trace	0.73	0.24
Dibromofluorescein	Commercial	Major	0.64	0.20
		Minor	0.30	0.29
		Minor	0.42	0.22
		Minor	0.79	0.20
		Trace	0.66	0.23
		Trace	0.83	0.15
Tetrabromofluorescein	99+%	Major	0.38	0.45
		Trace	0.41	0.34
Tetrabromofluorescein	Commercial	Major	0.44	0.37
		Trace	0.45	0.28
Dichlorofluorescein	Commercial	Major	0.71	0.25
		Minor	0.79	0.22
		Minor	0.44	0.32
Tetrachlorofluorescein	Commercial	Major	0.50	0.39
		Trace	0.54	0.27
Diiodofluorescein	Commercial	Major	0.56	0.34
		Minor	0.16	0.42
		Minor	0.36	0.36
		Minor	0.75	0.37
		Trace	0.60	0.41
		Trace	0.83	0.28
Tetraiodofluorescein	99+%	Major	0.22	0.52
Tetrachlorotetrabromo-fluorescein	99+%	Major	0.68	0.64
Dibromodiiodofluorescein	Commercial	Three or more fractions not separated completely.		
Dichlorotetraiodo-fluorescein	Commercial	Major	0.50	0.51
		Minor	0.57	0.44
		Trace	0.69	0.45
		Trace	0.00	0.92
		Trace	0.93	0.12
Dicarboxytribromo-fluorescein	Commercial	Major	0.67	0.31
		Minor	0.85	0.25
		Minor	0.79	0.31
		Minor	0.67	0.24
		Minor	0.40	0.34
		Trace	0.85	0.18
		Trace	0.79	0.23
Dicarboxypentabromo-fluorescein	Commercial	Three or more fractions not separated completely. A large one showing a color gradation and a trace.		
		Trace	0.82	0.28

was used. This allowed a solvent flow of about 17 cm in each direction. With solvent 1 one-dimensional chromatograms were tried with a solvent flow up to 40 cm; these did not seem to offer any advantages over a two-dimensional development of about 17 cm in each direction.

The R_F factors were calculated from the position of the leading edges of the initial and final spots. The R_F values are only approximate as no attempt was made to control such variables as temperature, concentration of the spot, etc. Experiments in this laboratory show that it is preferable to use parallel runs with a known color rather than to depend upon R_F factors for identification of unknown colors.

Several commercial samples and some purified samples of the fluorescein

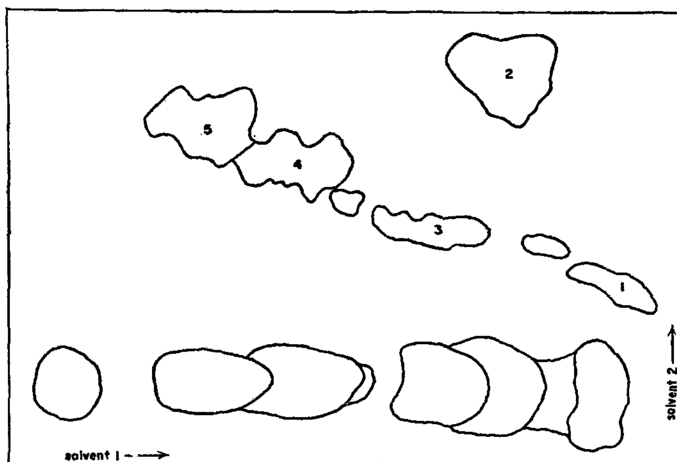

FIG. 1.—Mixture.

colors were chromatographed by the proposed technique. Table 1 gives the data obtained. In this table the largest spot is called the *major fraction.* Spots which were estimated as being due to 2 per cent or less of the colored material in the sample are called *traces.* Spots of intermediate strength are called *minor fractions.* In most of these samples, sufficient material was used to show traces of colored materials.

Figure 1 is a diagram of the chromatogram obtained from a mixture of purified samples of (1) Fluorescein, (2) Tetrachlortetrabromofluorescein, (3) Dibromofluorescein, (4) Tetrabromofluorescein, and (5) Tetraiodofluorescein. Two trace spots were barely noticeable; separate runs showed that one of these trace spots (between 1 and 3) was due to a trace fraction in the Dibromofluorescein and the other to a trace fraction in the Tetrabromofluorescein.

Figure 2 is a diagram of the chromatogram obtained from a commercial

sample of Diiodofluorescein. One of the minor spots is identified as Tetraiodofluorescein. A trace of Fluorescein is also present.

Figure 3 is a diagram of the chromatogram obtained from a sample of

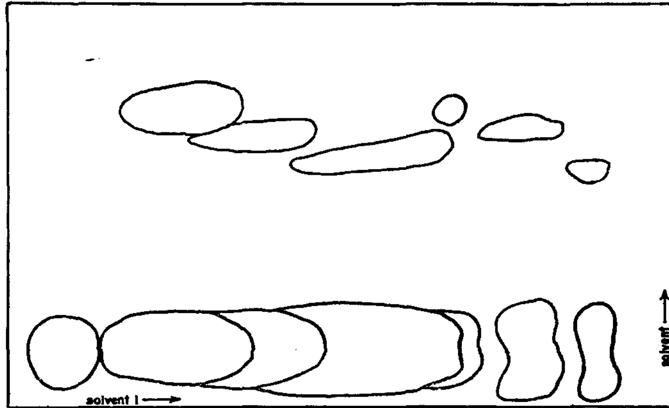

FIG. 2.—Commercial sample D&C Orange No. 11.

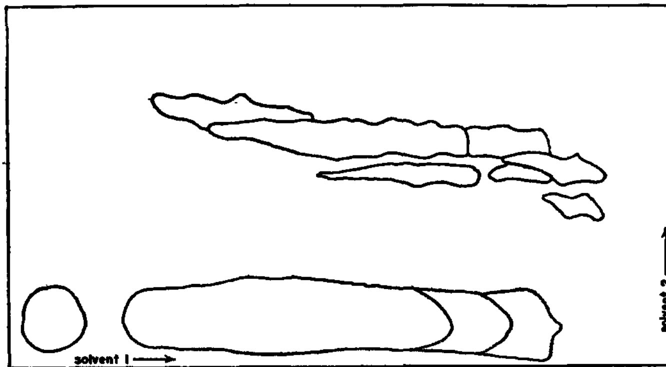

FIG. 3.—Commercial sample D&C Orange No. 14.

commercial Dicarboxytribromofluorescein. The chromatogram indicates the presence of three pairs of colors which have approximately the same R_F factors in solvent 1 but which have different R_F factors in solvent 2. The materials in each pair of spots seem to be almost identical in color.

Figure 4 is a diagram of the chromatogram obtained from a commercial sample of Dibromodiiodofluorescein. The outline is drawn where there was a noticeable change in color. It was shown by parallel runs with Dibromofluorescein and Diiodofluorescein that there cannot be noticeable

amounts of monohalogenated or dihalogenated fluoresceins present in this sample. However, there are at least nine tetrahalogenated and trihalogenated fluoresceins that might be present. The solvent systems do not seem to be adequate to separate the combination which we may expect to find in this color.

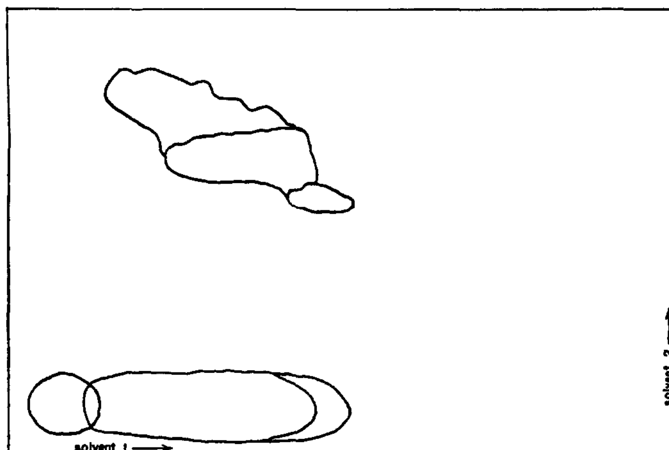

FIG. 4.—Commercial D&C Orange No. 16.

SUMMARY

Two solvent systems are suggested for paper chromatography of the fluorescein colors. Several colors of the fluorescein group were found to be satisfactorily separated by the proposed procedure.

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DETERMINATION OF DIMETHYLANILINE IN EXT. D&C BLUE NOS. 1 AND 2*

By KEITH S. HEINE, Jr. (Division of Cosmetics, Food and Drug Administration, Federal Security Agency, Washington 25, D. C.)

Dimethylaniline is an intermediate used in the production of Ext. D&C Blue No. 1 and No. 2. Separation of the intermediate from these colors is readily accomplished by steam distillation from a solution made alkaline

* Presented at the Annual Meeting of the Association of Official Agricultural Chemists, held at Washington D. C., October 1–3, 1951.

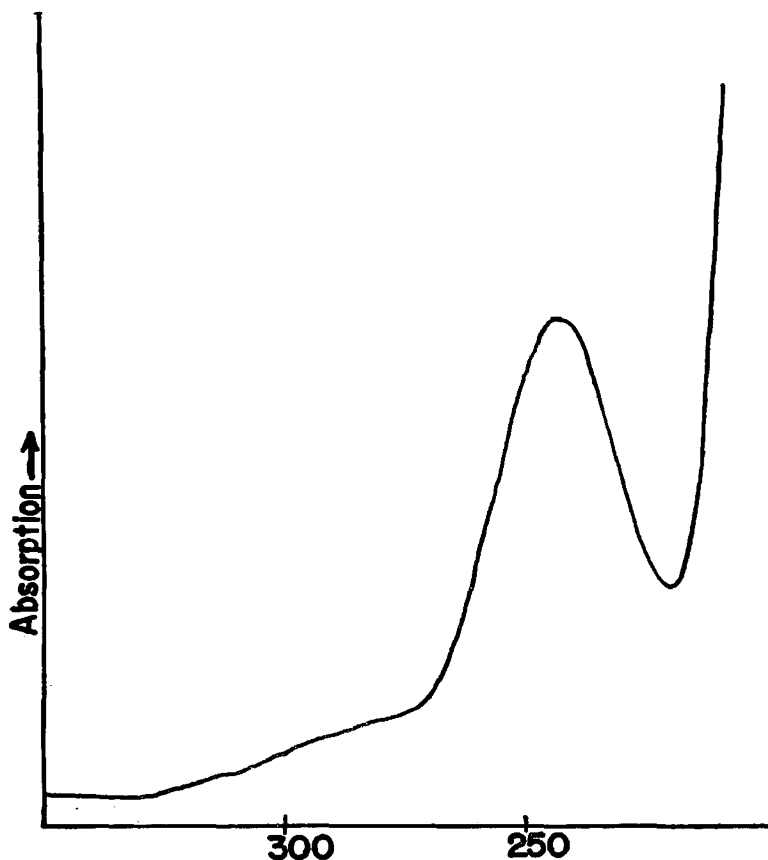

FIG. 1.—Absorption curve of dimethylaniline in dilute NH_4OH.

TABLE 1.—*Recovery of dimethylaniline from Ext. D&C Blue No. 2*

DIMETHYLANILINE ADDED	DIMETHYLANILINE RECOVERED, NET	RECOVERY
mg	*mg*	*per cent*
0.00	<0.01	—
2.22	2.10	95
2.22	2.15	97
1.11	1.01	91
1.11	0.97	87
0.55	0.63	115
0.55	0.60	109
	Average Recovery	99

with sodium carbonate. The intermediate, so separated, can be identified and determined spectrophotometrically.

METHOD

APPARATUS

A spectrophotometer suitable for use at 245 mμ.

REAGENTS

Standard dimethylaniline solution.—Place 20 mg of dimethylaniline in a 100 ml volumetric flask and dilute to 100 ml with 95% alcohol. Transfer a 10 ml aliquot to a second 100 ml volumetric flask, add ca 1 ml of conc. ammonia and dilute to 100 ml with water.

PROCEDURE

In a 500 ml round-bottom flask, place a few boiling chips, 0.50 g of Ext. D&C Blue No. 1 or No. 2, ca 250 ml of water, and ca 2 g of sodium carbonate. Fit the flask with two steam traps connected in series and a condenser. Distill at a rate of about 2 drops per second, collecting the distillate in a 100 ml volumetric flask containing 1 ml of conc. NH_4OH. Collect nearly 100 ml of distillate and then dilute to the mark with water. Determine the absorbancy of the standard and unknown solution at 245 mμ:

$$\text{Per cent dimethylaniline} = \frac{A_{unknown}}{A_{standard}} \times C_s \times \frac{1}{5}$$

where C_s = the concentration of the standard solution expressed in milligrams per 100 ml.

TABLE 2.—*Recovery of dimethylaniline from Ext. D&C Blue No. 1*

DIMETHYLANILINE ADDED	DIMETHYLANILINE RECOVERED, NET	RECOVERY
mg	*mg*	*per cent*
0.00	<0.01	—
2.22	2.15	97
1.11	0.95	86
0.55	0.53	96
		—
	Average Recovery	93

EXPERIMENTAL

The ultraviolet absorption curve of dimethylaniline in dilute ammonium hydroxide is shown in Figure 1. Solutions of dimethylaniline in 1:100 ammonia at concentrations ranging from 5.0 to 20.0 mg/l were found to obey Beer's law to within ±1 per cent.

A standard solution of dimethylaniline (Eastman Kodak, white label) was prepared and aliquots were added to 0.50 g samples of Ext. D&C Blue Nos. 1 and 2. These samples were analyzed by the proposed method; the results are given in Tables 1 and 2.

DETERMINATION OF UNCOMBINED p-AMINOACETANILIDE IN EXT. D&C RED NO. 1*

By K. S. HEINE, JR. and WM. J. SHEPPARD (Division of Cosmetics, Food and Drug Administration, Federal Security Agency, Washington, D. C.)

p-Aminoacetanilide is an intermediate used in the manufacture of Ext. D&C Red No. 1 (Amidonaphthol Red 6B, C. I. No. 57). In order to meet the requirements for certification by the Food and Drug Administration, batches of the dye may not contain more than 0.20 per cent of uncombined *p*-aminoacetanilide.

In this paper a convenient and reasonably accurate procedure for the determination of the intermediate in samples of Ext. D&C Red No. 1 is described.

METHOD

APPARATUS

A spectrophotometer capable of isolating a 5 mμ band when operating at 240 mμ.

REAGENTS

Dilute hydrochloric acid (1+99).

Standard p-aminoacetanilide (10 mg per l in dilute HCl).—Dissolve 100 mg of purified *p*-aminoacetanilide in exactly 100 ml of alcohol. Dilute a 10 ml aliquot of this soln to one liter with the dilute HCl.

DETERMINATION

Weigh 500 mg of Ext. D&C Red No. 1 into a 400 ml beaker, add 50 ml of diethyl ether, and mix thoroly. Filter the ether suspension thru a retentive filter paper. Rinse the beaker with 10 ml of ether and pass thru the same filter. Return the filter paper and residue to the beaker and re-suspend the dye in 50 ml of ether. Filter the ether suspension and wash the residue in the beaker and on the filter with two 10 ml portions of ether. Combine the ether solns and extract with five 20 ml portions of the dilute HCl. Heat the combined acid extracts ½ hour on the steam bath to remove dissolved ether. Cool, dilute to exactly 100 ml with the dilute HCl and determine the absorbancy of the standard and unknown solns at 241 mμ:

$$\text{Per cent p-aminoacetanilide} = \frac{A_{\text{unknown}}}{A_{\text{standard}}} \times C_s \times \frac{1}{50}$$

where C_s = concentration of standard *p*-aminoacetanilide soln in mg per l.

EXPERIMENTAL

Commercial *p*-aminoacetanilide (Eastman White Label) was recrystallized from water. The product melted at 162–164°C. (lit. 162° (2)). The absorption curve for *p*-aminoacetanilide in 0.1 *N* hydrochloric acid is shown in Figure 1. Solutions of *p*-aminoacetanilide in alcohol, water, or *N* HCl have been found to obey Beer's law to within ±1 per cent.

Aliquot portions of a standard solution of *p*-aminoacetanilide were added to 0.5 g portions of a sample of Ext. D&C Red No. 1 and the sol-

* Presented at the Annual Meeting of the Association of Official Agricultural Chemists, held at Washington, D. C., October 1–3, 1951.

