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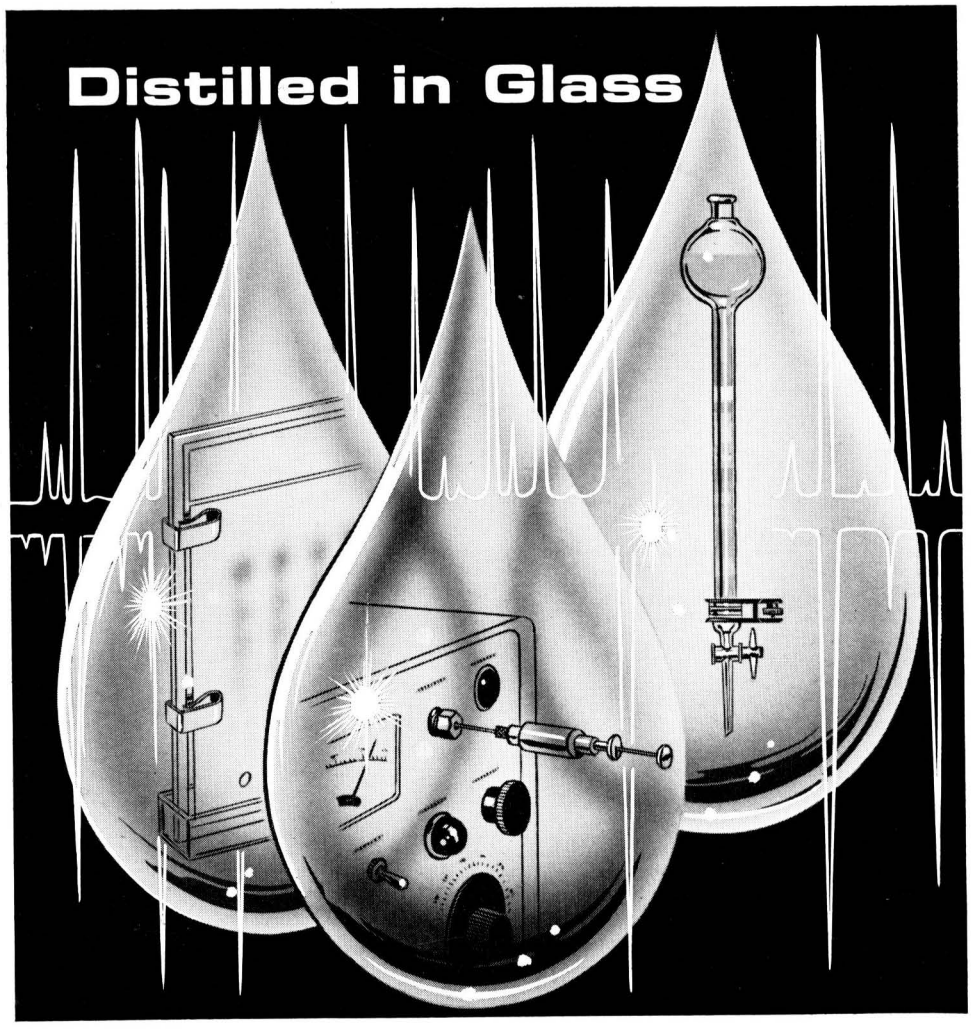

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FERTILIZERS

Determination of Total Fluoride and/or Fluosilicic Acid Concentration by Specific Fluoride Ion Electrode Potentiometry

By DONALD E. JORDAN (Continental Oil Co., Research and Development Department, Research Services Division, Ponca City, Okla. 74601)

A method is presented to determine total fluoride and/or fluosilicic acid concentrations directly, using the fluoride ion selective electrode. Samples can be analyzed at 20–30/hr with excellent precision; results are equivalent to those obtained with the AWWA standard test procedure No. B 703-60. Hydrofluoric acid, if present in the fluosilicic acid samples, interferes in the calculation for H_2SiF_6 concentration but not for total fluoride present. Specifically, the fluoride ion is freed from fluosilicate at pH 8.4–10.0; in addition, iron and aluminum hydrous oxides are formed at this pH. Then the solution is buffered to pH 5.7–5.9 at a fixed ionic strength with ammonium acetate buffer and the total fluoride is determined from a prepared calibration curve or computer program.

In recent months many publications have appeared, describing the application of the fluoride ion selective electrode to the determination of free fluoride in many difficult solution systems (1–4). Others have described the determination of low levels of fluoride in sea water and public water supplies (5–7). Crosby (8) has described equilibria of fluosilicate solutions at concentrations normally found in public water supplies and up to a concentration of 3000 ppm as F^-. No literature references were found that provided a method to completely free the fluoride from fluosilicate in concentrations ranging up to 29+% H_2SiF_6 whereby the fluosilicic acid could be directly and accurately analyzed by the direct determination of fluoride with the fluoride electrode.

A method is presented here to analyze fluosilicic acid production solutions up to 29% H_2SiF_6 by determination of total fluoride with the fluoride ion selective electrode and subsequent calculation to H_2SiF_6. The results can be obtained at 20–30 samples/hr with a precision equal to or better than that obtained with the American Water Works Association (AWWA) standard test method No. B 703–60. Specifically, the fluoride is freed from fluosilicate in basic solution according to:

$$H_2SiF_6 + 6MOH \rightarrow 6MF + SiO_2 + 4H_2O$$

Then a known volume of 3M ammonium acetate buffer solution (pH 5.7–5.9) is added to adjust the ionic strength and pH of the solution. Fluoride is determined with the electrode and calculated to H_2SiF_6.

Experimental

Apparatus and Reagents

(a) *pH meter.*—Ionalyzer Model 801 digital pH meter, fluoride ion electrode No. 94–09 (Orion Research, Cambridge, Mass.), and calomel reference electrode (Corning No. 476011, or similar electrode).

(b) *Ammonium acetate buffer solution.*—Adjust 231 g ammonium acetate 3M/L solution to pH 5.7–5.9 with glacial acetic acid.

(c) *Sodium fluoride solution.*—0.100*M*. Dissolve 2.099 g dried 99.9+% sodium fluoride in 200 ml water in 500 ml volumetric flask. Add 100 ml 3M ammonium acetate solution and dilute to 500.0 ml with more water. Prepare 0.01, 0.001, and 0.0001*M* F^- solutions by successively diluting 0.100*M* sodium fluoride solution. Be sure, however, that each solution contains only 100 ml 3M ammonium acetate buffer per 500.0 ml solution; store standards in tight plastic bottles.

Calibration Curve

Prepare calibration curve, using prepared standard solutions. Determine millivolts for each standard by immersing electrodes (rinsed with solution to be

measured) in standard fluoride solution. Read millivolts with meter in MV mode. It usually takes several seconds for reading to become constant. Plot initial calibration curve from measurements made in MV mode, then move selector switch to Rel. MV. Use standard solution near center of operating range for re-standardization in Rel. MV mode. Adjust calibration control until original reading observed in MV mode above is again observed in Rel. MV mode. Rel. MV calibration adjusts slope of calibration curve for day-to-day use, compensating for electrode drift and other solution phenomena between periods of measurements.

Plot concentration vs. millivolts on semilog paper with concentration on logarithmic axis. Slope is constant from 10^{-1} to $10^{-5}M$ (1900 to 0.19 mg F^-/L solution).

