CONTRIBUTED PAPERS

STUDIES ON COAL-TAR COLORS, VIII

FD&C YELLOW NO. 5

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

This is another in a series of papers dealing with the preparation of purified samples of various coal-tar colors as an aid in the development of methods of analysis for certifiable coal-tar colors. This paper gives the results obtained in the investigation of 3-carboxy-5-hydroxy-1-p-sulfophenyl-4-p-sulfophenylazopyrazole, the trisodium salt of which is certifiable as FD&C Yellow No. 5. (1) As in previous studies, the spectrophotometric properties of solutions of the purified color have been determined as an aid in the identification of this color in foods, drugs, and cosmetics, and for use in the analysis of samples of the color submitted for certification.

EXPERIMENTAL

Purification of Intermediates

Sulfanilic acid.—A commercial sample of sulfanilic acid was recrystallized twice from water and dried at 135°C.

Phenylhydrazine-p-sulfonic acid.—A commercial sample of phenylhydrazine-p-sulfonic acid was recrystallized twice from water and dried at 135°C.

Analysis:
S: Found, 17.04%; Calc., 17.04%
Equivalent Weight (as a Monobasic acid): Found, 186.4; Calc., 188.2

The semi-micro Kjeldahl procedure does not seem to be applicable to this compound; nitrogen analyses were low and variable.

Preparation of the monosodium salt of 1(4-sulfophenyl)-3-carboxy-5-hydroxy-pyrazolone.—The pyrazolone compound was prepared according to the method of Fierz-David (2). Phenylhydrazine-p-sulfonic acid, 18.8 grams (0.1 mole) was dissolved in 50 ml. of H_2O containing 6.0 grams sodium carbonate and the solution adjusted to neutrality (indicator paper) with dilute hydrochloric acid. To this solution 22.8 grams of 92% purity sodium ethyl oxalacetate (0.1 mole) was added, the mixture heated to 100°C. and stirred at this temperature for 45 minutes. The solution was cooled to room temperature, 5 ml. of alcohol and 18 ml. of conc. hydrochloric acid added, the ppt which formed was filtered with suction and washed with a little water. The product was recrystallized twice from ca 160 ml. of (1:1) alcohol-water and dried at 135°C. Yield, about 27%. Additional pure material was obtained by concentration of the filtrates.

The product obtained by this procedure is a white crystalline material, the analysis of which indicates that it contains one atom of sodium and titrates as a dibasic acid.

Analysis: Calc. for $C_{10}H_7O_6N_2S$ Na
Equivalent Weight: Found, 152.5; Calc., 153.1
Sulfur: Found, 10.28%; Calc., 10.47%
Sulfated Ash: Found, 22.87%; Calc., 23.19%

A sample dried at 135°C. showed no further loss in weight on drying at 138°C. and 1 mm. pressure.

Preparation of FD&C Yellow No. 5.—The 1(4-sulfophenyl)-3-carboxy-5-hydroxy-pyrazolone, 61.2 grams (0.2 mole), was dissolved in ca 500 ml. H_2O containing 64 grams of sodium carbonate and the solution cooled to 0–5°C.

To a solution of 35.0 grams (0.21 mole) of sulfanilic acid plus 11.5

TABLE 1.—*Analysis of purified FD&C yellow No. 5*
(Sample dried at 135°C, 1 atmosphere prior to analysis)

DETERMINATION	CALCULATED FOR PURE COLOR	FOUND	PURE DYE BY CALCULATION
	per cent	*per cent*	*per cent*
Nitrogen (Dumas)*	10.49	10.42	99.3
Sulfur	12.00	11.90	99.2
Titration, ml. 0.1 *N* $TiCl_3$ per gram	74.85 ml.	75.00 ml.	100.2
Sulfated Ash	39.87	39.51	99.2
Volatile Matter at 138°C. and 1 mm pressure		0.1	
NaCl		None	
Na_2SO_4		None	

* Analysis by Oakwold Laboratories, Alexandria, Virginia.

grams of sodium carbonate in ca 500 ml. H_2O, 64 ml. conc. hydrochloric acid was added, and the mixture cooled to 0–5°C. To this mixture 156 ml. of cold 10% (w/v) sodium nitrite soln. (0.22 mole) was added. The mixture was stirred at 0–5°C. for 1 hour, after which the excess nitrous acid was destroyed with sulfamic acid.

The solution of the diazonium compound was added to the pyrazolone solution; the mixture was maintained at 0–5°C. for 1 hour, then allowed to warm to room temperature, and finally heated on the steam bath for 1–2 hours.

The mixture was chilled in an ice bath, the precipitate filtered with suction and dried at 135°C. The product was dissolved in sufficient hot water to make a 10% solution, and an equal volume of conc. hydrochloric acid added. The mixture was cooled to room temperature and filtered with suction. The dye was dissolved in H_2O and reprecipitated with

hydrochloric acid. The recovered dye was dissolved in sufficient water to give a 10% solution, neutralized with sodium hydroxide to about *p*H 8, and an equal volume of alcohol added. The solution was cooled on an ice bath and the precipitate filtered with suction. The recrystallization from alcohol and water was repeated, and the final product dried at 135°C. Results of the analyses of the dried material are shown in Table 1.

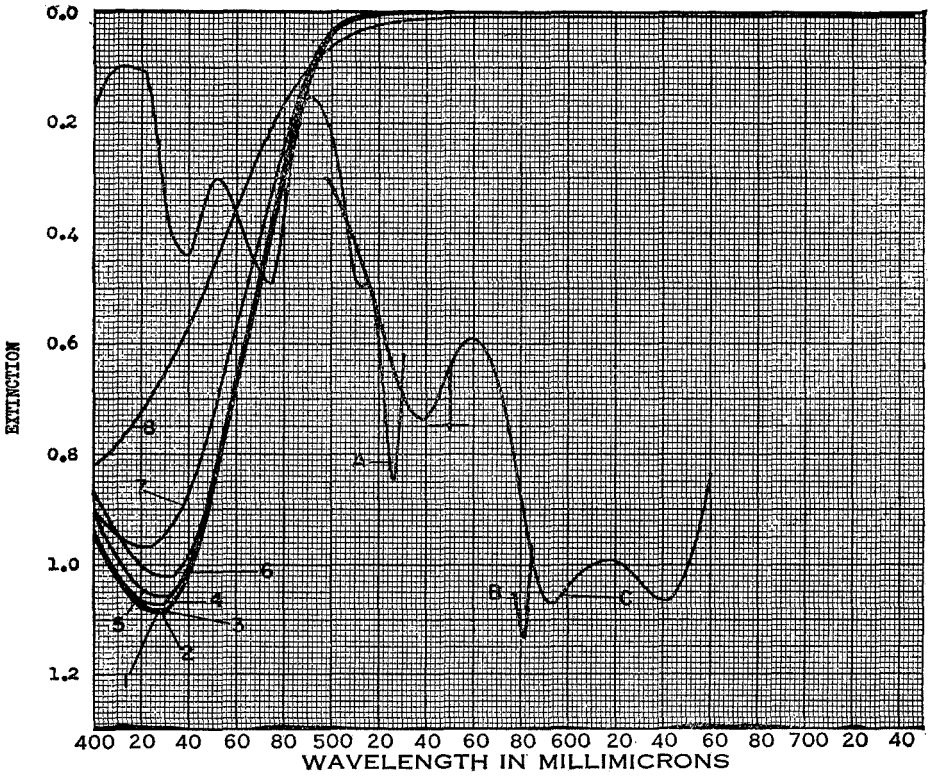

FIG. 1.—Absorption curves of FD&C Yellow No. 5 in aqueous solutions at various *p*H levels. (Conc. 19.90 mg. per liter.)

Curve 1—*p*H 6.0	Curve 5—*p*H 3.1
Curve 2—*p*H 7.0	Curve 6—*p*H 1.2
Curve 3—*p*H 5.1	Curve 7—*p*H 9.0
Curve 4—*p*H 8.0	Curve 8—*p*H 13.0 (calc.)

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

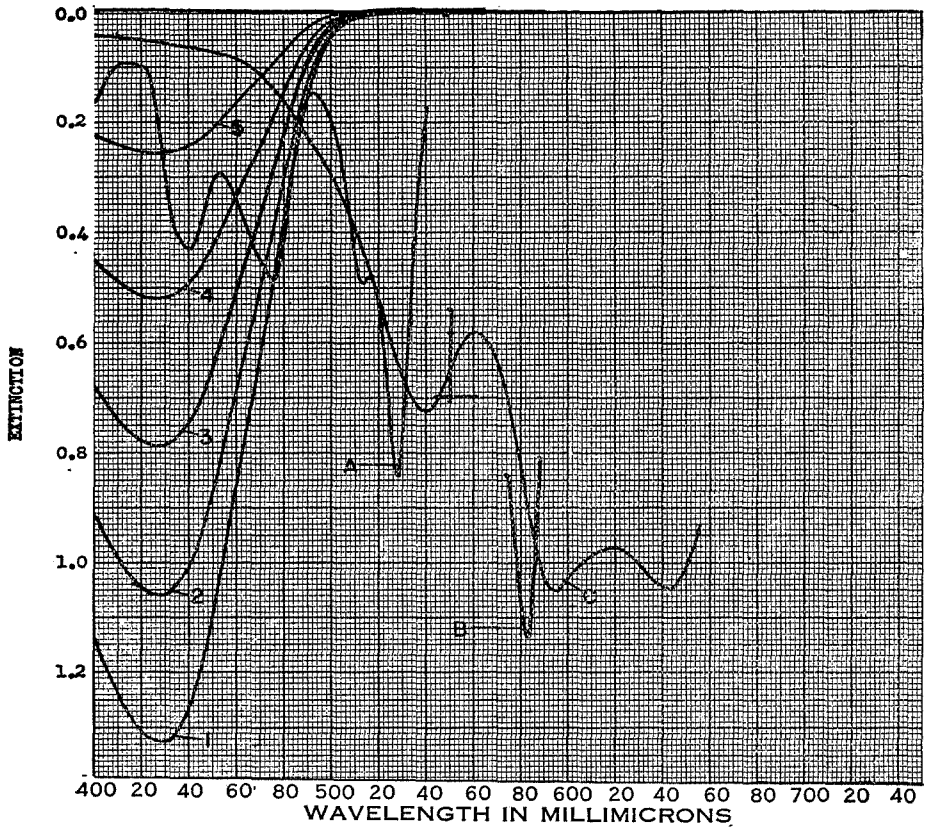

FIG. 2.—Absorption curves of FD&C Yellow No. 5 in water buffered with 0.02 *N* ammonium acetate.

Curve 1—24.61 mg/liter
Curve 2—19.69 mg/liter
Curve 3—14.77 mg/liter
Curve 4— 9.84 mg/liter
Curve 5— 4.92 mg/liter

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

SPECTROPHOTOMETRIC DATA

All spectrophotometric measurements were made with a General Electric recording spectrophotometer with an effective slit width of 8 mμ. Matched 1 cm. Pyrex cells were used.

A weighed sample of dried FD&C Yellow No. 5 was dissolved in a definite volume of water. Suitable aliquots of this solution were buffered with Clark & Lubs' buffer mixtures and diluted to volume. The spectrophotometric data obtained from these solutions are shown in Figure 1. The *p*H values are those obtained with a glass electrode *p*H meter.

Figure 2 and Table 2 show a typical set of the spectrophotometric data obtained on solutions of varying concentrations of FD&C Yellow No. 5 (buffered with 0.02 *N* ammonium acetate).

TABLE 2.—*Extinction values of purified FD&C yellow No. 5 in water with 0.02 N ammonium acetate*

CURVE NO.	CONCENTRATION MG/LITER	$E_{428\ m\mu}$	$\frac{E_{428\ m\mu}}{\text{CONCENTRATION}}$
1	24.61	1.328	0.0540
2	19.69	1.063	0.0540
3	14.77	0.792	0.0536
4	9.84	0.523	0.0531
5	4.92	0.260	0.0532
		Average	0.0536

DISCUSSION

The analytical data indicates that the purified sample of FD&C Yellow No. 5 has a pure dye content of over 99.2 per cent. The pure dye content obtained by the titanium trichloride titration of the purified FD&C Yellow No. 5 agrees, within the experimental error, with the other methods of analysis. The titanium trichloride titration is, therefore, a suitable procedure for the determination of the pure dye content of commercial samples of this color.

The absorption curve of FD&C Yellow No. 5 in water solution is practically unchanged from *p*H 5 to *p*H 7. There is slightly less absorption, and a shift in the wave length of maximum absorption, in strongly acid and strongly alkaline solutions. At *p*H 5 to 7, the wave length of maximum absorption of solutions of FD&C Yellow No. 5 is 428 ± 2 mμ. The average extinction per mg. per liter at *p*H 7, based on the results of seventeen determinations, was 0.0536. The average deviation from Beer's law in these determinations was 0.6%. Neutral solutions of FD&C Yellow No. 5 stored at room temperature for two weeks gave curves identical with freshly prepared solutions.

Twenty commercial samples of FD&C Yellow No. 5 were analyzed by titration with titanium trichloride, and by the spectrophotometric procedure using the purified FD&C Yellow No. 5 as the standard. The average value obtained by the spectrophotometric method was 0.6%

lower than that obtained by titration. The maximum deviation between the two methods was 1.4%.

A commercial sample of FD&C Yellow No. 5 was recrystallized by the procedure described (p. 938) beginning with "The product was dissolved in sufficient hot water to make a 10% solution***." The spectrophotometric properties of solutions of this material were identical with those of the purified color prepared in this laboratory.

SUMMARY

A sample of FD&C Yellow No. 5 has been prepared and purified. The analytical data indicates that the product has a pure dye content of 99.3–100.2%.

The data shows that the titanium trichloride titration is a suitable procedure for the determination of the pure dye content of samples of FD&C Yellow No. 5.

Spectrophotometric data for aqueous solutions of FD&C Yellow No. 5 have been presented. Neutral aqueous solutions of the color obey Beer's law to within ±0.6%.

For 20 commercial samples of FD&C Yellow No. 5 the agreement between the "pure dye" content as determined spectrophotometrically and by titration was very good.

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DETECTION OF FECAL MATTER IN FOOD PRODUCTS

II. AMYLASE ACTIVITY OF FECES AS A MEASURE OF FECAL CONTAMINATION

By J. W. Cook (Food and Drug Administration Federal Security Agency, San Francisco, Calif.)

Previously data was reported (1) of experiments on the use of the tryptic and alkaline phosphatase activities of mammalian feces as indices of fecal contamination of human food. These tests were not found satisfactory because fungi gave tryptic action which could not be differentiated from feces, and a phosphatase test that was sufficiently sensitive was not developed.

This paper reports experiments on starch splitting enzymes as indices of mammalian fecal contamination. Normal excreta are known to be potent sources of starch splitting enzymes. Plant materials, fungi, and

bacteria also contain amylases. However, plant materials in general have low concentration of amylases and can be differentiated from fungi and excreta under appropriate experimental conditions.

Wijsman developed a gelatin-starch plate technique in 1889, (2) to prove the existence of two types of amylase, alpha and beta. The method depended upon a difference in rate of diffusion, and a difference in action on starch, when the enzyme-containing materials were placed on stiff gelatin gels containing soluble starch. The enzymes were allowed to diffuse and hydrolyze the starch at room temperature. Enzyme action was detected by treating the surface of the gels with dilute iodine solution. The zones of diffusion around the enzyme source were revealed as characteristic rings. A colorless zone centered by the enzyme source indicated the presence of alpha-amylase, because beta-amylase alone does not hydrolyze starch to the achromic stage. A blue-violet or violet zone surrounding the colorless zone, or a violet zone alone, surrounded in either case by the blue of the unchanged starch, was said to indicate the presence of beta-amylase.

Giri (3) described a technique for the characterization of different amylases, including pancreatic and fungal. Bhargava and Giri (4) reported a method of differentiation of cereal flours based on the observations of Wijsman, but they used starch-agar instead of starch-gelatin plates. They incubated different amylase-containing materials on agar plates containing different varieties of starch. After allowing time for diffusion and hydrolysis, the surface of the plates was flooded with dilute iodine-potassium iodide solution. Color zones developed which were characteristic of either the type of amylase or the type of starch. Different varieties of starch or amylase yielded different combination of color in the zones of action.

It is generally accepted that both pancreatic amylase and *Aspergillus Oryzae* (the source of Takadiastase) amylase are alpha-amylases, whereas those of ungerminated plant material are predominantly beta-amylases. Alpha-amylases from different sources do not attack starches in exactly the same manner. The pancreas is reported not to have beta-amylase, whereas its presence or absence in *Aspergillus Oryzae* is still questionable. (2) Undamaged raw starch is much more resistant to attack than cooked starch. It is attacked by alpha-amylase but not by beta-amylase. However, cooked starch is attacked by either alpha- or beta-amylase, and better still by a combination of the two.

The reported difference in action between the amylolytic enzymes on cooked starch seemed to hold promise as a means for differentiating excreta from other amylase-bearing contaminants. It was theorized that the principal amylolytic action from excreta would be pancreatic; therefore its action would be distinguishable from the action of amylase derived from fungal or plant sources.

The work reported in the present paper demonstrates that some samples of excreta do show a distinct picture of pancreatic amylase action. However, some others show a picture similar to the activity of amylase from fungal and plant sources. Thus, the procedure to be described cannot always be depended upon to detect fecal contamination. If a sample of a contaminated food product yields the characteristic pancreatic amylase reaction, then in all probability the contaminant is excreta, but if it does not show such a picture, it may or may not be excreta.

EXPERIMENTAL

Starch Varieties and Color of Zones of Enzyme Action

Since Bhargava and Giri reported that different starches and amylases gave various color pictures, an experiment was run to determine which of a few common starches might give the best color conditions capable of differentiating excreta from fungal action.

Two per cent starch solutions were made from arrowroot, "soluble," potato, and corn starches. These were made by adding 2 grams of starch suspended in about 10 ml of cold water to 90 ml of boiling water. Boiling was not continued. Two- and one-half ml portions of these starches were made up to 25 ml with either hot 1.1 per cent agar solution or warm 16 per cent gelatin solution. This gave final solutions of 0.2 per cent starch in either 1 per cent agar or approximately 15 per cent gelatin. The mixtures were stirred well, poured into petri dishes, and allowed to gel.

Particles of mold bran (*Aspergillus Oryzae*), mouse excreta, pancreatin, insect excreta, and ground fennel seed (contaminated with smut), were added. The plates were stacked in a hydrating chamber consisting of a large desiccator containing a shallow layer of water and a few ml of toluene. The water vapor kept the plates from drying, and the toluene vapor was very effective in preventing mold and bacteria growth. After 24 hours at room temperature, which allowed for diffusion and enzyme action, the plates were flooded with approximately .01 *N* iodine-potassium iodide solution. The iodine solution was allowed to remain on the plate for 2–3 minutes and then poured off.

The gelatin-starch plates gave practically no color with this technique because the starch suspensions (except for soluble starch) did not mix with the gelatin. Soluble starch dispersed through the gelatin and gave a blue violet background color when treated with iodine. "Mold bran" (*Aspergillus Oryzae* grown on wheat bran) on a soluble starch-gelatin plate gave a proteolytic pit circled by a wide light blue zone. There was a similar light blue zone surrounding the active fennel seed particles. Surrounding the proteolytic pit produced by pancreatin, there was a very narrow colorless zone, then a narrow band of light blue (similar to, but not exactly like, the blue of " mold bran" action). The picture was

about the same in both large and small pancreatin diffusion zones, except that in the small zones the light blue area was wider in proportion to the colorless area. Mouse excreta gave a proteolytic pit, surrounded by a narrow light blue zone when the proteolytic pit was large. However, when the proteolytic pit was small, there was a colorless zone, then a light blue zone. In other words, this sample of excreta gave the same picture as pancreatin with gelatin-soluble starch when the pits were very small, but a somewhat different one when the pits were large. In either case, the picture was much different from "mold bran" or fennel.

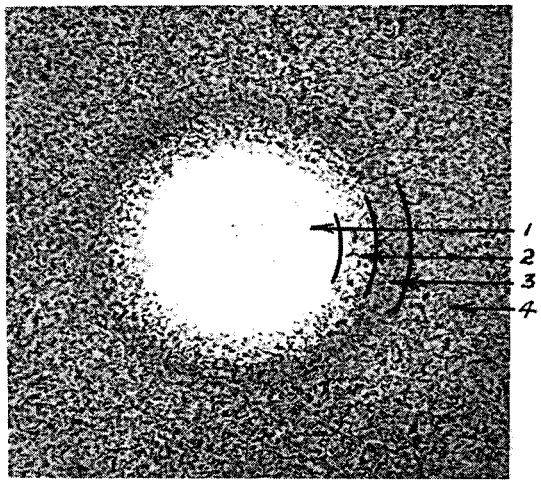

FIG. 1.—Colored Diffusion Zones from *Aspergillus Oryzae* on Potato Starch-Agar Plate.

1. Colorless zone —no spots.
2. Violet zone —spots are violet.
3. Dark blue zone—spots are dark blue.
4. Starch blue —spots are blue.

The different amylase-bearing materials gave considerably different pictures on starch-agar plates. On each of the arrowroot, potato, and corn starch-agar plates, mold bran, pancreatin, and mouse excreta and smut contaminated fennel each shows its own characteristic color picture. Figures 1, 2, and 3 show the characteristic zones developed on potato-agar plates. "Mold bran" (Fig. 1) gave a colorless central areas surrounded by a wide red violet band, then a blue zone darker than the general blue of the plate. Pancreatin and a mouse excreta sample (Fig. 2) each produced a colorless central area surrounded by a narrow red violet area, then a narrow light blue area, and then a dark blue area.

Smut-contaminated fennel seed (Fig. 3) gave a deep blue irregular area

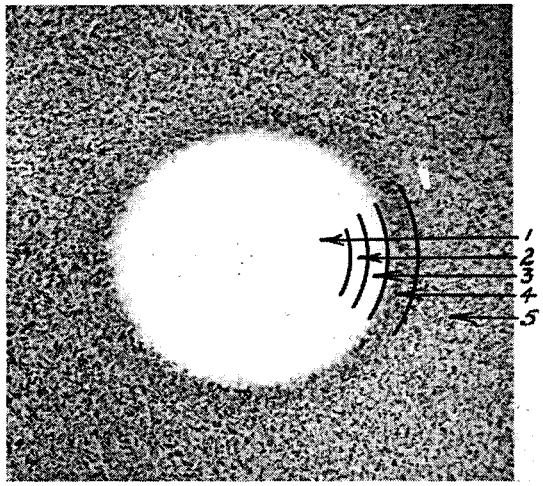

FIG. 2.—Colored Diffusion Zones from Mouse Excreta on Potato Starch-Agar Plate.

1. Colorless zone —no spots
2. Violet zone —no spots
3. Light blue zone—no spots
4. Dark blue zone—spots are dark blue
5. Starch blue —spots are blue

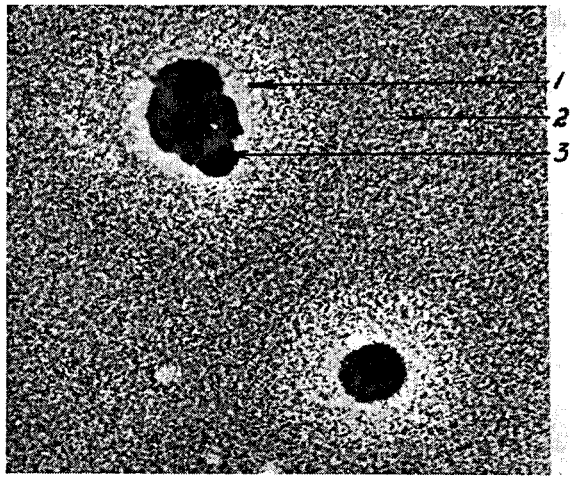

FIG. 3.—Colored Diffusion Zone from Fennel Seed on Potato Starch-Agar Plate.

1. Violet zone —spots are violet
2. Starch blue —spots are blue
3. Deep blue area—

surrounded by a narrow red-violet area. The deep blue central area was sharply outlined, but the red-violet area was not. Fennel seed placed on agar plates with no starch and held under similar incubation conditions, gave no such blue central color. Therefore, the deep blue central area was probably due to amylase action on the starch, but no attempt was made to determine whether the activity was due to the smut or the fennel seed. No action was evident from the insect excreta tested. The soluble starch-agar plates were violet-blue or purple so that there was very little difference in color zones due to the various enzyme sources.

The color zones were very similar on the different varieties of starch, but each starch gave a different shade of blue with iodine so that the color zones were viewed with different background colors. Therefore, the choice of starch for best differentiation depended on which gave the best contrast between color of zones of action and background color. Potato was chosen because it gave a dark, pure blue which gave good contrast with the red-violet zones. Both corn and arrowroot starch were more reddish-blue or purple, which blended rather than contrasted with the zones of action.

Appearance of Swollen Starch Granules

When raw starch is made into a 1–2 per cent starch paste by boiling, the starch granules swell and crowd together. The mass of swollen granules can be seen by use of a low power microscope, appearing as translucent "sacks" crowded together. When starch-agar plates, colored on the surface with iodine, are observed carefully macroscopically, or with a low power microscope, dark blue colored particles appear within a lighter blue, clear color. The particles of color are due to the "sacks" of the swollen and ruptured granules. These colored "sacks" are not present in soluble starch plates. Giri made no mention of them in his starch work. But, as is shown in Figures 1, 2, and 3, the appearance of these "sacks" or spots was different in the zones of action of different enzymes. The appearance of these spots is important in the differentiation of action of pancreatic and fungal amylases. They appeared as red-violet spots in the red-violet digestion zone from "mold bran" and fennel. They changed to blue color at the outer edge of the red-violet zone. No colored "sacks" appeared in the colored zones produced by pancreatin or mouse excreta. They were not visible until the blue background of the starch plate was reached.