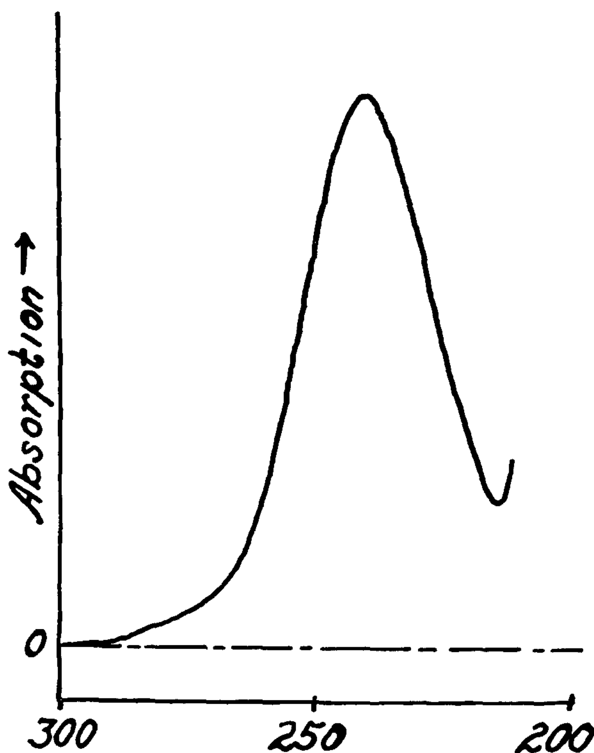

FIG. 1.—Absorption curve of p-aminoacetanilide in 0.1 N HCl.

TABLE 1.—*Recoveries of p-aminoacetanilide*

WEIGHT OF DYE	P-AMINOACETANILIDE		RECOVERY
	ADDED	FOUND	
g	*mg*	*mg*	*per cent*
0.0	1.00	1.00	100
0.0	1.00	0.98	98
0.50	0.0	0.01	—
0.50	0.50	0.42	84
0.50	0.50	0.43	86
0.50	0.50	0.48	96
0.50	1.00	0.87	87
0.50	1.00	0.90	90
0.50	1.00	0.91	91
0.50	1.00	0.93	93
0.50	2.00	1.78	89
0.50	2.00	1.88	94
	Av. recovery = 90		

vent allowed to evaporate at room temperature. These samples were analyzed by the proposed procedure with the results shown in Table 1.

SUMMARY

A method for the extraction and spectrophotometric determination of 0.1 to 0.4 per cent of uncombined *p*-aminoacetanilide in Ext. D&C Red No. 1 is described. Application of the procedure to samples of the dye containing added known amounts of the intermediate gave recovery values averaging 90 per cent.

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STUDIES ON COAL-TAR COLORS, X: EXT. D&C GREEN NO. 1*

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

The ferric salt of 6-sodiumsulfo-1-nitroso-2-naphthol (Naphthol Green B) is certifiable as Ext. D&C Green No. 1 (1). This paper describes the preparation of a carefully purified sample of Ext. D&C Green No. 1. Using this purified sample as a standard, the validity of the titanium trichloride titration procedure for the quantitative determination of the dye was investigated, and the spectrophotometric properties of solutions of the dye were determined.

EXPERIMENTAL

Schaffer's acid (2-naphthol-6-sulfonic acid) was prepared and purified as previously described (2). The Schaffer acid was converted to 6-sodiumsulfo-1-nitroso-2-naphthol by the method of Feldman (3). Schaffer's acid (0.1 mole) was dissolved in about 200 ml of water and just neutralized to phenolphthalein with 10 per cent sodium hydroxide solution. The volume was brought to 300 ml and the mixture was heated to 80° to dissolve the Schaffer salt. The warm solution was poured upon 300 g of ice contained in a 1500 ml beaker placed in a salt-ice bath. The contents of the beaker were stirred mechanically and sodium nitrite (14 g) was added. After about 15 minutes, 30 ml of conc. HCl was added slowly from a dropping funnel, the stem of which dipped below the surface of the liquid. The cold mixture was stirred for about 4 hours, then small portions of 30 per cent

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* Presented at the Annual Meeting of the Association of Official Agricultural Chemists, held at Washington, D. C., October 1–3, 1951.

sodium hydroxide solution were added until the solution was slightly alkaline as determined by a *p*H meter.

The nitroso compound was salted out of the solution by adding 150 g of sodium chloride, and collected on a sintered glass filter. The precipitate was transferred to a beaker, 200 ml of water was added, and the beaker was placed on the steam bath. After about 1 hour, 1 liter of alcohol was added, the mixture was allowed to cool to room temperature, and the precipitated nitroso compound was filtered off. This procedure was repeated until the filtrate gave no test for chlorides.

Preparation of Ext D&C Green No. 1.—The general procedure outlined in the Colour Index (4) was followed. The volatile matter present in the nitroso compound was determined by drying a sample at 135°, and 32.4 g of the nitroso compound (dry basis) was dissolved in 150 ml of hot water. A solution of 10 g of $FeCl_3 \cdot 6H_2O$ in 25 ml of water was added to the cooled solution of the nitroso compound. After four hours, the solution was made alkaline (*p*H meter) with 30 per cent sodium hydroxide solution. The solution was filtered and the filter paper washed with about 100 ml of hot water. The filtered solution was warmed to 50–55° and evaporated to a volume of about 150 ml under an air jet. Acetic acid (5 ml) was added, followed by 1250 ml of alcohol. After cooling to room temperature, the precipitated dye was collected on a sintered glass filter and washed with a 10:1 alcohol-water mixture.

The dye was digested with 100 ml of water plus 5 ml of acetic acid at 50° for about 1 hour, then precipitated by the addition of 1 liter of alcohol and filtered. This procedure was repeated several times, omitting the acetic acid, until samples from successive reprecipitations showed no increase in optical absorbancy.

ANALYTICAL DATA

All samples were dried in vacuo (2 mm Hg) at 130°, for 3 hours.

Iron.—The sample was digested with 2 ml portions each of sulfuric acid, nitric acid, and perchloric acid to destroy organic matter. About 100 ml of water was added, and after heating to dissolve the ferric sulfate, the Fe was precipitated with ammonium hydroxide. The ferric hydroxide was filtered off, dissolved in dilute hydrochloric acid, and reprecipitated with ammonium hydroxide. The precipitate was ignited in a platinum crucible. Found: Fe, 6.32, 6.34 per cent; Calc: for $(C_{10}H_5O_5NSNa)_3Fe$, 6.35 per cent.

*Nitrogen** *(Dumas).*—Found: N, 4.82 per cent, 4.73 per cent; Calc: 4.78 per cent.

Titration with titanium trichloride.—The A.O.A.C. procedure (5) was followed, except that sodium tartrate instead of sodium bitartrate was

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* Nitrogen by Oakwold Laboratories, Alexandria, Virginia.

used as the buffer. Stock solutions were prepared and aliquots were taken to prepare solutions containing about 0.1 g of dye plus 20 g of sodium tartrate in 150 ml of water. These solutions were boiled for several seconds and titrated slowly with 0.1 *N* titanium trichloride in an atmosphere of carbonic acid gas to the disappearance of the green color. Three series of titrations gave the following results in terms of 0.1 *N* $TiCl_3$ required per gram of dye: 134.5 (average of 2), 132.9 (average of 3), and 132.3 (average of 4). In each series, the titrations agreed within 0.1 ml of $TiCl_3$. Based on an equivalent weight equal to one-twelfth of the molecular weight these results correspond to 98.4, 97.3 and 96.9 per cent of pure dye, respectively.

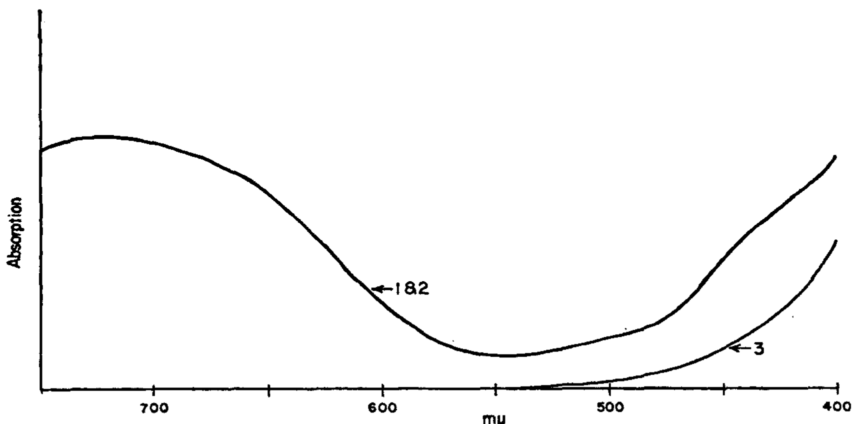

FIG. 1.—Ext. D&C Green No. 1
Concentration: 41.85 mg/liter
Solvent:
Curve 1—0.04 *N* $NH_4C_2H_3O_2$
Curve 2—0.1 *N* NaOH
Curve 3—0.1 *N* HCl
Cells—1 cm.

SPECTROPHOTOMETRIC DATA

Spectrophotometric measurements were made with a Cary recording spectrophotometer. A weighed sample of about 0.4 gram was dissolved in a liter of water and appropriate dilutions were made from this stock solution.

Figure 1 shows the effect of *p*H on the absorption curve of the color. Solutions 0.1 *N* with respect to sodium hydroxide and 0.04 *N* with respect to ammonium acetate have very nearly identical absorbancies; in 0.1 *N* hydrochloric acid the dye fades. In 0.04 *N* ammonium acetate solution, the average absorbancy at the wave length of maximum absorbancy was

0.0217 per milligram per liter. Absorbancies follow Beer's law. The solutions in ammonium acetate were stable for at least twelve hours.

DISCUSSION

Under the given conditions the reduction of Ext. D&C Green No. 1 with standard titanium trichloride yields consistent results. The equivalent weight would equal one-thirteenth of the molecular weight if the nitroso groups are reduced to amino groups and water, and the ferric iron to ferrous iron. Experimentally, the equivalent weight is found to approximate one-twelfth of the molecular weight. Using the latter value, the titration gives results which are 2–3 per cent low.

Six separate samples of commercial Ext. D&C Green No. 1 were analyzed as completely as possible. The average results for the six samples were as follows: pure dye (by titration), 83.6 per cent; volatile matter (135°), 11.6 per cent; and sodium chloride, 1.25 per cent, a total of 96.5 per cent. Carbonates, bicarbonates, and sulfates did not exceed 1 per cent. Organic intermediates were shown to be absent by the method of variable reference spectrophotometry in the ultraviolet region (6). These analyses confirm the low results obtained in the titration of the pure dye. Spectrophotometric analysis of the samples gave 86.5 per cent average pure dye content.

SUMMARY

A carefully purified sample of Ext. D&C Green No. 1 has been prepared.

The titanium trichloride titration gives slightly low results, but is a practicable method for the determination of Ext. D&C Green No. 1. Spectrophotometric data for aqueous solutions of Ext. D&C Green No. 1 have been presented. Aqueous solutions, buffered with ammonium acetate, obey Beer's law.

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(5) *Methods of Analysis, A.O.A.C.*, **34.21** (c) 7th Ed. 1950.
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REFRACTIVE INDICES OF RAFFINOSE HYDRATE SOLUTIONS*

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

The International Scale of refractive indices of sucrose solutions at 20° and 28°C. was adopted by the Association in 1938 (1). The tables of Jackson and Mathews (2) for the refractive indices of levulose solutions at 20° and 25°C. were included in *Methods of Analysis*, 6th edition (1945), p. 829, and were reprinted in the 7th edition (1950), p. 813. Those of Zerban and Martin for the refractive indices of dextrose and invert sugar solutions at 20° (3) were adopted as official by the Association in 1950 (4). The only other important sugar occurring in sucrose bearing plants used for industrial production, such as sugar beet, is raffinose, and the present report deals with the refractive indices of solutions of this sugar at 20°C.

The crude raffinose used in this work was supplied by H. W. Dahlberg, of the Great Western Sugar Company, to whom the writers express their thanks. The raffinose was repeatedly recrystallized from water and alcohol by the method described by the National Bureau of Standards (5). After three recrystallizations the specific conductance became constant. But according to a chromatographic test, performed by N. Albon, of the Tate and Lyle Research Laboratory at Ravensbourne, England, it still contained 0.15 per cent sucrose. It was therefore recrystallized a fourth time, and in this preparation Mr. Albon found less than 0.02 per cent of sucrose, *i.e.*, beyond the precision limit of the method. It was decided to recrystallize the raffinose once more, to reduce the sucrose to the nondetectable limit. The product from this fifth recrystallization was submitted to G. L. Clark, of the University of Illinois, for X-ray diffraction analysis. No sucrose could be detected by this method. The writers greatly appreciate the assistance of Mr. Albon and Dr. Clark in this investigation.

The final product was dried for about a month at room temperature during the dry winter period. It was spread out on a large sheet of filter paper and covered with another sheet of the same size, to allow access of air and assure freedom from dust. Twice a day or oftener the crystal mass was thoroughly worked over, to expose fresh crystal surfaces. The moisture determination was made by heating a 4-gram sample in a vacuum oven at 75° for eighteen hours and then for two- to three-hour periods to constant weight. The heating was then continued for two- to three-hour periods at 80°, 85°, and 100°. The constant weight under these conditions at 100° was 84.86 per cent of the original sample, against

* Presented at the Annual Meeting of the Association of Official Agricultural Chemists, held at Washington, D. C., October 1–3, 1951.

the theoretical of 84.84 per cent. It was concluded that the preparation had the composition of the pentahydrate. It was transferred to a glass stoppered bottle and used as such for the refractive index measurements.

The solutions of the raffinose hydrate were prepared, and the refractive indices were measured with a Bausch and Lomb Precision refractometer as described previously for dextrose and invert sugar (3), but it was not necessary to delay the measurements 24 hours because raffinose does not mutarotate.

The experimental results are shown in the second column of Table 1. From these values the following quartic equation was computed by the method of averages:

$$n_D^{20} = 1.33299 + 0.001218905\,p + 0.000006384354\,p^2 - 0.00000006844738\,p^3 + 0.0000000009595215\,p^4 \qquad (1)$$

The deviations from this formula are within 0.00003 up to a concentration of 17 per cent raffinose hydrate by weight, which is about 3 points higher than the saturation concentration of 14.14 per cent at 20°C. according to Hungerford and Nees (6). At higher concentration the deviations are somewhat greater. Solutions above 40 per cent would crystallize when placed in the refractometer prism.

The sum of the squares of the residuals for the forty experiments is 406×10^{-10}, showing a variance of 10.41×10^{-10}, and a standard error of estimate of 0.000032, the same as for the experiments on the refractive indices of lactose hydrate solutions (7) and slightly higher than that for dextrose solutions, 0.000029.

The only previous data on the refractive indices of raffinose solutions are those reported fifty years ago by Stolle (8), for 17.5°C. The results of the present work, at 20°C. and those of Stolle are shown in Table 2. The writers are indebted to S. M. Cantor for photostats of Stolle's table.