Procedure

Weigh 0.1–0.15 g fluosilicic acid sample into 100 ml volumetric flask. Add 10–20 ml water and 5 drops 1% phenolphthalein indicator. Add concentrated ammonia dropwise with swirling until color remains pink; then add 2 drops excess. Let stand ca 1 min, then add 20 ml ammonium acetate buffer, dilute to volume, and mix well. Determine and record millivolts on Rel. MV scale as described above.

Calculations

(a) Calculate mg fluoride manually or directly from slope of calibration curve. Calculation from slope is less cumbersome because it avoids extrapolation difficulties in determining differences of ±0.1 mv.

$$\% \ H_2SiF_6 = [1.263 \times 10^{-4} \times V \times \text{antilog} (S(\text{mv}) + \log F_i^-)]/\text{g sample}$$

where S = slope = $[\log (F_c^- - F_i^-)]/\Delta\text{mv}$, V = dilution volume, and F_c^- and F_i^- = mg fluoride at any mv reading and the intercept, respectively.

(b) For computer facilities, a simple program to calculate per cent fluoride is given below. This program is particularly applicable for time-share facilities where many determinations are required.

```
Data starts in 200
30 Let I = 0
40 Read M,W,D
50 Let I = I + 1
60 Let C = S *M + I
70 Let A = Exp (2.303 * C)
80 Let F = (A*D)/(W*10000)
90 Print I, "prcnt F = "; F
100 Go to 40
900 End
```

where M = millivolts, D = dilution, I = intercept, W = g sample, and S = slope of curve.

Discussion and Results

Fluosilicic acid and fluosilicates have traditionally been analyzed by a differential cold and hot titration, using bromothymol blue indicator and NaOH. The cold titration determines acid-ionizable hydrogen, including that from HF, H_3PO_4, H_2SO_4, etc., after fluosilicates have been precipitated as potassium fluosilicate. Heating the solution to boiling breaks down the fluosilicate which is titrated back to the same end point while boiling. Although the method is quite reproducible and precise, it is also slow and cumbersome, especially if only minute quantities of fluosilicic acid are present.

In our work on direct determination of fluosilicic acid, using the fluoride electrode, the fluosilicate was only partially dissociated in buffered or unbuffered solutions. Addition of buffer and ionic strength adjustment solution did not improve the measurable fluoride significantly. A brief study of dissociation characteristics of fluosilicate showed that hydrolysis to silica occurred in basic solution and the fluoride thus freed could be measured directly. Thus, addition of caustic to pH > 8.4 completely hydrolyzed fluosilicate to metal fluoride and insoluble silica. However, excess caustic made proper buffering difficult and the insoluble silica intermittently rendered the electrodes insensitive. Substitution of ammonium hydroxide for sodium or potassium hydroxide effectively controlled the hydroxyl ion concentrations, eliminating the difficulty with buffering. The electrode deactivation by silica was controlled by reducing the sample size to contain not more than 0.63 g SiO_2/L, whereby silica formed in a very finely dispersed state and had no measurable effect on the electrodes. Of course after several minutes the typical precipitate formed.

A great deal has been written about various single- and multiple-component buffer solutions used to free fluoride ions and fix the solution ionic strength. The total ionic strength adjustment buffer (4) is certainly useful in many systems but has limitations, at least to aluminum and iron in the system as shown by Harwood (6), who used total ionic strength adjustment buffer and cyclohexane diamine tetraacetate and Edmond (3), who used a citrate buffer, to complex aluminum and iron in order to correctly measure total fluoride. In the work described here it was observed that TISAB was not fully applicable, even with

Table 1. Comparison of H_2SiF_6 results by direct release and determination of total fluoride with the fluoride sensitive electrode vs. the AWWA differential titration

Sample	H_2SiF_6, %		
	Selective Electrode	AWWA	Diff.
1	27.25	27.75	−0.50
2	28.90	28.53	+0.37
3	23.49	23.75	−0.26
4	26.90	26.90	0
5	26.60	26.24	+0.36
6	27.91	27.36	+0.55
7	26.55	26.51	+0.04
8	27.62	27.43	+0.19
9	26.86	26.79	+0.07
10	28.41	28.41	0
11	27.96	27.84	+0.12
12	28.15	28.01	+0.14
13	29.83	29.64	+0.19
14	26.55	26.40	+0.15
15	26.75	27.09	+0.66
16	26.71	26.28	+0.43
17	27.14	26.88	+0.26

CDTA added, and citrate buffer has no apparent advantage over the ammonium acetate buffer used. Ammonium acetate buffer is easy to prepare, is an excellent buffer in the 5.5–6.0 pH range studied here, and is fully compatible in the system described. Further, after formation of hydrous oxide of any aluminum and iron during the fluosilicate hydrolysis, the acetate buffer prevents disproportionation to aluminum and/or iron fluoride complexes at the desired pH. Thus fluoride is maintained in solution in a measurable condition. Other buffer systems could undoubtedly be substituted with equivalent results but the acetate seemed to be desirable for this work.

The manufacturer of the fluoride electrode suggests that the calibration curve obtained in millivolts vs. concentration of fluoride should be linear from 10^{-1} down to at least $10^{-6}M$ with only slight deviation from linear to $10^{-7}M$ in fluoride. The calibration curve obtained in this work showed linearity only from 10^{-1} to $10^{-5}M$ in fluoride. Deviation from linearity below $10^{-5}M$ was small but significant; however, little or no error in sample measurements would be expected, because unknown samples are treated and the fluoride is measured in the same way as the calibration standards.

Table 1 shows the results obtained for fluosilicic acid production samples taken over a 4 month period. The results are compared to those obtained by the AWWA traditional differential titration procedure.

Theoretically the values for fluosilicic acid should be equal to or greater than those obtained by titration, because a very low concentration of HF is usually present in the production acid. Except for samples 1 and 3 the results show the expected trend. The agreement in these results by the 2 methods is generally excellent. Where the expected difference is greatest (samples 6 and 15), some HF is present in the samples, but even the differences shown in samples 6 and 15 are within the agreement one might expect between 2 different laboratories using 2 unrelated methods. An anomalous behavior shown by samples 1 and 3 could well be due to uncertainties in either procedure and the differences again are actually minor.

If samples of fluosilicic acid contained appreciable quantities of HF, the direct electrode method described here would only be applicable for total fluoride, because F^- from both H_2SiF_6 and HF would be determined.

Table 2. Precision of H_2SiF_6 determination using the selective fluoride electrode technique

Detn	Sample			
	2, %	3, %	11, %	17, %
1	28.78	23.30	28.36	27.09
2	28.80	23.57	27.87	27.01
3	28.99	23.43	28.11	27.03
4	28.89	23.47	27.96	26.88
5	28.87	23.46	28.43	27.30
6	28.99	23.26	27.72	27.10
7	28.65	23.83	27.47	27.21
8	28.97	23.81	27.89	27.25
9	28.54	23.55	28.20	27.20
10	28.97	23.53	27.62	27.33
Av.	28.90	23.49	27.96	27.14
Std dev.	0.21	0.20	0.32	0.14

Table 2 details the precision of the fluosilicic acid determination using the selective fluoride ion electrode. The precision of each sample is excellent, with the sample standard deviation ranging from ±0.5 to ±1.15% relative. The precision points out the applicability of the method for the rapid and precise direct determination of fluosilicic acid. The accuracy again is unknown because known standards are not available.

Acknowledgment

Dr. D. E. Monn prepared the computer program used in this work.