Variations in the preparation of starch solutions affect the predominance of these "sacks." A 0.2 per cent suspension of potato starch made by diluting a 2 per cent paste gave a more pronounced color to the "sacks" than one made with 0.2 per cent starch to begin with. Plates made by diluting 1–2 per cent suspensions to .2–.4 per cent, in agar produce spots which are readily seen with the unaided eye when the surface is treated with iodine.

Effect of pH

Plates prepared as above from C.P. potato starch had a *p*H of approximately 5.5. Additional plates were prepared from potato starch and 0.01 *M* potassium dihydrogen sulfate-sodium hydroxide, adjusted to *p*H of 6, 7, and 8 with sodium hydroxide solution. "Mold bran," pancreatin, mouse excreta, and fennel gave the same color-zone picture at all *p*H's, as was described previously. However, insect excreta gave positive action at *p*H 7 and 8 but not at 6. The color picture of insect excreta was also different from any described so far. There was a small colorless area surrounding the excreta. The colorless area faded into the blue of the plate without any red-violet zone.

Effect of Sodium-chloride

Animal amylases require low salt concentrations for activity. The plates described above which were adjusted to *p*H 6, 7, and 8 also contained 0.005 *M* sodium chloride, whereas those described previously had no salt added. There was no noticeable effect from the salt addition. Salt in the enzyme source, agar, or starch, was probably adequate for optimum action.

Analysis of Mouse Excreta Samples

A series of excreta samples (1) was tested by this method using potato starch-agar plates. The plates were treated with iodine solution after 24 hours' incubation. Fifteen samples were tested for activity. Ten gave color zone pictures like pancreatin, but five showed pictures similar to "mold bran" except there was no colorless central zone. This would seem to indicate that pancreatic amylase was not predominant in the five excreta samples.

Analysis of Powdered Sage Containing Mouse Excreta

One milligram of powdered mouse excreta was mixed with 10 grams of powdered sage leaves. A few milligrams of this mixture was sifted through a fine sieve onto one-half of a starch-agar plate. A similar amount of sage alone was added to the other half of the plate. The plate was held at room temperature over toluene vapors for 20 hours. Flooding with iodine solution revealed 8 spots typical of excreta, and one like "mold bran," in the area with sage plus excreta, but no spots appeared in the area with sage alone. Clumps of sage particles gave a general violet area, but no specific spots like the more potent enzyme sources.

Investigation of Starch-Gelatine Plate Technique

Balls and Schwimmer (5) reported that "Uncooked starch may be readily and completely digested by a mixture of extracts of hog pancreas and *Aspergillus Oryzae*. The breakdown of the starch granules may be observed without difficulty under the microscope."

It was theorized that if an unknown enzyme preparation was added to one containing *Aspergillus Oryzae* enzyme, the complementary action

reported by Balls and Schwimmer on raw starch would indicate whether or not the unknown enzyme was of pancreatic origin. It was hoped that the active principle from *Aspergillus Oryzae* could be incorporated into a gelatin plate without affecting the properties of the gelatin. Then, when a powdered food product was put onto the plate, any pancreatic enzyme added would give gelati. pits plus raw starch breakage, whereas fungal enzymes added would give gelatin pits without raw starch breakage. A successful technique was not accomplished, primarily because of the proteolytic activity of *Aspergillus Oryzae* preparations which destroyed the gelatin gels.

It was noted during this work that pancreatic amylase digested raw corn starch much more rapidly than fungal amylases. An attempt was made to use this action as a means of differentiation; however, only extremely potent excreta samples showed activity. The principal difficulty encountered was that optimum proteolytic pit formation took place at about *p*H 8–9 at which there was no raw starch breakage. Raw starch breakage was best at about *p*H 5, at which there was very little tryptic action except from the most potent sources. When the *p*H of the plate was adjusted half way between 5 and 8, excreta samples of low activity gave neither raw starch digestion nor proteolytic action.

As was shown earlier in this paper, the amylolytic enzymes diffuse faster in cooked starch-gelatin plates than do the proteolytic enzymes. Also it had been observed that raw starch breakage extended beyond the proteolytic pit in gelatin plates. An attempt was made to test the complementary effect of the two amylase sources by placing them close together on a raw starch-gelatin plate, thus allowing the two amylases to diffuse together outside of the zones of proteolytic action. No complementary action was noted under these conditions.

SUMMARY AND CONCLUSIONS*

The zones of digestion of cooked starch in agar plates, by pancreatic and some fungal amylases, are distinctively different. Smut-contaminated fennel seed, which was not distinguished from excreta by protease action (1) gave an unmistakably different starch digestion picture. Even *Aspergillus Oryzae*, another alpha-amylase, gave a picture much different from pancreatin and some mouse excreta samples. However, some excreta samples were found to give color pictures more like the fungal enzymes than like pancreatin; therefore, the technique as developed is not universally applicable for the detection of fecal matter.

The color zones described in this paper are not the same as those described by Giri. Using potato starch and takadiastase, he obtained a central colorless zone surrounded by a broad green zone, and finally a very distinct and deep violet colored ring at the fringe. Since takadiastase

* The author wishes to acknowledge indebtedness to Dr. Arthur N. Steers for helpful discussions and suggestions.

is prepared from *Aspergillus Oryzae*, it was expected that the spots should be the same.

It is difficult to attempt to explain these differences until more investigational work has been done. Factors arising from the method of manufacture of the starch, preparation of starch paste for the plates, *p*H, salts, accompanying enzymes, etc., may account for the differences. It would be necessary to study all of these for a full understanding of the procedure and to determine the possibilities of the technique.

The work reported here on raw starch breakage in gelatin plates is not promising. However, it may be possible to develop a cooked starch-raw starch-agar plate in which the cooked starch would reveal the place to search for raw starch breakage. This also poses problems: for example, (*1*) starch-agar plates have to be made at a higher temperature than starch-gelatin so that it is more difficult to prepare a "raw" starch-agar plate; (*2*) when the plates are flooded with iodine solution, all the raw starch granules turn deep blue: even those which show breakage are colored blue, and the breakage is thus completely masked unless extreme.

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DETECTION OF FECAL MATTER IN FOOD PRODUCTS

III. ALKALINE PHOSPHATASE SPOT TEST AS AN INDICATOR OF RODENT FECES

By J. W. Cook (Food and Drug Administration, Federal Security Agency, San Francisco, Calif.)

In another paper Cook and Steers (1) reported on the possible use of intestinal alkaline phosphatase as an indication of rodent feces in food products. Rodent excreta are a potent source of intestinal alkaline phosphatase, whereas food products, molds, and excreta of many insects have only acid phosphatases. Alkaline phosphatases hydrolyze monophosphoric acid esters in an alkaline medium, while acid phosphatases act on the same compounds in an acid medium. When phenolphthalein phosphate (2) is used as the ester in an alkaline medium, alkaline phosphatase activity is revealed by the appearance of red phenolphthalein. Acid phosphatase activity in an acid medium yields colorless phenolphthalein; therefore, the medium must be made alkaline after the incubation period to reveal activity.

Particles of rodent and insect excreta with about equal gelatin liquefying power showed a great difference in alkaline phosphatase activity (1). These excreta particles, incubated in drops of alkaline, buffered, phenolphthalein phosphate solution, produced a red-colored solution when of rodent origin, and no color when of insect origin. It was noted in the same publication that tl is test could be used in agar and gelatin plates, but tests run at that ti.ne were found satisfactory only when relatively large particles of excreta were present.

The present paper reports some preliminary work on a more sensitive test for alkaline phosphatase, using phenolphthalein phosphate on paper, or in gelatin or agar plates, and using the alkaline soda-borax buffer of Kolthoff and Vlesschhouwer (3).

EXPERIMENTAL

Control of pH by Buffers

The apparent lack of sensitivity of the spot test for alkaline phosphatase activity on gelatin plates (1) may have been due to inability of the buffer used to maintain a highly alkaline medium. It was also possible that the end products of gelatin hydrolysis (pit formation) might inhibit phosphatase activity or color formation. To clarify these points filter paper was moistened with a solution made 0.001 *M* with phenolphthalein phosphate and 0.05 *M* with the sodium barbital-sodium carbonate-hydrochloric acid buffer of King and Delory (about *p*H 10.5) (4). Mouse excreta particles gave bright red spots in from 1 to 10 minutes, whereas smut-contaminated fennel and "mold bran" (*Aspergillus oryzae*) gave no color. However, when these papers were exposed to ammonia fumes following the short incubation period, red color appeared from all three products. This indicated that the products themselves lowered the *p*H sufficiently to allow acid phosphatase activity.

Since the above concentration of buffer did not appear to maintain a sufficiently high *p*H, an experiment was run to study the effect of different buffer concentrations. Five buffer substrate solutions were prepared. All contained 0.001 *M* phenolphthalein phosphate, but the buffer varied from 0.025 *M* to 0.25 *M*, all at *p*H 10.5. Test papers were prepared by dipping filter paper into these solutions and placing the wet papers in the bottom of standard petri dishes. Various dry particles with high enzyme content were placed on the papers and were held for one hour at room temperature. At the end of the incubation period, particles which showed red spots were noted as shown in Table 1 in the section marked (a). Then the papers were held over an opened bottle of ammonium hydroxide and the colored spots again noted as in section (b) of Table 1.

It can be seen from the table that the high concentration buffer inhibited the acid phosphatase of pecan nut meats, but it also inhibited

TABLE 1.—*Effect of buffer concentration on phosphatase activity*
Sodium carbonate-sodium barbital buffer—*p*H 10.5 (4)
One hour incubation at room temperature

CONC. OF BUFFER	MOUSE EXCRETA	INSECT EXCRETA IN WALNUT MEATS	INSECT EXCRETA IN PECANS	PECAN NUT MEATS ALONE
	(a) *Before exposure to NH_3 fumes*			
0.25M	+	+	−	−
0.125	++	+	−	−
0.0625	+++	+	−	−
0.031	+++	+	−	−
0.025	+	+	−	−
	(b) *After exposure to NH_3 fumes*			
0.25M	+	+++	+	−
0.125	++	+++	−	−
0.0625	+++	+++	−	−
0.031	+++	+++	?	+
0.025	+++	+++	+	+

the action from mouse excreta. It also shows that the lower buffer concentration allows the *p*H to fall in the presence of excreta action, as shown by the increase in color when exposed to ammonia. The *p*H was not held high enough to inhibit the acid phosphatase of insect excreta in the presence of walnut meats.

A study of the published data on some alkaline buffers indicated that the alkaline soda-borax buffer of Kolthoff and Vlesschhouwer (3) would have a greater buffering capacity in the region of *p*H 9 to 10.5 than the more common buffers. Table 2 shows the results of adding particles of

TABLE 2.—*Effect of some food products and excreta in presence of food product, on phenolphthalein phosphate buffered by*
(a) sodium carbonate-sodium barbital, 0.0625 *M*
(b) sodium carbonate-sodium borate, 0.05 *M*

PRODUCT	REACTION FROM PRODUCT ALONE		REACTION FROM EXCRETA IN PRESENCE OF PRODUCT	
	a	b	a	b
Lima beans	+	−	++	+++
Lima beans	+	−	++	+++
Pole beans	+	−	++	++
Bush beans	+	−	+	+++
Dwarf peas	−	−	+	+++
Alaska peas	−	−	?	+++
Corn, golden bantam	−	−	+	++
Corn, sweet	−	−	+	++

food material and mouse excreta to papers containing phenolphthalein phosphate substrate and either (a) sodium carbonate-sodium barbital buffer, 0.0625 *M* or (b) sodium carbonate-sodium borate buffer, 0.05 *M*. The excreta particles were tested in the presence of food materials because of the tendency of the latter to reduce the *p*H and allow acid phosphatase activity.

It can be seen from Table 2 that soda-borax held the *p*H sufficiently high to inhibit action from the acid phosphatases of the food products and to allow more alkaline phosphatase activity from the rodent excreta.

Accutint Test Paper as Spot Test Paper

Accutint test paper No. 170 is a narrow range test paper which is blue at *p*H 9.1 and pink at *p*H 10.4. Strips of this paper were wet with the substrate-soda-borax buffer solution which turned the paper pink. Lima bean particles sprinkled on this moistened paper produced faint blue areas in a few seconds. Mouse excreta particles produced no immediate color changes, but a deep blue spot appeared following a short incubation period. The size of the spot and intensity of the blue color increased as the hydrolysis of the substrate continued, whereas the blue produced by lima bean powder gradually turned back to pink. The blue spot produced by the excreta was no doubt due to the liberation of phosphoric acid from the hydrolysis of phenolphthalein phosphate.

The size and intensity of the blue spot produced by the liberation of phosphoric acid from the hydrolysis of phenolphthalein phosphate seemed to be much greater than the size and intensity of the red spot produced by the phenolphthalein liberated. This indicated that the Accutint paper may be a more sensitive medium to use than one which depends on the development of red from phenolphthalein. With this in mind another substrate was tested. Disodium phenyl phosphate is commonly used as a substrate for phosphatase activity, in which case the liberated phenol is determined quantitatively. It is more readily hydrolyzed than phenolphthalein phosphate so it was thought that the sensitivity of the spot test would be increased by the increased rate of hydrolysis. However, when this substrate was used on Accutint test paper no blue spots developed. The factors responsible for this apparent lack of activity were not investigated.

Use of Sodium-Carbonate-Sodium Borate Buffer in Gelatin and Agar Plates

As was indicated previously (1) phosphatase activity, using phenolphthalein phosphate and barbital buffer in gelatin, gave negative results unless large fragments of excreta were present. Even when large fragments were present the red color that was formed faded quite rapidly. Similar plates made with soda-borax (0.025 *M*), *p*H 10.5, maintained the proper *p*H. Very small fragments of mouse excreta added to one plate

of this type now gave positive phosphatase action. Furthermore, the color remained indefinitely unless it diffused over too large an area to be seen. A small quantity of this gelatin-buffer-substrate was held over toluene and water vapor for 10 months. During this time the whole gel turned red and remained so. This indicates that the medium remained sufficiently alkaline to maintain phenolphthale n red, and yet was not sufficiently alkaline to destroy the phenolphthalein or the gelatin gel. The gradual development of color shows that the substrate, phenolphthalein phosphate, cannot be held under these conditions for over two months without breakdown.

Preliminary experiments with the above buffer-substrate, in agar, indicated that agar may be more satisfactory for use than the paper. The important factor probably is the increased buffering capacity of the larger volume of buffer.

Activity of Various Excreta Samples

The activity of thirty-three samples of rodent excreta (1) was tested on paper moistened with substrate-buffer solution (soda-borax buffer). Considerable variation in amount of action was evident, but all gave a definitely positive phosphatase test.

SUMMARY AND CONCLUSIONS

A buffer system that maintained a relatively constant and high *p*H proved to be one important factor in the development of a spot test capable of differentiating intestinal alkaline phosphatase activity of rodent excreta, from other enzymes such as those from some insect excreta or mold. A sodium carbonate-sodium borate buffer maintained a *p*H sufficiently high to inhibit acid phosphatases and allow alkaline phosphatase activity.

The use of Accutint test paper as a means of identifying the source of action appears to be well worth investigating. It also aids in the explanation of some features of the spot test. In conventional procedures, with test tube quantities of solutions, intestinal phosphatase has a *p*H optimum of about 9, with no action at *p*H 10 or above. However, in test paper and some agar plate work, the best results in differentiating phosphatases were obtained at *p*H 10.5. The formation of the blue spot on the Accutint test paper indicates that free phosphoric acid is liberated. This means that a very small amount of alkaline phosphatase activity automatically produces a zone which has a much more nearly optimal *p*H. Therefore the activity is enhanced. Furthermore, maintaining the *p*H at 10.5 completely stops acid phosphatases. This is very important, because if the food particle reduces the *p*H and allows some action from acid phosphatase, the *p*H is then further reduced to allow more acid phosphatase activity.

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ESTIMATION OF INSECT FRAGMENT CONTAMINATION IN CANNED SWEET CORN*

By R. G. TISCHER (Iowa State College, Ames, Iowa)

The movement of the European Corn Borer across the United States in recent years has made it increasingly difficult to prepare canned corn without strict attention to the removal of insect contamination from the raw product. Losses attributed to this pest alone have been estimated at three hundred and fifty million dollars in 1949. While considerable effort is being made to eliminate the corn borer and other insects in the field, only a limited amount of work has been done to improve plant practices, such as trimming and inspection, which might be expected to reduce the insect load and produce a clean product. Once the raw product is placed in the can, very little can be done to reduce the amount of contamination present. At this point and beyond, the problem is one of estimation of the extent of contamination.

Methods for the recovery and estimation of extraneous materials in foods have been the subject of continued efforts, mainly by members of the U. S. Food and Drug Administration, since its inception. These methods have been modified for use with a wide variety of products. While many are now published (1, 2, 3), much of the background work was set forth in the form of mimeographed Food and Drug Administration leaflets which are now out of print. Harris (4), in 1946, published an annotated bibliography concerning the methods used in the examination of foods for extraneous materials. A wide variety of commodities are represented in the literature cited. The apparent emphasis has been shared among dairy products, cereals, and fruits and vegetables. None of the publications listed dealt with canned corn. If frequency of occurrence of research effort is a fair criterion, very little stress has been placed on the methodology of insect fragment recovery: a segment of the field which is in need of serious consideration. Welch (10) has stated that there are approximately 100 procedures in existence for the demonstration of extraneous materials in food and drug products. Most of these methods are based upon one or more of three principles: (1) solution of the food product, leaving the insect contamination for enumeration, (2) separation

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by means of density differences, and (3) separation by means of preferential wetting with oily substances. The third method is used in separating insect fragments from canned corn.

J. D. Wildman (11) is credited with originating the gasoline flotation method for a separation of insects from food products. It was found that the incorporation of an oily substance, sucl as gasoline or kerosene, with the commodity being tested caused some types of insect larvae to rise with the oily layer, upon addition of water to the mixture. Separation was effected by decantation of the oily layer, leaving the vegetable material in the water phase. The method now in use is a modification of the original in which castor oil has been substituted for gasoline. Application of the flotation method has been made in many commodity fields and B. J. Howard (7, 8), through practical use in his extensive work on tomato products, made many improvements in the flotation method of estimation of insect contamination. Harris and O'Brien (6) and Harris and Knudsen (5), working with alimentary pastes and with flour, made comparisons of the efficiency of several modifications of methods in use for the separation of insect parts and rodent hairs from these foods.

In view of the economic importance of any contamination in food products, the accuracy of the method used for determining quantitatively, the extent of this contamination is of primary interest and further work on improvement of methods appears to be well warranted. Since prevention of contamination is the logical aim of the processor, methods are needed which will facilitate estimation of the probable extent of contamination in the processed product in terms of (1) field infestation, and (2) efficiency of removal in the processing operation. Accurate estimation of contamination in the product prior to processing, followed by the elimination of infested raw product from the processing operation is, of course, the most desirable procedure. Efforts toward this end have led directly to a consideration of the method of estimation available for this purpose. Work is in progress by several agencies on insecticides and other treatments for use in eliminating infestation in the field and for improvement of trimming, washing, and inspection during processing.

EXPERIMENTAL

In this laboratory, results from the analysis of canned cream style corn from a wide variety of sources indicate that the accuracy of the oil flotation method of estimation is open to some question in that this method may be expected to recover only approximately two-thirds of the number of fragments which may be demonstrated by other means.

The inconsistencies resulting from the A.O.A.C. method (1) suggested the investigation of other methods for the separation of insect fragments from canned corn. Separation by means of density differences and by

means of screening were investigated. The use of both stannic chloride and barium bromide was undertaken because of the high density of their solutions. Each allowed some separation of fragments from corn with some samples, but the irregularity of these results rendered the procedures unsatisfactory, primarily because corn density and insect fragment density vary from sample to sample. Attempts to find other media in which corn and insect fragments would be separated by means of density differences were unsuccessful. The use of screens, alone or in combination with a density separation, was equally unsatisfactory because in each trial the separations were incomplete.

Attempts were made to dissolve the corn, leaving the insect fragments intact for observation. Use was made of strong acids and bases, zinc chloride-iodine reagent, and several other substances known to dissolve cellulose. Since none of these procedures dissolved the corn completely no effective separation resulted.

With the failure of other methods to increase fragment recovery, attention was turned to an evaluation of, and possible improvement in, the accuracy of the A.O.A.C. method. As originally published (1), the A.O.A.C. method is based on use of a 200-gram sample of corn. The sample is placed in a two-liter Wildman trap flask where it is mixed with castor oil. Hot water (50°C.) is then added and after one-half hour has passed, the oil layer is trapped off. This process is repeated after an additional ten minutes. In each case the trap is washed with an alcohol-water mixture and the washings are added to the trapped material. The resulting liquid is passed through a ruled filter paper by means of suction applied to a Büchner funnel and the filter paper is examined for insect fragments using a low-power, wide field binocular microscope. Our experience has indicated that use of alcohol induces coagulation of starch which collects on the filter paper during filtration. This accumulation of starch material, when it occurs, retards filtration and makes the enumeration of insect fragments an extremely tedious task. The most effective method of applying the present A.O.A.C. modification (2) consisted in the addition of hydrochloric acid in the trapped fluid to bring the concentration up to approximately 0.2 per cent. The trapped fluid was then boiled on a hot plate for twenty to thirty minutes or until the starch was completely hydrolyzed as indicated by the absence of an iodine-starch reaction. When the resulting fluid was poured slowly over the filter paper, the filtration was usually completed in the course of a few seconds with a great saving of time, especially if a large number of samples were being handled. Repeated tests have indicated that no damage to the insect fragments results from this treatment and that observation of insect fragments on a starch free filter paper is relatively simple.

Use of the automatically emptying metal filter apparatus shown in Figure 1 also added efficiency by obviating the necessity for emptying filter flasks during the analysis.

SOURCES OF VARIATION

Use of the A.O.A.C. method for the determin tion of insect fragments in canned cream style corn has suggested the ex. tence of several impor-

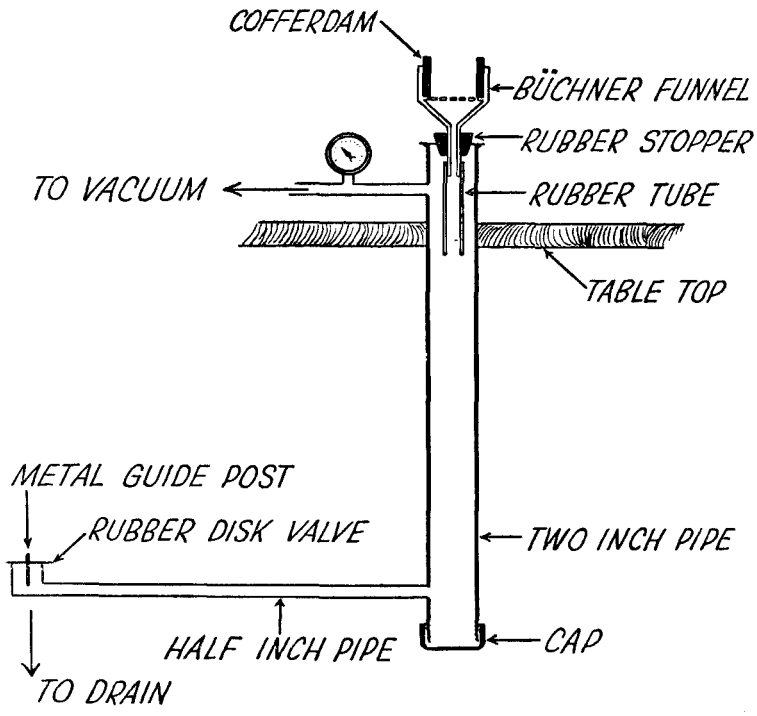

FIG. 1.—Automatic filter flask.

tant sources of variation: (1) source of the samples (geographic location, variety, weather, etc.), (2) among cans in a lot, (3) between duplicates from the same can, and (4) between analysts. As might be expected, lots of corn from various geographical locations in the United States vary both with respect to the number and kind of insect fragments found. In the West Coast States, the presence of corn earworm fragments is not uncommon, while in many of the Eastern and Middle Western States, aphids, gnats, etc., and corn borer fragments in addition to corn earworm fragments are found in contaminated lots.

A second source of variation is that among cans in a single lot. Sampling

at the rate of two cans per thousand cases indicates generally a very low content of insect fragments of all kinds. This small sample often results in disagreement between duplicates, and among samples within a lot, which may only be reconciled by further sampling. Counts have ranged from 0 to 50 fragments per can, with most samples containing approximately 0–2 fragments p r can (Table 1). Lots also exist in which almost all of the cans are prac ically free of fragments, with a small percentage of cans which may contain a whole borer or other whole insect, several large fragments, or a high count of microscopic fragments. The apparent inhomogeneity of such lots adds to the difficulty experienced in providing an accurate estimate of the contamination throughout such a lot.

Experience has provided proof that good agreement between duplicate tests within a can is at times difficult to obtain. When 200-gram samples

TABLE 1.—*Frequency distribution of naturally occurring insect fragments in canned corn*
By levels

NO. OF FRAGMENTS	NO. OF SAMPLES
0–2	361
2.1–4	66
4.1–8	38
8.1 and up	3
Total	468

were drawn from a single can after what was considered to be thorough mixing with a spoon, extreme variations were sometimes noted. One duplicate was often found to contain a large number of fragments, while the other contained none. No geometric orientation was noted, since the sampling was done immediately after the mixing was completed. In explanation, the conjecture has been made that the sample containing microscopic fragments must be mixed for a greater length of time if concordant results are to be expected from duplicate samples.