The temperature coefficients for 1°C. between 17.5 and 20°C. vary irregularly between -44 and $+120 \times 10^{-6}$, while they should be about $+84 \times 10^{-6}$ for water, and should gradually rise with the sugar concentration. The best available data for the temperature coefficient of the refractive indices of sucrose (1) and of levulose (2) are as follows:

	Temperature coefficients $\times 10^6$ *for 1°C.*			
Per cent sugar	0	10	20	30
Sucrose (20 to 28°C.)	100	115	128	138
Levulose (20 to 25°C.)	96	117	139	169

The temperature coefficients for dextrose between 17.5 and 20°C., based on the results of the writers (3) and those of Stolle (8) show similar irregularities as for raffinose, as shown in Table 3:

It is plain that the values obtained by Stolle must be in error. The refractive index of water (0.0000 per cent sugar) is reported to be 1.33310,

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

RAFFINOSE HYDRATE, WEIGHTS IN AIR, PER CENT	n_D^{20} OBSERVED	n_D^{20} CALCD. BY EQUATION 1	DEVIATION FROM FORMULA	SQUARE OF RESIDUALS TIMES 10^{10}
0.989	1.33418	1.33420	−0.00002	4
2.011	1.33547	1.33547	0.00000	0
2.914	1.33656	1.33659	−0.00003	9
3.848	1.33776	1.33777	−0.00001	1
4.749	1.33891	1.33892	−0.00001	1
6.110	1.34069	1.34066	+0.00003	9
6.538	1.34123	1.34121	+0.00002	4
8.063	1.34323	1.34320	+0.00003	9
9.096	1.34456	1.34456	0.00000	0
10.063	1.34584	1.34584	0.00000	0
11.063	1.34718	1.34718	0.00000	0
12.022	1.34846	1.34847	−0.00001	1
12.951	1.34972	1.34973	−0.00001	1
13.894	1.35100	1.35101	−0.00001	1
15.042	1.35262	1.35259	+0.00003	9
16.092	1.35404	1.35404	0.00000	0
16.553	1.35469	1.35468	+0.00001	1
17.053	1.35534	1.35537	−0.00003	9
18.117	1.35690	1.35686	+0.00004	16
19.135	1.35827	1.35830	−0.00003	9
20.176	1.35984	1.35978	+0.00006	36
21.233	1.36139	1.36129	+0.00010	100
22.298	1.36281	1.36282	−0.00001	1
23.064	1.36391	1.36393	−0.00002	4
24.165	1.36550	1.36553	−0.00003	9
25.109	1.36691	1.36692	−0.00001	1
26.337	1.36872	1.36873	−0.00001	1
27.198	1.36997	1.37001	−0.00004	16
28.181	1.37146	1.37148	−0.00002	4
29.018	1.37270	1.37274	−0.00004	16
30.165	1.37445	1.37448	−0.00003	9
31.019	1.37580	1.37579	+0.00001	1
32.015	1.37732	1.37732	0.00000	0
33.005	1.37887	1.37885	+0.00002	4
34.058	1.38046	1.38050	−0.00004	16
35.174	1.38224	1.38225	−0.00001	1
35.346	1.38257	1.38252	+0.00005	25
36.311	1.38411	1.38406	+0.00005	25
37.863	1.38648	1.38655	−0.00007	49
40.072	1.39018	1.39016	+0.00002	4
				406

TABLE 2.—*Comparison between Zerban and Martin's data at 20°C. and Stolle's data at 17.5°C. for the refractive indices of raffinose hydrate solutions*

RAFFINOSE HYDRATE, WEIGHTS IN AIR, PER CENT	n_D^{20} (ZERBAN AND MARTIN)	$n_D^{17.5}$ (STOLLE)	TEMP. COEFF. PER °C., TIMES 10^6
0	1.33299	1.33310	+ 44
1	1.33422	1.33448	+104
2	1.33546	1.33556	+ 40
3	1.33671	1.33683	+ 48
4	1.33797	1.33810	+ 52
5	1.33924	1.33941	+ 68
6	1.34052	1.34073	+ 84
7	1.34181	1.34204	+ 92
8	1.34311	1.34335	+ 96
9	1.34443	1.34464	+ 84
10	1.34576	1.34594	+ 72
11	1.34709	1.34723	+ 56
12	1.34844	1.34857	+ 52
13	1.34979	1.34992	+ 52
14	1.35116	1.35128	+ 48
15	1.35253	1.35263	+ 40
16	1.35391	1.35407	+ 64
17	1.35530	1.35552	+ 88
18	1.35670	1.35697	+108
19	1.35811	1.35841	+120
20	1.35953	1.35982	+116
21	1.36096	1.36122	+104
22	1.36239	1.36262	+ 92
23	1.36384	1.36403	+ 76
24	1.36529	1.36546	+ 68
25	1.36676	1.36691	+ 60
26	1.36824	1.36836	+ 48
27	1.36972	1.36981	+ 36
28	1.37121	1.37125	+ 16
29	1.37272	1.37269	− 12
30	1.37424	1.37413	− 44
31	1.37577		
32	1.37730		
33	1.37884		
34	1.38040		
35	1.38197		
36	1.38356		
37	1.38516		
38	1.38677		
39	1.38840		
40	1.39004		

whereas according to the best available information (9) it is 1.33320, considerably higher. Stolle's errors must have partly arisen from the fact that he determined the refractive indices of solutions containing weighed quantities of dextrose, raffinose, and other sugars in 100 ml of solution, calculated the refractive indices of solutions in per cent by weight on the basis of the densities of the solutions used, and reduced the results to weights in vacuo. It must be concluded that Stolle's reported results for 17.5° are affected by considerable errors.

TABLE 3.—*Comparison of results for dextrose*

DEXTROSE, PER CENT BY WEIGHT, IN AIR	$n_D^{17.5}$ (STOLLE)	n_D^{20} (ZERBAN AND MARTIN)	TEMP. COEFF. PER °C., TIMES 10^6
0.0000	1.33310	1.33299	44
0.9950	1.33473	1.33442	124
1.9898	1.33605	1.33585	80
3.9492	1.33890	1.33870	80
7.7849	1.34456	1.34440	64
11.5214	1.35026	1.35009	68
15.1145	1.35581	1.35570	44
18.6212	1.36164	1.36131	132
22.9045	1.36863	1.36836	108

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THE DETERMINATION OF MICRO QUANTITIES OF ISOPROPYL *N*-PHENYLCARBAMATE (IPC) IN HEAD LETTUCE

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The herbicidal activity of isopropyl *N*-phenylcarbamate (IPC) was first reported by Templeman and Sexton (1), who described the selective action of this compound against cereals and grasses. Additional evidence has been presented (2, 3, 4, 5) to substantiate such activity. More re-

cently, isopropyl *N*-phenylcarbamate has shown great promise for the control of ryegrass, mustard, and sour dock in alfalfa and similar forage crops. Chickweed in strawberries has been satisfactorily controlled (6) with this carbamate. It has also been found that this material will control annual blue grass, an abundant grassy weed encountered in such winter vegetable crops as lettuce and cauliflower.

The most effective time to apply IPC is when the weeds and roots are still in the embryonic stage. When thus applied early in the growing season, our studies (7) have shown that the carbamate will persist in the soil for only 2–3 weeks. Although this would automatically limit the amount of absorption of IPC by the food-stuff, it is still desirable to have a method for the determination of micro quantities of the chemical so that residue analyses can be made.

Examination of the structure of isopropyl *N*-phenylcarbamate suggested that if it could be hydrolyzed quantitatively to form aniline, isopropyl alcohol, and carbon dioxide, there should be a good possibility of measuring the carbamate in terms of the aniline by coupling the latter with another reagent to form a dye. The success of this process would depend upon the quantitative formation of aniline in the hydrolysis, a complete recovery of the aniline, and a sensitive method for indicating the presence of as little as 1 p.p.m. or less of aniline.

Preliminary attempts to hydrolyze the carbamate in various basic media met with failure due to incomplete hydrolysis. Concurrently with this work, L. Gard (8) demonstrated that a mixture of 85 per cent phosphoric acid, concentrated sulfuric acid and water would hydrolyze IPC quantitatively. In the present work it was found that a mixture of 91 per cent by volume of 85 per cent phosphoric acid and 9 per cent by volume of concentrated hydrochloric acid served the same purpose when aniline was to be determined. To maintain the carbamate in solution, 5 ml of glacial acetic acid was added to the hydrolyzing medium.

Several methods for determining aniline were investigated but were found unsuccessful because the color developed by the control on the reagents was of the same order of intensity as when aniline was present, and thus the sensitivity was poor. With the hypochlorite-phenol method (9), we were able to detect the presence of aniline in concentrations as low as 0.5 p.p.m. It was also demonstrated that aniline could be steam-distilled from an alkaline solution and quantitatively determined by this method, provided alcohols, amines, or ketones were not present. Apparently these latter types of compounds react with the hypochlorite reagent and the products interfere with the desired color formation, since the addition of large excesses of hypochlorite is not beneficial.

The feasibility of the above method was illustrated by hydrolyzing small quantities of IPC in acid solution, after which the aniline was removed by steam distillation from alkaline solution and determined by

the hypochlorite-phenol method. In four determinations the following results were obtained:

Mg of IPC Hydrolyzed	*Aniline Recovered, as mg of IPC*
0.10	0.09
0.52	0.50
1.04	1.00
5.00	4.82

After a procedure for measuring the aniline content of hydrolyzed IPC had been established, it was still necessary to develop a method for this hydrolysis and aniline measurement in the presence of vegetable material. When a weighed amount of isopropyl *N*-phenylcarbamate was added to one-fourth head of dried and untreated lettuce, and the mixture hydrolyzed in the usual way, then made alkaline and steam distilled, the distillate gave no test for aniline. Even when aniline was added to the hydrolyzed lettuce mixture, and the mixture made alkaline and steam distilled, no aniline could be found in the distillate. However, when aniline was added directly to the distillate, it could be measured quantitatively. This indicated that the aniline may have combined chemically with some product of the hydrolyzed lettuce. An extraction process to remove the IPC from the lettuce prior to hydrolysis was therefore employed. The previous development (10) of the Waring Blendor for similar extractions made this practicable. An ether solution containing 1.0 mg of the carbamate was added to 300 g of untreated lettuce. This corresponded to an IPC content on the lettuce of 0.33 p.p.m. After the ether was evaporated, the lettuce was extracted with methylene dichloride as outlined below and the extract was analyzed for aniline. A 96 per cent recovery of the carbamate, as aniline, was obtained. This procedure was then adopted as the standard method for the analysis of treated lettuce.

METHOD

APPARATUS

(1) *Waring Blendor.*

(2) *Filtration Equipment*: **a**. Five-inch basket centrifuge fitted with a glass cloth retaining screen. **b**. Centrifuge equipped with 250 ml centrifuge bottles.

(3) *Round bottom flask*, 100 ml, fitted with a reflux condenser.

(4) Unpacked distillation column, 1.5×10 cm, connected by a glass seal to a water-cooled condenser. (Column functions as a still-head.)

(5) *Volumetric flasks*, 50 ml.

(6) *Test tubes*, 20×150 mm.

REAGENTS

(1) *Methylene dichloride*, technical grade.

(2) *Diethyl ether*, A.R. grade.

(3) *Phosphoric acid-hydrochloric acid reagent.*—To 91 ml of 85% phosphoric acid, add 9 ml of conc. HCl. (The use of C.P. acids is preferred.)

(4) *Acetic acid*, glacial.

(5) *Sodium hydroxide soln.*—Dissolve 25 g of C.P. sodium hydroxide pellets in 75 ml of distilled water.

(6) *Calcium hypochlorite soln.*—Dissolve 5.0 g of calcium hypochlorite in 95 ml of distilled water at 60°C and filter while hot. (This soln should not be stored for more than 10 days.)

(7) *Phenol soln.*—Dissolve 5 g of phenol in a mixture of 5 ml of 28% ammonium hydroxide and 95 ml of distilled water. (This soln develops a blue color on standing and must be prepared daily.)

(8) *Hydrochloric acid soln.*—Dilute 12 ml of conc. HCl to 100 ml with distilled water.

PROCEDURE

Divide a head of lettuce into 300 g portions weighed to 0.1 g. (To obtain a uniform sample of the lettuce it should be cut into segments, or in half, so that each portion samples uniformly the interior as well as the exterior of the head.) Place the lettuce in a Waring Blendor and blend for two min. Add 200 ml of methylene dichloride and blend for ten min. more keeping the temp. below 35°C by the addition of chopped ice. Centrifuge the slurry with a basket centrifuge to remove the lettuce particles. Separate the methylene dichloride layer from the water phase with a centrifuge equipped with sedimentation tubes. Withdraw the water layer and discard. Rinse the equipment with about 100 ml of methylene dichloride and combine all extracts. Filter the combined extracts to remove last traces of solids, and evaporate the solvent by blowing a small stream of dry air over the surface of the soln. Transfer the residue quantitatively with ether to a small flask and remove the ether with a stream of air. Hydrolyze the residue with a mixture of 10 ml of the phosphoric acid-hydrochloric acid reagent and 5 ml of glacial acetic acid for one hour under reflux. Cool, dilute to about 50 ml with distilled water, and make alkaline with 60 ml of the 25% sodium hydroxide soln. Steam-distil the mixture thru the still head and collect the first 25–35 ml of distillate in a 50-ml volumetric flask. Dilute to volume at 20°C, mix, and transfer a 10 ml aliquot to a 20×150 mm test-tube. Add two drops of the freshly prepared 5% calcium hypochlorite soln and 2 ml of the hydrochloric acid and allow to stand for five min. Heat to boiling and add 5 ml of the freshly prepared phenol reagent; finally, dilute to 20 ml with distilled water. Allow the blue color to develop for 15 min and compare the intensity of the color with that obtained with known solns of the same volume, containing 0.01, 0.10, and 1.0 mg of aniline by looking down thru the top of the test-tubes. (A colorimeter may be used but is not essential since the changes in color intensity due to 0.01 mg variations of aniline are very easily distinguished by simple visual examination in samples containing 0.01 to 0.1 mg of aniline. Standard colors prepared from Toluidine Blue are recommended by the authors (9) of this method, but the use of standard aniline solns to develop the various color standards seemed more direct and was employed here.)

After determining in which range the aniline content of the unknown falls, prepare a second series of standards varying in concentration by 0.01 mg of aniline and compare these with the color developed by another aliquot of the sample soln. Apply a correction for the size of the aliquot, and convert this aniline content into the amount of IPC present in the lettuce by means of the following formula:

$$\text{p.p.m. of IPC} = \frac{\text{mg of aniline} \times 1.93 \times 1000}{\text{grams of sample}}$$

RESULTS

Arrangements were made with a commercial grower to treat lettuce with IPC at the recommended dosage for chickweed and ryegrass con-

trol. The IPC was combined with a DDT dusting powder to give a 21 per cent IPC dust. This dust was applied two weeks after the lettuce had been planted and when it was one-half inch out of the ground. The rate of application was 7 lbs. per acre. This treatment gave very effective control—90–95 per cent of the chickweed and 60–70 per cent of the rye grass. At maturity, forty-four days after treatment, approximately 15 lbs. of the harvested lettuce, both treated and untreated, was selected and stored in ice until analyzed.

(In another field, two applications of the IPC dust were made, corresponding to a treatment of 14 lbs. per acre. Since no additional benefits from the higher application was noted, the lettuce from these plots was not analyzed.)

The lettuce treated at the rate of 7 lbs. per acre was analyzed as already outlined. A 10-ml aliquot of the final steam distillate showed no color when tested by the hypochlorite-phenol method. The remaining 40 ml was then tested but again no color developed. Since the limit of sensitivity of this method of measuring aniline is about 0.5 p.p.m., or 0.02 mg of aniline in 40 ml of water, it can be said that the measurement indicated *less* than 0.025 mg in the entire 50 ml of solution. From this it can readily be calculated that the IPC content of the lettuce was less than 0.16 p.p.m. Actually, there was no evidence that any IPC was present.

Although we have not investigated other crops, the general principles of this method are probably applicable, but each crop would have to be tested for the presence of interfering substances just as was done with lettuce.

The common insecticides and fungicides would not be expected to interfere with this determination because aniline or its derivatives are not hydrolysis products of either class of compound. Actually, control plots of untreated crops should always be analyzed simultaneously, and these results would establish the presence of interfering substances, whatever their source.

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USE OF ION-EXCHANGE RESINS FOR THE REMOVAL OF NON-SUGAR REDUCING SUBSTANCES IN THE ANALYSIS OF FRESH LEAFY PLANT MATERIALS FOR SUGARS

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In previous papers it was shown that in the analysis of the edible portion of certain vegetables, ion-exchange resins were more effective than neutral lead acetate in removing non-sugar reducing substances (19, 20). In fact, no reducing materials, measurable by the copper method, remained after fermentation when ion-exchange resin clarification was used. This is a continuation of the study of the clarification procedure as applied to sugar analysis of the fresh leafy portions of a wide variety of plant materials. The ion-exchange (19), the conventional lead-phosphate, and the filter-aid (4, 20) procedures were compared. The nonfermentable (bakers' yeast) reducing substances were determined on all samples. Tentative identification of the sugars present in each plant material was made by paper chromatography.

PROCEDURE

(1) *Extraction of Sugars.*—An 80 per cent alcoholic extract of each sample was prepared and diluted to a definite volume (20).

(2) *Clarification.*—Aliquots of the extracts were evaporated on a steam bath to remove the alcohol. The concentrates were cooled, filtered thru a mat of Celite Analytical Filter-Aid[2] and diluted to a definite volume with distilled water (4, 20). These solutions will be referred to as the Celite-clarified solutions. Aliquots of these Celite-clarified solutions were used for further clarification by either the lead-phosphate (20) or batch-resin (19) procedure as previously described.

(3) *Fermentation.*—Washed bakers' yeast was used as previously described (19).

(4) *Hydrolysis.*—Invertase was used as previously described (20). Other aliquots of the solutions were made 1 N with hydrochloric acid, allowed to stand overnight at room temperature (1), and then neutralized with 6 N sodium hydroxide. There was no significant difference in the total reducing substances produced by the two methods of hydrolysis.