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Total Nitrogen Methods—Applicable to All Fertilizer Samples

By PAUL R. REXROAD and GARY F. KRAUSE (Agricultural Experiment Station, University of Missouri, Columbia, Mo. 65201)

An extensive collaborative study was conducted to evaluate a comprehensive nitrogen method and a Raney powder method for acceptance as official methods. Twenty-nine laboratories participated; 10 samples were used and the new methods were compared to the official method, 2.045 (sulfuric-salicylic acid). Evaluation of the data and overall consideration lead to the recommendation that both new methods be adopted as official. It is recommended that 2.047 (reduced iron) and 2.049 (chromium powder) be deleted as official methods.

Since all AOAC methods (1) for nitrogen in fertilizers are limited in their scope, a collaborative study was conducted to evaluate two new methods along with the official method, **2.045** (sulfuric-salicylic acid). The comprehensive nitrogen method (2, 3) and the Raney powder method (3–5) included in this study appeared to be applicable for the determination of total nitrogen in all fertilizer samples. The literature cited includes data covering ruggedness tests, extensive research, and applicability to samples with high Cl/NO_3 ratios or with a combination of nitrate and organic materials.

Because the timing of the study meant that the experimental work would be done during the busy fertilizer season, 54 laboratories were contacted as possible collaborators. Surprisingly, 34 laboratories expressed a willingness to collaborate. Due to a limited amount of certain samples, samples were sent to 30 laboratories; 29 submitted results from the study.

Experimental Design

Ten samples were selected for analysis (Table 1). All samples were given a single assay on each of 2 different days by each method. An exception was that the 28% nitrogen solution was not assayed by AOAC method **2.045**, which is not applicable to liquid samples containing nitrates (3). Duplicate blanks by each method were run with each set of assays. This resulted in a total of 70 assays/laboratory.

Sulfuric-Salicylic Acid Method[1]

See **2.042–2.045.**

Comprehensive Nitrogen Method[1]

(Applicable to all fertilizer samples)

Reagents

(a) *Chromium metal.*—100 mesh, low N (Fisher Scientific Co. No. C-318 or Sargent-Welch Scientific Co. No. SC11432 are satisfactory).

(b) *Alundum.*—Norton 14X (Arthur H. Thomas Co.).

(c) *Dilute sulfuric acid.*—Slowly add 625 ml H_2SO_4 to 300 ml H_2O. Dil. to ca 1 L and mix. After cooling, dil. to 1 L with H_2O and mix. Avoid absorption of NH_3 from air during prepn, particularly if stream of air is used for mixing.

(d) *Sodium thiosulfate or potassium sulfide soln.*—160 g $Na_2S_2O_3 \cdot 5H_2O$/L or 80 g K_2S/L.

For other reagents, *see* **2.049.**

[1] The section numbers within the method are those for the 11th ed., of *Official Methods of Analysis*, 1970 secs. **2.042–2.045** (11th ed.) = **2.036–2.038** (10th ed.), **2.049** = **2.042**, **2.050(a)** = **2.043(a)**, **2.051** = **2.044.**

Table 1. Samples for collaborative analysis by three total nitrogen methods

No.	Sample[a]	Description
1	6-24-24	Commercial
2	13-13-13	Commercial
3	4-0-55	Laboratory blend for high Cl/NO_3 ratio
4[b]	KNO_3	Fisher Scientific Co. P-383, theory 13.85% N
5[b]	$NH_4H_2PO_4$	NBS, single crystal, theory 12.17% N
6	28% soln	3-4-3 blend of urea, NH_4NO_3, and water
7	Urea-form	Commercial 38% N
8	Blood meal	Commercial
9	Tobacco stems	Supplied by University of Kentucky
10[b]	Nicotinic acid	Reagent grade, theory 11.38% N

[a] All samples were ready for analysis except for specified drying of 3 samples.
[b] To be dried 2 hr at 140°C.

Determination

Place 0.2–2.0 g sample contg $\leq$60 mg nitrate N in 500–800 ml Kjeldahl flask and add 1.2 g Cr powder. Add 35 ml H_2O or, with liqs, lesser amt to make total vol. of liq. 35 ml. Let stand 10 min with occasional gentle swirling to ensure soln of all nitrate salts. Add 7 ml HCl and let stand at least 30 sec but $\leq$10 min.

Place flask on preheated burner with heat input set at 7.0–7.5 min boil test, **2.050**(a). After heating 3.5 min, remove from heat and let cool.

Add 22 g K_2SO_4, 1.0 g HgO, and few granules Alundum. Add 40 ml dil. H_2SO_4, (c). (If adequate ventilation is available, 25 ml H_2SO_4 may be added instead of dil. H_2SO_4. If org. matter which consumes large amt of acid exceeds 1.0 g, add addnl 1.0 ml H_2SO_4 for each 0.1 g org. matter in excess of 1.0 g.)

Place flask on burners set at 5 min boil test. (Preheated burners reduce foaming with most samples. Cut back heat input if foam fills $\geq$⅔ of bulb of flask. Use variable heat input until this phase is past.) Heat at 5 min boil test until dense white fumes of H_2SO_4 clear bulb of flask. Digestion is now complete for samples contg ammoniacal, nitrate, and urea N. For other samples, swirl flask gently and continue digestion 60 min more.

Proceed as in **2.051**, second par., substituting *Reagent* (d) above for **2.049**(e).

Raney Powder Method[1]

(Applicable to all fertilizer samples)

Reagents

(a) *Raney catalyst powder No. 2813.*—50% Ni, 50% Al (W. R. Grace and Co., Raney Catalyst Division, 819 Hamilton National Bank Building, Chattanooga, TN 37402). *Caution:* Raney catalyst powders react slowly in water or moist air to form alumina; avoid prolonged contact with air or moisture during storage or use.

(b) *Sulfuric acid-potassium sulfate soln.*—Slowly add 200 ml H_2SO_4 to 625 ml of H_2O and mix. Without cooling, add 106.7 g K_2SO_4 and continue stirring until all salt dissolves. Dil. to ca 1 L and mix. Cool, dil. to 1 L with H_2O, and mix. Avoid absorption of NH_3 from air during prepn particularly if stream of air is used for mixing.

For other reagents, *see* **2.049.**

Determination

Place 0.2–2.0 g sample contg $\leq$42 mg nitrate N in 500–800 ml Kjeldahl flask (800 ml flask is preferred with samples which foam considerably, especially orgs). Add 1.7 g Raney catalyst powder, 3 drops *tributyl citrate*, and 150 ml H_2SO_4-K_2SO_4 soln. If org. matter exceeds 0.6 g, add addnl 2.5 ml of this soln for each 0.1 g of org. matter in excess of 0.6 g.

Swirl to mix sample with acid and place flask on cold burner. If burner has been in use, turn off completely at least 10 min before placing flask on burner. After flask is on burner, set heat input to 5 min boil test. When sample starts boiling, reduce heat to pass 10 min boil test. After 10 min, raise flask to vertical position and add 0.7 g HgO and 15 g K_2SO_4. (Contents of Kel-Pak No. 5 (Matheson Scientific, 1850 Greenleaf Ave., Elk Grove Village, IL 60007) without plastic container may be used.) Replace flask in inclined position and increase heat to 4–5 min boil test. (Cut back heat input if foam fills $\geq$⅔ of bulb of flask. Use variable heat input until this phase is past.) Heat at 4–5 min boil test until dense white fumes of H_2SO_4 clear bulb of flask. Digestion is now complete for samples contg only ammoniacal, nitrate, and urea N. For other samples, swirl flask gently and continue digestion another 30 min.