Another source of variation results from discrepancies between operators estimating the contamination in a single sample. It has long been recognized that analyses of this type require the attention of an experienced analyst whose ability to recognize insect fragments has been proven by repeated and exhaustive tests. To the operator who possesses even a minimum of experience, however, the recognition of many insect fragments is a simple matter. Using standard equipment, many fragments are so characteristic that they may be recognized at once. However, it has been the experience in this laboratory that some insect fragments, if finely enough divided, become difficult, if not impossible to identify, because of the lack of diagnostic characteristics which will appear definite

at the magnifications in common use. When the compound microscope is used extensively as an aid in identification, the added time required for each analysis becomes a very important factor. Conversely, efforts to reduce the time of observation for each determination below thirty minutes have resulted in great inaccuracies by all operators. If the

TABLE 2.—*Influence of operators on variations in insect fragment counts due to preparation and observation by two operators*

LOT NO.	1				2			
PREPARED BY OPERATOR	A		B		A		B	
READ BY OPERATOR	A	B	A	B	A	B	A	B
	1	1	0	0	4	4	3	4
	2	3	2	2	3	3	2	1
	2	3	4	6	2	3	4	4
	0	0	3	1	0	2	3	5
	5	4	4	5	3	4	6	8
	3	3	13	6	4	5	5	3
	1	1	5	4	3	2	0	0
	8	10	5	5	7	5	0	0
	3	3	1	2	3	5	4	7
	2	3	2	4	7	7	23	16
Total	27	31	39	35	36	40	50	48

TABLE 3.—*Influence of operators on variations in insect fragment counts due to preparation and observation by two operators*

Analysis of Variance

SOURCE OF VARIATION	d.f.	S.S.	M.S.
Lots	1	22.05	22.05
Error (a)	18	564.00	31.33
Preparation (P)	1	18.05	18.05
Error (b)	19	343.55	18.08
Readings (R)	1	0.05	0.05
R×P	1	2.45	2.45
Error (c)	38	115.40	3.03
Total	79	1065.55	

equipment recommended for use in this analysis could be clearly specified as a requirement in the procedure, the limits of variation due to the equipment could probably be brought closer together.

An example of typical data is shown in Table 2, with the analysis of variance in Table 3. Ten cans were drawn from each of two lots of corn, and duplicate samples were drawn from each can. One 200-gram sample

from each can was prepared by one operator, and the remaining duplicate from each can was prepared by a second operator. Both operators counted fragments on each 200-gram sample. The analysis of variance shows no significant differences due either to preparation or observation of samples by the two operators. However, the large mean squares associated with lots and preparation reflect the random variations which appear to occur about equally in both lots and in both preparations. The relatively small mean square for readings illustrates agreement between duplicates which decreases directly with sample size. Since both operators were in the same location, this example probably indicates much less operator variation than would be encountered with operators in different locations.

EFFICIENCY OF RECOVERY

Samples with a known content of insect fragments were needed to test the accuracy of the analytical procedure. In an effort to obtain insect fragments similar to those found in canned cream style corn, whole corn borer larvae were heated in an autoclave at 250°F. for times ranging from five minutes to 390 minutes to simulate the effect of commercial sterilization of the canned product. Following this treatment, the corn borers were placed in a Waring Blendor in a medium either of water or corn where they were blended for periods up to thirty minutes. Prolonged heating caused a slight shrinking and flattening of the larvae accompanied by the appearance of a brown color in some of the external portions, but caused very little separation of one part of the larvae from another. Attempts to dissect portions of the larva under the microscope after heat treatment were much simpler than similar operations on the fresh larva, chiefly due to the increased rigidity of the subject.

It may be surmised that the major share of corn borer fragments are produced commercially during the operation of cutting the kernels from the cob, with distribution of these fragments taking place in varying degrees during the remainder of the process. Treatment of heated larvae in the Waring Blendor was used to simulate the effect of the corn cutter in fragmenting larvae. While fragments could be produced by this means, their appearance under microscopic observation was that of fresh larva fragments, larger in size than those usually found, and discernibly different in appearance from those which occur naturally.

Despite the obvious differences between fragments prepared artificially in the laboratory and those occurring naturally in canned corn, these fragments were considered the best material available for quantitative tests of the efficiency of the oil flotation method of recovery. Following preparation, the fragments were separated into groups. Each group contained only one type of fragment. The fragments used in the analyses were leg fragments, head fragments, seta, sclerites, and false feet. These were added to 200-gram samples of corn in amounts of 5, 10, 20, and 30

per sample, each type of fragment being added at each level in duplicate. For every group of six samples, two of the six were devoted to control determinations to which no fragments were added, in an effort to establish

TABLE 4.—*Per cent recovery from cream-style corn of five types of corn borer fragments using oil flotation method*

FRAGMENTS	% RECOVERED AFTER ADDITION OF:				AVERAGE
	5 FRAG.	10 FRAG.	20 FRAG.	30 FRAG.	
Leg	40	70	70	48	57
Sclerites	60	55	58	65	60
Head	50	30	58	28	42
False foot	70	45	58	45	55
Seta	20	45	53	53	43
Average	48	49	59	48	51

TABLE 5.—*Frequency distribution of naturally occurring insect fragments in canned corn*

By Types

FRAGMENT	FREQUENCY
Antenna	6
Aphid legs	22
Breathing vent	15
Cornicle	4
False foot	6
Head	2
Head fragments	380
Larva fragments	116
Legs	81
Mandible	23
Seta	66
Sclerites	242
Skin	245
Tubular fragments	40
Wing	9
Whole borer	8
Whole insect	88
Half insect	12
Total	1,345

the absence of naturally occurring corn borer fragments. The per cent recovery for each type of fragment is indicated in Table 4. Although there were variations in results, no systematic variation is evident either among types of fragments or among the different levels. A frequency distribution

of the types of fragments occurring naturally (Table 5) indicates that head, sclerite, and skin fragments were found more often than the other types listed. This situation, however, could easily be the result of factors other than the specific efficiency of recovery inherent in the use of this method.

The oil flotation determination requires trapping of the oil layer after a one-half hour period, and again after an additional ten minutes. It was thought that repetition of this entire procedure on a sample might allow recovery of additional fragments. In an effort to test this idea, oil flotation determinations were made in duplicate, and were repeated five times on each of six samples to determine the efficiency of the oil flotation method

TABLE 6.—*Number and cumulative per cent of insect fragments recovered by consecutive oil flotations*

SAMPLE NO.	NUMBER OF FLOTATIONS										TOTAL FRAGMENTS
	1		2		3		4		5		
	No.	*Per cent*	*No.*	*Per cent*	*No.*	*Per cent*	*No.*	*Per cent*	*No.*	*Per cent*	
1	30	66.7	11	91.1	4	100	0	0	0	0	45
2	29	69.1	12	97.6	1	100	0	0	0	0	42
3	16	69.6	6	95.7	1	100	0	0	0	0	23
4	19	63.3	9	93.3	2	100	0	0	0	0	30
5	23	71.9	7	93.8	2	100	0	0	0	0	32
6	20	69.0	7	93.1	2	100	0	0	0	0	29
% Recovery Average		68.3		94.1		100	0	0	0	0	

in removing insect fragments for analysis. The use of several oil flotations on the same sample was made possible by removal of the excess water with heat upon completion of each determination. After each oil flotation the residue was returned to the Wildman trap flask and carried through the procedure again. The results are shown in Table 6. The averages indicate that approximately 70% of the fragments which can be removed by this procedure were removed by the first flotation; approximately 95% by two flotations; and, 100% by three flotations. The procedure was continued through the fifth flotation as an assurance that no fragments remained in the sample. In similar sets of samples where whole worms or large fragments were added, the over-all recovery was approximately 65%.

Two series of samples containing five cans in each sample were set up to determine whether any differences in recovery might be expected when the corn and oil were mixed directly, as compared with a procedure involving the mixture of 200 grams of corn with an equal amount of warm water, followed by addition of the castor oil. Two 200-gram samples were taken from each can. One of each pair was run by the first method and

the other by the second. Results indicated no significant difference between the two procedures. There is however, some advantage to be expected from the use of the mixture of water, corn and oil because of the ease in mixing.

DISCUSSION

The results obtained from the A.O.A.C. method for the estimation of insect fragment contamination in canned cream style corn indicate that the discrepancies observed resulted mainly from two general sources, (1) the inhomogeneity of the sample and (2) low recoveries inherent in the method. It seems likely that any corn borers or other insects which are cut apart in the process of manufacture remain fairly close together and are not homogeneously mixed in the product. Additional work is needed

TABLE 7.—*Analysis of variance*

COMPONENTS OF VARIANCE	ORIGINAL M.S.	TRANSFORMED M.S.
Operators×samples within cans	0.271	0.0161
Samples within cans	1.904	0.1176
Operators×cans	0.069	0.0299
Operators	0.006	0.0003
Cans	0.927	0.0714

to indicate whether thorough mixing of samples or batches may give more nearly homogeneous results. This applies specifically to the entire run of corn as it goes through the plant, and to the mixing of the can contents when duplicates are drawn from the same run.

An explanation of the low per cent recovery of insect fragments in the oil-wetting procedure may be that all fragments are not wet by the oil. It may well be that an investigation of the effect of thorough mixing on the wettability of insect fragments may facilitate improvement of this part of the procedure.

A statistical analysis of several large groups of insect fragment analyses has been made. The data were found to follow the Poisson distribution, and all values were transformed from Y to $\sqrt{Y+1}$ to facilitate an analysis of variance (9), which is shown in Table 7. These data indicate that the chief sources of variability were between cans and between duplicates. The extremely low variance among operators does not represent a true picture, because the operators collaborated closely in analyzing samples and reporting results.

In Table 8 is given the size of variation to be expected in terms of the standard error of the mean. It will be noted that this variation is dependent, not only on the number of cans sampled, but also on the average

number of insect fragments found per sample of 200 grams. It is interesting to note that, for an average of 2 insect fragments per sample of 200 grams, a sample of ten cans would be required to allow a standard error of .5344; while 1000 samples would be required for a standard error of .0774 at the same level of the contamination.

TABLE 3.—*Standard errors for sample means*

SAMPLE MEANS	NUMBER OF CANS SAMPLED				
	10	20	50	100	1000
0.5	.3779	.2694	.1732	.1273	.0550
1.0	.4363	.3111	.2000	.1470	.0632
1.5	.4879	.3478	.2236	.1463	.0707
2.0	.5344	.3811	.2449	.1800	.0774
3.0	.6171	.4400	.2828	.2078	.0894
4.0	.6899	.4919	.3162	.2324	.1000
5.0	.7558	.5389	.3464	.2546	.1095

Note.—Units in this table are fractions of insect fragments per sample of 200 g.

Although these data are sketchy in nature, they may be assumed to represent a point of departure for further research directed toward the definition of sampling procedures and sampling sizes to provide accurate estimation of insect fragment contamination.

SUMMARY

Investigation of the oil flotation method has indicated that the efficiency of recovery is approximately 70 per cent. The replacement of alcohol with water as a washing material and the addition of hydrochloric acid to hydrolyze starch, in conjunction with the use of an automatically emptying filtering apparatus, decreased the time necessary for each determination.

Preparation in the laboratory of samples containing 5, 10, 20, and 30 fragments of five types of corn borer parts indicated no well defined differences in the efficiency of recovery among types of fragments. Repeated oil flotations on the same sample allowed recovery of additional insect fragments with three flotations, indicating that approximately 70% of the insect fragments are recovered in the first flotation, 95% in the first two, and 100% with three flotations.

Variations between lots, between cans, and between duplicates within a can, were found to be much higher than variations resulting from preparation and observation by different operators. A table of standard errors has been presented by which it is possible to adjust the sampling requirements if the sample means are known.

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THE DETECTION OF SUN-CURED ALFALFA IN DEHYDRATED ALFALFA MEAL

By WILLIAM B. BREW*

INTRODUCTION

The regulations of the Association of American Feed Control Officials state (1) that the term "dehydrated" may not be applied to any alfalfa product containing an admixture of sun-cured alfalfa. The Trade Rules of the American Dehydrators Association (2) permit the term "dehydrated" to be used in describing an alfalfa product, provided that the "freshly cut alfalfa having a moisture content of not less than 50% has been artificially dried at a temperature of at least 100°C. or 212°F., that the drying process covers a period of not more than forty minutes, and that there be no admixture of sun-cured alfalfa." The use of sun-cured alfalfa mixed with dehydrated alfalfa meal and sold as the latter is clearly a case of adulteration.

Control and prevention of the above practice has been hampered by the lack of a specific test for the presence of sun-cured alfalfa meal admixed with dehydrated meal. As a result, adulteration of dehydrated alfalfa meal in this manner has been difficult to detect, and of too common occurrence.

* Ralston Purina Co., St. Louis 2, Mo.

Sun-cured and dehydrated alfalfa meal differ from each other in many respects, but in most cases the difference is in the degree with which some attribute is possessed. Such differences do not lend themselves readily for use as a qualitative test for the detection of sun-cured alfalfa in the presence of dehydrated alfalfa.

One demonstrable exception to the above statement, with limitations to be pointed out later, is the water-soluble, heat-coagulable fraction of the alfalfa protein. This fraction of the protein is denatured by the heat of the dehydration process and altered to a water-insoluble form. It is this protein fraction that serves as the basis for the test to be described.

Fourcroy (3) in 1789 gave an account of the heat-coagulable protein in the juices of various parts of many plants. More recent work by Osborne and co-workers (4) and a series of papers by Vickery (5) have described extensive experiments on the protein fractions of alfalfa. Such experiments have indicated the presence in fresh alfalfa of a group of water-soluble, nitrogen-containing compounds, part of which have the characteristics of albumin protein coagulable by heat, and the other part of which cannot be coagulated by heat.

Sun-cured alfalfa has been found to be similar to fresh alfalfa in that it contains the albuminous protein fraction in a water-soluble form. Under the conditions of the test to be described, cold water will readily extract about one third of the total nitrogen from ground samples of sun-cured alfalfa. Of this extractable portion, approximately one-half is in the form of albuminous protein susceptible to coagulation by heat, whereas the other half is made up of noncoagulable water-soluble nitrogen.

In contrast to sun-cured alfalfa, dehydrated alfalfa meal from high temperature dehydrators, as the result of heating during the dehydration process, does not have an appreciable albuminous protein fraction present in a water-soluble form. The temperature of dehydration is such that the albuminous protein fraction originally present in the fresh alfalfa is denatured and rendered insoluble in water. Hence, the nitrogen-containing materials extractable from dehydrated alfalfa meal are only those not susceptible to heat coagulation.

If cold aqueous extractions of sun-cured and of dehydrated alfalfa are made and clarified by filtration, the one from the sun-cured alfalfa will contain heat-coagulable proteins, whereas that from the dehydrated alfalfa will contain none of the heat-coagulable fractions. If both filtered extracts are heated to boiling for a short period of time, the extract from the sun-cured alfalfa will exhibit a heavy flocculent precipitate of coagulated protein, whereas the extract from the dehydrated alfalfa will remain essentially clear or only slightly cloudy.

METHOD

Add 25 ml of distilled water to 2 g. of the ground alfalfa to be tested and allow the mixture to stand with occasional shaking, but without heat, for 1 hour. (Shorter

periods of standing, and unweighed samples, will usually give a satisfactory test.) Filter the extract thru a filter paper into a test tube. Place the test tube containing the clear filtered extract into a boiling water bath and heat for 5 min. Observe the appearance of the heated soln immediately after removing from the water bath.

Any considerable precipitate is normally indicative of the presence of sun-cured alfalfa. The amount of precipitate is a rough indication of the amount of sun-cured alfalfa present. A little experience with known mixtures will soon enable the experimenter to interpret the test.

This test should not be applied without some knowledge of the type of dehydrating equipment used to produce the alfalfa in question. The test has been completely successful only on dehydrated alfalfa produced by high temperature dehydrators, usually of the drum type. A few low temperature dehydrators of the belt type, in combination with a degree of field drying, produce a product which gives erratic results with the proposed test. Possible causes of these erratic results are discussed in the following section of this paper.

APPLICATION OF METHOD

The above procedure has been applied to a number of mixtures of sun-cured alfalfa and drum-dried dehydrated alfalfa, both as known and

TABLE 1.—*Test for sun-cured alfalfa on blind samples*

SAMPLE NO.	RESULT REPORTED	ACTUAL COMPOSITION	
		DEHYDRATED	SUN-CURED
		per cent	*per cent*
1	Negative (no sun-cured)	100.0	00.0
2	Positive (over 25%, estimated)	66.7	33.3
3	Positive (over 50%, estimated)	50.0	50.0
4	Positive (over 50%, estimated)	25.0	75.0

as (to the analyst) unknown mixtures. In every case percentages of sun-cured alfalfa in excess of 10% were readily detected. The results on approximately 200 lots of alfalfa tested show no discrepancy between the results of this test and the verifiable information on the samples concerned. A typical set of results obtained on a set of samples submitted as unknowns, together with the composition as supplied by the submitting laboratory, are given in Table 1.

The following experiment was run in order to verify the fact that the test was the result of the effect of heat during dehydration:

A sample of sun-cured alfalfa meal, giving a heavy precipitate according to the method described, was subjected to dry autoclaving for 15 minutes at 15 lb. steam pressure. At the end of this treatment the sample of sun-cured alfalfa was retested and gave a completely negative test: that is,

no heat-coagulable protein could be extracted. This would indicate that the albuminous protein fraction, originally water-soluble in the sun-cured alfalfa, is denatured by heat and rendered insoluble to water in the dehydrated form.

The following experiment was run in order to establish what conditions of time and temperature during dehydration would effect the results of the test:

Samples of freshly cut alfalfa were dried in a large, forced-draft, hot-air

TABLE 2.—*Effect of drying conditions upon sun-cured alfalfa test*

TEMP.		DRYING TIME IN MINUTES					
		5	10	20	30	40‡	60
60°C.	Precip.	Heavy	Heavy	Heavy	Heavy	Heavy	Heavy
	Mois. %*	76.5	76.6	72.7	70.5	65.9	58.0
80	Precip.	Heavy	Heavy	Heavy	Heavy	Light	None
	Mois. %	78.1	74.8	62.5	55.7	50.8	16.0
90	Precip.	Heavy	Light	None	None	None	None
	Mois. %	75.7	75.2	56.7	39.8	23.4	2.9
100†	Precip.	Heavy	Light	None	None	None	None
	Mois. %	74.8	68.9	45.3	30.0	21.4	3.1
120	Precip.	None	None	None	None	None	None
	Mois. %	71.6	44.8	40.8	11.0	1.3	1.4

* Initial Moisture—82.3%.
† Minimum Temperature Allowed.
‡ Maximum Time Allowed.

oven for varying periods of time at various air temperatures. In each case the alfalfa was spread out in a thin layer on cheesecloth in a manner permitting the rapid passage of hot air over the alfalfa. Samples were removed at predetermined intervals and assayed for moisture and water-soluble, heat-coagulable protein. In order to test for the latter without using ground samples, each sample of whole alfalfa for assay was ground in a mortar with sand and water before proceeding with the test as usually carried out. Table 2 shows clearly that, with increased temperature and time, coagulation of the cold-water-soluble albuminous protein proceeds to the point at which no detectable precipitate is produced under conditions of the described test. In other words, no positive test for sun-cured

alfalfa could be obtained on any dry sample obtained under time and temperature conditions specified for the use of the term "dehydrated."

A possible explanation of a positive test for sun-cured alfalfa on some belt-dried products is that belt type dehydrators may produce a less uniform drying action. In drum dehydrators the alfalfa is constantly in motion; in belt dehydrators uneven loading of the belt may permit some alfalfa to pass thru the dehydrator so protected by a heavy layer of surrounding alfalfa that it may never have been heated sufficiently to coagulate the protein. Hence, some samples may not give a test for a completely dehydrated product, despite the fact that the definition of dehydrated meal has been literally met. This explanation would seem to best account for variable results obtained on lots of alfalfas presumably processed alike.

From the foregoing it may be seen that the test should be limited in application to dehydrated alfalfas known to have been produced in high temperature dehydrators. Since these constitute the great bulk of dehydrators now in operation, this limitation is not serious.

SUMMARY

A method has been developed capable of detecting sun-cured alfalfa as an adulterant in dehydrated alfalfa meal in amounts in excess of 10%.

ACKNOWLEDGMENTS

The author wishes to acknowledge his gratitude to E. L. Schneider and J. T. Stickley, Ralston Purina Co., St. Louis, Mo., for their assistance in performing many of the analytical tests, and to L. M. Faris, W. J. Small Co., Kansas City, Mo., for the unknown samples tested.

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THE CO-DETERMINATION OF INDOLE AND SKATOLE

By CHARLES S. MYERS*

The following procedure presents experimental data for pure solutions, whereby (A) indole may be determined in the presence of skatole, and (B) skatole may be estimated by calculations based on the difference between two spectrophotometric readings. This procedure is based on the well known Ehrlich reaction of indole with *p*-dimethylamine-benzaldehyde as modified by Chernoff (1).

In an early publication, Heimrod and Levene (2) described the use of phosphoric acid plus ferric chloride as the condensing reagent for Ehrlich's reaction. Later, Winkler (3) reported the use of this acid for the condensation of tryptophane and glyoxylic acid, and Morgan and Elson (4) note that glacial acetic acid enhanced the color development in the reaction between glucosamine and *p*-dimethylaminobenzaldehyde in the presence of mineral acid.

Chernoff's modification involves a more radical departure from the conditions of the original Ehrlich reaction, since it uses 85% orthophosphoric acid as the condensing acid, and glacial acetic for color development. The concentrated orthophosphoric acid also functions as a convenient extractant, from chloroform solution, of what is evidently a colorless form of the indole-aldehyde condensation product. On addition of glacial acetic acid to the orthophosphoric acid extract, the characteristic color rapidly develops to a maximum and remains stable for several hours.

Although providing advantages with respect to convenience, reproducibility, sensitivity, and ready adaptability to a wide variety of samples, Chernoff's modification possesses little, if any, greater specificity than the original, very non-specific Ehrlich reaction. Clarke *et al.* (5) noted the presence of an interfering substance on applying the procedure to butterfat distillates. More recently, Beacham (6) mentioned the presence of interfering substances in fresh and canned oysters, and clams, on applying the modification; Duggan and Strasburger (7) also noted interference in a study of the indole content of shrimp.

In a rather extensive study of the application of the reaction to a variety of fish extracts, an account of which is not within the scope of this report, the writer (8) attempted to find means of improving the specificity of the Chernoff modification. One of the more successful results of this attempt is presented herein, in a somewhat preliminary form, with the hope that the procedure might find some practical application to bacterial cultures and perhaps to other types of material. Incidentally, it is of interest to note that Braun and Silberstein (9) have employed an indicator paper impregnated with methanol, orthophosphoric acid, and

* Pennsylvania Department of Health, Harrisburg. Formerly with U. S. Fish & Wildlife Service, College Park, Md.

p-dimethylaminobenzaldehyde for the detection of indole in bacterial cultures. These authors used orthophosphoric acid because of its nonvolatility (hydrochloric acid is usually employed in the conventional test). Their objective was a general, convenient qualitative test rather than a specific quantitative method.

REAGENTS

1(A) *For indole and/or skatole.*—Dissolve 4.0 g of pure *p*-dimethylaminobenzaldehyde in 5 ml of glacial acetic acid by warming on the steam bath. Add 100 ml of 85% H_3PO_4 and thoroly mix. (If the aldehyde is not a pale yellow in color with a mp. of 73–75°C., it should be recrystallized from acetic acid soln after the hot soln has been filtered from an activated charcoal treatment. Alternately, steam distillation, followed by recrystallization from acetic acid or dilute (about 50%) ethanol, may be used.)

1(B) *For indole only.*—Dissolve 5 g of the aldehyde, prepared as above. To this add a mixture of 100 ml of 85% H_3PO_4 plus 25 ml of H_2O. Thoroly mix for use.

2. *Glacial acetic acid U.S.P. with particular reference to the test for oxidizable material).*—Test about 50 ml according to the method described below for indole and skatole. If any color is formed, reflux the acid with $KMnO_4$ and distill.

3. *Chloroform, alcohol-free.*—Wash U.S.P. chloroform several times with H_2O to remove alcohol.

(It might be well to mention the corrosive action of the final H_3PO_4-glacial acetic acid mixture on the skin. Although apparently benign, and practically unnoticeable at the time of original contact, painful burns may occur if the acid mixture is not immediately and thoroly removed. Therefore, tubes or pipettes controlled preferably by aspirator pump or similar device, are recommended for transfer of the final colorimetric soln to the cells. For the protection of the instrument it is advisable to cover the cells while readings are being taken.)

COLOR REACTION FOR INDOLE AND/OR SKATOLE

To 40–50 ml of a $CHCl_3$ soln of the standard, or extract of the sample, contained in a suitable glass stoppered vessel (graduated cylinder or volumetric flask), add 5 ml of reagent **1(A)** and shake vigorously for *one minute.* (For quantitative results, this reaction time is more critical for skatole than for indole.) After the H_3PO_4 layer has separated cleanly, draw off as much of the $CHCl_3$ as possible, without disturbing the lower layer. (This may be conveniently accomplished by means of a tube attached to a water pump, with the end of the tube drawn out into a curved capillary.) Dilute the H_3PO_4 layer to 50 ml with the glacial acetic acid, and thoroly mix. Allow to stand for ca 5 min, then read the resultant color at 565 mμ. (With 1 cm cuvettes, as supplied with the Beckman spectrophotometer, indole concentrations up to about 100 micrograms per 50 ml and skatole concentrations up to about 300 micrograms per 50 ml are conveniently determined.)