(5) *Sugar Measurement.*—Reducing sugars were determined by the A.O.A.C. Somogyi micro copper method (2). All of the values shown in the table are the average of closely agreeing replicates, with a maximum variation of ± 2 per cent in all except the low values where ± 0.01 per cent absolute errors were tolerated.

(6) *Paper Chromatography.*—The paper chromatographic technique

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

was essentially that used by Partridge (13, 14). All of the chromatograms were made on Whatman No. 1 filter paper. The solvent mixtures used for irrigating the chromatograms were *n*-butanol-ethanol-water (10:1:2) and ethyl acetate-pyridine-water (8:2:1) (7, 14). The ethyl acetate-pyridine-water chromatograms were thoroughly rinsed with anhydrous ethyl ether prior to drying and application of the spray reagent.

The initial spots were placed on the paper by means of platinum loops which delivered from 1 to 5 microliters of solution according to the size of loop used. All chromatograms were irrigated at room temperature (25°C.) and the time varied from 6 hours to 72 hours dependent upon the solvents used and the sugars under investigation.

The positions of the sugars were determined by spraying the dried papers with one of the following reagents: An acidic solution of resorcinol (14), aniline acid phthalate (15), aniline trichloracetic acid (17), an alkaline solution of 3,5-dinitrosalicyclic acid (6, 11) or a *n*-butanol solution of trichloracetic acid and orcinol (8). The orcinol spray gives a yellow color with certain sugars, but gives a blue color with ketoheptoses, which is reported to be specific for this latter group of sugars (8). The specificity of the orcinol spray reagent has been investigated in this laboratory and a report will appear elsewhere.

Stachyose, raffinose, sucrose, glucose, fructose, galactose, arabinose, and xylose on the chromatograms were tentatively identified by authentic samples of known sugars as controls. The presence of stachyose and raffinose was confirmed by a previously described technique (18).

DISCUSSION OF RESULTS

The data show that the ion-exchange method of clarification is equal to or more effective than the neutral lead acetate method for all of the samples investigated. The sugar values obtained by all three methods of clarification are given in Table 1.

Although the color of the clarified solutions may not contribute to the reducing value of the solution in the determination of sugars it does affect the visibility of the end point in titration methods. This is especially true when ferricyanide methods are used. The ion-exchange resin treatment gave water-clear solutions in all of the tests made for this report but the neutral lead acetate clarification procedure did not remove all of the color from several of the solutions.

The data in Table 1 also show that after fermentation appreciable amounts of reducing substances remained in the extracts of the leaves of the fig, peach, and squash (*zucchini*) even after ion-exchange resin clarification. It had been anticipated that plant materials would be found which would contain non-fermentable reducing substances since bakers' yeast will not ferment all sugars. Some of these non-fermentable reducing sugars, such as the pentoses, the heptoses, galactose, and others, are

TABLE 1.—*Percentages of reducing substances*[1] *originally present, after hydrolysis, after fermentation, and the tentative identification of the sugars by paper chromatography*

PLANT MATERIAL	METHOD OF CLARIFICATION			SUGARS IN ADDITION TO SUCROSE, GLUCOSE AND FRUCTOSE
	CELITE	LEAD-PHOSPHATE	ION-EXCHANGE RESINS	
Alfalfa				None
Originally present	0.30	0.27	0.22	
After hydrolysis	0.89	0.84	0.78	
After fermentation	0.10	0.07	0.03	
Fermentables	0.79	0.77	0.75	
Red beet tops				None
Originally present	0.57	0.59	0.54	
After hydrolysis	0.96	0.95	0.93	
After fermentation	0.07	0.03	0.00	
Fermentables	0.89	0.92	0.93	
Carrot tops				None
Originally present	0.89	0.74	0.75	
After hydrolysis	1.47	1.24	1.29	
After fermentation	0.11	0.02	0.01	
Fermentables	1.36	1.22	1.28	
Endive				None
Originally present	1.35	1.31	1.29	
After hydrolysis	1.47	1.44	1.40	
After fermentation	0.02	0.02	0.02	
Fermentables	1.45	1.42	1.38	
Fig leaves				Galactose, 3 ketoheptoses,[2] and traces of arabinose and xylose.
Originally present	1.62	1.55	1.47	
After hydrolysis	3.06	2.93	2.95	
After fermentation	0.29	0.24	0.19	
Fermentables	2.77	2.69	2.76	
Grass (unidentified)				Two or more fermentable polysaccharides having ketose structure and fructofuranoside linkages.
Originally present	0.27	0.18	0.14	
After hydrolysis	1.11	1.03	0.95	
After fermentation	0.16	0.08	0.00	
Fermentable	0.95	0.95	0.95	
Mustard greens				Traces of raffinose, arabinose and xylose.
Originally present	1.59	1.56	1.40	
After hydrolysis	1.76	1.69	1.60	
After fermentation	0.07	0.08	0.00	
Fermentables	1.69	1.61	1.60	
Peach leaves				
Originally present	1.07	0.88	0.73	A ketoheptose and traces of arabinose and xylose.
After hydrolysis	2.46	2.39	2.15	
After fermentation	0.50	0.25	0.18	
Fermentables	1.96	2.14	1.97	
Swiss chard				Raffinose
Originally present	2.07	1.91	2.04	
After hydrolysis	2.62	2.41	2.53	
After fermentation	0.09	0.04	0.01	
Fermentables	2.53	2.37	2.52	
Squash (*Zucchini*) leaves				Raffinose and stachyose
Originally present	0.32	0.30	0.29	
After hydrolysis	0.47	0.43	0.45	
After fermentation	0.12	0.11	0.10	
Fermentables	0.35	0.32	0.35	

[1] Calculated as glucose.
[2] Three distinct blue-green spots were obtained with the orcinol spray. Only two ketoheptoses have been reported to occur in nature. The specificity of the reagent and further identification of the materials giving the test are under investigation by the authors.

known to be present in some plant materials. It seemed desirable to determine whether there were any non-fermentable reducing sugars present in the plant extracts studied. Therefore, paper chromatograms of the plant extracts, before and after fermentation with bakers' yeast, were prepared and the sugars present tentatively identified. The data are also given in Table 1.

The non-fermentable reducing substances in the fig leaf extracts were equivalent to 0.19 per cent of glucose. The pentoses, present in traces, did not contribute an appreciable reducing sugar value. However, it is quite possible that galactose and the ketoheptoses account for it. So far as the authors have been able to ascertain from the literature, this is the first report of the occurrence of ketoheptoses in tree leaves. An investigation of a wide variety of tree leaves is in progress to determine whether ketoheptoses are commonly present. The occurrence of sedoheptulose in succulent plants has been well established (10, 12, 16) and recently it has been identified in other plants (3). Mannoheptulose has been reported in the avocado (9).

The non-fermentable reducing substances in peach tree leaves, equivalent to 0.18 per cent glucose, is due, at least in part, to the ketoheptose present. The pentoses are not present in sufficient amount to be a factor.

It is of interest to note that free pentoses were not detected in seven of the plant materials studied and they were present in the other three in traces only, *i.e.*, in the order of 0.005 per cent as estimated by visual comparison with known amounts of arabinose and xylose on the chromatograms.

Melibiose (residue from raffinose) and manninotriose (residue from stachyose) were identified by paper chromatography, in the fermented squash leaf extract. These reducing sugars were responsible in part, at least, for the 0.1 per cent of reducing material remaining after fermentation.

The fermentable polysaccharides in the grass extract were not identified but may be similar to the levan-like substance isolated from rough-stocked meadow grass, *Poa trivialis* (5).

The data in this and previous reports (19, 20) have demonstrated the applicability of selected ion-exchange resins for the removal of non-sugar reducing substances. In all of the tests they have been equal or superior to the neutral lead acetate method of clarification. They have also been effective in the removal of color which may interfere in determining the end point in certain titration methods. The resins are convenient to use and save working time.

SUMMARY

1. Selected ion-exchange resins were found to be equal or superior to neutral lead acetate for the removal of non-sugar reducing substances and color in the analysis of plant materials for sugars.

2. Non-fermentable reducing substances were found in certain leaf extracts after clarification by ion-exchange resins. These reducing substances were composed, at least in part, of non-fermentable reducing sugars.

3. The sugars present in the plant materials studied were tentatively identified by paper chromatography.

4. This is the first identification (tentative) of ketoheptoses in tree leaves.

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DETERMINATION OF CAROTENE IN ALFALFA MEAL

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In comparing the A.O.A.C. method for determining carotene in alfalfa meal with the Silker, Schrenk, and King procedure used in this laboratory (1), it was noted that the A.O.A.C. method consistently gave lower results. Since this work was started, Thompson and Bickoff (2) reported a similar discrepancy between the A.O.A.C. method and a modification used

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by them. The data presented herein confirm and extend the work of Thompson and Bickoff.

EXPERIMENTAL

Methods. The A.O.A.C. method consists of refluxing a 2–4 g sample of meal for 1 hour with 30 ml of 30 per cent acetone in Skellysolve B, filtering, diluting the filtrate to 100 ml with Skellysolve B, pouring the extract on a column of magnesium oxide-supercel mixture, and washing the column with 50 ml of 9 per cent acetone in Skellysolve B. In the Silker, Schrenk, and King method, the sample is soaked overnight with 60 ml of 30 per cent acetone in Skellysolve B, the sample is filtered, the residue is washed with Skellysolve B, and the extract is concentrated to 40 ml. The extract is poured on a magnesium oxide-supercel column and the carotene is eluted with 4 per cent acetone in Skellysolve B. Elution is continued until the eluate is colorless. Hence, the volume of eluting agent may vary from one sample to another.

TABLE 1.—*Comparison of the A.O.A.C. and Silker, Schrenk, and King methods for the determination of carotene in alfalfa meal*

SAMPLE	A.O.A.C.		SILKER, SCHRENK, AND KING
	50 ML ELUANT	100 ML ELUANT	
	mg/100 g	*mg/100 g*	*mg/100 g*
1	5.9	6.3	6.9
2	15.1	16.7	16.7
3	9.1	16.7	17.1
4	10.1	17.7	18.3
5	19.8	21.2	21.9
6	20.2	21.8	22.1
7	24.7	27.4	29.0
8	26.3	27.8	29.1

Comparison of methods. In using the A.O.A.C. method, it was observed that with some samples carotene was still being eluted after 50 ml of 9 per cent acetone had passed through the column. A number of samples were analyzed by the Silker, Schrenk, and King method, the A.O.A.C. method, and the latter procedure modified to employ 100 ml of eluting agent instead of 50 ml. The data, presented in Table 1, show that when the volume of eluting agent is limited to 50 ml, the values obtained are low compared to those obtained by the Silker, Schrenk, and King method. When 100 ml of eluting agent was used, the values are in much better agreement, although with most of the high carotene meals results by the A.O.A.C. method are still appreciably lower. Spectral examination of the eluant solutions with a Beckman spectrophotometer indicated that the pigment removed by the second 50 ml portion was carotene. Hence, part of the discrepancy between the two methods was due to incomplete elution of carotene.

TABLE 2.—*Comparison of the Silker, Schrenk, and King Method with a combination of the Silker, Schrenk, and King and A.O.A.C. methods*

SAMPLE	SILKER, SCHRENK AND KING	COMBINATION
	mg/100 g	*mg/100 g*
1	6.9	6.2
2	10.8	9.7
3	22.1	21.8
4	22.6	22.2
5	25.1	24.7

In view of these data, all other comparisons in this study which involved the A.O.A.C. method were made by modifying it to employ 100 ml of eluting agent.

To investigate further the differences between these methods, alfalfa meal was extracted by refluxing one gram with 30 ml of 30 per cent acetone in Skellysolve B for 1 hour as in the A.O.A.C. method. The sample was filtered and the analysis completed according to the Silker, Schrenk, and King method. This procedure, listed as "Combination" in Table 2, gave values which were lower than those obtained by the Silker, Schrenk, and King method. These results indicate that part of the discrepancy between the A.O.A.C. and Silker, Schrenk, and King methods occurs in the extraction of the sample. Either extraction was incomplete, or slight carotene destruction occurred during refluxing. Since both methods require heat during the analysis, the difference probably is not due to differences in destruction or isomerization.

The extraction phase of the A.O.A.C. method was investigated further by soaking the sample in 30 ml of 30 per cent acetone in Skellysolve B overnight, instead of refluxing it for 1 hour. The analysis was completed according to the A.O.A.C. method, except that 100 ml of eluting agent was used instead of 50 ml. This modification thus is similar to the alternative A.O.A.C. method (3), and differs only in the volume of eluting agent used. The data (Table 3) show good agreement between the Silker,

TABLE 3.—*Comparison of the modified A.O.A.C. and the Silker, Schrenk, and King methods with the modified alternative A.O.A.C. method*

SAMPLE	MODIFIED A.O.A.C.	SILKER, SCHRENK, AND KING	MODIFIED ALTERNATIVE A.O.A.C.
	mg/100 g	*mg/100 g*	*mg/100 g*
1	13.8	14.3	14.4
2	16.1	16.6	16.5
3	16.6	16.9	16.8
4	31.2	32.4	32.8

Schrenk, and King method and the modified alternative method, and further illustrate the inadequacy of the one hour extraction.

Extraction by percolation. The A.O.A.C. method states that the Goldfisch extractor is suitable for extracting carotene if a thimble is not used. Apparently, the intent is to assure a hot extraction rather than a relatively cold extraction by the condensing solvent. Samples were analyzed by the modified A.O.A.C. method both with and without thimbles. Thus, in one case the carotene was extracted by reflux and in the other by percolation of the distillate through the meal contained in the thimble. Table 4 indicates that percolation was as efficient as refluxing in removing carotene. In addition, percolation has certain advantages. It eliminates the necessity of filtering before diluting the extract to 100 ml. Also, under reflux the loose alfalfa particles often cause bumping and this may result in some of the meal being trapped in the condenser. This could lead to incomplete extraction.

TABLE 4.—*Comparison of carotene extraction by percolation and by reflux in conjunction with the modified A.O.A.C. method*

SAMPLE	PERCOLATION	REFLUX
	mg/100 g	*mg/100 g*
1	3.2	3.5
2	24.6	24.3
3	24.7	24.6
4	25.5	25.7
5	31.0	30.7
6	31.6	32.3
7	32.0	32.1

Extraction by soaking. A disadvantage of the Silker, Schrenk, and King procedure is that samples must be soaked overnight in the extracting solvent. The analysis of many samples must be completed the same day they are received. It was of interest to determine the minimum time of soaking that would still yield complete extraction of carotene.

Samples of meal were soaked in 60 ml of 30 per cent acetone in Skellysolve B at temperatures of 4°, 25°, 37°, and 45°C. and at room temperature (30–32°C.). Samples from each series were analyzed after 2, 6, 12, 18, 24, 36, and 48 hours. The results, presented in Table 5, show that maximum carotene extraction at room temperature occurred in 18 hours. At 37°C. and 45°C. the maximum occurred in about 12 hours. At temperatures below 25°C. extraction was not complete after 48 hours of soaking. No destruction of carotene occurred upon prolonged extraction, even at the higher temperatures. It is apparent that the soaking time cannot be shortened sufficiently to permit completion of an analysis on the same day the sample is received at the laboratory.

Effect of sample size. Alfalfa meal which has been in storage for several

TABLE 5.—*Effect of temperature and time of soaking on carotene determination by the Silker, Schrenk, and King method*

TEMPERATURE °C.	HOURS OF EXTRACTION						
	2	6	12	18	24	36	48
	(*Results as mg/100 g*)						
4	10.7	11.7	12.1	12.3	12.0	12.8	13.2
25	12.0	13.5	13.5	13.8	13.9	14.0	14.1
Room	11.9	13.5	13.6	14.2	14.1	13.9	14.3
37	12.9	13.4	14.2	14.2	14.3	14.4	14.6
45	13.4	14.1	14.5	14.5	14.6	14.5	14.7

months may contain only a small amount of carotene. With such meal it is necessary to use from 2 to 4 g for analysis in order to obtain the desired accuracy. To determine the effect of sample size, a meal with a low carotene content was analyzed by the modified A.O.A.C. method and the Silker, Schrenk, and King procedure, using 2, 3, and 4 g samples. From Table 6 it will be seen that sample size had no effect on the results obtained. No difficulties were encountered with the method of Silker *et al.*, but serious bumping occurred when 3 and 4 g samples were analyzed by the modified A.O.A.C. method.