Proceed as in **2.051**, second par. If 800 ml Kjeldahl flasks have been used, add 300 instead of 200 ml H_2O.

Results

Early returns called attention to Sample 3. It was the 4–0–55 laboratory blend of NH_4NO_3 and KCl to give a high Cl/NO_3 ratio. Microscopic examination and chemical analyses established that the sample was not uniform in composition. Efforts to keep a known composition had left the sample poorly blended or prone to segregate. The reported results showed a coefficient of variation of 19.54%. Therefore, no further consideration was given to this sample.

When we were ready to apply statistical analyses to the data, results had been received from 27 laboratories. One laboratory had not followed the methods specified. Three laboratories had incomplete results because weighed portions con-

tained too much nitrate, it was difficult to keep organic sample in the flasks, or modifications made it difficult to interpret certain results. It was felt that restricting the statistical study to the results from 23 laboratories with complete data would be adequate.

Statistical Analysis and Discussion

Two linear models were used to study the data, resulting in 2 analyses of variance on each sample or 18 total. The following model was fit to the per cent N data:

$$X_{ijk} = \mu + L_i + M_j + LM_{ij} + e_{ijk}$$

where i = 1, 2, ... number of laboratories; j = 1, 2, 3 (except for Sample 6 when method **2.045** was not used); k = 1, 2; X_{ijk} = reported per cent N from the i^{th} laboratory using j^{th} method for k^{th} run; μ = overall mean; L_i = effect of i^{th} laboratory; M_j = effect of j^{th} method; LM_{ij} = interaction between i^{th} laboratory using j^{th} method; e_{ijk} = random effect, assumed to be normally distributed with mean zero and variance σ^2. The model $X_{ij} = \mu + L_i + M_j + e_{ij}$ (symbols except X_{ij} have same definitions as above) was fit to the absolute differences between runs for each laboratory-sample-method combination to study repeatability.

The data were analyzed on a per sample basis to avoid the need for transformation. *F*-tests and Duncan's new multiple-range test were used in the usual way to identify any effects which would be expected to occur less than 5% of the time by chance (type I error rate). There were no missing values among the data; therefore, each effect was estimated from balanced data.

Our study of repeatability indicated that each method was equally precise with one exception. With Sample 2, the average absolute deviation per method was 0.0843, 0.0443, and 0.1238 for **2.045**, comprehensive, and Raney methods, respectively. The mean for the Raney powder method was significantly higher than that for the comprehensive nitrogen method, but not significant from the mean for **2.045**. The 2 small means were not significantly different. Generally, no major troubles with precision were found for any method, since the average absolute differences were quite small.

The statistical data from 23 laboratories were treated to the ranking test for laboratories, as recommended by Youden (6) (Table 2). Four

Table 2. Summary of ranked results

Nonrefractory Samples[a]		Refractory Samples[b]	
Lab.	Coll. Score	Lab.	Coll. Score
12	17[c]	7	8
7	18[c]	20	10
17	22[c]	6	15
2	37	9	26
9	46	13 labs	27–44
8	57	5	45
16	60	22	47
13 labs	71–91	1	48
22	108	18	59
1	117	8	60
18	138[c]	23	62

[a] Samples 1, 2, 4, 5, 6, 7.
[b] Samples 8, 9, 10.
[c] Significant.

scores showed significance as being beyond the upper or lower 5% region. The score of 138 by Laboratory 18 was the maximum score possible. This meant that the mean of his 6 assays on a given sample was the lowest such mean on each of the 6 nonrefractory samples. As suggested by Youden, the authors decided to set aside all the results from this laboratory. This collaborator tended to get low results on the refractory samples also.

It can be noted that Laboratories 7 and 9 tended to get high results on both sets of samples. In addition to Laboratory 18, Laboratories 1 and 22 tended to get low results on both sets of samples.

Another approach at evaluation was to prepare tables for each of the 9 samples, listing means for each laboratory method. The statistical results supplied by the computer were then applied to the tables to indicate significant differences. These were based on Duncan's new multiple-range test. Table 3 is an example of 1 of the 9 tables and contains data of special interest. Nicotinic acid has frequently been used in Kjeldahl work as a substance on which it is difficult to approach theory. Low results are not rare, but the data reported by Laboratory 23 in Table 3 are unusual. The 3 reported means each represent the average of 3 pairs of precise results. The first and second runs were made about a month apart. This enhances the unusual nature of these values. This laboratory also obtained significantly high data by the Raney powder method on the monoammonium phosphate sample and showed the most frequent pattern of significant differences

Table 3. Per cent nitrogen method means by laboratories for Sample 10[a]

Lab.	2.045	Compr. N	Raney
1	11.24	11.18	11.13
2	11.41[b]	8.80[c]	11.45[b]
3	11.26	11.24	11.24
4	11.37	10.97	11.26
5	11.31	10.62	11.30
9	11.30	11.30	11.46
10	11.23[b]	9.18[c]	10.87[b]
11	11.29[b]	10.26[c]	11.34[b]
16	11.33	11.27	10.62
17	10.03[b]	11.21[c]	10.95[c]
22	11.18	11.17	11.24
23	2.14[b]	4.58[c]	13.96[d]
Overall	10.84[b]	10.66[c]	11.34[d]

[a] Table is incomplete but includes all data of significance; Sample 10 is nicotinic acid, theory 11.38% N.
[b, c, d] Different superscripts show significant differences in a horizontal plane.

Table 4. Significant within-laboratory differences[a]

Sample	2.045 H	2.045 L	Compr. N H	Compr. N L	Raney H	Raney L	Total per Sample
1	0	0	0	2	0	0	2
2	0	0	1	1	0	1	3
4	0	3	0	1	0	0	4
5	0	1	0	3	0	0	4
6	X	X	1	1	0	3	5
7	0	1	0	1	0	2	4
8	2	1	0	3	1	0	7
9	0	3	0	0	0	1	4
10	0	1	0	3	0	0	4
Subtotal	2	10	2	15	1	7	
Total	12		17		8		37
Adjusted total[b]	11		10		7		28

[a] H and L denote the number of laboratories with significantly high or low within-laboratory values, comparing this method vs. the other methods. X = method not applicable to this sample.
[b] Deleting Laboratory 4 and Sample 10.

between methods. From this accumulation of irregular data it was decided to exclude all data from Laboratory 23 from the basic collaborative study.

The data from 21 laboratories was then processed by the computer. The 9 tables were reevaluated for significant differences, using Duncan's new multiple-range test. A summary of the frequency of the significant differences is given in Table 4.

As would be expected, most of the differences were to the lower side. The differences that were found between methods were more often found with the comprehensive nitrogen method. Two special factors contributed to this. Three of the differences were low values on Sample 10, nicotinic acid, and 4 of the low values were from Laboratory 4 having low results from the comprehensive nitrogen method. The adjusted total in Table 4 shows this. On this basis the new methods performed as well as **2.045**, with the Raney method having the best rating.

Of the 546 means for pairs of assays from the 21 laboratories, there were only 9 statistically isolated low means and no isolated high means. Two were from the comprehensive nitrogen method on the nicotinic acid. (This was not unexpected as the method has not proven extremely rugged for nicotinic acid in previous studies (2, 3).) Two of the other low means were made up by pairs of a low and a normal assay by the comprehensive nitrogen method on Sample 1. The Raney method gave a pair of low values on Sample 2 (13–13–13) where 1.6 g sample was weighed. The Raney method also gave a pair of low values on Sample 6, the 28% nitrogen solution. Perhaps the sample weighed was too large and the reducing capacity of the Raney powder was exceeded. Surprisingly, 3 laboratories had pairs of low results on Sample 5, the monoammonium phosphate. Two of them used the comprehensive nitrogen method and one used **2.045**.