Prepare a reagent blank in the same manner, starting with 30–50 ml of $CHCl_3$.

COLOR REACTION FOR INDOLE ONLY

With another aliquot of the $CHCl_3$ standard soln, or extract of the sample, repeat the above procedure, substituting 5 ml of reagent **1(B)** for reagent **1(A)**. (Although there should be no appreciable difference between the two reagent blanks, it is advisable to prepare a separate blank for reagent **1(B)** in the same manner as above.)

DISCUSSION

The concentration of reagent used in both of the above procedures is considerably higher than that recommended by Chernoff. This was

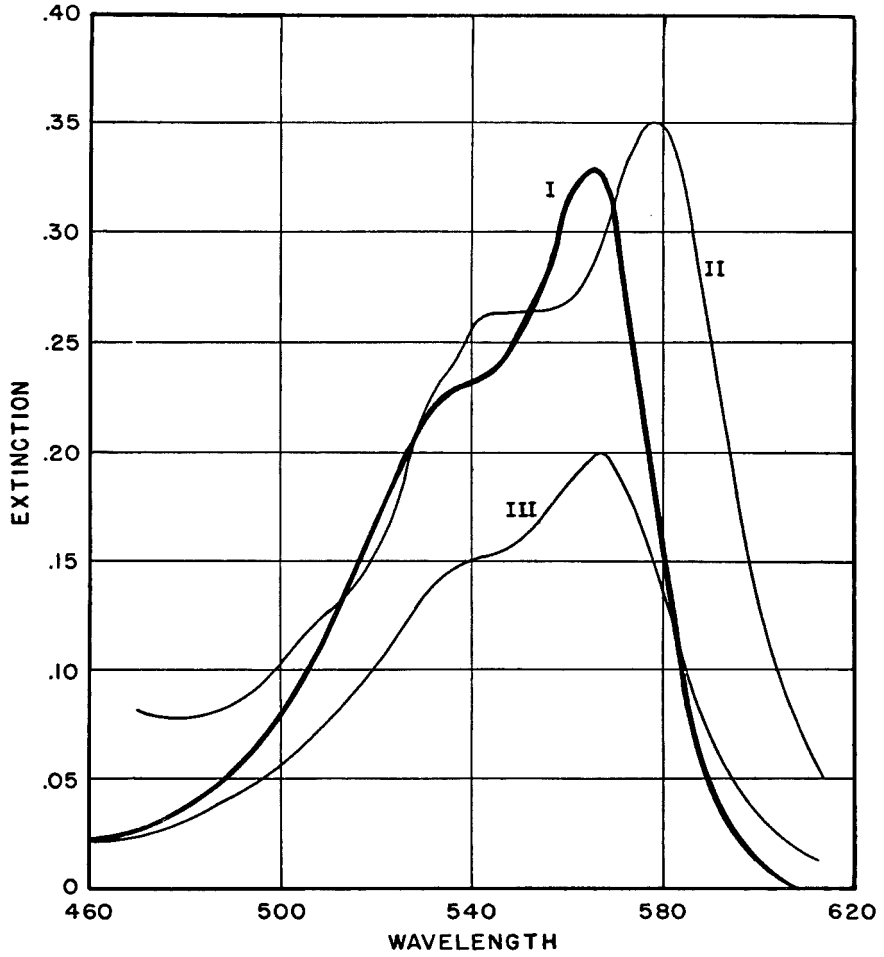

FIG. 1.—Absorption curves of indole, skatole, and their mixture, using reagent 1B.

Curve I, 25 mmg Indole per 50 ml Solution.
Curve II, 80 mmg Skatole per 50 ml Solution.
Curve III, 10 mmg Indole plus 20 mmg Skatole per 50 ml Solution.

found essential for good reproducibility of results for skatole. For samples of biological origin it is desirable to separate the chloroform and orthophosphoric acid layers, and to dilute the latter with the glacial acetic acid as soon as a clear separation has occurred on standing. In the case of sample extracts, centrifuging at a mild rate of speed may hasten this step.

This work was done with chloroform solutions of pure indole, skatole,

and their mixtures. A Beckman spectrophotometer with the tungsten lamp as source of light, and 10-mm. corex glass cuvettes were used. Absorption curves for each substance, and for known mixtures, were determined through most of the visible range, after reaction by each of the two methods described above. In addition, standard curves for both compounds were prepared at their respective absorption maxima.

Figure 1 gives the absorption curves of indole, skatole, and their mixture, using reagent **1(A)**. It is believed that these data provide a reliable basis, first, for testing the purity of indole and skatole, and, second, for selecting the proper filter-photometer assembly for quantitative routine assay if an abridged instrument is employed.

Similar data are plotted in Figure 2, except that reagent **1(B)** was used. Curve IV of Figure 2, however, is simply that for the mixture represented in Figure 1, transferred for convenient comparison of the curves. Further curves (not shown) prove conformity with Beer's law when both the indole and skatole colors are developed with reagent **1(A)** and read, respectively, at 565 and 578 mμ.

The skatole absorption curves possess the same general shape as the indole curves (Fig. 1). The only real difference is that the characteristic "plateau," appearing at the 535–555 mμ region, is somewhat more pronounced; otherwise the effect of the methyl group is according to the general rule for such a substitution: *i.e.*, simply a shift of the entire curve toward the longer wave-length portion of the spectrum.

It is evident that it takes about three times as much skatole as of indole to produce equivalent optical densities throughout the " plateau" region; and also at the respective absoprtion maxima for the two colored compounds. It thus appears that a filter photometer with filters centering at about 535 and 570 mμ, respectively, should yield satisfactory quantitative results by this procedure.

Judging from general appearance and melting point, the sample of crystalline skatole from which standard solutions were prepared for this work was not absolutely pure. However, from previous experience, it appears unlikely that further purification would have any material effect on the results herein reported. Nevertheless, the ratio between the indole and skatole absorption coefficients, and perhaps the position of the skatole absorption maximum, might conceivably be slightly inaccurate. It would, therefore, be desirable to repeat the basic data with a skatole sample as pure as it can be made by the rather tedious steam distillation-multiple recrystallization process.

The mechanism of the co-determination of indole and skatole is apparent from a consideration of the curves of Figure 2. If, for example, curve III (10 mmg indole; reagent **1(B)**) is subtracted from curve IV (10 mmg indole; 20 mmg skatole; reagent **1(A)**) near the point of maximum density, skatole can be estimated from the data of Figure 1.

The general parallelism of the curves for the pure compounds (Fig. 1) indicates the difficulty of determining them individually by the conventional method of simultaneous equations obtained by taking readings at two selected wave lengths. The proposed simpler co-determination appears to possess considerably more value for rapid accurate assays.

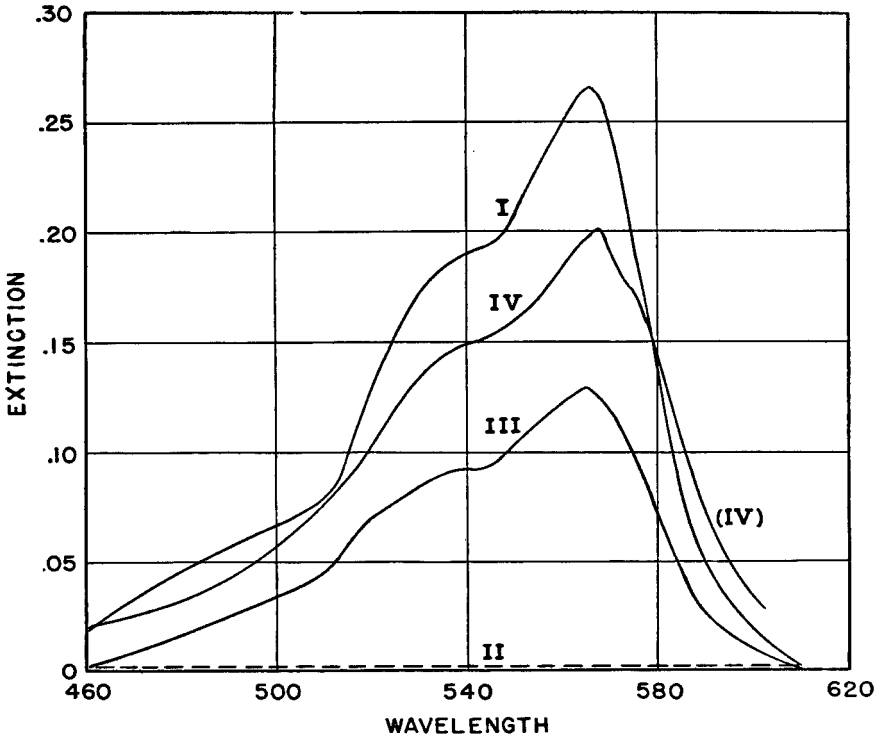

FIG. 2.—Absorption curves of indole, skatole, and their mixture, using 1A.

Curve I, 20 mmg Indole per 50 ml Solution.
Curve II, 100 mmg Skatole per 50 ml Solution.
Curve III, 10 mmg Indole plus 50 mmg Skatole per 50 ml Solution.
Curve IV, 10 mmg Indole plus 20 mmg Skatole (From Figure I: reagent 1A).

Considering that the Ehrlich's reaction involves a condensation with the elimination of H_2O, it is logical to expect that an increase in the concentration of water in the reagent would have an inhibiting effect on the condensation. Furthermore, as shown by Burr and Gortner (10) the reactivity of the hydrogen atoms of the pyrrole ring of indole (and its derivatives) decreases in the following order: beta, alpha, and imide, with the latter rarely involved in condensation reactions. Therefore, the combination of an optimum amount of water in the reagent, plus the effect

of substitution of the beta-hydrogen atom with a methyl group, seems responsible for the observed difference in reactivity between indole and skatole. While the optimum range for the orthophosphoric acid concentration of reagent **1(B)** has not been accurately determined, the concentration given above represents closely the mid point of a total range of approximately 4%; with less than about 72% orthophosphoric acid the indole reaction is affected, while with more than about 76% orthophosphoric acid skatole contributes materially to the color produced by the reagent **1(B)**.

If interfering substances are absent or easily removed, the application of this "differential" procedure to bacteriological cultures appears promising, and it is hoped that it may find usefulness in this field.

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THE DETERMINATION OF GAMMA BENZENE HEXACHLORIDE IN UNHOMOGENIZED MILK AND IN BEEF FAT

By L. E. TUFTS, G. W. DARLING, and R. H. KIMBALL*

The problem of analyzing a rather large number of milk samples, resulting from studies of the use of formulations containing the pure gamma isomer of benzene hexachloride (now known as lindane) in the dairy industry, has led to the development of a relatively simple procedure for the extraction of the butter fat carrying the lindane from the milk sample. This is accomplished by shaking the sample with aqueous potassium hydroxide, followed by a single extraction with hexane. The hexane is removed by evaporation, and the residual butter fat is hydrolyzed with potassium hydroxide in methanol, to convert the fat to soaps and the lindane to trichlorobenzene. After replacement of the methanol with

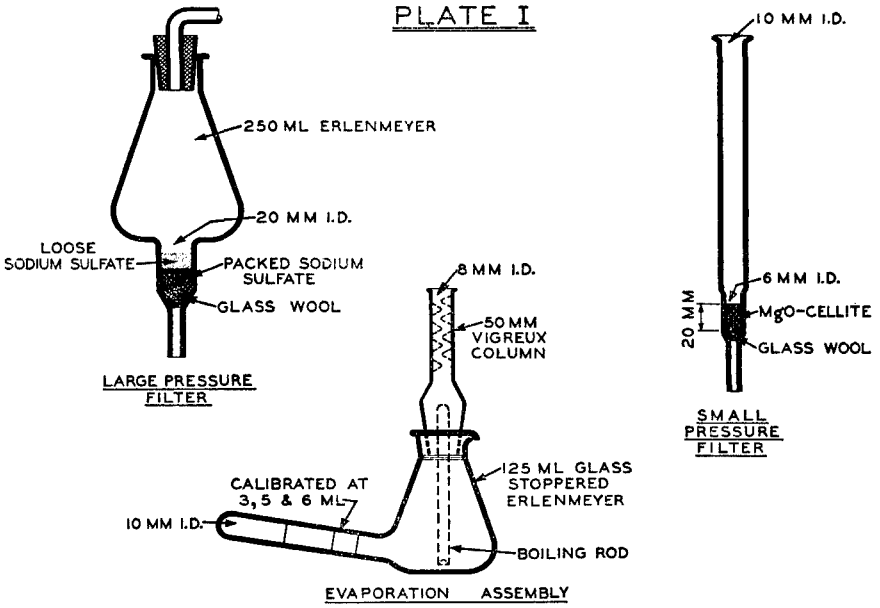
* Hooker Electrochemical Company, Niagara Falls, New York.

hexane, the soaps are washed out with water, and the hexane solution containing the trichlorobenzene and unsaponifiables is treated chemically to remove interfering substances, and concentrated to small volume, in which the trichlorobenzene is determined by ultraviolet absorption.

The method was also applied to the analysis of beef fat. Initial attempts to macerate the fat samples directly with hexane and with ether were unsatisfactory because of the water content of the fat. Acetone was more successful, and gave good recovery of added lindane. However, it was found that direct saponification of the entire fat sample was simpler and gave comparable results.

In general the method is an adaptation of the analysis for benzene hexachloride in animal tissues developed by B. Davidow and G. Woodard of the Division of Pharmacology, Food and Drug Administration, Washington D. C. (1). The same is true of the method of Frawley and Davidow (2).

PLATE I



I. APPARATUS

The special apparatus is shown in Plate 1. The pressure filters make it possible to filter hexane solutions without evaporation losses. The evaporation assembly is used to concentrate the purified hexane solution of trichlorobenzene to exactly 6 ml. without loss of trichlorobenzene. Globular separatory funnels are preferred to the ordinary Squibb type. No grease should be used on either the glass stoppers or the stopcocks.

II. SPECIAL REAGENTS

Glass wool.—Fine Pyrex glass wool, in a porcelain evaporating dish, is brought up to 500°–600°C. in a muffle furnace over 2–3 hours; then the furnace is turned off and allowed to cool. This treatment removes any organic dust or oil.

Methanol.—This should be redistilled over NaOH in all-glass apparatus through a column such as the Snyder, discarding the first and last fractions. This methanol is used to make up the alcoholic potassium hydroxide, and for rinsing apparatus.

Alcoholic potassium hydroxide, 2 N.—132 grams of CP or reagent grade pellet KOH (minimum 85% KOH) is dissolved and made up to 1 liter with redistilled methanol.

Normal hexane.—Commercial grade of normal hexane, from Phillips Petroleum Company. This gave as satisfactory results as their more expensive technical grade. Purification by 28–200 mesh size silica gel, from Davison Chemical Corporation, gave consistent results. It was advantageous to work on a two liter cycle. The hexane was percolated through a 100-cm. column of silica gel in a section of 40 mm (I.D.) glass tubing having a 10-mm bottom outlet. A pad of glass wool in the constricted portion of the tube supported the gel and filtered the effluent hexane. The first liter collected was satisfactory for use in the analytical procedure; the second liter was fed to the column at the start of the next cycle. After each cycle the gel was removed from the column, washed with successive 500 ml. portions of distilled water until no organic layer formed on the water, then reactivated by heating to 350–400°C. for two hours, and transferred to a covered jar while still above 100°C.

Magnesium oxide adsorption mixture.—An intimate mixture of equal weights of Johns-Manville Celite analytical filter aid, with Westvaco Chemical Division adsorptive powdered magnesia.

Anhydrous sodium sulfate.—Eimer and Amend powdered reagent grade.

III. SAMPLING

Milk should be sampled as soon as possible after milking, making sure that it is well stirred to insure an even distribution of the butter fat, which carries the lindane. Convenient containers are one pint glass stoppered bottles, which should be cleaned with ordinary soap and rinsed with distilled water, avoiding synthetic detergents. From 190–220 ml of milk is placed in each bottle, and preserved with 2 ml of 40% formalin per quart of milk. The samples are stored at room temperature, and for best results should be at least 16 hours old before extraction. The procedure described will not extract the butter fat from homogenized milk.

The fat samples were taken by biopsy from test animals, and held in frozen storage. Some additional work was done on suet from local markets to establish the nature of the blanks.

IV. PROCEDURE FOR THE ANALYSIS OF MILK

The bottle neck and stopper are carefully cleaned with methanol and water, and the gross weight is determined to 0.5 gram. To the sample at room temperature, 17 ml of 12 *N* aqueous potassium hydroxide is added, and the bottle is weighed again. It is then shaken steadily, end to end, until the milk coagulates into large curds. This usually takes three to five minutes, but has required up to fifteen minutes in a few cases. Two hundred ml of hexane is then added, and the bottle is weighed again. It is then shaken violently for three minutes, poured into a 500 ml globular separatory funnel, and allowed to settle for ten to twenty minutes, but not longer. With proper handling, about 160–180 ml of hexane separates as a clear layer, and the lower layer of milk can be drawn off and discarded. If an emulsion layer is present, it is retained with the hexane, and is broken by a short vigorous shaking followed by swirling. Any final doubtful portion should be discarded with the milk. Failure to shake the milk with KOH long enough to produce large curds can cause excessive emulsion at this point. Additional extractions usually run into severe emulsion troubles; therefore, only one extraction is used, and the sample weight is corrected for that portion of the hexane which is lost with the milk. Finally, the sample bottle is cleaned, dried, and weighed to give the tare weight used in the calculations.

The large pressure filter is packed with a wad of glass wool under 10 grams of anhydrous sodium sulfate, pressed down firmly. Five grams of sodium sulfate is placed loosely on top of the packed portion of the filter. The hexane layer is shaken violently with 6 to 8 grams of sodium sulfate to remove entrained water, and is poured from the top of the separatory funnel into the filter. It is then forced through the filter into a tared 300 ml standard taper extraction flask containing 2 or 3 boiling chips, by means of carbon dioxide or nitrogen pressure above the solution. (Preferred to air pressure, because less danger of impurities). The clear filtered hexane solution is weighed to ± 0.1 gram. The hexane is boiled off on a steam bath. It is desirable to recover this hexane for repurification with silica gel, since it is considerably purer than commercial hexane. When boiling has nearly ceased, the condenser is removed from the flask. Heating is continued for ten minutes after the mouth of the flask becomes dry. Under these conditions approximately 1.0 gram of hexane remains in the flask, and lindane is not lost. The flask is then cooled and weighed. The weight of residual fat and the weight of hexane evaporated are calculated. The weight of residual hexane in the flask may be checked if desired by running a blank, from which the last of the hexane is removed by blowing a gentle current of carbon dioxide into the mouth of the open flask for five minutes. It can be allowed for in the calculation of the percentage of butter fat. Usually 10 to 20% of the hexane added to the sample is lost by entrainment in the milk and retention in the filter. To

compensate for the incomplete recovery of hexane, the sample weight is reduced by the same proportion as the loss of hexane.

For each gram of residual butter fat in the extraction flask, 7 ml of 2 *N* potassium hydroxide in methanol is added. The flask is attached through the ground joint to a reflux condenser, and refluxed on the steam bath for one hour. One hundred ml of water followed by 50 ml of hexane is added through the condenser, and the mixtur is washed into a 1 liter globular separatory funnel and made up to about 800 ml with water. After thorough shaking and settling, the water layer is drained off into another separatory funnel and washed in the same way with 50 ml of fresh hexane to insure complete extraction of the trichlorobenzene. The second hexane layer is combined with the first. A high velocity stream of water, when directed into the separatory funnel, will cause the formation of such small droplets of hexane in the water layer that excessive time of settling is required. To avoid this, the distilled water is best supplied from a reservoir with a hydrostatic head of two to four feet. Even this slow stream of water should be directed to flow down the inner wall of the funnel, with gentle swirling of the contents.

The combined hexane layers are washed in this way with four 600–700 ml portions of water. To aid in the complete removal of fatty acids, 5 ml of 4 *N* aqueous potassium hydroxide is added and shaken vigorously. Another portion of water is run in and separated, and the treatment with potassium hydroxide and water is repeated. Finally, the hexane solution is washed with five ml of 6 *N* sulfuric acid, and run into a 250 ml flask with a standard taper stopper. Twenty ml of 6 *N* sulfuric acid and twenty ml of 5% potassium permanganate are shaken in the funnel and drained into the flask, which is stoppered and shaken on a machine for thirty minutes. The contents of the flask are then rinsed back into the separatory funnel and diluted to 600–800 ml with water without shaking, and the water layer is discarded. Five ml of 6 *N* sulfuric acid and five ml of 10% CP sodium metabisulfite solution are added. The funnel is stoppered and shaken violently, and 600–800 ml of water is added with swirling, settled and discarded. At the beginning of this separation, the stopcock is loosened to allow the reducing solution to remove any permanganate which has seeped into the joint. The hexane is then washed with 600–800 ml of water. The stopcock is rinsed again as the water layer is drained off. The hexane layer is washed twice with potassium hydroxide as directed above, and finally with three 600–800 ml portions of water, rinsing the stopcock as the water is separated. At the end of the last separation the hexane layer is allowed to just fill the bore of the stopcock. The water in the stem of the funnel is washed out with methanol which in turn is washed out with hexane. The hexane layer is then drained into the special evaporation flask (Plate 1) and the funnel is rinsed down with 10 to 20 ml of hexane. The boiling rod and the evaporation head are set in place,

and the assembly is heated on the steam bath over a 2 inch diameter opening. The sample is allowed to boil down to a volume of 3 to 5 ml for about 20 minutes. After cooling, the evaporation head is removed, the boiling rod is rinsed down with a few drops of hexane, and the solution is made up to 6 ml with hexane and mixed thoroughly. It is then poured into the small pressure filter, which is prepared with a three-quarter inch layer of tightly packed magnesium oxide-Celite absorption mixture above a small plug of glass wool. Eight to ten pounds of nitrogen pressure is used to force the solution through the filter. The first 1 ml of filtrate is discarded, and the remainder is collected in a small flask. This is the solution in which the concentration of 1,2,4-trichlorobenzene is determined by ultraviolet absorption.

V. PROCEDURE FOR THE ANALYSIS OF BEEF FAT

Twenty ± 0.10 grams of the fat sample, sliced into one-fourth inch strips, is weighed into a tared 300 ml standard taper extraction flask containing three boiling chips. Eighty ml of 2 *N* potassium hydroxide in methanol is added. The flask is attached to a reflux condenser, and refluxed on the steam bath for one hour. Then 100 ml of cold water, followed by 50 ml of hexane, is added through the condenser. The mixture is reheated nearly to boiling and filtered hot without delay. For this purpose the large pressure filter is packed with a wad of glass wool, the lower portion being pressed down firmly, the upper layer being packed loosely to trap the gelatinous, fibrous unsaponifiable matter in the sample. Without separation, the hexane and aqueous layers are filtered into a 1-liter globular separatory funnel, using six to eight pounds of gas pressure above the sample. The flask and filter are washed into the separatory funnel with water at 40–50° until free from soap. Water is added to bring the total volume to approximately 800 ml, and the funnel is shaken thoroughly for two minutes. After settling for fifteen minutes the water layer is drained off into another separatory funnel and washed in the same way with 50 ml of fresh hexane to insure complete extraction of the trichlorobenzene. The second hexane layer is combined with the first. The combined hexane layers are washed four times with 50 ml portions of 2 *N* potassium hydroxide, then with five ml of 6 *N* sulfuric acid, treated with permanganate and carried through the remainder of the analysis just as in the determination of lindane in milk.

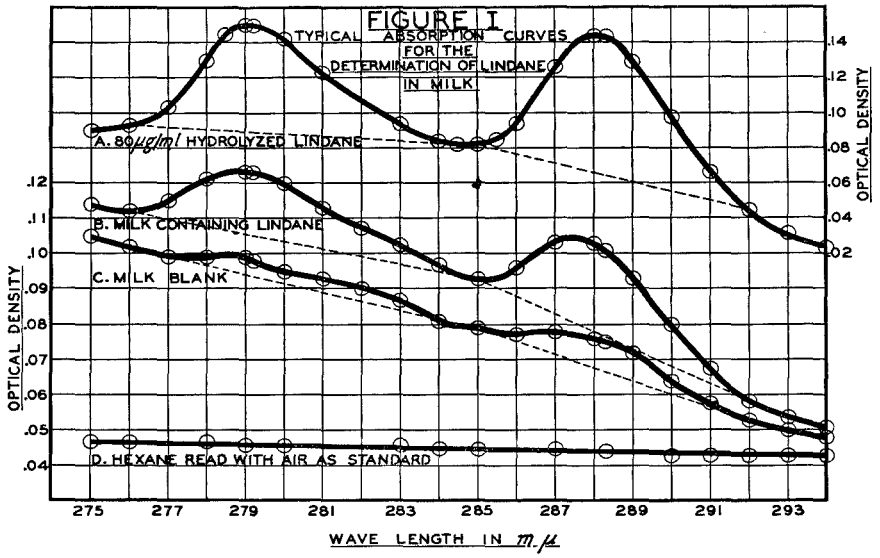
The ultraviolet absorption measurements are made as described for the milk samples in Section VI. Blank determinations should be run, on the most significant samples available, and used to correct the determinations. In the limited number of samples studied, the blanks appear to be somewhat more variable than the milk blanks. A few negative values were obtained, in which the optical densities at 279.2 and 288 mμ were as much as 0.007 below the base line. Other blanks were as high as 0.02 above the

base line. This corresponds to an extreme variation of ±4 p.p.m. of lindane.