Pelleted meal. Frequently it is necessary to determine the carotene content of dehydrated alfalfa pellets. It was observed that samples prepared by grinding alfalfa pellets were much more dense than meals which had not been pelleted. It seemed possible that such samples would be more difficult to extract and that fineness of grinding might be important. To determine this, pellets were ground to pass 10, 20, 40, and 60 mesh screens and carotene was determined by the modified A.O.A.C. and the Silker, Schrenk, and King methods. The data (Table 7) show that fineness of grinding had little effect on the results obtained by the Silker, Schrenk, and King procedure. With the modified A.O.A.C. method, however, the best extraction was obtained when the sample was ground to 60 mesh. In this laboratory it previously had been found that 20 mesh grinding was adequate for unpelleted meals when the soaking technique was employed. By this procedure the more dense particles from pellets apparently are as easily extracted as unpelleted meals. However, Zscheile and Whitmore (4) reported that meals should be ground to 40 mesh

TABLE 6.—*Effect of sample size on carotene determination by the modified A.O.A.C. and the Silker, Schrenk, and King methods*

WEIGHT OF SAMPLE	MODIFIED A.O.A.C.	SILKER, SCHRENK, AND KING
g	*mg/100 g*	*mg/100 g*
2	2.8	3.0
3	2.8	3.0
4	2.7	3.0

when reflux extraction was employed. Thus, in some cases particle size may be partially responsible for the incomplete extraction encountered with the A.O.A.C. method.

TABLE 7.—*Effect of fineness of grinding on the determination of carotene in alfalfa pellets by the modified A.O.A.C. and by the Silker, Schrenk, and King methods*

GRIND	MODIFIED A.O.A.C.	SILKER, SCHRENK, AND KING
mesh	*mg/100 g*	*mg/100 g*
10	20.6	22.6
20	20.4	22.6
40	20.8	22.6
60	21.3	22.4

DISCUSSION

Incomplete extraction and elution of carotene limit the use of the A.O.A.C. method, especially with meals of high carotene content. The Silker, Schrenk, and King method also will be of limited use for control purposes when an analysis must be completed on the same day the sample is received. The modification of Thompson and Bickoff may eliminate the shortcomings of the A.O.A.C. method. However, it also employs overnight soaking to remove carotene from the sample and thus may be objectionable to control chemists. Another disadvantage of this modification is that it also employs 9 per cent acetone for elution. Non-carotene pigments undergo appreciable movement with this concentration of acetone, and column dimensions and volume of eluting agent must be controlled closely to avoid elution of non-carotene pigments while achieving quantitative removal of carotene. Failure to observe these requirements may result in poor agreement between laboratories. Further work is needed to evaluate such factors and to devise a procedure which will permit the analyst to adapt the conditions of analysis to the circumstances of time and type of sample.

SUMMARY

Lower values for carotene in alfalfa meal were obtained by the A.O.A.C. method of analysis than were obtained by the Silker, Schrenk, and King procedure. The lower results were due mainly to incomplete elution of the carotene from the adsorbent. The error was corrected by increasing the volume of eluting agent from 50 ml to 100 ml.

Percolation extraction was as efficient as reflux extraction, and was more useful because it eliminated bumping. When 3–4 g samples were used, bumping was especially pronounced with reflux extraction.

Temperature and time are important factors in the soaking method of carotene extraction. Maximum extraction at room temperature was obtained in 18 hours. At 37° and 45°C., extraction was complete in 12 hours.

At temperatures below 25°C. extraction was not complete after 48 hours of soaking.

Grinding to 20 mesh is satisfactory for preparing alfalfa pellets for analysis by the Silker, Schrenk, and King method. Grinding to 60 mesh gave the highest results with the modified A.O.A.C. method.

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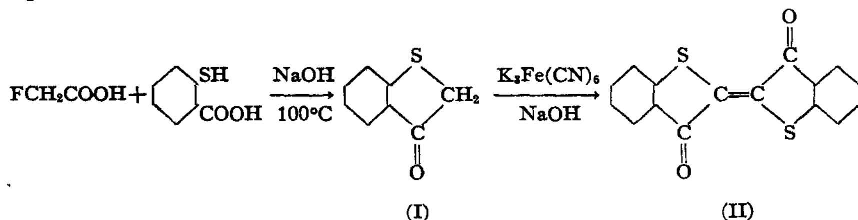
A NEW QUALITATIVE TEST FOR MONOFLUOROACETIC ACID*

By L. L. Ramsey and W. I. Patterson (Food & Drug Administration, Federal Security Agency, Washington 25, D. C.)

Positive analytical results for monofluoroacetic acid by the quantitative method of Ramsey and Clifford (1) are not conclusive evidence that this acid is actually present. Although specific for fluorine-containing organic acids this method also measures, in addition to monofluoroacetic acid, such acids as difluoroacetic and trifluoroacetic. Therefore in conjunction with the quantitative method a confirmatory test is essential. Monofluoroacetic acid can be detected by the qualitative indigo test for monochloroacetic acid (2), but for a positive test at least 3 mg of the fluoroacetic acid is usually required. Because of the extreme toxicity of monofluoroacetic acid, the detection of this poison at very low levels is of utmost importance. Thus, for the detection of small amounts of 1080 (sodium salt of the acid, used as a rodenticide) in foods which may have been accidentally contaminated, and for certain biological studies, the need for a specific qualitative test more sensitive than the indigo test is quite evident.

The test described here is based upon the formation of thioindigo (3), a water-insoluble red dye. The well-known reactions are indicated by the equation below:



* Presented at the Annual Meeting of the Association of Official Agricultural Chemists, held at Washington, D. C., October 1–3, 1951.

Since thioindigo (II) is an analog of indigo, a comparison of the two tests is of interest. In the indigo test the formation of indoxyl, the precursor of indigo, starting with anthranilic acid and monofluoroacetic acid, requires rather drastic treatment—an alkali fusion at 310°–320°C. In the thioindigo test the analogous formation of thioindoxyl (I) occurs readily when thiosalicyclic acid and monofluoroacetic acid are heated with alkali at 100°C. Although colorless indoxyl is readily converted to the blue indigo by oxidation with air, the reaction with thioindoxyl is slow and is achieved more quickly with potassium ferricyanide. The sensitivity of the thioindigo test is considerably greater than that of the indigo test; as little as 20 micrograms of pure monofluoroacetic acid, under optimum conditions, will give a positive thioindigo test. The test is also more rapid since the water bath step and the fusion step are not required.

Development of simple but specific tests for trace substances in biological materials is difficult, especially as they may be associated with closely related chemical compounds of widely different physiological activity. Thus, the task of separating such closely related compounds as monofluoroacetic and difluoroacetic acids in milligram quantities is tedious by whatever approach the chemist might use (4). However, both the indigo and thioindigo tests are quite specific; only alpha-halogen acetic acids will give a positive test. Negative results were obtained by the thioindigo test with difluoroacetic and trifluoroacetic acids and with alpha- and beta-chloropropionic acids. Thus, for the thioindigo test the separation of monofluoroacetic acid from other fluorine-containing acids is not required. However, excessive amounts of extraneous organic acids decrease the sensitivity of the test.

The thioindigo test described here is suitable for foods and biological materials containing 1080 at a level as low as 1 p.p.m. if an ether extract containing 0.5 mg or more of the compound can be obtained.

METHOD

In general, follow the directions of Ramsey and Clifford (1) for sample preparation and ether extraction. If convenient, extract a sample of sufficient size to obtain 2–10 mg of 1080. With very low levels of 1080, *e.g.*, 1–5 p.p.m., extract a sample of sufficient size to obtain at least 0.5 mg of 1080. Separate the ether extract from any aqueous sludge which may have been carried over in the extraction, add ca 5 g anhydrous sodium sulfate and 1 g decolorizing carbon per 100 ml ether, and shake vigorously. (Nuchar C 190N appears to adsorb less 1080 than some other carbons.) Allow to stand ca 15 min. at room temperature with occasional shaking, and decant thru a fluted filter into a separatory funnel. Add ca 25 ml water and sufficient NaOH soln (ca *N*) to make the aqueous layer alkaline after vigorous shaking (outside test paper). Draw off the aqueous layer into a 125 ml Erlenmeyer flask and aerate to remove the dissolved ether. Using a *p*H test paper and ca *N* solns of H_2SO_4 and NaOH, adjust to *p*H 5–7. Add 1 g carbon and place on the steam bath for 15 min. Cool under the tap and filter thru a fluted filter into a test tube ca 25 mm×150 mm in size. Add 1 ml of thiosalicylic acid soln (300 mg thiosalicylic acid[1] + 2 ml *N*

[1] Eastman's technical grade was used.

NaOH + 18 ml H_2O), 2 drops NaOH 1 + 1, and mix. Concentrate the soln to a small volume by placing on a steam bath under a gentle current of air. Completely dry the residue in a drying oven at 130°C. or, if time is not a factor, in a 100°C. oven. (When convenient, overnight drying is quite satisfactory, with or without prior concentration of the soln.) Dissolve the *thoroly* dry residue in 2–3 ml water, add 1 ml of potassium ferricyanide soln (20 mg/ml), and mix. The appearance of a red color is a positive test for 1080. A red precipitate forms at once when 1 mg or more of 1080 is present, or upon standing when only a fraction of a mg is present.

Employ chromatographic instead of carbon purification in the following instances:

1. With pineapple juice when less than 2 mg of 1080 can be extracted.
2. With grape juice even when 2 mg or more of 1080 can be conveniently extracted, and
3. With any food or material when 1080 is strongly suspected and a negative test is obtained using the carbon purification technic.

For chromatographic purification, follow the procedure of Ramsey (4) for separating the 1080 from other acids. Discard the forerun which may contain acetic acid and other extraneous materials. Collect a percolate fraction large enough to contain all of the 1080 as determined by a preliminary run. Extract the fluoroacetic acid from the chromatographic percolate with 25 ml water and sufficient alkali to cause the aqueous layer to retain its alkalinity after vigorous shaking (outside test paper). Draw off the organic layer and discard. Draw off the aqueous layer into a 125 ml Erlenmeyer flask and aerate to remove the chloroform. Pour the soln into a test tube and continue as in the procedure with carbon purification, beginning with "Add 1 ml of thiosalicylic acid soln . . . ."

For monochloroacetic acid follow the directions above with this exception: use only the carbon purification technic.

RESULTS AND DISCUSSION

The results of applying the thioindigo test to biological tissue and a variety of foods are tabulated in Table 1. The fruit juices were included as test materials because with these maximum interference from extraneous coloring matter could be expected. Certain materials, presumably acidic, which may be present in the alkaline extract of the 1080 from the ether extract of the sample, usually decrease the sensitivity of the test. With small quantities of 1080 this interference is sometimes quite marked. For example, when 350 ml of pineapple juice containing 6 p.p.m. of added 1080 was extracted to isolate about 2 mg of 1080, a positive test was obtained using a single carbon treatment; but when a 350 ml sample containing 1.5 p.p.m. of added 1080 was extracted to isolate about 0.5 mg of 1080, a negative test was obtained even with two carbon treatments. When chromatographically purified, the latter sample gave a positive test.

With a given amount of monofluoroacetic acid, a procedure which gives a positive test on one food may be negative on another food. For example, a positive test was obtained when an ether extract of H_2SO_4-acidified grapefruit juice containing 2 mg of 1080 was treated with decolorizing carbon. On the other hand, negative tests were obtained when the same purification procedure was applied to grape juice and raspberry juice. In

TABLE 1.—*Thioindigo test for 1080 employing three purification procedures: (1) carbon treatment of the ether extract only, (2) double carbon treatment as described in the method, and (3) partition chromatography as described in the method. Positive test, +; negative test, 0*

PRODUCT	1080* ADDED	1080 EXTRACTED	RESULTS		
			SINGLE CARBON TREATMENT	DOUBLE CARBON TREATMENT	CHROMA-TOGRAPHY
	p.p.m.	*mg*			
Flour	20	1.0	+		
	10	0.5			+
Hamburger	10	0.5		+	
Rat Carcass	†	0.8			+
Apple Juice	6	2.0	+		
	1.5	0.5			+
Beer	6	2.0	+		
	1.5	0.5			+
Blackberry Juice	6	2.0		+	
	1.5	0.5		+	
Grape Juice	6	2.0	0	0	
	1.5	0.5			+
Grapefruit Juice	6	2.0	+		
	1.5	0.5			+
Lemon Juice	6	2.0		+	
	1.5	0.5			+
Orange Juice	6	2.0	+		
	1.5	0.5	0		+
Pineapple Juice	6	2.0	+		
	1.5	0.5		0	+
Raspberry Juice	6	2.0	0	+	
	1.5	0.5		+	

* Commercial 1080 was used in this work.
† See Table 2 in *J. Pharm. Exptl. Therapeutics*, **99**, 433 (1950). Rats were fed 3.3 mg 1080/kg, and died within 24 hrs. Analyzed 12/12/49 and found 1.8 p.p.m. of 1080. Extracted sufficient tissue to obtain ca 0.8 mg and tested qualitatively 6/28/51. Carcasses were preserved during the interim by freezing.

order to obtain a positive test with the grape juice a chromatographic purification was required, but with the raspberry juice two carbon treatments sufficed, one on the ether extract and one on the aqueous alkaline extract of the ether. The interfering material in grape juice which is

removed by chromatography but not by carbon treatment has not been identified. It is not a pigment since all except a trace of pigment can be removed by the double carbon treatment.

Obviously when a positive qualitative test for 1080 is obtained, an adequate positive test for fluorine must also be obtained in order to be sure that monochloroacetic acid is not responsible for the test.

Spectrophotometric absorption curves* for the colored product obtained from commercial 1080 and from a sample of flour to which 1080 had been added indicated that the dye formed in both cases was thioindigo.

The thioindigo test described here is equally suitable for monochloroacetic acid, except that only the carbon purification technic is applicable. Very little purification of monochloroacetic acid is achieved by the chromatographic method. Amounts of monochloroacetic acid in the range 0.5 to 2.0 mg extracted from flour, hamburger, orange juice, pineapple juice, and raspberry juice all gave a positive test.

SUMMARY

A new, sensitive, and highly specific qualitative test for 1080 in foods and biological tissue is described. The test is based on the formation of thioindigo, using thiosalicyclic acid as the reagent to obtain thioindoxyl, which is then oxidized to thioindigo with potassium ferricyanide. The test is also applicable to monochloroacetic acid in foods and biological tissue.

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THE IDENTIFICATION OF AZO DYES BY SPECTROPHOTOMETRIC IDENTIFICATION OF THEIR REDUCTION PRODUCTS†

I. COMPOUNDS WHICH GIVE SIMPLE AMINES OR DIAMINES ON REDUCTION

By J. H. Jones and L. S. Harrow (Division of Cosmetics, Food and Drug Administration, Federal Security Agency, Washington 25, D. C.)

The identification of azo compounds by identification of their reduction products is an established technique. Koch (1, 2, 3, 4) and others (5, 6) have published procedures for the reduction of several types of azo dyes and for the identification of the reduction products. Koch identifies the

* Courtesy of Lee Harrow, Division of Cosmetics, Food & Drug Administration, Washington, D.C.
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reduction products by the preparation of suitable derivatives and must, therefore, use relatively large samples of dye. In this laboratory, it is frequently necessary to identify a dye when only a few milligrams of material are available. We have therefore developed procedures for the spectrophotometric identification of the reduction products obtained from small samples of dyes. Spectrophotometric identification of aromatic compounds is at least as conclusive as is identification by the classical procedure of preparation and identification of suitable derivatives.

In our hands, the identification of azo colors by identification of their reduction products is used to supplement, rather than replace, identification based on the spectra of the colors themselves. Most colors can be identified from the absorption spectra of their solutions; however, the spectra of some closely related compounds are so similar that positive identification of an unknown in this manner is difficult. There are also cases in which the curve of an unknown color does not correspond with any of the curves available for comparison. In such situations, identification of the reduction products is of considerable value in establishing the identity of unknown materials.

When an azo color is reduced, there is obtained the amine originally diazotized and used in the coupling reaction (or a reduction product of this amine) as well as an amino derivative of the compound to which the diazo component was coupled. This paper deals with the identification of those reduction products that are simple amines or diamines (*i.e.*, amines that contain no other reactive or solubilizing groups). These amines can be extracted from aqueous solution with ethyl ether; some of them readily distill with steam.

(The identification of other types of reduction products, such as amino-naphthols, amino-sulfonic acids, and other water-soluble compounds, will be discussed in another report.)

Since, in this procedure, the amines are identified spectrophotometrically, it is necessary to have available for comparison the absorption spectra of authentic samples of the amines. Authentic samples of many of these are available in most laboratories. Unusual amines, such as some of the diamines, can be readily prepared.