A search for a correlation relating to the factors influencing the isolated low results was not too fruitful. One factor is that Laboratory 4 got 2 of these low results. This laboratory had 5 significant between-method variations in the overall study of means in Table 4. It was significantly low by the comprehensive nitrogen method on 4 of the 9 samples. The Associate Referee is not certain that the weakness is primarily within the laboratory concerned or more a function of the method. If the results of Laboratory 4 and the nicotinic acid sample are removed from the data, then there are only 5 significantly isolated low means; 2 are from 1 laboratory. There does not appear to be a firm basis for criticism or for choosing between methods by isolated low means.

A summation of the data is shown in Table 5. All samples had respectable coefficients of variation except 9 and 10. Sample 9 gave special problems with foaming and was the least uniform in composition based on physical appearance. Statistically all samples except Sample 7, the

Table 5. Summary of data from all laboratories

Sample	% N			Coeff. of Var.[a]	Range of Means[b]	Range of Means[c]	AAFCO Tolerances[d]
	2.045	Compr. N	Raney				
1	6.50	6.45[e]	6.51	1.65	0.55	0.24	0.52
2	13.40[f]	13.34	13.34	0.66	0.84	0.48	0.61
4	13.64[e]	13.73	13.73	1.00	0.86	0.86	0.61
5	12.06	12.04	12.14[f]	1.15	1.32	0.52	0.61
6	——	27.72[f]	27.65[e]	0.55	1.75	1.22	0.83
7	37.90	37.89	37.77	1.01	2.19	2.19	0.88
8	12.47	12.40[e]	12.46	0.83	0.53	0.53	0.61
9	3.60[e]	3.74[f]	3.68	3.58	0.68	0.68	0.49
10	11.23	10.94[e]	11.24	3.11	2.73	1.49	—
Sum[g]	127.26	127.31	127.28				

[a] All data for each sample.
[b] Range of the 63 lab. means by sample and method (42 for Sample 6).
[c] Adjusted with 9 isolated low means deleted.
[d] Recommended AAFCO tolerances for testing for deficiency (7).
[e] Significantly low.
[f] Significantly high.
[g] Excluding Sample 10 and using 27.69 for method **2.045**, Sample 6.

urea-form material, show significant differences between methods. Even then the data do not lend themselves to any easy choice in clearly picking one method over the other.

The Associate Referee was especially interested in the performance for Samples 4 and 5, the standard reference materials. Significant between-method differences did appear. Method **2.045** was low for Sample 4 (KNO_3), and the Raney method was high for Sample 5 (monoammonium phosphate). The new methods gave more results above theory than the Referee expected. Seven means, ranging from 13.89 to 14.14, were obtained on the KNO_3 (theory 13.85). Thirteen means between 12.21 and 12.35 were obtained on the monoammonium phosphate (theory 12.17). These above theory values were about equally divided between the 2 new methods. Method **2.045** had no value above 13.87 on Sample 4 and only 2 values above 12.21 on Sample 5. There were no isolated significantly low means on Sample 5, but the 3 that occurred on Sample 6 are not easily understood or explained. This extension of the range or tailing out of results is certainly a function of an expanded 21-laboratory study. The range of values in the 3-laboratory 1957 study (3) for monoammonium phosphate was 0.31 versus the adjusted range of means of 0.52 for the current study. In a large study the natural negative biases will occasionally be overshadowed by some other sources of bias that can give either high or low results. An evaluation of the blanks reported with the study reveal no unusual pattern. Irregular blanks were rare. It might be noted that the blanks were of a ratio of 1, 2, and 3, respectively, for the methods. This should create no problems, as even the significantly higher blanks by the Raney method were consistent in magnitude.

The Associate Referee decided to include the AAFCO recommended tolerances in Table 5 to compare them with the corrected range of means. Although considerably different factors are involved in these 2 columns of data, some comparisons seem worthy of mention. The magnitude of the ranges versus the AAFCO tolerances indicates a problem area for Sample 6, the 28% nitrogen solution, and Sample 7, urea-form. It appears that the AAFCO tolerances are relatively small compared to the magnitude of the analytical variability in assaying the samples in this study. The Referee hopes that the variability in assaying these high analysis materials may be decreased. Meanwhile, the lower analysis materials seem to enjoy a distinct advantage in the AAFCO tolerances.

Three overall points are of major interest in Table 5: the magnitude of the differences between method means, the rather random pattern of significantly high and low results, and the close agreement of the sums. The conclusion is that the 2 new methods are practically equivalent, on the basis of statistical data, for their intended use. They are also practically equivalent to **2.045**, where it is applicable.

Comments of Collaborators

Six of the 21 laboratories indicated that no real effort was made to meet the specified boil test because single heat digestion burners were used. This factor did not significantly affect their data. Probably the boil tests are not as critical as would be expected, but because of the research in method development, especially with certain samples, it is felt best to leave the specified boil tests in the written procedures.

The collaborators were not asked to state a preference or to rate the methods. One section of the data reporting form did ask for comments. Most of the 29 laboratories did not state a preference. Of those that did, many still prefer **2.045** to either of the new methods. Preferences were made for the Raney method over the other 2, for the comprehensive method over the Raney method, and for other methods not in the study.

The most common complaint was more foaming with organics with the new methods. The Raney method was criticized somewhat more on this point than the comprehensive method.

All 3 methods at times gave low results on the nicotinic acid sample. Additional low results on this sample were submitted under comments. The comprehensive method gave the most trouble.

Two laboratories commented on the extra fumes encountered with the comprehensive method. Other comments were on the large amount (150 ml/determination) of *Reagent* (**b**) and low amount of nitrates used by the Raney method.

Conclusions and Recommendations

Both new methods meet the goal of being applicable to all fertilizer samples. Certain factors such as simplicity call for choosing a single official method for total nitrogen, but a clear choice of the one best method is not indicated to the Referee at this time. Some of the factors contributing to this that have not been mentioned in this paper are the variability in the percentage of samples containing organic matter in various parts of the country and the disadvantage of being limited to one source of a key reagent such as the Raney catalyst powder.

It is recommended (*1*) that the comprehensive nitrogen method included here be adopted as official first action; (*2*) that the Raney powder method included here be adopted as official first action; (*3*) that **2.046** and **2.047** (reduced iron) and **2.048** and **2.049** (chromium powder) be deleted; (*4*) that the second sentence in **2.043**(**a**) be changed to read: "Conduct digestion over heating device adjusted to bring 250 ml H_2O at 25° to rolling boil in ca 5 min or other times as specified in method"; (*5*) that the second paragraph of **2.044** be changed by substituting "enough" for "25 g" after first parenthesis.

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Collaborative Study on an Automated Method for K_2O in Fertilizers

By LARRY G. HAMBLETON (Department of Biochemistry, Purdue University, Lafayette, Ind. 47907)

An automated method for K_2O in fertilizers utilizes basic Technicon equipment and the Technicon range expander. The range of 35–55 ppm was expanded to read 20 to 80% *T*, respectively. Nine laboratories studied the automated method and the official STPB method 2.085 for ten fertilizer samples and a potassium nitrate standard. The study lacked participation from laboratories having AutoAnalyzer equipment. The method is recommended for further study.