It should be remembered, however, that the same variation in the lindane content of the 4–5% of butter fat in a milk sample would correspond to a variation of only ±0.1–0.2 p.p.m. in the milk.

VI. ULTRAVIOLET ABSORPTION MEASUREMENTS

The trichlorobenzene produced by alkaline hydrolysis of lindane is known to consist predominatingly of the 1,2,4 isomer, which is the one determined by ultraviolet absorption. The need for exact knowledge of the isomeric composition is avoided by standardization against known amounts of lindane which have been subjected to alkaline hydrolysis;



the concentration of 1,2,4 trichlorobenzene then serves as a measure of the lindane originally present in the milk.

The instrument used was a model Du Beckman Spectrophotometer with quartz prism and cells. The purified hexane to be employed should be checked for transparency by reading the ultraviolet absorption as directed under Standardization; the absorption curve should be almost flat, as illustrated by curve D in Figure 1.

Standardization.—0.0320 gram of pure gamma isomer is weighed into a 250 ml standard taper extraction flask and dissolved in 25 ml of methanol. Five ml of 2 *N* KOH in methanol is added, and the solution is refluxed on the steam bath for an hour. (Use of a much larger amount of KOH, equivalent to that used in the determination, gave identical

results.) One hundred ml of water and 50 ml of purified hexane are added through the condenser. The sample is washed into a liter separatory funnel with water, and water is added to bring the total volume to 600 ml. The funnel is shaken thoroughly for two minutes to assure complete extraction of the trichlorobenzene into the hexane layer. After discarding the water layer, the hexane layer is washed with six 500–600 ml portions of water. The hexane layer is then filtered into a 100 ml volumetric flask through a layer of sodium sulfate supported in a funnel by a plug of glass wool. After rinsing the separatory funnel and the filter thoroughly with hexane, the filtrate is made up to 100 ml with hexane. This solution contains trichlorobenzene equivalent to 320 micrograms (0.00032 gram) of lindane per ml. From this a solution containing trichlorobenzene equivalent to 80 micrograms of lindane per ml is made by diluting a 25 ml aliquot to 100 ml with hexane.

Optical density measurements throughout the range from 275 to 294 millimicrons are made on this 80 micrograms per ml solution, with the matched cell filled with hexane as zero. The slit width is held at 0.6 mm for all measurements both in standardization and determinations. (A narrow slit width considerably accentuates the maxima and minima.) A characteristic set of optical density readings are plotted as Curve A in Figure 1. Absorption maxima are shown at 279.2 mμ and 288 mμ, with minima at 276, 285 and 292 mμ. Since the instrument used had not been carefully calibrated, the maxima and minima may fall at slightly different wave lengths on other instruments. Careful check readings are made of the optical density at the wave lengths of the five maxima and minima. Similar check readings at the same wave lengths are made on hexane solutions containing 50, 100 and 200 micrograms of hydrolyzed lindane per ml. At least two of these solutions should be prepared from a second hydrolysis of lindane.

The optical density increments at the two maxima can be obtained by the familiar base line method, either graphically or by the following calculation:

A = optical density at 276 mμ
B = optical density at 279.2 mμ
C = optical density at 285 mμ
D = optical density at 288 mμ
E = optical density at 292 mμ

For the 279.2 mμ band, the optical density increment will be:

$$B-(C+0.644(A-C))$$

and for the 288 mμ band:

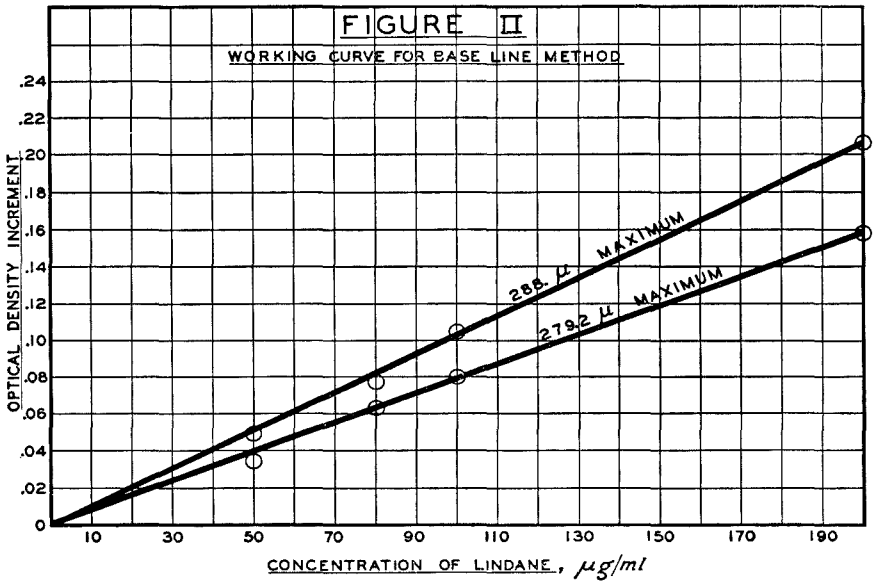
$$D-(E+0.571(C-E))$$

The values for the optical density increments at both bands are plotted against the micrograms of lindane per ml of hexane solution, to give the working curves shown in Figure 2.

In the determinations, the absorption of the 6 ml of hexane solution obtained from each sample of milk is measured by the same procedure

used in the standardization, at a slit width of 0.6 mm with hexane as zero. Duplicate optical density readings at the five wave lengths should check within ± 0.001. The optical density increments at 279.2 mμ and at 288 mμ are calculated as described. Curve B in Figure 1 is the absorption curve of a milk sample containing about 0.45 p.p.m. of lindane.

Milk samples which are known to be free of lindane nevertheless always show small "blank" optical density increments at both absorption maxima. Curve C in Figure 1 is typical of the absorption shown by a



blank milk sample. The optical density increment at 279.2 mμ is 0.0047, and at 288 mμ is 0.0082. In spite of much experimental work these blanks have not been eliminated nor made as constant as would be desirable. Evidence points to both the milk and the reagents (probably the methanol), as contributing to the blank absorption. In size, the blank at each peak may correspond to 0.1 to 0.6 p.p.m. of lindane in the milk, averaging about 0.45. Whenever control samples are available, the optical density increments at each peak shown by the blank are subtracted from the optical density increments obtained from the sample, and the corrected values are used to read the micrograms of lindane per ml of hexane from the working curves in Figure 2. These values are multiplied by 6 and divided by the sample weight (adjusted for loss of hexane) to give the parts per million of lindane in the milk.

Following are the calculations for the determination which gave curve B corrected by the blank which gave curve C:

Optical Density	*Blank*	*Determination*
A = 276 mμ	.102	.112
B = 279.2 mμ	.0985	.123
C = 285 mμ	.079	.093
D = 288 mμ	.076	.102
E = 292 mμ	.053	.059

For the peak at 279.2 mμ:

$$B-(C+0.644(A-C))$$

Blank: $.0985-(.079+.644\ (.102-.079))=.0047$

Det: $.123\ -(.093+.644\ (.112-.093))=.0178$

$.0178-.0047=.0131$ = O.D. increment for lindane

From Figure 2, .0131 = 16.2 μg per ml

Corrected weight of samples = 208.5 g

$$\frac{16.2\times6}{208.5}=0.47 \text{ p.p.m. lindane}$$

For the peak at 288 mμ:

$$D-(E+0.571\ (C-E))$$

Blank: $.076-(.053+.571\ (.079-.053))=.0082$

Det: $.102-(.059+.571\ (.093-.059))=.0236$

$.0236-.0082=.0154$ = O.D. increment for lindane

From Fig. 2, .0154 = 15.0 μg per ml

$$\frac{15.0\times6}{208.5}=0.43 \text{ p.p.m. lindane}$$

Average of 0.47 and 0.43 = 0.45 p.p.m. lindane in the milk.

It is felt that the chance of overlooking interference from abnormal contaminants is greatly diminished by plotting a few spectrograms of both blanks and determinations, and by the fact that the lindane content is determined at two absorption maxima, which should be expected to give fairly concordant results. No abnormal interference has been noted in this laboratory in the analysis of milk from nineteen sources.

VII. DETERMINATION OF KNOWN QUANTITIES OF LINDANE IN MILK

Known quantities of lindane were added to milk in the form of a methanol solution containing 0.01 gram of lindane per 50 ml. Analysis gave the following recoveries:

Added p.p.m.	*Found p.p.m.*	
	at 279.2 Peak	*at 288 Peak*
0.5	0.45	0.49
1.0	0.94	0.84
5.0	4.7	5.0

In the determinations, the results at the two peaks normally agree within 0.2 p.p.m.

VIII. DETERMINATION OF KNOWN QUANTITIES OF LINDANE IN FAT

Known amounts of lindane in methanol solution were added to the fat sample prior to the hydrolysis. The first determination was carried out by acetone extraction, the second by direct hydrolysis of the fat.

Added	*Blank*		*Corr. Determination*	
5.0 p.p.m.				
	279.2 mμ	*288 mμ*	*279.2 mμ*	*288 mμ*
O.D. increment	.0049	.0123	.0119	.0186
Found p.p.m.			5.1	5.2
Added				
10.0 p.p.m.				
O.D. increment	.0165	.0244	.0202	.0274
Found p.p.m.			7.7	8.3

With the use of a control blank it is believed that results showing 0.2 p.p.m. or more of lindane in milk, or 4 p.p.m. in fat, can be considered significant. It is obvious that the same results should be interpreted with considerable caution when no control blank is available.

IX. REFERENCES

(1) B. Davidow and G. Woodard, *This Journal*, **32**, 751 (1949).
(2) Frawley and Davidow, *Ibid.*, **32**, 758 (1949).

A STUDY OF THE USE OF ION-EXCHANGE RESINS FOR THE REMOVAL OF NON-SUGAR REDUCING SUBSTANCES IN THE ANALYSIS OF FRESH AND DEHYDRATED VEGETABLES FOR REDUCING SUGARS

By Kenneth T. Williams, Arthur Bevenue, and Barbara Washauer
(Western Regional Research Laboratory,[1] Albany, Calif.)

The presence of non-sugar reducing substances constitutes a major source of error in the determination of reducing sugars in some plant materials, and these substances are not removed by clarification with neutral lead acetate (5, 8, 9, 10, 13). It has been shown (13) that in the analysis of dehydrated potatoes non-sugar reducing substances were still present in extracts that had been clarified by the lead-phosphate-carbon procedure, even though the clarified solution was water-clear. The use of ion-exchange resins for the removal of non-sugar reducing substances resulted in a major improvement in the clarification procedure (13). The investigation has been continued and this report outlines a simplified technique for the use of ion-exchange resins, including a study of the

[1] Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

comparative effectiveness of various commercially available resins, and includes fermentation data to indicate the removal by the resins of non-fermentable reducing substances.

PROCEDURE

(1) *Ion-exchange resins.*—The resins were used in the form activated by the manufacturers, with the exception of Amberlite IRA-400, Dowex 50, and the Duolites.[2] The exceptions were activated as recommended by the manufacturer. All of the resins were thoroughly backwashed with deionized water and air-dried prior to use.

The time required for the resins and the plant solutions to reach equilibrium was studied. It was found that a contact period of two hours provided a sufficient margin of safety, and this amount of time was used in all of the experiments.

(2) *Extraction of sugars.*—An 80% alcoholic extract of each vegetable sample was prepared and diluted to a definite volume (13).

(3) *Clarification.*—Aliquots of the extracts were evaporated on a steam bath to remove the alcohol. The concentrate was cooled, filtered through a mat of Celite Analytical Filter-Aid and diluted to a definite volume with distilled water (13). The volume was chosen so that the reducing sugar concentration was about 0.4 mg. per ml. of solution. Aliquots of this water solution were used for further clarification. The carbon, lead-phosphate-carbon, and the ion-exchange column procedures have been previously described (13). For the batch-resin procedure, 2.0 g. each of cation- and anion-exchange resins were intermixed in a 250-ml. Erlenmeyer flask, a 50-ml. aliquot of the water solution of the plant extract was added, and the mixture agitated about every 10 minutes for a period of 2 hours. After the 2-hour contact period, the solution was filtered through a fluted filter paper, discarding the first few ml. of the filtrate.

(4) *Fermentation.*—Ten grams of bakers' yeast was suspended in 80 ml. of distilled water. The suspension was centrifuged and the supernatant liquid was discarded. The yeast was washed four times by this procedure and then suspended in 100 ml. of distilled water. A buffer solution was prepared containing 34 g. sodium acetate ($3H_2O$), and 15 ml. glacial acetic acid in 500 ml. of solution. A 50-ml. aliquot of the solution to be fermented was placed in a 100-ml. volumetric flask, together with 2 ml. of the acetate buffer, 2 ml. of 0.2 *M* KH_2PO_4, 6 ml. of the yeast suspension, and 7 drops of Difco invertase. After mixing, the flask was lightly plugged with cotton, and the bulb of the flask was immersed in a water-bath maintained at 38°C. The samples were agitated 5 or 6 times during a 3-hour fermentation period. The fermented solutions were filtered, with suction, through a sintered glass funnel containing a mat of Celite.

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

It was found by fermenting 50-ml. aliquots of a solution of the pure sugars, containing a mixture of 0.02% levulose (d-fructose), 0.02% dextrose (d-glucose), and 0.04% sucrose, for various periods of time, that fermentation was complete in about 2 hours, as determined by the absence of reducing substances. Fermentation of a similar solution of the pure sugars, after treatment with neutral lead acetate and disodium hydrogen phosphate (as used for clarification) was also completed in this period of time.

TABLE 1.—*Determination of reducing sugars*[1] *in extracts of dehydrated white potatoes after clarification with different ion-exchange resins*[2]

METHOD OF CLARIFICATION		METHODS USED TO EVALUATE REDUCING SUGARS					
		HASSID (6, 7)			A.O.A.C. SOMOGYI (2)		
		SAMPLE NO. 1	SAMPLE NO. 2	SAMPLE NO. 3	SAMPLE NO. 1	SAMPLE NO. 2	SAMPLE NO. 3
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Celite[3]		0.86	2.39	2.77	0.65	2.18	2.36
Lead-phosphate-carbon[3]		0.74	2.13	2.24	0.59	2.09	2.15
Amberlite IR-100H, Amberlite IR-4B[4]		0.46	1.88	1.76	0.43	1.93	1.76
RESIN MIXTURES[5]							
CATION EXCHANGER	ANION EXCHANGER						
Duolite C3	Duolite A4	0.44	1.83	1.87	0.40	1.81	1.87
Zeo-Rex	Duolite A4	0.45	1.81	1.87	0.41	1.72	1.87
Zeo-Karb	Duolite A4	0.47	1.81	1.86	0.43	1.72	1.84
Amberlite IR-100H	Ionac A293M	0.46	1.88	1.88	0.43	1.77	1.81
Amberlite IR-120	Duolite A4	0.45	1.84	1.89	0.41	1.76	1.88
Dowex 50	Duolite A4	0.45	1.87	1.90	0.41	1.76	1.86
Ionac C200	Permutit S	0.43	1.89	1.93	0.39	1.75	1.87
Ionac C200	Duolite A4	0.43	1.93	1.92	0.39	1.77	1.90
Amberlite IR-100H	Duolite A4	0.56	1.93	1.94	0.47	1.78	1.88
Amberlite IR-100H	Permutit S	0.54	1.95	1.95	0.45	1.75	1.92
Amberlite IR-100H	Ionac A300	0.54	—	1.97	0.45	1.75	1.96
Amberlite IR-100H	Amberlite IRA-400	0.57	1.93	1.97	0.48	1.86	1.94
Amberlite IR-100H	De-Acidite	—	2.17	—	—	—	—
Amberlite IR-100H	Amberlite IR-4B	0.71	2.03	2.05	0.52	1.93	1.99
Duolite S-30[6]		—	2.38	—	—	2.20	—

[1] Results calculated as percentage of dextrose.

[2] Sources of ion-exchange resins—
American Cyanamid Co., New York, N. Y.: Ionac C200, A293M, and A300.
Chemical Process Co., San Francisco, Calif.: Duolites C3, A4, S-30.
Dow Chemical Co., Midland, Mich.: Dowex 50.
Permutit Co., New York, N. Y.: Zeo-Karb, Zeo-Rex, Permutit S, and De-Acidite.
Rohm and Haas Co., Philadelphia, Pa.: Amberlites IR-100H, IR-4B (both of "Analytical Grade" quality), IR-120, IRA-400.

[3] Celite: Dealcoholized plant-concentrate filtered through a Büchner funnel containing a thin mat of Celite Analytical Filter-Aid (13).
Lead-phosphate-carbon: Water-solution of plant extract treated with excess neutral lead acetate, allowed to stand 15 minutes, then filtered through a filter paper of analytical grade. The excess lead was removed from the filtrate with disodium hydrogen phosphate and the precipitate was removed by filtration. The filtrate was treated with Baker & Adamson, Code 1551, decolorizing carbon (5 mg. carbon per ml. of solution) for ten minutes, and the carbon was removed by filtration.

[4] The resins were used (the cation and the anion resins in separate columns) as described in previous publications (12, 13).

[5] 2 g. cation and 2 g. anion exchange resins (intermixed) per 50 ml. of dealcoholized plant extract; the period of contact was 2 hours.

[6] Described by the manufacturer as a resinous adsorbent which "has no ion-exchange properties in the usual sense."

(5) *Sugar measurement.*—Reducing sugars were determined by the A.O.A.C. Somogyi micro copper method (2) and the Hassid micro ferricyanide method (6, 7). All of the values shown in the tables are the averages of closely agreeing replicates, with a maximum variation of $\pm 2\%$. At intervals during this investigation, all of the analyses were repeated, and the results confirmed the original findings, with the exception of the dehydrated "heat-damaged" carrot (see footnote 4, Table 4).

DISCUSSION

In a previous paper it was shown that potato extracts were more effectively clarified by passing the solution through ion-exchange columns

TABLE 2.—*The effect of ion-exchange resins on the recovery of sugars from a solution containing 0.01% dextrose, 0.01% levulose, and 0.02% sucrose*

RESIN MIXTURE[1]		RECOVERY[2]
CATION EXCHANGER	ANION EXCHANGER	
		per cent
Amberlite IR-100H	Duolite A4	100.5
Amberlite IR-100H	Ionac A293M	98.5
Amberlite IR-100H	Amberlite IR-4B	98.6
Zeo-Karb	Duolite A4	101.5
Zeo-Rex	Duolite A4	98.6
Ionac C200	Permutit S	100.3
Ionac C200	Ionac A293M	97.8

[1] See footnotes 2 and 5, Table 1.
[2] The same recovery was obtained when the sugar solution contained a mixture of 80 mg. KH_2PO_4, 106 mg. $CaCl_2 \cdot 2H_2O$, and 131 mg. $NaC_2H_3O_2 \cdot 3H_2O$ per liter.

of Amberlites IR-100-H and IR-4B, respectively, than by the neutral lead acetate procedure (13). To further simplify the method, these resins were tested in a batch procedure, and it was found that they were not satisfactory for use in this manner (see Table 1).

Since commercially available ion-exchange materials were becoming more numerous and more varied in their properties, and because of the success reported for a batch procedure in the clarification of cane molasses for sugar analysis (11), tests were made on a variety of resin combinations by the batch method. The results of a series of such tests, together with three other clarification procedures, are given in Table 1. The data show that many of the resin combinations were as effective as the column procedure. Other mixtures did not remove all of the non-sugar reducing substances, indicating that any selected resin mixture must be first experimentally verified for its efficiency as a clarification agent.

The data in Table 2 show that the resins, as used in this investigation. did not affect the concentration of pure sugar solutions. This was also

TABLE 3.—*Percentages of reducing sugars*[1] *in dehydrated vegetables*[2] *as determined by different methods of clarification and evaluation*

METHOD OF CLARIFICATION[3]	MINIMUM AMOUNT OF HEAT DAMAGE[4]		HEAT DAMAGED[4]	
	A.O.A.C. SOMOGYI (2)	HASSID (6, 7)	A.O.A.C. SOMOGYI (2)	HASSID (6, 7)
	CABBAGE			
Ionac C200—Ionac A293M	36.6	36.0	34.1	35.7
Amberlite IR-100H—Ionac A293M	36.0	36.8	34.4	36.0
Zeo-Karb—Duolite A4	37.1	37.3	34.8	35.8
Zeo-Rex—Duolite A4	36.4	37.1	—	36.3
Amberlite IR-100H—Duolite A4	37.3	37.3	35.7	36.4
Amberlite IR-100H—Amberlite IR-4B	37.1	37.7	36.6	38.3
Ionac C200—Permutit S	38.6	38.0	36.1	37.3
Amberlite IR-100H—Amberlite IR—4B[5]	36.2	36.6	34.3	35.7
Carbon[6]	39.5	39.7	38.9	39.3
Lead-phosphate-carbon	39.6	40.3	36.6	38.5
Lead-phosphate	42.1	43.2	41.9	44.1
Celite	41.1	43.8	41.1	43.2
	ONION			
Ionac C200—Ionac A293M	30.4	32.0	24.6	26.3
Amberlite IR-100H—Ionac A293M	29.4	32.1	22.6	26.3
Zeo-Karb—Duolite A4	30.3	32.3	22.9	26.5
Zeo-Rex—Duolite A4	30.7	32.4	24.1	26.4
Amberlite IR-100H—Duolite A4	30.7	33.3	22.9	26.8
Amberlite IR-100H—Amberlite IR-4B	30.9	32.7	25.3	28.1
Ionac C200—Permutit S	32.1	34.0	26.0	28.0
Amberlite IR-100H—Amberlite IR-4B[5]	30.1	33.3	23.7	26.3
Carbon[6]	32.6	33.6	26.6	29.1
Lead-phosphate-carbon	30.9	33.7	25.6	28.6
Lead-phosphate	34.0	36.7	28.6	34.0
Celite	33.7	36.2	29.2	33.4
	CARROT			
Ionac C200—Ionac A293M			6.41	6.59
Amberlite IR-100H—Ionac A293M			5.39	5.99
Zeo-Karb—Duolite A4			5.62	6.20
Zeo-Rex—Duolite A4			5.84	6.10
Amberlite IR-100H—Duolite A4			5.73	6.24
Amberlite IR-100H—Amberlite IR-4B			6.10	6.59
Ionac C200—Permutit S			6.76	7.06
Amberlite IR-100H—Amberlite IR-4B[5]			5.62	5.96
Carbon[6]			7.14	7.58
Lead-phosphate-carbon			6.37[7]	7.61[7]
Lead-phosphate			7.44	8.54
Celite			7.22	8.37

See next page for footnotes [1–7].

true when small amounts of salts (*ca.* the quantity of mineral matter present in potato extracts prepared in this investigation) were added to pure sugar solutions. In marked contrast, as much as 70% of the sugars were adsorbed by the resins when solutions of the same sugar concentration contained 80% ethy' alcohol.

There was no apparent correlation between the exchange capacity of each resin and its effectiveness as a clarifying agent.

The clarification study was extended to include fresh and dehydrated cabbage, carrot, and onion; the fresh and dehydrated materials were from different lots. The dehydrated cabbage and onion samples were prepared from the same lots, respectively, of which one portion had been dried under carefully controlled conditions of time and temperature, to minimize the amount of "heat-damage" to the finished product; the other portion had been subjected to excessive heat treatment to obtain a finished product that would be normally considered "heat-damaged."

The values obtained by the two methods of sugar measurement, after clarification of the dehydrated vegetable extracts by various procedures, are given in Table 3. The data show that lead-phosphate clarification does not remove the non-sugar reducing substances from the dehydrated vegetable extracts. When this procedure was followed by treatment with Baker and Adamson Code 1551 decolorizing carbon some of the reducing substances were removed. It has been previously shown that this selected carbon does not adsorb significant amounts of reducing sugars under the conditions used in these tests (3). The values obtained by the column and batch procedures, when certain resins were used, were in agreement and lower than those obtained by the other methods of clarification. The sugar values of the dehydrated vegetables (Table 3) obtained by the A.O.A.C. Somogyi copper method were lower than those determined by the Hassid ferricyanide method, an exception being the "minimum heat-damaged" cabbage sample. The difference between the values obtained by the two analytical methods indicate the presence of non-sugar reducing substances oxidizable by the ferricyanide but not by the cupric ion. In addition, there may be non-sugar reducing substances present that are measured by both of the methods. Fermentation studies were made to obtain information regarding the presence of these substances; the results are given in Table 4.

Footnotes to Table 3.

[1] Calculated as dextrose.
[2] Moisture content 4–8%.
[3] See footnotes 3 and 5, Table 1.
[4] Under simulated commercial dehydrating conditions one portion of the fresh vegetable was dried for a period of time at a temperature to give the "minimum amount of heat damage" as measured by the color of the dried product. Another portion of the same vegetable was given excessive heat treatment, giving a dried product intensely dark in color, such product being called "heat-damaged."
[5] The resins were used (column procedure) as described in a previous publication (13).
[6] An aliquot of the Celite-filtered solution was treated with Baker & Adamson, Code 1551, decolorizing carbon (5 mg. carbon per ml. of solution) for ten minutes, and the carbon was removed by filtration.
[7] See footnote 4, Table 4.