In many cases, an organic compound can be identified by the general shape of the absorption curves and the location of the absorption peaks. Absorbancy ratios are often useful in distinguishing between compounds that have similar curves. For example, although the curves of the two toluidines in acid solution are quite similar, the ratio of the absorbancy at 260 mμ to the absorbancy at 270 mμ is different for the two pure compounds. Other absorbancy ratios, such as the ratio for the two peaks in alkaline solution, or the ratio of the major peak in alkali to the peak in acid solution, will be of aid.

PROCEDURE

APPARATUS

A spectrophotometer capable of isolating a 5 mμ wave length band in the region 220–400 mμ.

REAGENTS

Sodium hydrosulfite.—c.p.
Titanium trichloride.—ca. 0.1 *N*.

REDUCTION

(a) *Water soluble colors.* Place 5–10 mg of color in a small beaker and dissolve in a minimum amount of water. Heat the soln on a steam bath and add 1 mg por-

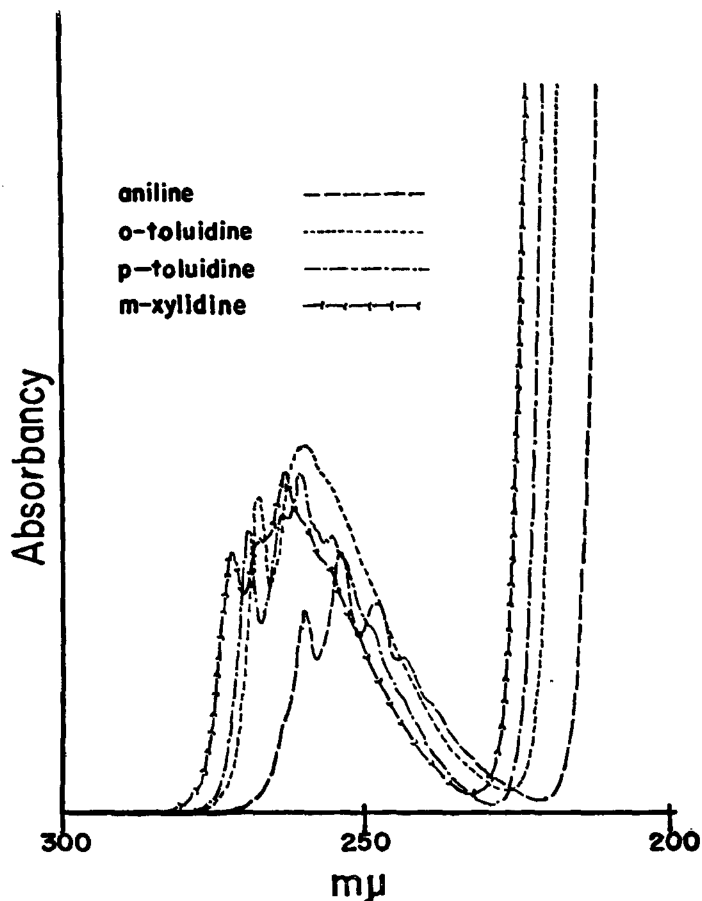

FIG. 1.—Absorption curves of several aromatic amines (200 mg per liter in 0.1 *N* HCl).

tions of $Na_2S_2O_4$ until all the color has disappeared, or until there is no further noticeable change of the color on heating for an additional 15 min.

(b) *Oil-soluble colors.* Dissolve 5–10 mg of the color in a small amount of hot alcohol, add 2 ml of 10% (w/v) NaOH soln and reduce with $TiCl_3$ soln under a stream of CO_2. Add 2 mg of $Na_2S_2O_4$ to stabilize the reduction products.

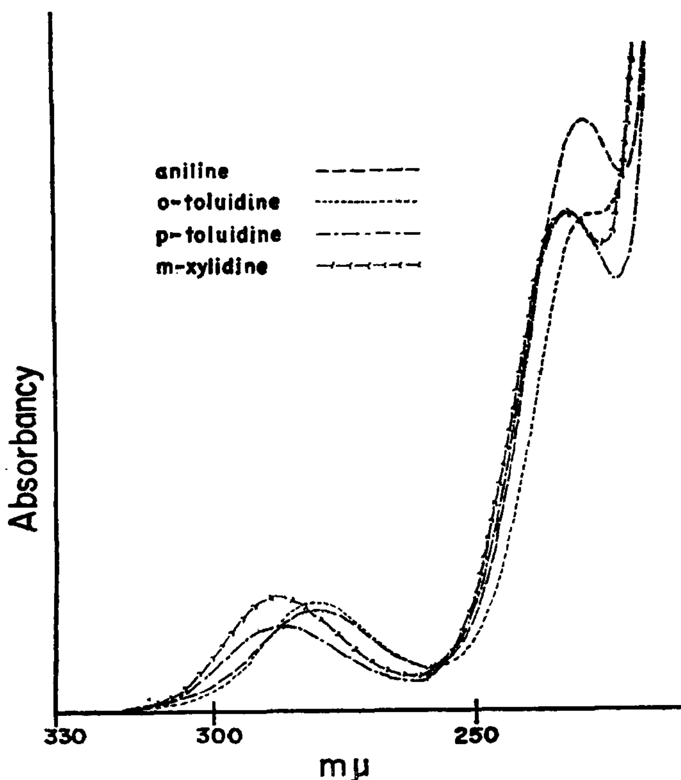

FIG. 2.—Absorption curves of several aromatic amines (10 mg per liter in 0.1 *N* NaOH).

DISTILLATION OF VOLATILE AMINES

When reduction is complete, transfer the soln to a 300-ml round-bottom flask, add ca 200 ml of H_2O, connect to a suitable distilling apparatus, and distill into a container in which 1 ml of conc. HCl and 10 ml of water have been placed. After ca 150 ml has been collected, transfer the distillate to a separatory funnel, make alkaline (litmus) with NaOH soln, and extract with two 40-ml portions of ether. Wash the combined ether extracts once with 20 ml of water and then extract with three 20-ml portions of ca 0.2 *N* HCl. Warm the combined acid extracts on a steam bath for 15–30 min to expel the last traces of ether. Determine the ultraviolet absorption spectra of the recovered amine in this dilute HCl soln and in ca 0.1 *N* NaOH (add

a roughly calculated excess of NaOH soln), and compare with the spectra obtained from known samples of amines that might be present. (Volumes are not critical as absorption curves are considered mainly in their qualitative aspect. An acidity of 0.1 *N* was found ample to develop fully the acid phase of all amines examined and a corresponding alkalinity is sufficient for the measurement of the alkaline phase.)

EXTRACTION OF NON-VOLATILE AMINES

Cool the reduced soln (or the residue from the steam distillation), make defi-

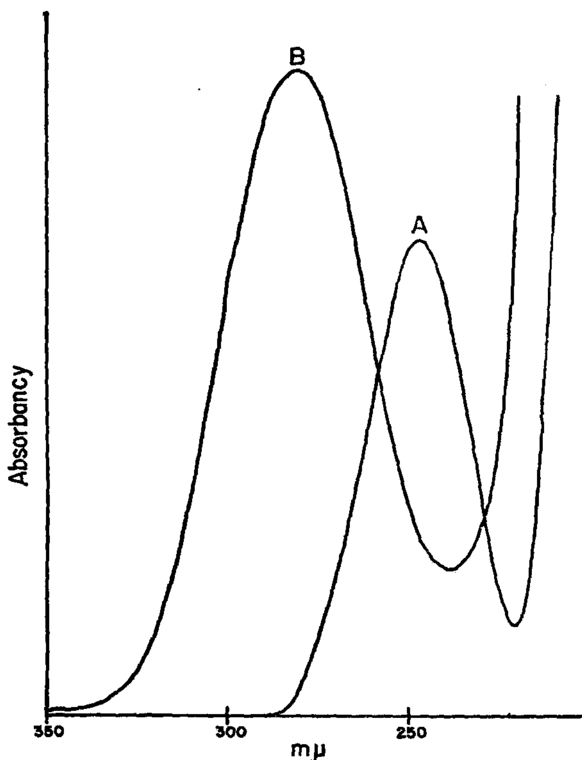

FIG. 3.—Absorption curves of benzidine (10 mg per liter). Curve A.—in 0.1 *N* HCl. Curve B.—in 0.1 *N* NaOH.

nitely alkaline with NaOH soln and continue as directed above, beginning, "extract with two 40-ml portions of ether . . . ."

DISCUSSION

The procedure may be used to identify the colors used in a variety of products. As with most methods for the identification of coal-tar colors, application of the reduction procedure must be preceded by a reasonably

good separation of the unknown color from other materials in the samples to be analyzed. Since it would obviously be impossible to describe all applications of the proposed procedure, a few typical examples are discussed.

A problem that frequently arises is that of determining whether an unknown color was prepared from aniline or from one of its homologues. In many cases, the substitution of toluidine or xylidine for aniline in an

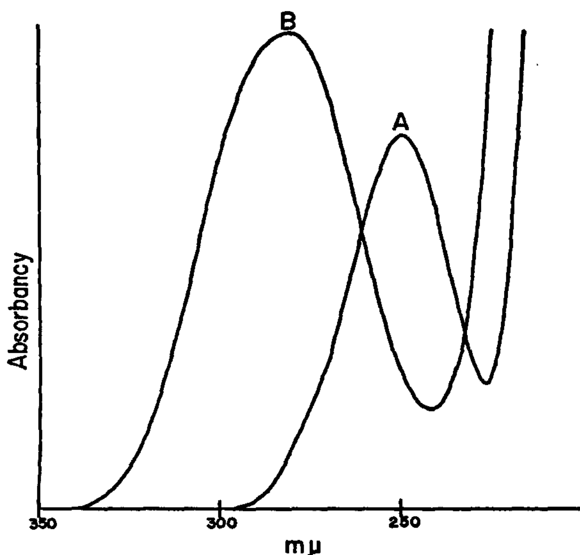

FIG. 4.—Absorption curves of *o*-tolidine (10 mg per liter). Curve A.—in 0.1 *N* HCl. Curve B.—in 0.1 *N* NaOH.

azo color will have little effect on the spectra of the color. If, however, the compound is reduced and the volatile amine isolated by steam distillation, differentiation between the homologues is relatively easy.

The ultraviolet absorption curves of aniline, *o*-toluidine, *p*-toluidine, and *m*-xylidine are shown in Figures 1 and 2. As would be expected, the absorbancy curves for these four amines are quite similar in general appearance. The curves in acid solution, however, show readily perceptible differences. The curves in alkaline solution can be used to confirm the identification made from the curves in acid solution.

As indicated by the curves in Figure 1, in 0.1 *N* hydrochloric acid solution, a concentration of about 100 mg per liter of these amines is needed to obtain a good curve. If only a small amount of the unknown material is available, the amount of acid used in the extraction of the ether layer may be reduced to two 10 ml portions; a recovery of as little

as 2 mg of the amines will be adequate for identification. In alkaline solution, a concentration of 10 mg of the amine per liter will give a satisfactory curve.

The xylidine curve shown in Figures 1 and 2 is that of *m*-xylidine

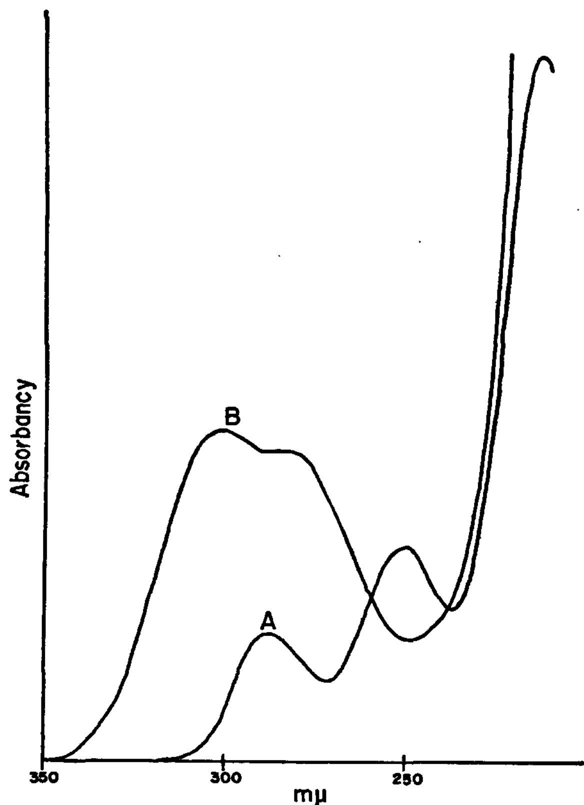

FIG. 5.—Absorption curves of dianisidine (10 mg per liter). Curve A.—in 0.1 *N* HCl. Curve B.—in 0.1 *N* NaOH.

(2,4-dimethylaniline). Commercial xylidine is usually a mixture consisting chiefly of 2,4- and 2,5-dimethylaniline. The curve of this mixture is readily distinguished from the curves of aniline or those of the toluidines.

A large number of azo colors are prepared by coupling benzidine, tolidine, or dianisidine with naphthols, naphthylamines, or their derivatives. These amines do not steam distill readily but are recovered by extraction with ether from basic aqueous solution. The absorption curves of these amines are shown in Figures 3, 4, and 5. Obviously, they can be readily differentiated. Since a concentration of 10 mg per liter is ade-

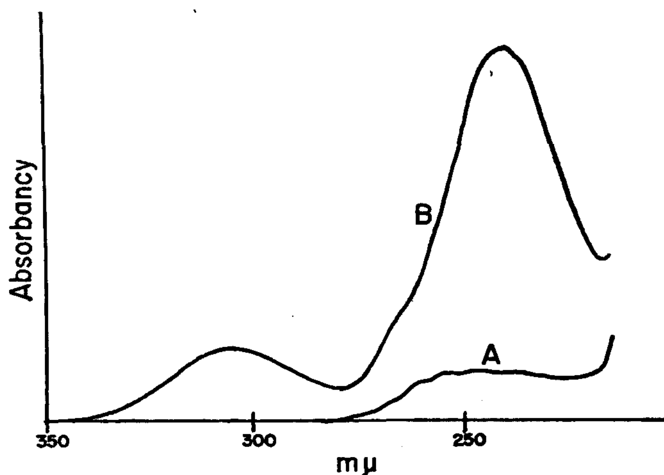

FIG. 6.—Absorption curves of p-phenylenediamine. Curve A.—50 mg per liter in 0.1 *N* HCl. Curve B—10 mg per liter in 0.1 *N* NaOH.

quate for spectrophotometric identification of these diamines, less than 1 mg of the amine is sufficient for identification.

Colors such as bis-azo compounds derived from amino-azo-benzene, amino-azo-toluene, etc., give both a monamine and a diamine on reduction. The monamine can be recovered by steam distillation and the diamine subsequently recovered by extraction with ether from the non-

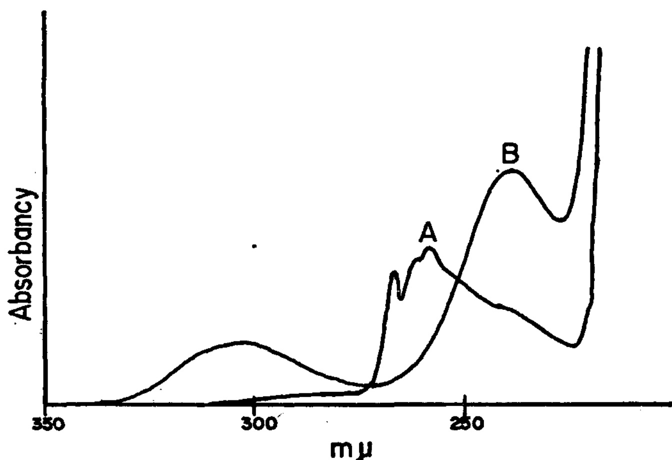

FIG. 7.—Absorption curves of 2,5-diaminotoluene. Curve A.—200 mg per liter in 0.1 *N* HCl. Curve B.—10 mg per liter in 0.1 *N* NaOH.