In 1964 Gehrke, Ussary, and Kramer (1) published an automated flame photometric method, using the Technicon AutoAnalyzer for the analysis of K_2O in fertilizers. The sampling rate was 40/hr; a propane-oxygen flame photometer was used with a lithium nitrate internal standard. The method covered a range of 0–20 ppm potassium, using potassium nitrate to standardize the instrument. They reported that phosphate was the only anion present in fertilizers that caused a significant interference in the potassium-lithium intensity ratio and concluded that fertilizers containing less than 16% K_2O could be analyzed directly without anion exchange resin treatment. For samples with a K_2O content greater than 16%, more accurate results were obtained when sample aliquots were passed through an anion exchange column.

Ussary and Gehrke (2) published an automated flame photometric method in 1965 for the analysis of potassium in fertilizer-grade potassium salts. This was a modification of the original method (1). The pumping manifold was redesigned to

operate in the range of 35–65 ppm K_2O. The Technicon recorder was replaced with a Leeds and Northrup zero adjustable range recorder. This replacement allowed the lower end of the working curve to be suppressed and the upper portion expanded to read full scale. A 2.5 K potentiometer was placed between the sample and reference recorder input leads. When the signals from the potassium-lithium detectors were equalized with the potentiometer, the effect of the pumping pulsations was eliminated. Later, Ussary and Gehrke (3) applied the method to regular-grade fertilizers.

The method used in this collaborative study was a modification of the Ussary and Gehrke method (3). The same pumping rates were used, with the addition of a small mixing coil after the pump to mix $LiNO_3$ and water before sample was added. This addition increased the stability of the flame photometer and also decreased the noise level. The Technicon recorder was used with the addition of the Technicon range expander. With a 2× expansion, the range of 35 to 55 ppm was expanded to read 20–80% T, respectively. Potassium dihydrogen phosphate was used to prepare the standard solutions in place of potassium nitrate. Potassium dihydrogen phosphate used as the standard reduced interferences due to phosphate anion. The method incorporates the ammonium oxalate and the ammonium citrate extraction methods.

Evaluation of the method was made with samples used in the 1968 collaborative study on potassium in fertilizer. For this evaluation, a Technicon flame photometer III which uses natural gas and air for the flame was used. The samples were analyzed 5 times each and the means from these analyses were compared to the means reported by the official STPB method **2.085** for the samples in the collaborative study. These means did not show a significant difference at the 5% level. The standard deviations were approximately 0.1 for all samples.

It was found that the use of cylinder air in place of air from a compressor reduced the noise level of the recordings. The range expander makes it necessary to clean the recorder slide wire more frequently to obtain good precision.

This report of the Associate Referee was presented at the 83rd Annual Meeting of the AOAC, Oct. 13–16, 1969, at Washington, D.C.

Collaborative Study

Ten mixed fertilizer samples and a potassium nitrate primary standard were sent to each of 9 collaborators. The study was designed according to the plan for closely matched pairs as discussed by Youden (4). The following samples were sent to collaborators:

Pair 1	6–24–24
	6–24–25
Pair 2	15–15–15
	14–14–14
Pair 3	15–40–8
	15–40–6
Pair 4	0–15–40
	0–15–44
Pair 5	0–0–60
	0–0–62

KNO_3 primary standard (46.59% K_2O theory)

The collaborators were requested to make a single determination on each sample by the automated flame photometric method and by the official STPB method **2.085**. The samples were to be analyzed by both extraction methods and the collaborators were requested to include their standard curves and recording charts with their reports. The collaborators were also requested to include 2 standards at the end of the run to check the drift of the instrument. If drift was excessive, they were to rerun sample solutions and standards.

METHOD

Reagents and Apparatus

(a) *Technicon AutoAnalyzer.*—Basic Technicon equipment with flame photometer and range expander (Fig. 1).

(b) *Ammonium oxalate solution.*—4%. Dissolve 50 g $(NH_4)_2C_2O_4$ in 1 L water.

(c) *Ammonium citrate solution.*—Prepare as in **2.032**(a).

(d) *Lithium nitrate solution.*—Dissolve 0.6894 g dried $LiNO_3$ (2 hr at 105°C) in 1 L water.

(e) *Standard stock solution.*—1.4447 g dry (2 hr at 105°C) KH_2PO_4/L water (0.5 mg K_2O/ml).

(f) *Working standards.*—35, 38, 41, 44, 47, 50, and 55 μg K_2O/ml. Use 50 ml buret to measure appropriate amounts of stock solution into 500 ml volumetric flasks containing 0.2 g $(NH_4)_2C_2O_4$ for sample preparation (a), or 12 ml ammonium citrate solution **2.032**(a) for sample preparation (b). (If am-

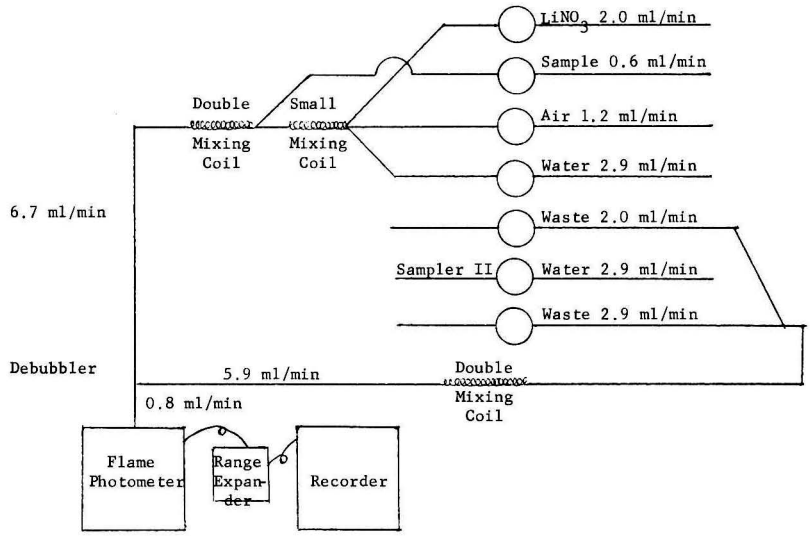

FIG. 1—Flow schematic for autoanalysis for K_2O in fertilizers.

monium citrate standards are to be held overnight, add 3–4 drops $CHCl_3$.)

Sample Preparation

(a) *Ammonium oxalate extraction.*—Weigh 1 g sample into 500 ml volumetric flask, add 50 ml 4% $(NH_4)_2C_2O_4$ and 125 ml water, boil 30 min, and cool. Dil. to volume with water, mix, and filter or let stand until clear.

(b) *Ammonium citrate extraction from direct available P_2O_5 extract.*—Prepare as in **2.037**. Add 3–4 drops $CHCl_3$ to sample solutions if they are to be held overnight.

Instrument Calibration

Pump 55 μg K_2O/ml standard continuously through AutoAnalyzer system. Set range expander to 1× position and adjust calibration control on flame photometer to read 85% T on recorder. Pump 35 μg K_2O/ml standard continuously and set range expander to 2× position. Turn adjustable range positioner on range expander to obtain 23% T on recorder. Range of 35–55 μg K_2O/ml will read ca 20–80% T on recorder with range expander set at 2× position.

Determination

Pipet aliquot of sample solution, according to tabulation below, into 250 ml volumetric flask. Dilute to volume with water and mix. Read sample and standards at the rate of 40/hr with AutoAnalyzer. Prepare standard curve of emission against concentration of K_2O and read μg K_2O/ml from graph.