TABLE 4.—*Percentages of reducing substances*[1] *in fresh and dehydrated vegetables, before and after fermentation, as determined by different methods of clarification and evaluation*

METHOD OF CLARIFICATION[2]	CELITE		LEAD-PHOSPHATE-CARBON		IR-100H–A293M		ZEO-KARB—A4	
METHOD OF ANALYSIS	A.O.A.C. SOMOGYI	HASSID	A.O.A.C. SOMOGYI	HASSID	A.O.A.C. SOMOGYI	HASSID	A.O.A.C. SOMOGYI	HASSID
Fresh Vegetables								
Cabbage								
Before fermentation	3.07	2.88	2.96	2.89	2.88	2.69	2.98	2.85
After fermentation	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fermentables	3.07	2.88	2.96	2.89	2.88	2.69	2.98	2.85
Carrot								
Before fermentation	3.06	3.09	3.04	3.13	2.98	3.00	3.07	3.08
After fermentation	0.0	0.11	0.0	0.09	0.0	0.11	0.0	0.11
Fermentables	3.06	2.98	3.04	3.04	2.98	2.89	3.07	2.97
Onion								
Before fermentation	3.15	3.18	3.02	3.09	2.91	3.14	3.00	3.07
After fermentation	0.05	0.35	0.0	0.16	0.0	0.31	0.0	0.31
Fermentables	3.10	2.83	3.02	2.93	2.91	2.83	3.00	2.76
Potato, white								
Before fermentation	1.67	1.81	1.70	1.73	1.59	1.63	1.64	1.65
After fermentation	0.0	0.14	0.0	0.06	0.0	0.06	0.0	0.0
Fermentables	1.67	1.67	1.70	1.67	1.59	1.57	1.64	1.65
Dehydrated Vegetables[3]								
Cabbage, minimum heat damage								
Before fermentation	41.1	43.8	39.6	40.3	36.0	36.8	37.1	37.3
After fermentation	2.2	4.8	0.5	3.4	0.0	1.2	0.0	0.9
Fermentables	38.9	39.0	39.1	36.9	36.0	35.6	37.1	36.4
Cabbage, heat-damaged								
Before fermentation	41.1	43.2	36.6	38.5	34.4	36.0	34.8	35.8
After fermentation	1.8	7.1	0.0	4.0	0.0	1.8	0.0	1.5
Fermentables	39.3	36.1	36.6	34.5	34.4	34.2	34.8	34.3
Carrot, heat-damaged								
Before fermentation	7.22	8.37	[4]	[4]	5.39	5.99	5.62	6.20
After fermentation	1.85	2.93			0.0	1.10	0.13	0.99
Fermentables	5.37	5.44			5.39	4.89	5.49	5.21
Onion, minimum heat damage								
Before fermentation	33.7	36.2	30.9	33.7	29.4	32.1	30.3	32.3
After fermentation	1.2	5.4	0.0	2.3	0.0	1.9	0.0	2.1
Fermentables	32.5	30.8	30.9	31.4	29.4	30.2	30.3	30.2
Onion, heat-damaged								
Before fermentation	29.2	33.4	25.6	28.6	22.6	26.3	22.9	26.5
After fermentation	4.7	9.6	0.7	4.5	0.0	3.1	0.0	2.8
Fermentables	24.5	23.8	24.9	24.1	22.6	23.2	22.9	23.7
Potato, heat-damaged #3								
Before fermentation	2.36	2.77	2.15	2.24	1.81	1.88	1.84	1.86
After fermentation	0.46	0.92	0.20	0.45	0.0	0.29	0.0	0.22
Fermentables	1.90	1.85	1.95	1.79	1.81	1.59	1.84	1.64

[1] Calculated as dextrose.
[2] See footnotes 3 and 5, Table 1.
[3] Moisture content 4–8%.
[4] The measurements on the solutions clarified with lead-phosphate-carbon, obtained at intervals during this investigation, were not reproducible as shown by the following data. For the before fermentation, after fermentation, and fermentable values, respectively, the A.O.A.C. Somogyi method gave: 7.10 −1.20 =5.90; 6.37 −0.84 =5.53; 6.48 −0.80 =5.68; 5.89 −0.86 =5.03.
The Hassid ferricyanide method gave: 7.22 −2.12 =5.10; 8.01 −1.99 =6.02; 7.61 −2.19 =5.42.

The data in Table 4 show that there are no unfermentable reducing substances present in the extracts from the fresh vegetables that are measurable by the copper method (a minor exception was the onion, which showed less than 2% of unfermentable reducing substances). This fact is not true for the ferricyanide method of analysis. The data do not

show conclusively whether the reducing material, measured after fermentation, was present in the original solution or was formed during the fermentation process (14). It has been previously shown that the lead clarification procedure is not essential for the determination of reducing sugars in many types of fresh vegetables (4).

The data in Table 4 also show that resin treatment of the dehydrated vegetable extracts removed all of the non-fermentable reducing substances, as measured by the copper method (a minor exception was the dehydrated carrot, which, after treatment with the resins Zeo-Karb and Duolite A4, still showed about 2% unfermentable reducing substances). The sugar values of the solutions treated by the two different resin mixtures were in agreement, when determined by the copper method. The values for the fermentable substances of the resin-treated dehydrated onion and cabbage extracts were in agreement when determined by either the copper or ferricyanide method. However, in general, there was no consistent agreement between the values obtained by the different clarification procedures when reducing substances remained in the extracts after the completion of the fermentation period. The behavior of these non-sugar reducing substances could be expected to vary throughout the procedures, since they need not be of the same type in the various vegetable extracts.

The analytical data on the dehydrated vegetables indicate that non-sugar reducing substances are formed during the drying process, even though precautionary measures are exercised in preventing so-called "heat-damage." A comparison of the reducing-sugar content of "heat-damaged" samples of cabbage and onion with that of samples damaged a minimum amount shows that a large loss of sugar resulted from the heat treatment. These facts suggest that plant materials should not be dried (as is sometimes practiced) prior to the extraction of the sugars; instead, as directed by the official method of the A.O.A.C. for plants (1), the fresh material should be immersed in "hot redistilled alcohol."

The ion-exchange resins did not change the concentration of pure sugar solutions or similar solutions containing small amounts of salts. Furthermore, there is no loss of sugar from the extracts of fresh vegetables when such materials are treated with ion-exchange resins. Therefore, the authors believe that the resin clarification procedure, followed by the A.O.A.C. micro copper method for the determination of the sugars, gives the most reliable sugar values obtained in this investigation. Also, the batch-resin procedure for clarification is, by far, the simplest from the manipulative standpoint.

The use of ion-exchange resins for the removal of non-sugar reducing substances from extracts of dried vegetables may provide, in part, a method for studying such materials for possible toxic properties.

CONCLUSIONS

In the preparation of plant materials for sugar analysis, the samples should not be dried prior to the extraction procedure. Instead, the fresh material should be immersed in hot redistilled alcohol, as directed by the official method of the A.O.A.C.

Selected ion-exchange resins, used in a batch procedure, provide a simple and effective method for the removal of non-sugar reducing substances from extracts of dehydrated plant materials preparatory to their analysis for sugars.

The determination of sugars in resin-clarified plant extracts by the A.O.A.C. micro copper method gave the most reliable sugar results in this investigation.

Experimental evidence indicates that non-sugar reducing substances are formed in vegetables during the drying process, even though precautionary measures are exercised in preventing so-called "heat-damage."

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THE USE OF ION-EXCHANGE RESINS IN THE DETERMINATION OF FRUIT ACIDS*

By JOHN B. WILSON† (Food and Drug Administration, Washington 25, D. C.)

The A.O.A.C. methods for fruit acids are subject to certain limitations as to the quantities of sugar and acid which may be present in the sample taken for analysis. It is specified that the portion taken for analysis may contain not over 20 grams of total sugars, nor more acid than the equivalent of 3 ml of normal alkali. In the case of the various acids, 3 ml of normal is equivalent to 192 mg. of anhydrous citric, 201 mg. of malic, and 225 mg. of tartaric.

While these limitations have little effect upon the determination of the predominating acid in most fruit products, they are a distinct disadvantage when the analyst wishes to determine an acid of minor importance such as citric acid in apples, blackberries or cherries, which contain only small amounts of this acid. Such a project might be of considerable importance in presenting authentic analyses to demonstrate addition of citric acid to commercial products made from these fruits. The same limitations would preclude the possibility of determining l-malic acid in the fruits listed, together with their acid content and other pertinent data, in Table 1.

TABLE 1.—*l-malic acid content of various fruits*

FRUIT	L-MALIC ACID*	TOTAL ACIDITY*	ADMISSIBLE SAMPLE	L-MALIC ACID PRESENT
	g/100 ml	*g/100 ml*	*Wt. in grams*	*mg*
Blueberry	0.10	1.66	12	12
Currant	0.05	2.35	8	4
Loganberry	0.08	2.10	9	7
Pineapple	0.12	0.96	20	24
Raspberry	0.04	1.34	15	6
Strawberry	0.10	1.01	20	20

* Nelson, E. K. "The Acids of Fruits," American Medicine, New Series, 22: 812–815 (1928).

In the procedure 1°V. = 30.1 mg of l-malic acid contained in the portion taken for analysis. All readings obtained on the samples described in Table 1 would be less than one degree Ventzke, a very unsatisfactory reading from the analyst's point of view.

Maple products form another important field in which the determination of l-malic acid is of value. The "Malic Acid Value (Cowles)," **34.13** in the *Book of Methods,* has earned the reputation of being one of the best

* Contribution from Division of Food (Food and Drug Administration), W. B. White, Chief.
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criteria upon which the analyst may base an estimate of maple content in mixed sirups. If the analyst is in a position to analyze a maple sugar or maple sirup, and mixtures made from it with cane sugar sirup or corn sirup, it will be found that the malic acid value is roughly proportional to the percentages of true maple used.

The malic acid value of a maple product is not due entirely to malic acid, since any other acid forming a calcium salt precipitated by alcohol under these conditions and forming calcium carbonate upon ashing, would increase the malic acid value accordingly. Some years ago Hartmann reported analyses of five maple sirups, correlating malic acid value with l-malic acid determination by a precursor of the present A.O.A.C. method, and concluded that the actual determination of malic acid had no advantage over malic acid value. As this report is not available to readers the results are given in Table 2.

TABLE 2.—*Hartmann's correlation of malic acid value and malic acid determination**

GRADE	COLOR	ORIGIN	% SOLIDS	MALIC ACID VALUE		MALIC ACID	
				WET BASIS	DRY BASIS	WET BASIS	DRY BASIS
1	Light Amber	New York	62.8	0.77	1.22	0.72	1.15
2	Dark Amber	New York	62.6	0.70	1.15	0.68	1.08
2	Dark Amber	Pennsylvania	67.8	0.58	0.85	0.51	0.75
2	Dark Amber	Canada	68.8	0.59	0.86	0.40	0.57
3	Dark Brown	Vermont	68.0	0.72	1.06	0.54	0.80

* Analyses made by Fred Hillig.

Here again determinations of l-malic acid on mixed maple sirups are unsatisfactory because of the low readings obtained. Using the highest malic acid content reported in Table 2, the expected reading on a 10% mixture would be less than 1°V.; on a 15% mixture, about 1.15°V. With this same pure sirup the reading would be only about 8°V. using the A.O.A.C. method. In order to increase the malic acid readings to a satisfying degree through the use of larger samples, the writer turned to the use of ion exchange resins for the isolation of the malic acid, and performed the experimental work for the most part on synthetic samples, maple sirup and mixtures of maple with other sirups.

A number of commercial processes are now in use which call for the use of ion-change resins for the removal of acid and basic constituents from fruit products. Matchett, *et al.* (1) devised a process for recovery of tartaric acid from grape waste materials such as pomace from grape juice, and pomace and still slop from wine and brandy making. Buck and Mottern (2) removed malic acid from apple juice in the manufacture of sirups and later (3) produced malic acid as a by-product. Ion-exchange

resins are also used in the recovery of citric acid and bland sugar sirups from the skin and other inedible parts of pineapples (4) which formerly went to waste. Gore (5) has suggested their use for acid removal in the preparation of sirups from orange and grapefruit juices.

In the laboratory ion-exchange resins were used by Barnes and Kennedy (6) in their study of the products of the reaction between glucose and glycine, by Benson and Calvin (7) in their research on photosynthesis, and by Haas and Stadtman (8) in their study of the compounds involved in browning of apricots. All of these workers used the resins in fractionating the mixtures resulting from these various reactions into acid, basic, and non-ionic fractions as a preliminary step in identifying their constituents.

The writer chose Duolite A-3 anion exchange resin and Duolite C-3 cation exchange resin for the purpose, since several of the workers mentioned above recommended them as being less reactive toward sugars than others. When the order was placed the manufacturer (Chemical Process Co., San Francisco, Calif.) supplied Duolite A-4, a newer and improved product which has displaced Duolite A-3.

It was calculated that quantities suited to the purpose would approximate 35 ml of resin for each of the two columns. Quantities of normal alkali needed to activate the cation column, and of double normal acid needed to activate the anion column, and the quantities of water needed for proper washing were determined by experiment. Those recommended are slightly greater, in the interest of safety, but during the work the wash water was tested frequently to show that the columns were properly washed. To test the cation column for proper washing, pass 10–15 ml of water through the column into a test tube containing about 1 ml of 0.1 *N* silver nitrite soln. If no precipitate of silver chloride is formed the column is ready for use. To test the anion column pass 10–15 ml of water through the column and immerse a piece of *p*H testing paper in the effluent. If the *p*H is below 8.5, the column is in proper condition for use.

Several solutions were prepared to approximate the composition of maple sirup as follows: 50 mg of calcium carbonate and 35 mg of potassium carbonate to supply the cations representative of the insoluble and soluble ash of maple sirup were placed in a beaker, 26 ml of H_2O was added, followed by the quantity of malic acid given in Table 3. The mixture was warmed and gave off gaseous carbon dioxide, and all of the ingredients dissolved. When clear, 50 g of sugar was added and the mixture stirred until all was dissolved. After cooling, determinations of malic acid were made as described below.

Three samples of maple sirup and two samples of mixed maple and cane sugar sirups were obtained on the open market and analyzed by the method. Malic acid value (Cowles) was determined on all samples; l-malic acid was determined on the maple sirups by the shortened version

TABLE 3.—*Malic acid in synthetic solutions*

MALIC ACID ADDED	POLARISCOPIC READING	MALIC ACID FOUND	RECOVERY
mg	°V.	*mg*	*per cent*
52	5.2	50.4	97
100	10.4	100.9	100.9
300	31.0	300.7	100.2
504	50.0	485.0	97.0
600	59.5	575,2	95.9

of the A.O.A.C. method, similar to that used by Hillig in obtaining the results reported in Table 2. The procedure used consisted of the following steps: 1. Heat 30 grams maple sirup to 50°C. and add 3 ml 1 *N* sulfuric acid to decompose salts of l-malic acid. 2. Transfer to a 250 ml volumetric flask using about 25 ml of H_2O, dilute to the mark with alcohol, and filter. 3. Precipitate the lead salts as in the A.O.A.C. method. 4. Decompose lead precipitate with hydrogen sulfide, and filter. 5. Evaporate the filtrate; shake with uranium acetate and read in the polariscope in the usual manner. Results are given in Table 4.

As a test of the clarity of the directions, Sidney Hess of the Division of Food analyzed one synthetic sample and two of the commercial samples by the method. In the synthetic sample he found 332 mg of l-malic acid

TABLE 4.—*Malic acid value and l-malic acid determinations on sirups*

METHOD	ON SIRUP BASIS PER CENT			ON DRY BASIS PER CENT		
	COWLES	HARTMANN	WILSON	COWLES	HARTMANN	WILSON
Sirup						
Pure 1	0.37	0.251	0.255	0.55	0.382	0.382
	0.38		0.250	0.57		0.373
Pure 2	0.44	0.291	0.285	0.66	0.434	0.428
	0.42	0.295	0.282	0.63	0.441	0.421
Pure 3	0.69	0.590	0.560	1.03	0.866	0.836
	0.68	0.579	0.566	1.01	0.863	0.845
			0.565	1.01		0.843
Mixed 1	0.11		0.067	0.16		0.100
	0.11		0.065[a]	0.16		0.097[a]
			0.061[a]			0.092[a]
Mixed 2	0.11		0.072	0.16		0.108
	0.11		0.071[a]	0.16		0.106[a]

[a] 250 g sample used; 400 ml H_2O to dilute; other volumes same as with 50 ml sample.

when 353 mg had been added; in "Pure 3" he found 804 mg per 100 g (dry basis); in "Mixed 1" he found 97 mg per 100 g, using a 50 ml sample, and 97 mg per 100 g using a 250 g sample.

A bottle of commercially prepared apple juice was obtained. Titration of the acidity showed the presence of 0.38 g malic acid per 100 ml. l-malic acid was determined by the shortened Hartmann method as described above, using 50 ml of sample, and by the proposed method using 100 ml of sample; l-malic acid found was 0.464 g per 100 ml by the Hartmann method; 0.427 g and 0.411 g per 100 ml by the proposed procedure.

Another bottle of apple juice prepared in a former year by the same firm was found to contain 0.56 g malic acid per 100 ml by titration; 0.653 and 0.643 g per 100 ml by the Hartmann method, using 35 ml sample; 0.646 g and 0.635 g per 100 ml by the proposed method, using 100 ml sample; and 0.669 g per 100 ml by the proposed method, using a 50 ml sample. In the case of the 100 ml samples a second alkaline extraction of the anion column gave a solution containing 0.016 g and 0.015 g of malic acid which, when added to the quantity found by a single extraction, increased the above determinations to 0.662 g and 0.650 g per 100 ml. A second alkaline extraction of the anion column in the case of the 50 ml sample gave a reading of 0°V., showing no malic acid had been left in the column by the single extraction.

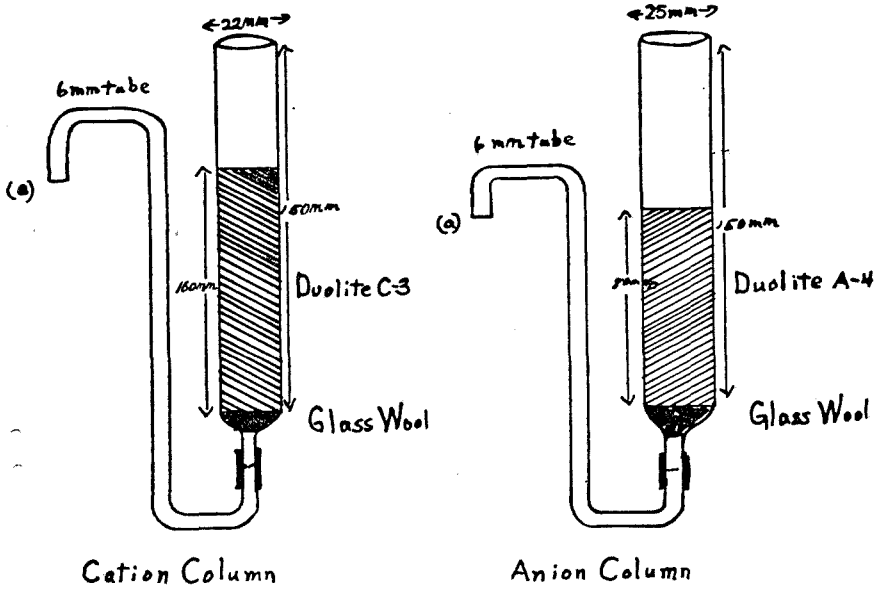

FIG. 1.—Ion-exchange columns.

Assemble the tubes as in Fig. 1, connecting the outlet S-tube with rubber tubing; place enough glass wool in bottom of each to prevent the resins from passing into the outlet tube, and support with a clamp on a ring stand. Place a rubber band around the outlet tube to hold it vertically against the clamp. Fill with water to just cover the glass wool. Now add 37 ml H_2O and mark the large tube at the meniscus. Place beaker under and touching outlet so that effluent will run down the side of the beaker. Add the proper wet resin to the tube until filled to the mark of the meniscus. Fill tube with water and let it drain to equilibrium. Tap sides of tube gently and let it come to equilibrium. The meniscus should be just level with the top of the charge of resin. If the meniscus lies above the top of the resin, the outlet tube must be lengthened at (a); if below, it must be shortened at (a) until just opposite the top of the resin.

ACTIVATION OF THE COLUMNS

In the case of the cation column: activate the resin by passing 100 ml of 2 *N* HCl thru the column and follow with water until the effluent gives no test for chloride with $AgNO_3$. (It was found that 170–180 ml of water was needed; in all future instances a wash of 200 ml of H_2O was used.) Pass 25 ml of *N* NaOH thru the column and follow with sufficient H_2O to cause the *p*H of the effluent to fall to about 5. Repeat the complete cycle 2 or 3 times, reactivate with 2 *N* HCl, wash with 200 ml H_2O, and the column is ready for use.

In the case of the anion column: activate the resin by passing 100 ml of *N* NaOH thru the column and follow with H_2O until *p*H falls to 5 or below. (About 200 ml of H_2O was found necessary to give a *p*H of 5. In all future cases a wash of 240 ml of H_2O was used.) Now pass 100 ml of 2 *N* HCl thru the column, followed by 240 ml H_2O. Repeat this cycle 3 times, reactivate the column with *N* NaOH, wash with 240 ml H_2O, and the column is ready for use.

DETERMINATION

Weigh a 50-ml graduated cylinder on the rough balance, fill to the 50 ml mark with maple sirup and again weigh. Transfer the sample to a 250-ml beaker using about 100 ml of H_2O. Pass the mixture thru the cation column into a 400-ml beaker, follow with 100 ml of H_2O in 7 installments of ca 15 ml each, collecting the washings in the same beaker. Mix the effluent and at once pass thru the anion column and collect in a liter beaker. Now pass 8 30-ml portions of H_2O first thru the cation column, then thru the anion column, washing down the sides of the 400-ml beaker with the water as it issues from the outlet from the cation column. Reserve the effluent from the anion column for study of the sugars, or discard as desired.

Reactivate the cation column with 100 ml of 2 *N* HCl, followed by 200 ml of H_2O, reserving the effluent for a study of the cation fraction, or discard as desired.

Place a 150 ml beaker in position at the outlet of the anion column so that the liquid will run down the side of the beaker. Add 25 ml of *N* NaOH to the anion column in 10 to 12 installments of 2–3 ml each, allowing each addition to pass into the column before the next is added. Follow with 100 ml of H_2O in ca 5 ml installments in the same manner. Tap the side of the column to prevent further drainage after removal of beaker. When no more liquid issues, remove the beaker, mix the contents, and pass thru the cation column, collecting the effluent in a 600-ml beaker. Pass 30 ml of H_2O thru the anion column into the 150 ml beaker, turning the beaker in such a way as to wash down its sides. Tap to complete drainage. Then pour contents of the 150 ml beaker thru the cation column into the 600-ml beaker. Repeat the washing 7 times (using 240 ml H_2O altogether). Add a few glass beads to the 600

ml beaker and evaporate carefully over a flame to ca 10 ml. Transfer the residue quantitatively to a 25 ml volumetric flask containing 0.5 g of sodium acetate. When the flask is nearly full, add 0.5 ml glacial acetic acid; fill to the mark and mix.

Place 0.5 g "Darco G 60" in a 50-ml conical flask, add the above soln, let stand for 30 min. with occasional mixing. Filter thru a 589 SS white ribbon filter paper into a second 50-ml flask. To the filtrate add 2 g uranyl acetate, stopper, wrap in a towel to exclude light, and shake for 10 min. Filter into a third 50 ml conical flask thru a 589 SS blue ribbon filter. Fill a 200 mm polariscope tube with the filtrate and read in degrees Ventzke. Allow the tube to remain at 20°C. overnight, protected from light, and again read. Degrees V. times 9.7 = mg malic acid in sample. Calculate to g malic acid per 100 g sample on the dry basis. (If desired the analyst may check the figure 9.7 by treating weighed quantities of pure l-malic acid as directed above for the sample after evaporation to 10 ml.)

RESULTS

Encouraged by the results of l-malic acid, the writer prepared synthetic samples containing tartaric acid and citric acid with a view to applying the usual procedures for the determination of these acids after isolating the acid fraction by means of the ion exchange resin columns used for l-malic-acid.

A neutralized solution containing 207 mg of tartaric acid by titration was passed through the isolation procedure for malic acid. After evaporation to 20 ml, the solution was neutralized with potassium hydroxide and the tartaric acid determined as in 26.33 in the *Book of Methods*, beginning "Add 2 ml of acetic acid, etc." 195 mg of tartaric acid was found.

The anions were isolated from the neutralized solutions of citric acid as described in the proposed method. The solution was evaporated to about 75 ml and the citric acid determined as directed in the *Book of Methods*, **26.37**, beginning "Cool, and add 10 ml of H_2SO_4 (1+1). . . . " The results are given in Table 5.

TABLE 5.—*Citric acid in water solutions by the proposed method*

ANHYDROUS CITRIC ACID	
ADDED	FOUND
22.9 mg	20.9
45.6	44.5
94.0	84.5
91.6	86.0
184.9	168.5
376.0	364.0
370.5	358.0
376.0	361.0

In the case of the last three results a correction of 20 mg was added to each determination, since it was found that a second alkali extraction of the anion column yielded 20 mg, 18 mg, 20 mg of citric acid. Since the

precipitate of pentabromacetone from 350 mg or more citric acid is quite voluminous, the isolated anions of the last two samples were made to volume and the citric acid determined in an aliquot representing 40 per cent and 25 per cent, respectively, of the entire amount.

While the method has not been applied to mixtures of the acids, the writer sees no reason why any difficulty would be experienced in applying it to mixtures of citric and malic acid, or citric and tartaric acid. However, in case of mixtures of malic and tartaric acids, it would be necessary to remove tartaric acid before polarizing l-malic acid, since both acids have a rotation. It is possible, in the case of mixtures of l-malic and d-tartaric acids, that the l-malic acid can be calculated from the difference in polariscopic reading between two isolated anion solutions, one of which has been treated with uranium acetate and the other has not been so treated.