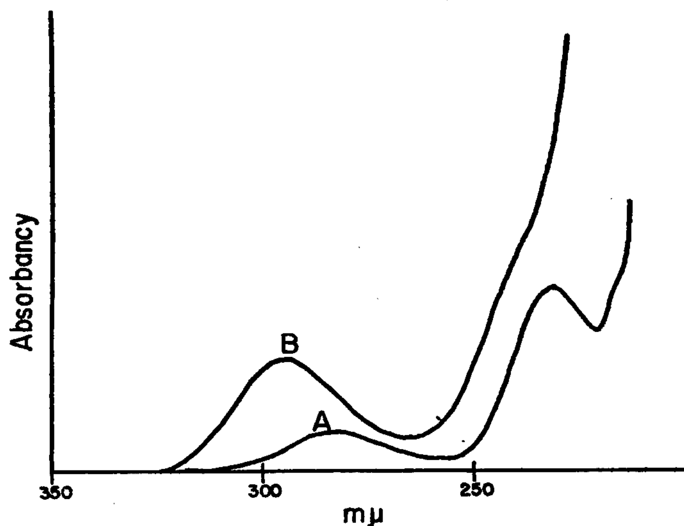

FIG. 8.—Absorption curves for 2,4-diaminotoluene. Curve A.—10 mg per liter in 0.1 *N* HCl. Curve B.—10 mg per liter in 0.1 *N* NaOH.

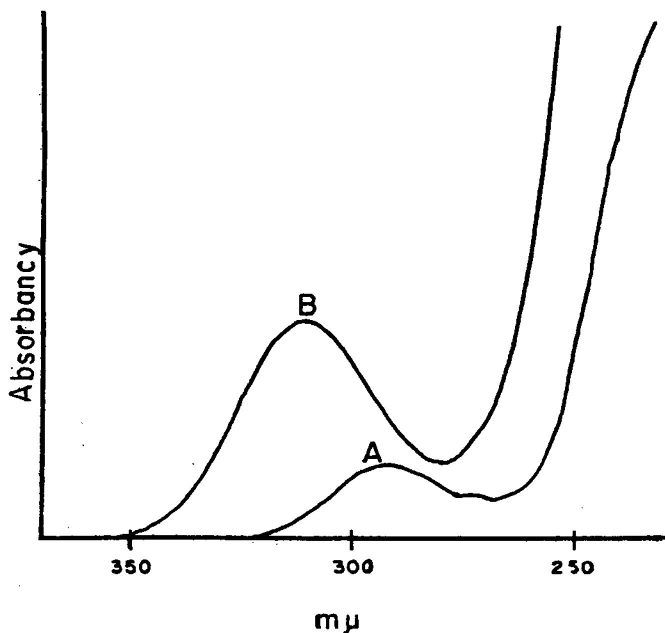

FIG. 9.—Absorption curves for 2-chloro-1,4-phenylenediamine. Curve A.—100 mg per liter in 0.1 *N* HCl. Curve B.—10 mg per liter in 0.1 *N* NaOH.

volatile fraction. Azo colors prepared from nitro-amines give the corresponding diamines on reduction. Many of these diamines can be recovered by extraction with ether. The curves of several diamines isolated by the

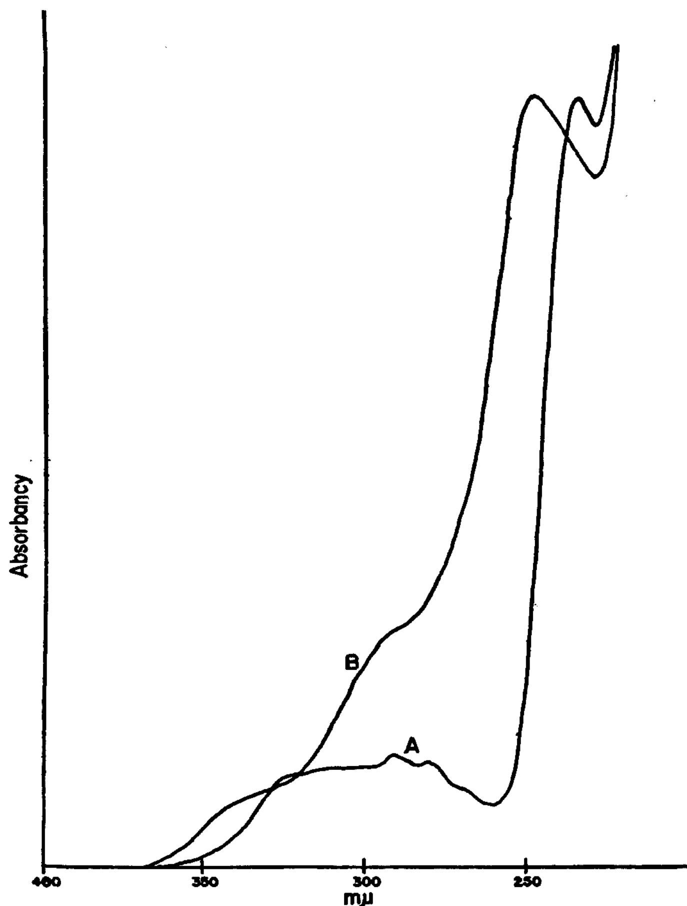

FIG. 10.—Absorption curves for 1,2-diaminonaphthalene. Curve A.—10 mg per liter in 0.1 *N* HCl. Curve B.—10 mg per liter in 0.1 *N* NaOH.

proposed procedure are shown in Figures 6, 7, 8, 9, and 10. In most cases, 1 mg of the diamine is sufficient for identification.

APPLICATIONS

Table 1 lists a number of azo colors from which one or more reduction products have been isolated and identified by the proposed procedure.

TABLE 1.—*Separation and identification of amines from azo compounds*

COLOR	AMINE SEPARATED	METHOD OF SEPARATION
FD&C Yellow No. 3	Aniline	Steam distillation from alkaline solution
	1,2-diamino-naphthalene	Et_2O extraction from alkaline solution
FD&C Yellow No. 4	*o*-Toluidine	Steam distillation
	1,2-diamino-naphthalene	Et_2O extraction
FD&C Red No. 1	Pseudocumidine	Steam distillation
D&C Red No. 5	Xylidine	Steam distillation
D&C Red No. 17	Aniline	Steam distillation
	p-Phenylenediamine	Et_2O extraction
D&C Red No. 18	*m*-Xylidine	Steam distillation
	1,4-dimethyl-3,6-diaminobenzene	Et_2O extraction
D&C Red No. 31	Aniline	Steam distillation
FD&C Red No. 32	Xylidine	Steam distillation
D&C Red No. 33	Aniline	Steam distillation
D&C Red No. 35	3,4-diaminotoluene	Et_2O extraction
D&C Red No. 36	2-Chloro-p-phenylenediamine	Et_2O extraction
D&C Red No. 38	3,4-diaminotoluene	Et_2O extraction
FD&C Orange No. 2	*o*-Toluidine	Steam distillation
D&C Orange No. 3	Aniline	Steam distillation
D&C Brown No. 1	Xylidine	Steam distillation
D&C Black No. 1	Aniline	Steam distillation
	p-Phenylenediamine	Et_2O extraction
Ext. D&C Yellow No. 3	Aniline	Steam distillation
Ext. D&C Yellow No. 5	3,4-diaminotoluene	Et_2O extraction
Ext. D&C Red No. 9	Naphthylamine	Et_2O extraction
Ext. D&C Red No. 11	Aniline	Steam distillation
Ext. D&C Red No. 13	Aniline	Steam distillation
	p-Phenylenediamine	Et_2O extraction
1-Phenylazo-2-naphthol C.I. 24	Aniline	Steam distillation
1-(4-Methylphenylazo)-2-naphthol	*p*-Toluidine	Steam distillation
C.I. 518	*o*-Anisidine	Et_2O extraction
C.I. 520	*o*-Anisidine	Et_2O extraction
C.I. 477 (Trypan Blue)	Tolidine	Et_2O extraction
C.I. 370 (Congo Red)	Benzidine	Et_2O extraction

Some problems satisfactorily solved through application of the procedure described have been:

1. An oil-soluble dye was found to have an absorption spectrum which could be that of D&C Red No. 17, D&C Red No. 18, or C.I. 258. The volatile amine obtained on reduction of the dye was identified spectro-

photometrically as *o*-toluidine. The dye in question was therefore C.I. 258.

2. Two colors, each of which was labeled with the same name, were found to have different absorption spectra. From one of the samples, a volatile amine was separated and identified as aniline by the proposed procedure. No volatile amine was obtained from the other sample. Further analysis of the second color proved that anthranilic acid instead of aniline had been used in its preparation.

3. A blue color, isolated in small amounts from a biological stain, could not be identified with available standard samples. The spectrophotometric curve indicated that the color might be related to Trypan Blue, C.I. 477. Identification of the reduction products showed that the color was prepared from dianisidine. With this much information, authentic samples of dyes containing dianisidine were obtained. The absorption spectrum of the unknown compound was found to be identical with that of C.I. 518.

SUMMARY

Procedures have been described for the separation and spectrophotometric identification of simple amines and diamines obtained by reduction of azo compounds. The procedures are applicable to samples containing only a few milligrams of azo color.

Examples of the practical application of the procedures are given.

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A COMPARISON OF VARIOUS ANALYTICAL METHODS ON TOBACCO CONTAINING NON-NICOTINE ALKALOIDS*

By R. N. JEFFREY[1]

INTRODUCTION

Markwood and Barthel (1) first reported that Maryland Medium Broadleaf and the Cash variety of flue-cured tobacco contained considerable amounts of nornicotine, and additional information has been obtained since of the frequent occurrence of this alkaloid. However, the published evidence seems to indicate that nicotine has been the predominant alkaloid in most commercial tobacco in this country up to the present time. Recently, the interest in using wild species of *Nicotiana* as sources of disease resistance in breeding has been increasing. Smith and Smith (2) have stated "In crosses between *Nicotiana tabacum*, which contains mostly nicotine, and species whose alkaloidal complex was made up largely or entirely of nornicotine, the hybrids contained mainly nornicotine together with small amounts of nicotine."

The results presented here indicate that the conventional methods of nicotine analysis give seriously conflicting results when applied to samples in which the alkaloid is not predominantly nicotine. It is not possible to determine by any one of the extensively-used quantitative methods, taken alone, whether the alkaloid present in a given sample is nicotine or not. It is shown that, with certain samples, some methods of "nicotine" analysis give results two and one-half times as high as with other methods. It seems necessary to call attention at this time to the necessity for caution in accepting results of usual methods as indicating either the true nicotine or total alkaloid content, particularly when applied to material of mixed heritage. However, a completely satisfactory method has not been devised up to the present time.

METHODS

The tobacco samples used in this study were derived chiefly from the F_2 generation of crosses between type 31-V low-nicotine burley (3) and flue-cured (bright or Virginia) tobacco. Samples of both parent lines and of low-nicotine cigar tobacco from seed obtained from Germany and similar to the low-nicotine ancestor of the low-nicotine burley were also included. The tobacco was grown at Beltsville, Md., in 1949 and was not topped or suckered until just before harvest. After air curing, it was stripped, dried, ground and stored in screw topped glass jars and analyzed in the spring of 1950. Much of it was re-analyzed in the spring of 1951

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after continued storage in an air-dry condition in the dark at room temperatures.

The samples were analyzed in several ways as follows:

1. By steam distillation in the Griffith-Jeffrey apparatus (4) with 1 ml of 30% sodium hydroxide and 1 g of sodium chloride into 2 ml of 1+4 hydrochloride acid. "Nicotine" was determined by the ultra-violet method of Willits and co-authors (5).

2. By steam distillation as above, followed by making up to 250 ml, and precipitation of the alkaloids from one 100 ml aliquot of distillate with 1 ml of 12% silicotungstic acid. This material was then heated on the steam bath to dissolve most of the precipitate, cooled slowly to room temperature, and then placed in the refrigerator at 7°C. overnight to obtain the maximum yield of filterable precipitate according to Avens and Pearce (6). The precipitates were filtered cold through Selas crucibles, heated 1 hour at 650°C., cooled and weighed.

3. Another 100 ml aliquot of the distillate obtained under *2* was evaporated nearly to dryness and then treated with acetic acid and sodium nitrite according to the method of Bowen and Barthel (7) and steam distilled using magnesium oxide (8).

4. By steam distillation using excess magnesium oxide followed by determination in the spectrophotometer.

5. By the method of Garner (9) as later modified (10). This involves extraction into petroleum ether from the strongly basic tobacco sample and from the organic solvent into a standard acid water solution. Excess acid is determined by a back-titration.

The results were calculated in the manner given in the papers concerned except that the results of steam distillation from sodium hydroxide and sodium chloride were calculated both on the assumption that all the silicotungstic acid precipitate was nicotine, as in the Official Method (11) and by the sum of the nicotine and nornicotine results according to Bowen and Barthel (7).

EXPERIMENTAL RESULTS

The results obtained in 1950 are shown in Table 1. These samples have been segregated according to the results of the Bowen and Barthel analyses into three classes:

a. Those samples in which 85–100% of the total alkaloid appears to be nicotine and in which the total alkaloid content is over 0.20%. These are referred to as normal type samples, and appear to be similar in alkaloid composition to the majority of the samples of commercial tobacco currently used.

b. Those samples in which the values obtained for nornicotine by this method are equal to, or higher than, those obtained for nicotine and in which the total alkaloid content is over 0.20%. These are referred to as mixed alkaloid type samples.

TABLE 1.—*Alkaloids in tobacco by different methods of analysis.* Results expressed as percentage of air-dry weight

Distillation with NaOH+NaCl		Bowen-Barthel method			Distillation with MgO	Garner titration	Ratio of nicotine to total alkaloid
Ultraviolet absorption	Silicotungstic acid precipitation	Nicotine	Nor-nicotine	Total	Ultraviolet absorption		
Normal Type Tobacco							
1.26	1.24	1.21	0.03	1.24	1.23	1.25	0.98*
1.22	1.20	1.14	0.06	1.20	1.19	1.26	0.95*
1.31	1.27	1.20	0.07	1.27	1.27	1.32	0.95*
1.08	1.04	0.94	0.06	1.00	1.02	1.06	0.94
0.66	0.65	0.60	0.04	0.64	0.62	0.72	0.94
1.56	1.47	1.36	0.10	1.46	1.46	1.57	0.93*
1.60	1.59	1.47	0.11	1.58	1.52	1.58	0.93*
1.73	1.79	1.61	0.17	1.78	1.63	1.68	0.91*
1.92	1.90	1.69	0.19	1.88	1.80	1.89	0.90*
1.81	1.77	1.56	0.19	1.75	1.72	1.82	0.89*
1.70	1.68	1.47	0.19	1.66	1.58	1.60	0.89*
1.82	1.80	1.54	0.23	1.77	1.77	1.86	0.87*
1.56	1.60	1.38	0.20	1.58	1.52	1.60	0.87*
1.56	1.59	1.35	0.23	1.58	1.46	1.62	0.85*
Mixed Alkaloid Type							
1.48	1.36	0.64	0.65	1.29	0.99	0.99	0.50
2.34	2.32	1.02	1.18	2.20	1.66	1.74	0.46
2.12	2.00	0.78	1.11	1.89	1.26	1.35	0.41
0.91	0.91	0.33	0.54	0.87	0.86	0.86	0.38
0.42	0.37	0.11	0.23	0.34	0.28	0.26	0.32
1.91	1.86	0.55	1.20	1.75	1.20	1.20	0.31*
1.28	0.97	0.23	0.68	0.91	0.53	0.63	0.25*
0.48	0.40	0.09	0.28	0.37	0.36	0.90	0.24
0.94	0.84	0.18	0.61	0.79	0.48	0.50	0.23*
2.07	1.64	0.35	1.19	1.53	1.10	0.97	0.23*
0.97	0.70	0.14	0.52	0.66	0.54	0.50	0.21*
1.19	0.91	0.17	0.68	0.85	0.54	0.64	0.20*
0.33	0.30	0.05	0.22	0.27	0.17	0.15	0.19
0.69	0.33	0.06	0.26	0.32	0.34	0.28	0.19
1.37	1.13	0.20	0.85	1.05	0.58	0.55	0.19*
0.34	0.40	0.05	0.32	0.37	0.21	0.24	0.14
1.32	0.86	0.10	0.70	0.80	0.50	0.42	0.13*
1.60	1.21	0.13	0.98	1.11	0.76	0.66	0.12*
0.64	0.73	0.05	0.62	0.67	0.34	0.42	0.07*
2.01	1.59	0.07	1.39	1.46	0.91	0.76	0.05*
1.95	1.51	0.06	1.33	1.39	0.96	0.72	0.04*
Low Alkaloid Type							
0.09	0.09	0.05	0.03	0.08	0.10	0.10	0.63
0.12	0.11	0.06	0.05	0.11	0.09	0.06	0.55
0.19	0.15	0.07	0.07	0.14	0.15	0.13	0.50
0.10	0.11	0.05	0.05	0.10	0.09	0.12	0.50
0.14	0.12	0.05	0.06	0.11	0.10	0.12	0.45
0.12	0.12	0.03	0.08	0.11	0.09	0.10	0.27
0.29	0.20	0.03	0.15	0.18	0.18	0.18	0.17

* Reanalyzed one year later.

c. Those samples in which the total alkaloid content is less than 0.20% are referred to as low alkaloid samples. Since the results obtained for nicotine and nornicotine are so low as undoubtedly to approach the limit of accuracy of the method, any attempt to segregate these samples on the basis of the proportion of their alkaloids would lead to classifications of doubtful reliability. With the exception of this last group, no samples were found which had an intermediate proportion (50–85%) of the alkaloid present as nicotine. This absence of samples of intermediate proportion was observed also by Valleau (3). This point is being investigated further on account of its genetic implications.