% K_2O = μg K_2O/ml from standard curve × appropriate factor

% K_2O Expected in Sample	*Aliquot*	*Factor*
2	no diln	0.0500
3–4	150	0.08333
5–6	100	0.1250
7–8	75	0.1667
9–13	50	0.2500
14–16	40	0.3125
17–20	30	0.4167
21–25	25	0.5000
26–30	20	0.6250
31–43	15	0.8333
44–65	10	1.250

Results and Discussion

Five collaborators reported results by the automated flame photometric method. Two collaborators reported results using only the STPB method. Of the 5 laboratories that reported flame photometric results, 3 used Model III flame photometers, 1 used a Model II, and 1 a Model I. The AutoAnalyzer recording charts showed very little drift when compared with the 2 standards placed at the end of the run. The laboratories using the Model III flame photometers were able to obtain the proper expansion with the range expander. The expansion obtained by the Model II instru-

Table 1. Collaborative results for automated and official STPB methods for K_2O in fertilizers[a]

	Av. Found, %			s_r Precision			s_b Systematic			s_d		
Pair	Oxalate	Citrate	STPB	Oxalate	Citrate	STPB	Oxalate	Citrate	STPB	Oxalate	Citrate	STPB
1	25.17	25.20	25.12	0.21	0.10	0.09	0.14	0.38	0.17	0.29	0.55	0.25
2	14.74	14.77	14.74	0.12	0.07	0.20	0.07	0.15	0.09	0.15	0.22	0.23
3	7.84	7.91	7.87	0.13	0.08	0.13	0.17	0.23	0.13	0.27	0.32	0.23
4	42.39	42.65	42.40	0.45	0.50	0.12	$-s_b$	0.71	0.23	0.22	1.12[b]	0.35
5	61.16	61.60	61.41	0.30	1.07[c]	0.25	0.28	0.75	0.57	0.50	1.51[d]	0.85
Std, 46.59% K_2O	46.91	46.14	46.53							0.57	0.85	0.48

[a] Results of a single determination on each pair by each collaborator.
[b] 0.26 when laboratories using flame Models I and II omitted from calculations.
[c] 0.47 when laboratories using flame Models I and II omitted from calculations.
[d] 0.22 when laboratories using flame Models I and II omitted from calculations.

ment was less than that obtained by the Model III. The collaborator using the Model I flame was unable to obtain proper expansion with a 2× expansion; therefore, he used 4× expansion and a range of 44–55 ppm K_2O.

The data were ranked by Youden's ranking criteria (4) to determine whether the Model I and II flame photometers gave consistently higher or lower results when compared with the results from the Model III. With the oxalate extraction method, all laboratories fell within the critical scores at the 5% level, but the laboratory using the Model I flame fell outside these scores when the citrate extraction was used. The expansion with the flame Model I was greater with the standards used for the oxalate extraction than for the standards used for the citrate extraction.

Two-sample X-Y charts were prepared for each pair of samples as indicated by Youden (4). For the oxalate extraction method, the pairs showed a compact cluster of the data points. For the citrate extraction method, the results by the flame photometer Model I fell outside the cluster of data points for all pairs except No. 2. For pairs Nos. 4 and 5, the laboratory with the Model II instrument also fell outside this cluster of data points. In Table 1, results are shown that contain the data from all of the laboratories and the effect, when significant, on the data of omitting the laboratories using the Model I and II flame photometers. The data for sample pairs 4 and 5 with citrate extraction, where noted, represent only the laboratories using the flame photometer Model III. The results of omitting these laboratories show the effect of the Model I and II flame photometers in raising the standard deviations.

The estimate of the standard deviation of how successfully repeat determination can be made in the same laboratory under the same conditions (s_r) compares favorably for the automated oxalate extraction method with the s_r for the STPB method for each sample pair. s_r for the automated citrate extraction method also compares favorably with the STPB method with the exception of the laboratories using the flame Models I and II on pair 5.

The estimate of the standard deviation for the distribution of systematic error between laboratories is given under s_b in Table 1. For the automated citrate extraction method, s_b^2 is significant at the 5% level, when compared to the STPB method by the F-test for pairs 1, 2, and 3. It is not significant for the automated oxalate extraction method.

The standard deviation for the data, s_d, is given in Table 1. When s_d^2 is compared by the F-test to s_d^2 for the STPB method, there is a significant difference at the 5% level for sample pair 4 by the automated citrate extraction method. The other pairs do not show a significant difference at this level.

The means from the automated oxalate and the automated citrate extraction methods were compared to the means of the STPB method by the t-test at the 5% level and did not show a significant difference.

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Conclusions and Recommendations

The automated method using ammonium oxalate and ammonium citrate extraction appears tenable for the flame photometer Model III. A comparison of the means for the automated method and the official STPB method did not show a significant difference; s_r, s_b, and s_d compare closely to those obtained by the STPB method.

These estimates for the flame photometer Models I and II are acceptable for all pairs, using ammonium oxalate extraction. The Model I instrument gave unacceptable results on sample pairs 4 and 5, using the ammonium citrate extractant. The Model II photometer also gave erratic results on these sample pairs with ammonium citrate extraction. The range expansion obtained by both instruments, using the citrate extraction, was considerably less than the expansion obtained, using the oxalate extraction. With samples containing large amounts of K_2O, a small error in reading the recordings provided by the instrument would introduce a large error in K_2O content when the range is not fully expanded.

The collaborative study lacked participation from laboratories having AutoAnalyzer equipment. A larger participation from laboratories with Models I and II flame photometers is needed to determine adequacy of this method with these instruments. While evaluation of data obtained from Model III instruments indicates satisfactory results may be obtained, the participation of only 3 laboratories equipped with this instrument indicates further study before adoption as an official method by the Association.

It is recommended that study be continued on the automation of the analyses of K_2O in fertilizers and that the modified STPB method be adopted as official final action.

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The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee A and were adopted by the Association. See *JAOAC* **53**, 377–378 (1970).

DRUG RESIDUES IN ANIMAL TISSUES

GLC Determination of Ethopabate in Chicken Tissues by Derivative Formation

By P. R. HANDY[1] and F. J. HOLZER (Eli Lilly and Co., Greenfield Laboratories, P.O. Box 708, Greenfield, Ind. 46140)

The published method for the determination of ethopabate residues in chicken tissues has been improved by formation of the 2,4-dinitrophenyl derivative of *m*-phenetidine, which is produced by hydrolysis of ethopabate. The derivative is suitable for gas-liquid chromatography, using an electron affinity detector, and allows a simpler cleanup procedure. The sensitivity of the method is about 0.05 ppm; average recoveries at 0.5 ppm range between 66 and 97% for all tissues.

Ethopabate (4-acetamido-2-ethoxybenzoic acid, methyl ester) is a feed additive used as an aid in prevention of coccidiosis in chickens (1). The published residue method specifies the hydrolysis of ethopabate and most of its metabolites to *m*-phenetidine (2), an extensive extraction–cleanup procedure, and final determination by GLC, using argon ionization detection. The cumbersome nature of the extraction steps and the unavailability of an argon ionization detector in our laboratory led to the investigation of an alternative procedure based on conversion of the *m*-phenetidine to its 2,4-dinitrophenyl (DNP) derivative. This derivative is suitable for GLC with electron affinity detection (3, 4) which, due to its specificity and sensitivity, permits a simpler cleanup procedure.

Experimental

Reagents

(Solvents and solutions, unless specified, are analytical reagent grade.)