SUMMARY

A method is presented in which l-malic acid is isolated from maple sirup, mixed maple sirups, and apple juice by means of ion-exchange; and the malic acid determined by polarization in the presence of uranium acetate.

Similar technique is applied to solutions of tartaric acid and citric acid after which the tartaric acid is determined by the bitartrate method, and citric acid by the pentabromacetone method.

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A METHOD FOR DETERMINING TOTAL NITROGEN IN FERTILIZERS CONTAINING CHLORIDES*

APPLICABLE IN THE PRESENCE OF ORGANIC MATTER, CYANAMID, OR UREA

By PHILIP SHUEY (Shuey and Company, Savannah, Ga.)

The investigation of losses in nitrogen caused by the presence of chlorides in fertilizer mixtures was taken up at the 1947 fall meeting of the A.O.A.C., and since that time evidence has been presented to show that some losses occur in certain types of mixtures when using the present official method.

The odor of nitrous fumes after the addition of the sulfuric-salicylic acid solution is a very common observation. Then too, the determination of nitrate nitrogen in waters by the phenol-sulphonic acid method, the tolerance for chlorides is only 100 parts per million (1).

Losses in nitrogen in fertilizer mixtures containing chlorides and nitrates have also been investigated by Dyer and Hamence (2), and by the author (3). It appears that the losses are due to the formation of some nitrosyl chloride (NOCl) which is not fixed by the salicylic acid. Dyer and Hamence experimented with various types of mixtures and found losses by the Jodlbauer method of from 0.12 to 0.90% nitrogen, equivalent to 5 to 20% of the nitrate nitrogen present.

In general, the higher the nitrates and chlorides, the greater will be the loss by the present official method, but it has been found that mixtures very high in chlorides and containing only 2% nitrate nitrogen undergo very considerable losses, in some cases as high as 25% of the nitrate nitrogen present.

Fertilizers very high in chlorides are used in Arkansas, Ohio, Indiana, Wisconsin, and Michigan, and to some extent in the Southeast, and the indications are that this tendency will probably increase. As the tolerances allowed in the various States are usually rather narrow, the importance of making accurate determinations of nitrogen under all conditions is at once apparent.

Various attempts have been made by the author to obtain satisfactory results by reducing the nitrates in an acid solution previous to digestion by the Ulch or similar methods. In general such methods have yielded low results.

The method finally developed is somewhat similar in principle to the Dyer-Hamence method (2), and the Davisson-Parson method (4). It differs in that the water is not boiled off in an acid medium, but is used for distilling the nitrate and ammoniacal nitrogen in an alkaline medium. No additional equipment, such as traps, etc., is required, and no rinsing

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is necessary. The method is considered simple and accurate, and the time required is very little longer than by the official method, since the first step of the distillation may be conducted in about the same length of time that the flasks are required to stand in order to dissolve the nitrates before making the reduction. Further, the digestion is more rapid than with the ordinary procedure.

It should be mentioned that cyanamid-nitrate mixtures in the presence of moisture also have a tendency to yield low results by the official method. This has been thoroughly investigated by Kenneth D. Jacob and Walter J. Geldard (5) using the Davisson-Parsons method. The presence of cyanamid may have been a contributing factor by increasing the temperature on the addition of the sulfuric-salicylic acid solution. Since nitrates and chlorides, and also nitrates and cyanamid in the presence of moisture, cause losses by the present official method, it is reasonable to assume that the losses might be increased when all are present. The method here proposed is applicable to any and all mixtures containing nitrates, cyanamid, and moisture, as well as chlorides.

METHOD

(1) Transfer the weighed sample (0.5000–1.000 g according to the nitrogen content) to a Kjeldahl flask.

(2) Add sufficient Devarda's alloy, 40 mesh and finer, to reduce the nitrate nitrogen present (1 g for each 35 mg of nitrate nitrogen; 2 g is usually sufficient).

(3) Wash down the neck of the flask with ca 125 ml distilled water (150 ml if nitrate content high).

(4) Add a few drops of mineral oil and ca 1 ml of tributyl phosphate to control foaming.

GROUP NO. 1:

	THEORY	A.O.A.C. METHOD	AUTHOR'S NEW METHOD	DIFFERENCE BETWEEN METHODS
	per cent	*per cent*	*per cent*	*per cent*
Grade 8-8-4½		7.96	8.14	
Nitrate Nitrogen	8.00	7.94	8.22	
7.59% and Chlorine 4.32%		Ave. 7.95	Ave. 8.18	0.23
Grade 6-8-6		5.99	7.17	
Nitrate Nitrogen	6.02	6.09	6.29	
5.59% and Chlorine 5.76%		Ave. 6.04	Ave. 6.23	0.19
Grade 14-8-17		13.14	13.90	
Nitrate Nitrogen	13.86	13.24	13.99	
6.45% and Chlorine 16.80%		13.32	13.91	
		Ave. 13.23	Ave. 13.93	0.70

GROUP NO. 2:

	A.O.A.C. METHOD	AUTHOR'S NEW METHOD	DIFFERENCE BETWEEN METHODS
	per cent	*per cent*	*per cent*
(Florida)			
Sample No. 61842			
Total Nitrogen 12%	12.05	12.46	
Nitrate Nitrogen 11.74%	11.97	12.42	
Chlorine 5.60%	Ave. 12.01	Ave. 12.44	0.43
(Florida)			
Sample No. 61715			
Total Nitrogen 12%	11.68	12.04	
Nitrate Nitrogen 5.31%	11.64	12.04	
Chlorine 11.20%	Ave. 11.66	Ave. 12.04	0.38
(Florida)			
Sample No. 61757			
Total Nitrogen 10%	9.75	10.06	
Nitrate Nitrogen 5.15%	9.75	9.98	
Chlorine 7.10%	Ave. 9.75	Ave. 10.02	0.27
(Florida)			
Sample No. 61809			
Total Nitrogen 4%	4.15	4.18	
Nitrate Nitrogen 0.71%	4.14	4.14	
Chlorine 3.70%	Ave. 4.15	Ave. 4.16	0.01
(Virginia)			
Sample No. 1 14-0-14	13.16	13.46	
Nitrate Nitrogen 6.71%	13.16	13.38	
	Ave. 13.16	Ave. 13.42	0.26
(Virginia)			
Sample No. 2 7-7-7	6.95	6.95	
Nitrate Nitrogen from 1.5 to	6.93	7.12	
2.0%	Ave. 6.94	Ave. 7.03	0.09
(Virginia)			
Sample No. 3 5-10-10	5.10	5.27	
Nitrate Nitrogen 0.60%	5.10	5.24	
	Ave. 5.10	Ave. 5.26	0.17

(5) Add 5 ml of strong caustic soda, ca 50%, such as is used regularly in nitrogen determinations.

(6) Connect at once with the regular condenser and distil until only 10–25 ml remains in the flask. (Sufficient standard acid should have been placed in the receiver flask for the total nitrogen present. Kjeldahl and receiver flask should be marked identically to avoid mix-up. Heat applied moderately for first five (5) min. This distillation is completed in ca thirty (30) min. additional.) See note 1.

GROUP NO. 3:

		A.O.A.C. METHOD		AUTHOR'S NEW METHOD	DIFFERENCE BETWEEN METHODS
		per cent		*per cent*	*per cent*
Sample No. 4 (Georgia) 2.16-6.56-30.04		2.04		2.15	
Nitrate Nitrogen 0.25%		2.00		2.15	
Chlorine 29.25%	Ave.	2.02	Ave.	2.15	0.13
(Mississippi) 12-8-8					
Sample No. 5		11.79		12.17	
Nitrate Nitrogen 1.32%		11.75		12.13	
Chlorine 7.68%	Ave.	11.77	Ave.	12.15	0.38
(Arkansas) 5-9-27					
Sample No. 6		4.98		5.25	
Nitrate Nitrogen 1.96%		4.99		5.29	
Chlorine 21.0%	Ave.	4.98	Ave.	5.27	0.29
(Arkansas) 6-9-27					
Sample No. 7		5.44		5.99	
Nitrate Nitrogen 2.13%		5.44		6.03	
Chlorine 21.64%	Ave.	5.44	Ave.	6.01	0.57
(Arkansas) 8-8-8					
Sample No. 8		6.62		6.94	
Nitrate Nitrogen 2.13%		6.66		6.79	
Chlorine 7.50%	Ave.	6.64	Ave.	6.86	0.22

(7) Cool flask somewhat and add 5 ml dilute (1:1) sulfuric acid and twirl; then add 25 ml of conc. sulfuric acid and ca 0.7 g HgO ($CuSO_4$ may be used in place of the HgO as the catalyst, if desired. K_2SO_4, is purposely omitted to prevent caking on cooling).

(8) Boil off the small remaining quantity of water over strong heat, and complete the digestion without lowering the flame (ca thirty min. when mercury is used).

(9) Finish as in the regular Kjeldahl method.

NOTE 1. To check complete reduction of nitrate transfer a drop of residual liquid by means of a long capillary glass tube to a spot plate. Add a minute quantity of brucine sulfate followed by a drop of conc. H_2SO_4. No coloration shows complete absence of nitrates. There should be complete absence of the odor of ammonia when the flask has been disconnected.

NOTE 2. Be sure to connect the Kjeldahl for final distillation to the corresponding receiver used in step 6 above.

NOTE 3. About 1 g 30-mesh granular zinc or about 2½ g broken pumice ranging from size of a small pea to dust may be used to prevent bumping. Zinc dust does not prevent it.

NOTE 4. A mixed indicator made by dissolving 1.250 g methyl red and 0.825 g methylene blue in one liter of alcohol may be used instead of methyl red alone. The mixed indicator appears more sensitive.

NOTE 5. The Devarda's Alloy metal at present furnished by J. T. Baker Chemical Company, Phillipsburg, New Jersey, is 20-mesh and finer. The 40-mesh and finer metal may easily be obtained by sieving, using a 40-mesh sieve, or grinding the coarse metal in an iron mortar to pass the sieve. The finer metal causes quicker action and is almost entirely acted upon by the alkali. Experiments have shown a reduction as high as 52 mg of nitrate nitrogen for one g of the alloy.

In order to show the accuracy of the method, various groups of samples were analyzed by this method, and also by the Official Method, *Methods of Analysis*, A.O.A.C. 1945, Sec. **2.28,** using a gram of salicylic acid in 30 ml H_2SO_4, as specified). The first group shown consists of samples that were prepared in the laboratory, thus furnishing a theoretical analysis. The second group consists of four samples furnished by Gordon Hart, Assistant State Chemist of Florida, and three samples sent by Dawson B. Bates, of Virginia. The third group is comprised of current samples received from Arkansas, Georgia, and Mississippi.

As the last sample given was found to contain no organic matter, cyanamid, nor urea, it was also analyzed by the Devarda method (*Methods of Analysis*, A.O.A.C., 6th Ed. **2.31**) and found to contain 6.79% and 6.87% nitrogen, with an average of 6.83%.

It is observed that in all cases when the nitrates and chlorides were high the results by the A.O.A.C. methods were considerably lower. In the case of Sample No. 7, 6-9-27, this difference was 0.57%. If this difference represents the loss in nitrate nitrogen due to the very high chlorides (21.64% chlorine), as appears to be the case, it amounts to almost 27% of the nitrate nitrogen present.

It will be noticed that in the samples that are relatively low in either nitrates or chlorides, the difference found between the two methods is not so great. When the nitrate nitrogen drops to 0.71% and the chlorine to 3.70% (Florida Sample No. 61809), there was found to be no difference between the two methods.

Attempts to reduce the losses by chilling the sulfuric acid-salicylic acid solution in ice water and combining the chlorine as silver chloride by the use of silver sulfate were without success.

TEST FOR APPLICABILITY OF OFFICIAL METHOD

Add approximately 6 grams of sample to a 400 ml beaker, add 30 ml of the sulfuric-salicylic acid solution, cover quickly with a watch glass, twirl and look through the beaker towards the sky. If no yellow nitrous fumes are observed after about two minutes, there will not be any great loss of nitrogen by the present official method. This test has been found sufficiently delicate to show a positive reaction when 38 milligrams of nitrate nitrogen, along with high chlorides, are present. For example, the third sample given in the table showed a positive test with as little as 0.6 gram. Lack of time prevented the author from doing further work in developing a more sensitive qualitative method.

SUMMARY

It has been shown that there is a tendency to obtain low results for nitrogen by the present official method, in fertilizers containing nitrates and chlorides. When chlorides are high large losses may occur. There is an apparent tendency toward higher nitrates and chlorides in fertilizers.

Analytical data on various mixtures is furnished, and the differences between the official method and the proposed method are shown. No loss was found to occur in the case of the presence of 0.71% nitrate nitrogen and 3.70% chlorine, and the present official method is accurate under these conditions.

A rapid qualitative method is furnished which enables one to determine approximately when the present official method will not apply. The official Devarda method should always be used in the absence of organic matter, cyanamid, or urea, but digestion is essential when any of these is present. A method is proposed that is believed to be accurate under all conditions.

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HYDROGEN-ION ADJUSTMENT WITH *p*H METER FOR CALCIUM PRECIPITATION IN NITRIC-PERCHLORIC ACID DIGESTS OF BIOLOGICAL MATERIALS*

By B. D. HITES and C. W. ACKERSON (From the Department of Agricultural Chemistry, Agricultural Experiment Station, Lincoln, Nebraska)

The method described by Gerritz (1) for the digestion of biological materials with nitric and perchloric acids to prepare samples for the determination of calcium and phosphorus has been found to be simpler, more convenient and time saving in our laboratory than the dry ashing procedure. The method used in the A.O.A.C. *Methods of Analysis* (2) for adjusting the hydrogen ion concentration of an aliquot of the diluted acid digest to a pink color with methyl red indicator, for the precipitation of calcium oxalate, has often proved difficult. Incomplete elimination of the

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strong oxidizing substances causes decolorization of this indicator or may convert others to permanent colors that do not change when ammonium hydroxide is added.

It was found that when methyl red was added to aliquots of solutions made from samples of digested hay, some of the solutions decolorized the indicator before the proper amounts of ammonium hydroxide could be added, and therefore the desired *p*H was not attained. To insure the proper hydrogen ion adjustment in the presence of oxidizing substances, a *p*H meter equipped with glass and calomel electrodes is necessary for adjusting the solutions to the desired *p*H of 5.0.

METHOD FOR ADJUSTING *p*H

Transfer 200 ml (or suitable aliquot) of the soln from the diluted acid digestion to a 400-ml beaker, insert the glass electrodes of a *p*H meter in the soln and add 1 to 1 ammonium hydroxide soln from a burette, while stirring the soln, until a *p*H reading of 3.0 is obtained. Raise the electrodes above the surface of the soln,[1] wash them with cold water, and while stirring the soln introduce 10 ml of a saturated soln of ammonium oxalate in the 400-ml beaker. Re-insert the electrodes and add either normal ammonium hydroxide or normal hydrochloric acid soln (if necessary) a drop at a time until a *p*H of 5.0 is indicated. Raise the electrodes and rinse them thoroly with cold water. Bring the contents of the beaker to boiling and boil slowly for 10 min. Cool and allow to stand at least four hours. Filter, wash, and determine the calcium by the procedure as given in *Methods of Analysis* (2).

TABLE 1.—*Results for calcium*

CALCIUM DIGESTED WITH SUCROSE	
I	II
Calcium Added	
39.6 mg	49.5 mg
Calcium Found	
39.6	49.2
39.8	49.4
39.6	49.2
39.6	49.3
Average 39.6+	49.3−

Table 1 gives the results for the calcium as determined from the nitric-perchloric acid digests in two series of samples. Each series contained a different amount of calcite and one gram of sucrose before digestion. The greatest variation among any of the four determinations shown in column

[1] The electrodes are removed from the solution and washed to prevent calcium oxalate from being precipitated on them. Tests showed that if they remained in the solution during the addition of the ammonium oxalate, some calcium oxalate was deposited on the electrodes, which was not removed by washing.

1 is 5 parts per thousand, and for the data given in column II the variation is 6 parts per thousand.

Six determinations of residual calcium were made on a prairie hay; six on this hay after 49.5 mg of calcium as a calcite had been added, and six after 99.2 mg of calcium had been added. The data given in Table 2 shows the accuracy of method.

TABLE 2.—*Determination of residual calcium in prairie hay*

DETM. NO.	15 GRAMS PRAIRIE HAY ONLY	15 GRAMS PRAIRIE HAY	
		+49.5 MG Ca	+99.2 MG Ca
	Mg Ca in Hay	*Mg of added Ca recovered*	
1	66.0	49.5	99.0
2	66.5	49.5	98.5
3	66.5	49.0	99.0
4	66.0	49.5	99.0
5	67.0	49.5	100.0
6	66.5	49.0	99.0
Average	66.4+	49.3+	99.1−

The method insures accurate adjustment of the hydrogen ion concentration in solutions containing strong oxidizing agents that tend to alter the indicator when calcium is to be precipitated as an oxalate from solutions containing phosphates. It does not require any more time for the adjustment of the hydrogen ion concentration with the *p*H meter than when an indicator is used.

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SEPARATION OF ACETIC, MONOFLUOROACETIC, DIFLUOROACETIC, AND TRIFLUOROACETIC ACIDS*

By L. L. RAMSEY (Division of Food,† Food and Drug Administration, Federal Security Agency, Washington, D. C.)

Partition chromatography, employing a buffer solution as the immobile phase and an immiscible organic solvent as the mobile phase, has been used to separate the volatile fatty acids (1, 2) and to separate and purify the penicillins (3). The method described here for the separation of acetic acid and the three fluoroacetic acids is based upon the same general

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† W. B. White, Chief.

principle. The separation is made on a silicic acid column using a 2 *M* phosphate buffer of *p*H 2 as the immobile phase, and chloroform containing varying percentages of tertiary amyl alcohol as the mobile phase. The order of elution from the column is: 1. acetic, 2. monofluoroacetic, 3. difluoroacetic, and 4. trifluoroacetic acid. The separation of the acids from each other is complete, blank percolate fractions being obtained between any two of the acids. Recovery of the acids from the column is essentially quantitative. The working range of the method with the size column described here is 1 to 15 mg of each acid, the lower limit being determined by the accuracy of the titration. Best results appear to be obtainable in the range 3 to 10 mg. Each acid is tentatively identified by its threshold volume and this tentative identification is confirmed, if desired, by suitable means. Acetic acid may be confirmed by chromatography upon a water-chloroform-butanol column (4) or by preparation of the mercurous salt (5). The fluoro acids may be confirmed by fluorine analysis; however, when at least 5 mg of monofluoroacetic acid can be isolated, it is best identified by the highly specific indigo test of Wilson (6) for monochloroacetic acid.

A few other fluorine containing acids have been tested with this procedure; none interfere with the determination of the fluoroacetic acids. Monofluorophosphoric and hexafluorophosphoric acids do not interfere because they will not elute under the conditions employed. Heptafluorobutyric acid elutes largely with acetic acid, the threshold volume of heptafluorobutyric acid being slightly smaller. Seven or eight 5-ml blank fractions are obtained between heptafluorobutyric and monofluoroacetic acids. The heptafluorobutyric acid can be differentiated from acetic acid and estimated by fluorine analysis. Lactic acid elutes almost entirely with trifluoroacetic acid.

Preliminary experiments indicate that the method can not be applied directly to the extracts of foods and biological materials; small amounts of unidentified acids present in these materials apparently change the threshold volumes somewhat and also make it difficult to follow the elution of the acids from the column by titration.

METHOD

APPARATUS

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

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

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

REAGENTS

(1) *Silicic acid.*[a]—For best results dry to constant weight in an oven at 100°C. (ca 24 hrs) or in a desiccator over P_2O_5.

[a] Mallinckrodt's analytical reagent grade precipitated powder was used.

(2) *Mobile solvents.*—Remove the alcohol from U.S.P chloroform by washing it three or four times with distilled water, using a quantity of water each time ca equal to one-half the volume of chloroform. To three portions of the washed chloroform add sufficient tertiary amyl alcohol[b] to give 3, 8, and 15% of the alcohol by volume, hereafter designated as CA-3, CA-8, and CA-15, respectively.

(3) *Immobile solvent.*—To a 2-M solution of potassium dihydrogen phosphate add sufficient 2 M phosphoric acid to give a *p*H of 2.0 as determined by a *p*H meter.

(4) *Cresol red soln*, 0.05%.

PROCEDURE

(1) *Preparation of the chromatographic column*:

To 5 g of silicic acid in a mortar add the maximum amount of the phosphate buffer that the gel will hold without becoming sticky (ca 60–80% of its weight). Mix well with a pestle, add ca 25 ml of CA-3 and work up into a slurry. (This should be smooth; if the silica agglomerates in the solvent, too much buffer soln was used.) Place a small cotton plug in the bottom of a chromatographic tube and pour in the slurry, tilting the tube slightly to avoid air bubbles. Allow the silicic acid to pack down under 2–10 lb. pressure applied thru a gas pressure regulator. When excess solvent has drained thru (column firm and viscous enough to resist pouring when tipped), the column is ready for use. (In this step care must be exercised to avoid cracking or drying out of the gel; this is caused by leaving the pressure on after the column has packed down and all the solvent has sunk into the gel.)

(2) *Testing the silicic acid for its suitability and standardization of the chromatographic column*:

To a mixture of ca 5 mg each of acetic, monofluoroacetic, difluoroacetic, and trifluoroacetic acids present as their dry sodium salts in a glass-stoppered test tube (22 mm×150 mm) add 5 ml of CA-3, 2 or 3 drops of H_2SO_4(1+1), and 4 or 5 glass beads.

Shake gently until all of the salts have been thoroly wetted with the sulfuric acid. Using an eyedropper pipet, transfer the soln of the acids to the column and force the soln into the gel under pressure. Place a 25-ml graduated cylinder under the column to catch the percolate. Wash the test tube with three 3-ml portions of CA-3, allowing the wash solvent to sink into the gel each time under pressure before the next portion is added. Fill the tube with the CA-3 solvent and adjust pressure so that the rate of percolation is from 3–5 ml per min. (A separatory funnel or other suitable container may be fitted in the chromatographic tube to serve as a larger reservoir for the solvent.) After 25 ml of percolate have been collected, collect 5-ml fractions in 5-ml graduated cylinders. Transfer the 25-ml fraction to a glass-stoppered Erlenmeyer flask, add ca 20 ml of water and 1 or 2 drops of the cresol red indicator, and aerate with a stream of CO_2 free air or nitrogen for ca 3 min. to remove the CO_2. Titrate with 0.01 *N* NaOH, stoppering the flask and shaking vigorously near the end point. Add the succeeding 5-ml percolate fractions to the same flask and titrate each one. Record the cumulative volumes and cumulative titrations for these fractions. When in this series of fractions (acetic acid) the titer, after rising to a peak, falls to 0, change to a fresh titration flask. To this flask add ca 20 ml of water and the next percolate fraction, aerate to remove CO_2, and titrate. Continue the collection and titration of the percolate fractions. After all of the second acid (monofluoroacetic) has been collected, as shown by the titer for a fraction dropping

[b] Sharples Chemicals, Inc., technical product was used.

to 0, change to a fresh titration flask and change the eluant to CA-8. After all of the third acid (difluoroacetic) has been eluted, change to a fresh titration flask and change the eluant to CA-15. Continue the collection and titration of fractions until the titer falls to a constant value, the blank for CA-15 (ca 0.05 ml of 0.01*N*/5 ml percolate). By inspection of the data determine the threshold volume for each of the acids, considering the threshold volume of an acid to be the cumulative volume of percolate just prior to the first fraction containing such acid. This determination of the threshold volume for each of the four acids constitutes the standardization of the column for 5-mg quantities of the acids. Correct the total titer for each acid by subtracting blanks obtained in a run with no acid added. Calculate the quantity of each acid recovered. Consider the silicic acid suitable if, upon following the above procedure, there are blank fractions between the acids and the recoveries of the added acids are within 5%.

(3) *Determination and identification*:

Determine the acids in an unknown exactly as described above in the standardization and suitability procedure. (It is imperative that exactly the same reagents be employed and that the same amount of buffer soln be added to the silicic acid.) Compare the threshold volumes of the unknown acids with those of ca the same quantities of known acids and make a tentative identification. Confirm the tentative identification of acetic acid, if desired, by chromatography upon a water-chloroform-butanol column (4) or by preparation of the mercurous salt (5). Confirm the tentative identification of fluoroacids by elementary fluorine analysis of the appropriate fractions. For mono- and difluoroacetic acid use the method of Ramsey and Clifford (7). For trifluoroacetic acid use the same general procedure except substitute the sealed tube fusion step specified in the method of Elving & Ligett (8) for the ashing step in the Ramsey and Clifford procedure.

RESULTS AND DISCUSSION

The data in Table 1 show the threshold volumes and the recoveries obtained with mixtures of the four acids in the range 1–15 mg. Recoveries ranged from 87–105%; duplicates were in good agreement. Recoveries of the fluoro acids based upon F determinations are generally lower than those based upon direct titrations. The threshold volume of a given acid is quite reproducible for the same quantity of acid; however, the threshold volume of a small quantity of an acid is generally higher than that of a substantially larger quantity of the same acid. For example, the threshold volume of 1 mg of difluoroacetic acid (mixture A) was found to be 200; whereas the threshold volume of 15 mg (mixture D) was found to be 155. Table 2 shows the effect of the *p*H of the immobile phase and of the percentage of *n*-butanol (*n*-butanol instead of *t*-amyl alcohol was used in the preliminary work) in the mobile phase upon the threshold volume of monofluoroacetic acid.