A study of Table 1 will show that fairly satisfactory agreement was obtained between the five methods on the samples of the first or normal type. Because of the small quantities present, we cannot be certain of any significant differences in the last or low alkaloid type samples. On the other hand, it is seen that values obtained by the different methods on the mixed alkaloid type samples do not approach satisfactory agreement. This extends the observations of Bowen (12), who found that widely different results were obtained by distillation from weak NaOH, and from strong NaOH+NaCl, particularly on samples which contained a considerable proportion of non-nicotine alkaloids. The relations between results obtained by the different methods on the mixed alkaloid samples are discussed below.

1. Ultraviolet vs. silicotungstic acid methods.—Of the 21 mixed alkaloid samples, the results obtained by the ultraviolet method are higher in 18 cases than the results obtained by silicotungstic acid precipitation, slightly lower in 2 cases, and equal in one case. In the case of 33 other mixed alkaloid samples not separately reported here (the two methods were applied to aliquots of the same steam distillate) the ultraviolet results were 0.01 to 0.27% higher in 31 cases (average 0.12% higher), 0.02% lower in one case, and equal in one case. This shows conclusively that higher results are generally obtained by the ultraviolet method with this type of sample. Willits and co-workers (5) pointed out that an error exists in their method when nornicotine is present, but a similar error, equal to about 10 per cent of the amount of the nornicotine, also results from the use of the nicotine silicotungstate factor, as is done in the Official Method. This difference is shown in Table 1 (*cf.* the second and fifth columns). However, the difference between the first two columns is, on the average, about two to three times as large as the difference between the second and fifth columns.

The following investigations were undertaken in an attempt to find the cause of this difference:

a. Extinction coefficients.—The specific extinction coefficients of nicotine and nornicotine given by Willets *et al.* have been essentially confirmed in this laboratory, so this factor does not appear to be a significant cause of the difference.

b. Solubility of nornicotine silicotungstate.—The solubility of nornicotine silicotungstate was tested by precipitating 2.04 mg of nornicotine with the same amount of silicotungstic acid and hydrochloric acid as in the analytical procedure, but in total volumes of 10, 100, and 1000 ml. The precipitates were heated and then cooled in the refrigerator overnight, and the solubility was calculated from the difference in weights of the precipitates as obtained from the different volumes. The solubility of the nornicotine silicotungstate was found to be about 115 mg per liter at 7°C., compared to a similarly determined value of about 11 mg per liter for nicotine silicotungstate. Avens and Pearce (6) reported values which would correspond to 12 mg of nicotine silicotungstate per liter at 0°C. and 19.5 mg at 25°C. Spies (13) reports about 7.0 mg at 25°C., and the corresponding acid concentration. This value was obtained by determining the equilibrium solubility, whereas, the preceding ones were determined by filtering the precipitate and washing as in regular analytical work. No previous values on nornicotine silicotungstate have been found in the literature. Thus, it would appear that nornicotine silicotungstate was about ten times as soluble as nicotine silicotungstate at low temperatures. The solubility at elevated temperatures was estimated by observing the temperatures at which the Tyndall cone disappeared on heating. Values of approximately 193 and 386 mg per liter were found at 51° and 75°C., respectively, for nornicotine silicotungstate, as compared to 170 mg per liter at 75°C. for nicotine silicotungstate. These results indicate that any silicotungstate precipitation method would give low values on mixed alkaloid samples and explain at least in part the lower values obtained by this method when compared to those by ultraviolet absorption. The error in the Official Method due to the use of too high a factor for nornicotine is in the opposite direction, so these errors tend to compensate each other, but cannot be expected to do so satisfactorily in all cases.

The Official Method states that aliquots containing as little as 0.01 g of nicotine may be precipitated from samples containing very small quantities of nicotine. Valleau (3) and others have shown that in tobacco samples containing very small quantities of total alkaloids there is an increased probability that much of it will be nornicotine, or at least not nicotine. The Official Method does not set any limit on the volume in which the 0.01 g of alkaloid should be contained. The present results indicate that if it were all nornicotine 0.011 g per liter would remain in solution. Thus, no precipitate would be obtained if the volume was over 900 ml and only 89% of the 0.01 g would be precipitated, even with the special precautions with respect to lower temperature and acidity described below, if the volume was 100 ml.

Other things being equal, the errors introduced due to solubility are likely to be greater in a semimicro method, such as was used in the present investigation, than in a macro method like the Official Method. The following precautions were taken to limit this effect:

The precipitate was heated on a steam bath and then slowly cooled. This gives larger crystals which are more readily filterable.

The samples stood overnight in the refrigerator as recommended by Avens and Pearce (6), instead of at room temperature, as a means of decreasing the solubility effect.

Less acid was used, viz., 2 ml of 1+4 HCl in 250 ml (an acid concentration of about 0.02 *N*) instead of the 3 ml of 1+4 HCl per 100 ml (a concentration of about 0.072 *N*) prescribed in the Official Method. Spies (13) found that the equilibrium solubility of nicotine silicotungstate at 25°C. was about 11 mg per liter when the acid concentration was 0.07 *N*, as compared to 7 mg at 0.02 *N* acid.

Since it seemed possible that the high solubility of nornicotine silicotungstate had been previously overlooked because of co-precipitation with nicotine, various mixtures of nornicotine and nicotine (between 1 to 9 and 9 to 1) were precipitated as before. The results indicate that the solubility of the mixed silicotungstic acid precipitate starts to increase almost immediately in progressing from pure nicotine silicotungstate toward pure nornicotine silicotungstate. However, the solubility of the mixed silicotungstates is not quite as great as would be predicted on the basis of an assumption of no interaction.

2. Distillation with NaOH and NaCl vs. MgO.—The results (Table 1) obtained by the MgO distillation method, shown in column 6 are distinctly lower on the mixed alkaloid samples than are the results of total alkaloid determinations, whether they be those presented in columns 1, 2, or 5. On the other hand, the results by MgO distillation are very much higher than the results for nicotine by the Bowen-Barthel procedure. Thus, the statement which is often quoted, especially from the older German literature, to the effect that nicotine is distilled with MgO but other alkaloids are not, is far from true under these conditions. Since the still used in this work was designed to give the maximum rate of distillation of all volatile substances, it cannot operate as an efficient fractionating column. Tests with pure nornicotine and MgO showed a recovery in the distillate of about 35 per cent. When MgO is used with the distillation equipment ordinarily used in the Official Method, some of the nornicotine from pure solution also distills over, though less than the present apparatus. Thus, the results obtained on mixed alkaloid samples with the usual apparatus plus MgO would be lower than those reported here, but still higher than the nicotine values obtained by the Bowen-Barthel procedure, and lower than the total alkaloid content.

3. Distillation vs. solvent extraction.—The results obtained by the Garner method shown in column 7 are very similar to those obtained by MgO distillation, and on these particular samples correspond to the nicotine values plus about one-half of the nornicotine values as derived by the Bowen-Barthel procedure. This seems also true of the MgO distillation

results. But it would be surprising if two methods based on entirely different chemical principles, both in the separation and the analysis steps, should give such similar results on samples differing considerably in composition if they are measuring the concentration of one compound plus a portion of another compound. An alternative explanation will be presented below.

Effect of storage of samples. Partly as a check upon the technique of new analysts some of the samples discussed above were later re-analyzed. It was soon found that the reproducibility of results was about the same, but that agreement with former results was not uniformly good. Hence, a group of 12 normal samples and 12 mixed alkaloid samples were re-

TABLE 2.—*Average analytical results obtained on tobacco samples after storage (as percentages on air-dry basis)*

METHOD	NORMAL TYPE TOBACCO[1]			MIXED ALKALOID TYPE TOBACCO[2]		
	1950	1951	CHANGE	1950	1951	CHANGE
NaOH-NaCl Distillation:						
Ultraviolet absorption	1.59	1.56	−0.03	1.44	0.90	−0.54
Precipitation	1.58	1.57	−0.01	1.16	0.72	−0.44
Bowen-Barthel:						
Nicotine	1.41	1.39	−0.02	0.19	0.17	−0.02
Nornicotine	0.15	0.17	+0.02	0.90	0.51	−0.39
Total	1.56	1.56	0.00	1.08	0.67	−0.41
MgO Distillation	1.51	1.51	0.00	0.70	0.67	−0.03
Garner	1.59	1.59	0.00	0.66	0.71	+0.05

[1,2] 12 Tobacco samples of normal type and 12 samples which contained principally non-nicotine alkaloids were analyzed in the spring of 1950, stored in air-dry condition in closed containers and re-analyzed in the spring of 1951.

analyzed by all methods. These samples were arbitrarily selected on the basis of the amount of sample on hand, sufficient alkaloid content so that significant changes could be observed and, in the case of the mixed alkaloid samples, low nicotine to total alkaloid ratios. (The samples which were re-analyzed in 1951 are starred in Table 1.)

The average results on these samples by each method in each year are summarized in Table 2. It is seen that results (by different analysts) on the normal type samples do not differ significantly from those obtained a year earlier. In the case of the mixed alkaloid samples, the results obtained for nicotine by the Bowen-Barthel procedure, those by the MgO distillation, and those by the Garner method did not change markedly. However, the total alkaloid results fell very sharply, whether determined by ultraviolet absorption or silicotungstic acid precipitation. Most of this change occurred in the fraction designated as nornicotine by the Bowen-Barthel procedure. Since results by the total alkaloid methods fell, when those by the MgO distillation and Garner methods did not,

it is obvious that the equations which had been devised to express the relationship between nicotine and nornicotine, as determined by the Bowen-Barthel procedure and by these other two methods, were no longer satisfactory. The absolute reduction in the ultraviolet absorption values of the mixed alkaloid samples is greater than the reduction in the silicotungstic acid precipitation values, but the ratio of the former to the latter is about the same in both years, and averages about 1.25. Most of the individual samples have ratios of 1.2 to 1.3. Those which differ are generally high or low in both years.

These results make it appear probable that the mixed alkaloid samples contained, at least at the first analysis, a third substance in addition to nicotine and nornicotine. This substance appears to be distilled, in considerable part at least, in the presence of strong NaOH and NaCl but not in the presence of MgO. It absorbs ultraviolet light in a manner similar to the known nicotine alkaloids. It does not partition into organic solvents in the free form and into water in the salt form as does nicotine. It would appear to be much less stable than nicotine or nornicotine, as with these samples it disappeared to the extent of nearly a third of the total alkaloid content in the course of one year's air-dry storage at room temperature. Bowen and Barthel state (of their method)—"this method is based on the assumption that anabasine or other steam-volatile secondary-amine alkaloids aside from nornicotine are absent; such alkaloids have been found only in traces in *Nicotiana tabacum*." Thus, they make no claim that their method will give satisfactory results for nornicotine in the presence of such a substance as is indicated here. So far, we have not isolated and identified any substance meeting the above requirements, but work is being continued along this line. Evidence has been found that such a substance is not restricted to this particular genetic type of tobacco, but is contained in other types which show considerable amounts of nornicotine by the Bowen-Barthel procedure. The discovery of the relatively high solubility of nornicotine silicotungstate does not assist in explaining this loss in alkaloid content in storage, even though it could explain a large part of the difference between the ultraviolet and precipitation results in a given year.

SUMMARY

Samples of tobacco, some of which contained considerable quantities of non-nicotine alkaloids, were analyzed for alkaloids by 5 different methods. Satisfactory agreement was obtained between all methods on those samples which did not yield high values for nornicotine by the Bowen-Barthel procedure. On samples which did yield high values for nornicotine, the values obtained by use of published "nicotine" methods were in some cases as much as two and one-half times as high by one method as by another.

On such mixed alkaloid samples, distillation with strong alkali and salt gave much higher values than weak alkali distillation; this confirmed previous findings. On these samples the use of an ultraviolet spectrophotometric method of determination gave higher results than silicotungstic acid precipitation. This difference was found to be due at least in part to the much higher solubility of nornicotine silicotungstate as compared to that of nicotine silicotungstate.

Evidence is presented for the presence in these mixed alkaloid samples of an additional substance besides nicotine and nornicotine which reacts to some but not all of the alkaloid methods used. This unknown substance disappears from air-dry samples on storage and results in a change in alkaloid values obtained with methods similar to the present official methods, but not with weak alkali distillation methods or solvent extraction methods.

Though certain commercial varieties of tobacco now contain a considerable proportion of non-nicotine alkaloids, most of them do not. However, attention is drawn to the possible increase of mixed alkaloid varieties due to the use of "nornicotine type" wild species of *Nicotiana* in disease resistance breeding.

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BOOK REVIEWS

Ion-Exchange Resins. By ROBERT KUNIN and ROBERT J. MYERS. 212 pages. 1950. John Wiley & Sons, Inc., 440 Fourth Ave., New York 16, N. Y. $4.75.

The authors have culled and condensed the voluminous literature on this relatively new but increasingly important subject into a brief, well organized treatise. The brevity of the book is somewhat compensated for, however, by its comprehensive bibliography of 615 references. The book is readable and is well illustrated with tables, charts, graphs, and photographs—there is a total of 30 tables and 104 figures. The titles of the 12 chapters are: 1. Introduction and historical review, 2. The theory and mechanism of ion exchange, 3. Cation exchange resin characteristics, 4. Anion exchange resin characteristics, 5. The synthesis of ion exchange resins, 6. Applications, general considerations, 7. Water softening by ion exchange, 8. Deionization of water and other electrolyte solutions, 9. Ion exchange in analytical chemistry. 10. Miscellaneous applications, 11. Methods of studying ion exchange resins, and 12. The design of ion exchange units.

As is indicated by the chapter headings, a considerable portion of the book (4 chapters totalling 67 pages) is devoted to such industrial uses of ion exchange resins as water softening, deionization of aqueous solutions, sugar refining, and desalting of sea water. To the analytical chemist, the chapter on ion exchange in analytical chemistry, consisting of only 16 pages, will probably be disappointingly sketchy.

Although its emphasis is on the commercial applications of ion exchange, the book will be of considerable value to the chemist desiring a general knowledge of the subject. For most details on procedures employing ion exchange resins, however, he will need to consult the original references.

L. L. RAMSEY

The California Wine Industry. A Study of the Formative Years. 1830–1895. By VINCENT P. CAROSSO. University of California Press, Berkeley and Los Angeles. 241 pages.

Those interested in the history of wine making, particularly as it developed in California from its very earliest days, will enjoy this fascinating and absorbing little volume. It consists of a 2 page preface, a 5 page prologue, 8 chapters of 153 pages, 9 pages of epilogue, 24 pages of notes, 16 pages of bibliography, and a 14 page index.

This small book rather settles the controversy as to who were first to grow grapes, to make and sell wine in California, and to what extent.

For grape growers, wine makers and merchants, the reviewer believes that it is a valuable text in that it outlines the pitfalls and perils of wine growing. While historic in scope, it serves as a sort of preview of what could or may occur again in California, unless proper steps are taken to avoid overplanting, overproduction, floods, plant epidemics, depressions, improper tariffs (prohibition and wars).

In the prologue is discussed the romance of the early days leading up to the year 1830. After this follow the great names: Vinges, Wolfskill, Wilson, Kellar, Rowland, White, Vallejo, Hilgarde, Husman and Frohling. Special chapters deal with Kohler, the pioneer wine merchant, and Haraszthy, the father of the modern California wine industry. All in all, it is a very thorough volume, correctly and entertainingly written.

PETER VALAER

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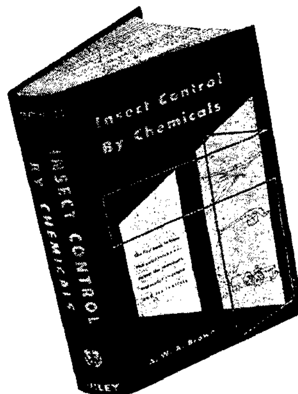

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