The data in these tables were obtained with acids of a high degree of purity. For the purpose of recovery calculations they were considered to be 100% pure. The acetic acid (Eastman grade) was from a lot used in a previous investigation and found to contain none of its homologs (5). The monofluoroacetic acid was from the same purified lot used in a previously

reported work (7). The difluoroacetic acid was purified by extracting an acidified solution of the commercial sodium salt with alcohol-free ether, drying the ether extract, distilling off the ether, and fractionally distilling the acid about six times in a simple distillation flask. The fraction finally collected boiled at 132.0–132.5°C. Although the neutral equivalent found

TABLE 1.—*Recoveries and threshold volumes of acetic, monofluoroacetic, difluoroacetic, and trifluoroacetic acids when present in admixture*

ACID NAME	ADDED	FOUND, MG		THRESHOLD VOLUME
		BY TITRATION	BY F DETN.	
	mg			*ml*
		Mixture A		
Acetic	1.00	0.87		45
Monofluoroacetic	1.00	0.95	0.92	105
Difluoroacetic	1.00	0.97	0.87	200
Trifluoroacetic	1.00	0.91	0.93	275
		Mixture B		
Acetic	5.00	4.9, 4.8		40, 40
Monofluoroacetic	5.00	4.8, 4.8		105, 105
Difluoroacetic	5.00	5.0, 5.0		180, 190
Trifluoroacetic	5.00	5.0, 5.2		260, 265
		Mixture C		
Acetic	10.00	9.9, 9.8		40, 40
Monofluoroacetic	10.00	9.9, 9.8	9.2, 9.2	100, 105
Difluoroacetic	10.00	10.0, 10.0	9.4, 9.5	180, 180
Trifluoroacetic	10.00	10.0, 10.1	9.3, 9.2	250, 255
		Mixture D		
Acetic	15.00	14.9		35
Monofluoroacetic	15.00	14.2		100
Difluoroacetic	15.00	15.7		155
Trifluoroacetic	15.00	14.6		240
		Mixture E		
Acetic	10.00	9.9		40
Monofluoroacetic	1.00	0.9	0.92	115
Difluoroacetic	10.00	9.9		180
Trifluoroacetic	10.00	10.0		250

for the acid was 100.7 (calcd. 96.0), fluorine analysis indicated that all the acid titrated was difluoroacetic (F found, 97.7% of this titn. value). Apparently the acid contained a small amount of water. The trifluoroacetic acid was purified by the sulfuric acid method of Swarts (9) beginning with the commercial sodium salt. The fraction distilling at 71.5–72.0°C. was found to be pure. The neutral equivalent found was 113.7

TABLE 2.—*Effect of the pH of the buffer solution (immobile phase) and of the percentage of n-butanol in the chloroform (mobile phase) on the threshold volume of monofluoroacetic acid*

BUFFER, pH	*n*-BUTANOL IN CHLOROFORM	THRESHOLD VOLUME
	per cent	
1.0	5	25
1.5	1	90
1.5	2	70
1.5	5	30
2.0	2	80
2.0	5	40
2.5	2	90
2.5	5	40
3.0	1% to 95 ml, then 5%	145
3.0	5	50
4.2	5% to 100 ml, then 10%	120
6.6	5	not eluted in 200 ml

(calculated 114.0). The fluorine found by the method of Elving and Ligett (8) was 48.0% (calculated 50.0%).

SUMMARY

A method employing partition chromatography is described for the separation of acetic, monofluoroacetic, difluoroacetic, and trifluoroacetic acids. The acids are separated upon a column of silicic acid using a phosphate buffer of *p*H 2 as the immobile phase, and chloroform containing varying percentages of *t*-amyl alcohol as the mobile phase. The separation is followed by titration of percolate fractions of suitable volume with standard alkali. The determination of the acids is based upon direct titration and, in case of the fluoro acids, also upon the fluorine content. The data indicate that the separations are complete and the recoveries essentially quantitative.

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DETERMINATION OF ALCOHOL IN WINES AND LIQUEURS*

By A. D. ETIENNE and G. F. BEYER (Laboratory, Alcohol Tax Unit, U. S. Bureau of Internal Revenue, Washington 25, D. C.)

A method is presented for the determination of ethyl alcohol by volume in wines and liqueurs which is both rapid and of sufficient accuracy ($\pm 0.5\%$) for use, either in wineries and rectifying plants, where the facilities of a chemical laboratory are not readily available, or as a preliminary check in the laboratory.

The Williams method and apparatus for determining the percentage of ethyl alcohol by volume in alcoholic solutions[1] has been in use for some time, but its use has been more or less restricted to distilled spirits in which the solid content is relatively low and the alcoholic content is high. The method is based on the principle of extracting the alcohol from the sample by an immiscible solvent in a specially calibrated tube, and reading the percentage of alcohol from the position of the meniscus of the line of separation of the two liquids.

When the above method is used on wines, vermouths, and liqueurs, where the solids are much higher and the alcoholic content is much lower than in distilled spirits, it is inaccurate by reason of several factors: The standard reagent does not give an accurate reading due to the solids; the decrease in volume of the lower layer caused by the extraction of alcohol into the upper layer is not sufficient to be readily observed or accurately measured on the present tube; a great many wine products form an emulsion which will not break.

The above drawbacks have been overcome by several means:

(1) The extracting reagent has been modified and is made up as follows:

70 ml.	Pentasol (Synthetic Amyl Alcohol)
28 ml.	Toluene
1.8 ml.	HCl 10%

(2) The volume of sample has been increased from 7.5 ml. to 10 ml., and the volume of extracting reagent has been increased from 7.5 ml. to 20 ml. This provides a larger volume of alcohol available for extraction and a more complete extraction of the available alcohol.

(3) The apparatus or tube (Fig. 1) has been redesigned and calibrated to read from 5% to 22% in 1% intervals, with sufficient distance between each calibration for fairly accurate interpolation for fractions of 1%.

(4) The difficulty encountered with many wines, particularly ports, where emulsions formed that would not break into two distinct layers, has been overcome by a preliminary treatment of the sample with activated carbon (Darco S-51).

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* Presented at the Annual Meeting of the Association of Official Agricultural Chemists, held at Washington, D. C., October 2–4, 1950.

[1] *Ind. Eng. Chem.*, 18, 841 (1926).

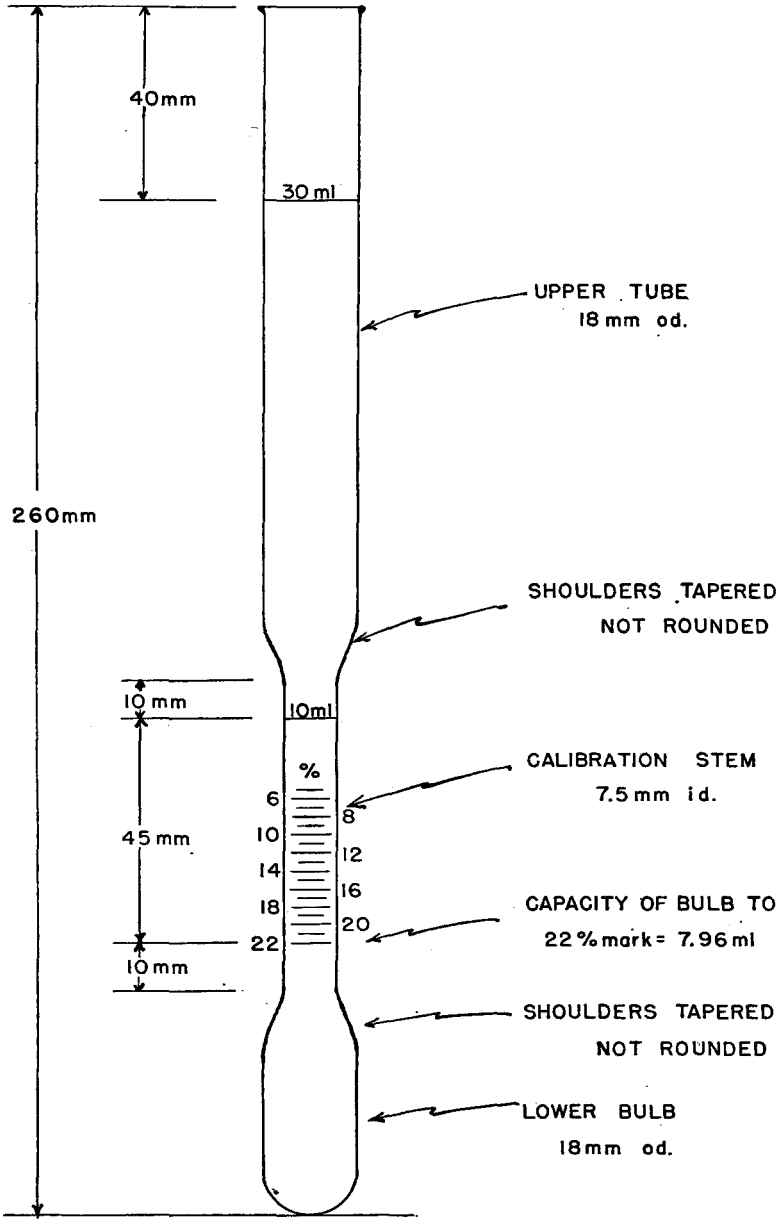

FIG. 1.—Etienne tube.

Design and Calibration of Tube

The tube was designed to give the greatest distance between the smallest calibration and at the same time maintain in the tube at a convenient working size consistent with the volumes used.

The capacity of the lower bulb is such that a volume of 7.96 ml. (22%) will come to a point about 10 mm. above the juncture of the lower bulb and the calibration stem. The calibration stem is 7.5 mm. ID. The 10 ml. mark should be about 10 mm. below the juncture of the calibration stem and the upper tube. The distance between the 7.96 ml. (22%) mark and 10 ml. mark should be 45 mm. A distance of about 40 mm. should be provided between the 30 ml. mark and the top of the upper tube to facilitate mixing.

The tube is calibrated at 25°C. by accurately measuring into the tube the number of ml. of distilled water as indicated by Table 1, for the various percentages. An accurate 10 ml. burette graduated in 1/20 ml. is used. The tip of the burette is extended so that the desired amounts can be introduced well into the tube without splashing or wetting the sides. After each addition the tube is marked with its corresponding percentage mark. (A No. 10 rubber stopper bored to a close fit makes a convenient stand for the tube.)

TABLE 1.

5%—9.45 ml.	16%—8.92 ml.	17%—8.40 ml.
6 —9.36	12 —8.83	18 —8.31
7 —9.27	13 —8.75	19 —8.22
8 —9.18	14 —8.66	20 —8.13
9 —9.10	15 —8.57	21 —8.05
10 —9.01	16 —8.48	22 —7.96

PREPARATION OF SAMPLE

(1) *Carbon treatment.*—Place 30–40 ml of wine into any convenient size flask and add sufficient carbon (about 0.5 g.) to fairly well decolorize the wine. Stopper and shake for about 30 seconds. Pour the entire contents onto a dry filter paper. If first portions of filtrate contains suspended carbon, refilter until filtrate is clear. (Small traces of carbon do not interfere. This volume provides sufficient sample for rinsing and duplicate tests if desired.)

(2) *Liqueurs and cordials.*—Proceed with the carbon treatment as above and prepare a 1–1 dilution with water. Proceed with the regular method, multiplying the result by 2.

METHOD

Introduce 10 ml of the wine sample, straight or prepared as given above, into the tube with a pipette. Accurately adjust the bottom of the meniscus to coincide with the 10 ml mark. Remove any excess sample on the sides of the tube above the 10 ml mark by means of a swab or a roll of filter paper. Introduce 20 ml of reagent to the 30 ml mark. Stopper the tube with a close-fitting rubber stopper and invert

TABLE 2.—*Per cent alcohol by volume*

TYPE	SOURCE	LABELED	PYCNOMETER	TREATED	UNTREATED	TREATED AND DIL. 1–1
Fortified Wine						
Port	New York	19.0	19.1	19.2	Emul.	
Port	Calif.	19.5	20.03	20.2	Heavy Emul.	
Port	Calif.	20.0	19.9	20.0	Heavy Emul.	
Port	Calif.	20.0	19.14	19.0	Heavy Emul.	
Port	Ohio	20.0	19.1	19.0	Sl. Emul.	
Sherry	Ohio	20.0	18.9	19.2	20.0 Estimated	
Sherry	New Jersey		20.0	20.0	20.25	
Sherry	Maryland	19–21	20.32	20.2	20.8	
Concord Sweetened & Fortified	Ohio		20.03	20.0	Sl. Emul. 21.0 Estimated	
Concord Unfinished & Fortified	Ohio		19.84	19.6	Heavy Emul.	
Muscatel	Calif.	19–21	20.09	20.0	20.3	
Catawba	Ohio		20.3	20.2	20.5	
Apple	Kentucky	20.0	17.8	17.5		
Unfortified Wine						
Loganberry	Calif.	12.0	10.77	10.5	Emul.	
Peach	Georgia	12–14	11.88	12.0	12.2	
Blackberry	Florida	12–14	11.33	11.0	Sl. Emul. 10.1 Estimated	
American Red Grape	Georgia	14	12.53	12.5	Emul.	
Zinfandel	Calif.	12–14	12.45	12.5	Sl. Emul. 12.0 Estimated	
Sauterne	Calif.	12–14	10.83	11.0	11.1	
Barberone	Calif.	12–14	12.85	12.5	Sl. Emul. 12.0 Estimated	
Liquers						
Alcohol	240 gm. sugar	1000 ml.	29.8			14.8×2=29.6
Sugar Sol.	300 ml. abs. Alc.					
Peach Liqueur		30.0	30.2			15.2×2=30.0
Blackberry		30.0	29.7			15.0×2=30.0
Cherry		30.0	30.0			15.2×2=30.4
Creme de Menthe		23.0	22.4			11.3×2=22.6

the tube a number of times with moderate shaking to insure an intimate mixing of the solutions. Stand the tube in an upright position and allow separation to take place. When separation is complete shake down the globules of the lower solution that adhere to the sides of the upper tube and stopper, by flipping and twirling the tube between the thumb and fingers. When settling and drainage is complete, usually 5 minutes, read the percentage of alcohol where the meniscus between the two layers falls on the calibration marks. Repeat the above operation of mixing and settling and read again.

NOTE: (1) The tube should be cleaned frequently with a detergent solution of hot soap and water. (2) The mixing operation should cover a period of about 2 minutes.

RESULTS

A number of samples of fortified wines, unfortified wines, and liqueurs were tested by the A.O.A.C. method[2] and the new method both before and after carbon treatment. The results are tabulated in Table 2 and indicate a greater accuracy than claimed by the method.

When the wines were used without preliminary carbon treatment and slight emulsion remained after settling amounting to 3 or 4 mm., the percentage was estimated by reading the mid-point of the emulsion.

More accurate and satisfactory results are obtained by using the preliminary carbon treatment. The mixing and shaking can be more vigorous and the separation is more rapid and cleaner.

Work is being conducted on the adaptation of this method for determining the alcoholic content of distillery beers and will be reported on in a subsequent paper.

Acknowledgment is made to R. L. Ryan, Chief of the Laboratory Division, for his original suggestion and assistance during the course of the development.

ANNOUNCEMENT

The formal statements in the proceedings, listing the subjects for which no reports were made, were omitted in the August Journal, because of its size. The titles of these subjects have been recorded in the Index.

CORRECTIONS

May *Journal*, page 331: Robert L. Tenney, Wahl-Henius Institute, change address to 1135 Fullerton Avenue, Chicago, Ill.

August *Journal*, page 662: Under "Summary and conclusions," second line in text, the word "perchloric" should have been "sulfuric."

Page 729: The date given for publication of the paper entitled "Water-Insoluble Fatty Acids and Butyric Acid in Cream and Butter," should have been "1949."

Page 845: The reference numbered "5" in the text, line 6 from the end of the page, should be deleted; footnote 5 should be on page 843.

Page 889: In paper "Spectrophotometric Method for the Quantitative Estimation of Technical Chlordane," by Bernard Davidow, change the word "compensated," in title of Table 1, to "components."

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[2] *Methods of Analysis, A.O.A.C.*, 1945 182.

NOTE ON THE DETERMINATION OF MOISTURE IN STARCHES BY THE KARL FISCHER REAGENT

By ELIZABETH A. MCCOMB (Western Regional Research Laboratory,[1] Albany, California)

Fosnot and Haman (1) included the analysis of corn starch in a study of moisture in cereal and cereal products by the Karl Fischer reagent. The samples were dispersed in dry methanol, heated to the boiling point, cooled and titrated. Porter and Willits (2) investigated the use of the Karl Fischer reagent for moisture in potato starch after extraction in dry methanol for eight hours. Johnson (3) determined the moisture in wheat and potato starch after extraction in dry methanol at 60°C. for one-half hour. Schroeder and Nair (4) refluxed corn starch samples in dry methanol in a 90°C. bath for 5 minutes or longer, cooled, and titrated.

TABLE 1.—*A comparison of the Fischer reagent and vacuum oven method for moisture in starch samples*

STARCH SAMPLE	KARL FISCHER REAGENT		VACUUM OVEN, 100°C., 5 HOURS	
	per cent		*per cent*	
Bean	13.38, 13.35, 13.30	(13.35)*	13.52, 13.49	(13.51)
Cassava	11.35, 11.22, 11.22	(11.26)	11.46, 11.44, 11.47	(11.46)
Corn	11.03, 10.89, 11.05	(10.99)	11.07, 11.05, 11.10	(11.07)
Pea	10.04, 10.01, 9.99	(10.01)	9.97, 9.99, 9.98	(9.98)
Potato	11.96, 12.02, 11.99	(11.98)	11.95, 11.96, 11.97	(11.96)
Sweet Potato	9.97, 9.91, 9.88	(9.92)	9.95, 9.87, 9.95	(9.92)
Wheat	9.05, 9.01, 9.09	(9.05)	9.12, 9.09, 9.11	(9.11)

* Values in parentheses are averages.

By use of a technique previously described for the determination of moisture in some protein materials (5), it has been found that dry methanol plus Karl Fischer reagent completely extracts the moisture from starch samples in 15 minutes at room temperature.

METHOD

Samples of approximately 300 mg. were weighed into dry glass-stoppered 250-ml. Erlenmeyer flasks containing a stirrer, and 25 ml. of dried methanol were added to each sample. A moderate excess of Fischer reagent was added to the sample in the alcohol, and the contents of the flasks were agitated by a magnetic stirrer for 15 minutes. An excess of reagent was maintained during the stirring and if the original amount added was insufficient, as indicated by the appearance of the yellow color of spent Fischer reagent, more reagent was added. The determination was completed by back-titrating with standard water solution.

An optimum stirring time of 15 minutes was selected because a 10-minute period showed slightly low results, and 30 minutes gave no increase in percentage of moisture found.

This method combines the advantages of speed, simple manipulations at room temperature, good precision, and accuracy comparable with a vacuum oven method (6).

[1] Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

ACKNOWLEDGMENT

The author wishes to thank Jack Guggolz of this Laboratory, who furnished the starch samples.

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BOOK REVIEWS

The Chemistry of Industrial Toxicology. By HERVEY B. ELKINS. John Wiley & Sons, Inc. Price $5.50.

This concise and informative book is a thorough introduction to the chemical aspects of industrial toxicology. The author's primary aim is to emphasize the industrial poisons and the industrial processes in which they are involved rather than their physiological action on the human body. This book is written primarily for the hygienist, chemist and engineer interested in the problems of occupational hygiene. The subject matter is presented in an interesting and orderly manner and the book is very readable. Throughout the text much of the material is summarized in tables. Some 366 references to pertinent literature are included in the bibliography.

The first two chapters are devoted to a general discussion of the poisons which are industrial hazards and to methods used for detecting them. Of special interest to the occupational hygienist are Chapter 3–11, which offer an intensive study of over 200 industrial poisons. Included in these chapters are the physical properties of the various poisons; their harmful physiological effects, maximum permitted tolerances and methods of determining the existence of a hazard.

The author then deals with the industrial operations and processes in which toxic substances are produced; the hazards involved are analyzed and preventive measures are given. A list of the maximum allowable concentrations of more than 200 compounds in air and 12 in the urine constitute part of the text. One chapter includes some of the unsolved problems of occupational hygiene.

The last two chapters are devoted to apparatus required for testing toxic substances. Analytical methods and procedures for the determination of many of the more common toxicants both in air and urine are included.

Although a great deal of information is packed in this text yet the style is always clear and unambiguous.

HERBERT A. BRAUN

Organic Syntheses—An Annual Publication of Satisfactory Methods for the Preparation of Organic Chemicals, Vol. 30. A. C. COPE, Editor in Chief, VI+110 pages. 1950. John Wiley & Sons, Inc., 440 Fourth Avenue, New York 16, N.Y. $2.50.

Volume 30 conforms with earlier volumes of this series of tested directions for the laboratory preparation of a variety of organic compounds. It contains 39 such procedures. For those who have the need to try new and commercially unavailable

reagents, some of these preparations may be of interest. The value of checking analytical procedures is well known to regulatory chemists; a similar check is applied to each compound whose preparation is given in "Organic Syntheses."

Volume 30 lists the following compounds:

9-Acetylanthracene, 3-Aminopyridine, p-Aminotetraphenylmethane, DL-Aspartic Acid.

Benzoylcholine Iodide and Chloride, *n*-Butylacetylene.

β-Carbethoxy-γ,γ-Diphenylvinylacetic Acid, Chloroacetonitrile, trans-2-Chlorocyclopentanol.

4,4′-Dichlorodibutyl Ether, Diethyl cis-Δ^4-Tetrahydrophthalate and Diethyl cis-Hexahydrophthalate, 1,4-Diiodobutane.

Ethanedithiol, Ethylenimine, 5-Ethyl-2-Methylpyridine, Ethyl Phenylcyanoacetate.

Fumaronitrile, Glutaric Acid, Hexahydro-1,3,5-Tripropionyl-s-Triazine, 2-Iodothiophene.

2-Mercaptobenzimidazole, Methanesulfonyl Chloride, N-Methyl-2,3-Dimethoxybenzylamine, 1-Methyl-3-Ethyloxindole, Methyl-β-Thiodipropionate.

1-Naphthaldehyde, o-Nitroacetophenone.

Phenylacetylene, trans-1-Phenyl-1,3-Butadiene, α-Phenyl-α-Carbethoxyglutaronitrile, α-Phenylglutaric Anhydride, Phenylsuccinic Acid, 2,3-Pyrazinedicarboxylic Acid.

1,2,3,4-Tetrahydrocarbazole, cis-Δ^4-Tetrahydrophthalic Anhydride, Tetraphenylarsonium Chloride Hydrochloride, o-Tolualdehyde.

Vanillic Acid, Vinyl Laurate and other Vinyl Esters.

W. I. PATTERSON

Phenomena, Atoms and Molecules. By IRVING LANGMUIR. Philosophical Library, New York, 1950. 436 pages. Price $10.00.

As the title itself might suggest, Dr. Langmuir's book is a collection of selected topics rather than the treatment of a single theme. The chapters consist of individual papers published during the long career of this versatile Nobelist. The scope of the book is as broad as the author's interests.

The first three chapters are devoted to a general discussion of the philosophy of science, National science legislation, the problem of increased incentives for research, and the control of atomic energy. During his trip to Moscow at the end of the war, Dr. Langmuir was favorably impressed by both the physical equipment and the eager spirit of the Russian scientists. It would be interesting to know if he is still so hopeful of mutual understanding, and of the ultimate success of the U.N.O.

In chapters 11 and 18 he seeks to explain the physical properties of compounds in terms of their molecular structure. In his interpretations he uses the Lewis cubical atom and the octet theory, which were at the height of their popularity when these papers first appeared. The contrast between these ideas and the more recent theories on structure, as presented by Pauling in "The Nature of the Chemical Bond," demonstrates the rapid advances in theoretical chemistry.

The remaining chapters discuss such topics as absorption of gases on solids, physical forces in surface bonding, molecular orientation in liquid films, and studies on atomic hydrogen. Even the most mathematical parts are well organized and readable. Experimental methods and detailed interpretation of data clarify each step in the development of a theory. Although the subject material is not new, the book gives an excellent historical picture of fundamental research.

C. S. PRICKETT

German-English Dictionary for Chemists. By AUSTIN M. PATTERSON, 3rd Edition, John Wiley & Sons, New York, 1950. xviii+541 pages. Price $5.00.

The first impression one gets of this new edition is the increase in size over the first edition published in 1917. In fact, comparison shows that it is almost doubled; with its 541 pages as compared to the 316 of the original. Many of the chemical profession recall the sensation caused by the appearance of the first edition. A full measure of success in chemistry was not to be expected without some ability to read the extensive German literature, but how inadequate were the general German-English dictionaries, used in the classroom to translate Goethe and Schiller, as aids in the accurate translation of technical and scientific words, as well as those combination terms so popular with the German scientist!

The output of scientific and technical literature in German is below that of those early years, but it may not always be. However that may be, there is such a wealth of chemical knowledge already published in that language that the scientist, if he is to profit from this storehouse of information, will wish to read that pertaining to his work. This 3rd edition will enable him to do so more easily and efficiently, not only by virtue of its comprehensiveness, but also because of the retention of out-moded or even obsolete terms. This work is more than a chemical dictionary. It is sufficiently complete for use by the chemist to obviate any necessity for the consultation of a general dictionary. It is designated as for the chemist, but workers in other scientific fields will find it a valuable asset which they can ill afford to be without. The growth of the book is due not only to new terms in the fields of technology electronics and warfare, but more importantly to additional meanings for many of the words in earlier editions. One is not surprised, therefore, to find under "Nahr" 54 words and combinations from "Nahragar" to "Nahrzufuhr" whereas only 24 are to be found in the first edition.

HENRY A. LEPPER

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