(a) *1-Fluoro-2,4-dinitrobenzene (DNFB).*—(Eastman No. 6587). Purify by fractional crystallization as follows: Slowly cool DNFB in refrigerator until most of solution has solidified; then decant remaining liquid. The solid DNFB is then ready for use after warming to room temperature.

(b) *Cyclohexane.*—Practical (Matheson, Coleman and Bell). Purify by percolating 500 ml through column containing 40 g silica gel (0.2–0.5 mm) (Brinkmann Instruments Corp.).

(c) *m-Phenetidine-DNP reference standard.*—Add 1 g *m*-phenetidine (Eastman No. 1399) to 50 ml 2.5% aqueous sodium borate solution. Add 50 ml *p*-dioxane (Spectroquality, Matheson, Coleman and Bell) containing 2 ml DNFB and heat mixture 45 min in 60°C water bath. Cool mixture and collect precipitate by vacuum filtration. Wash precipitate with 0.1*M* Na_2CO_3 and dry, using vacuum. Recrystallize from ethanol-water and dry again, using vacuum. Melting point should be 151–152°C. Use 1 μg/ml solution in purified cyclohexane to establish retention time of *m*-phenetidine-DNP.

Gas Chromatography

Use either Jarrell-Ash Model 28-700 universal chromatograph, Hewlett-Packard Model 402 gas chromatograph, or similar instrument. Pack 4′ × 3 mm id glass column with 3% OV-17 on 80–100 mesh Gas Chrom Q. Conditions and operating parameters for Jarrell-Ash instrument: tritium electron affinity cell, column temperature 242°C, flash heater 280°C, detector 220°C, nitrogen carrier gas 80 ml/min, electrometer 0.1×10^{-9} AFS, and chart speed 4 min/inch. Conditions and operating parameters for Hewlett-Packard instrument: nickel-63 electron affinity pulsed detector, column temperature 252°C, flash heater 280°C, detector 295°C, argon-methane (90 + 10) carrier gas 100 ml/min, electrometer range 1, attenuation 32, pulse 150, and chart speed 4 min/inch. Use any sample injection procedure listed in *Pesticide Analytical Manual* (5). *m*-Phenetidine-DNP has a retention time of 6 min and yields symmetrical peaks.

Sample Preparation

Blend representative 5 g sample chicken muscle, liver, kidney, fat, or skin in 100 ml deionized water and rinse blended tissue into 400 ml beaker. Add 5.0 ml concentrated HCl, cover beaker, autoclave 90 min at 121°C, and cool rapidly in ice bath.

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Extraction

Filter hydrolysate through Whatman No. 2V paper into 500 ml separatory funnel. Rinse beaker and filter with deionized water. Adjust pH to 8–9 with 50% NaOH, using pH paper. Extract with 50 ml carbon tetrachloride (CCl_4) and let stand until phases separate. Filter clear CCl_4 layer and any light emulsion through granular Na_2SO_4 into 250 ml separatory funnel containing 30.0 ml 0.5*N* HCl. Repeat extraction of hydrolysate with 2 additional 50 ml portions of CCl_4. Drain heavy emulsion after third extraction, taking care to distribute it evenly over Na_2SO_4 to prevent channeling. (Alternatively, centrifuge emulsion layer and then drain clear CCl_4 through Na_2SO_4.) Rinse Na_2SO_4 with 25 ml CCl_4 and shake combined CCl_4-HCl mixture at least 30 sec. Let layers separate and discard lower CCl_4 layer. Pipet 15.0 ml HCl layer into 125 ml Erlenmeyer flask.

Derivatization

Neutralize HCl aliquot to pH 7.5–8.5 with 2*N* NaOH and pH paper. Add 10 ml borate solution and 1 drop DNFB and agitate mixture on shaker table 30 min. Transfer flask to 60°C water bath and heat 30 min. Add 2 ml 2*N* NaOH, mix, and continue heating 50 min. Cool sample and quantitatively transfer to narrow-neck, 50 ml volumetric flask with deionized water. Pipet 1.0 ml purified cyclohexane into flask, shake vigorously 1 min, let stand 5 min, and shake again. Add deionized water to flask to bring cyclohexane layer into reach of syringe and inject 1–2 μl for GLC analysis.

Analyze a standard recovery sample consisting of control tissue sample fortified with 0.50 ppm ethopabate with each set of experimental samples. Also analyze the following process standard: Add 2.5 μg ethopabate to 100 ml water and process exactly as sample beginning with addition of HCl.

Calculations

Measure peak height (PH) of standards, recovery samples, and experimental samples and determine ethopabate:

ppm ethopabate =
(sample PH/recovery sample PH) (0.05)

Calculate per cent recovery in recovery sample as follows:

% Recovery = (recovery sample PH/process standard PH) (100)

Discussion

Ethopabate and most of its metabolites are converted by acid hydrolysis to a common compound, *m*-phenetidine. The *m*-phenetidine is extracted with CCl_4 from the hydrolysate after pH adjustment. Emulsions tend to form with liver and kidney samples. However, we have found that nearly quantitative extraction can be obtained by filtering the emulsion through Na_2SO_4 or centrifuging and washing the salt bed with CCl_4. Extraction from filtered CCl_4 into 0.5*N* HCl is essentially quantitative.

The conversion of *m*-phenetidine to the DNP derivative at levels equivalent to 0.1 ppm ethopabate is unreliable in the presence of solvents such as acetone or *p*-dioxane in the DNFB-buffer mixture because of low level solvent impurities. This led to the direct addition of DNFB to the buffered sample and subsequent shaking to effect dispersion of the reagent. Conversion of *m*-phenetidine to the DNP derivative is about 80% and is reproducible as indicated in Table 1. Quantitation is made by comparison of samples to standards derivatized simultaneously. Some care

Table 1. Recovery of standard added at various steps in procedure

Sample	Step	*m*-Phenetidine Added, μg	Peak Height, cm	Rec., %
1	reagent blanks	0.0	nil	
2		0.0	nil	
3	acid before CCl_4 extraction	3.18	10.05	83
4		3.18	9.90	82
5	CCl_4 fraction	3.18	10.10	83
6		3.18	10.50	86
7	acid aliquot after CCl_4 extraction	1.59	9.85	81
8		1.59	9.75	80
9	flask after derivative formation	0.74	5.90	104
10		0.74	5.70	101
Standard		0.74	5.65	

[a] Values represent amount of *m*-phenetidine base added. In samples 3–8, this was added as the hydrochloride and in samples 9–10, as the DNP derivative.

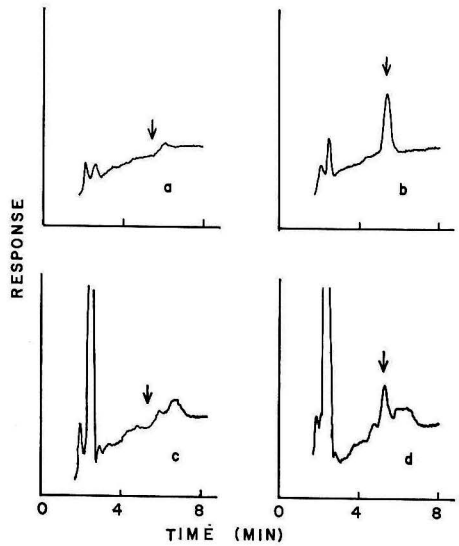

FIG. 1—Typical chromatograms of: a, process reagent blank; b, process ethopabate standard, 0.1 ppm; c, liver control; d, liver control plus ethopabate standard, 0.1 ppm.

is required in the extraction of the derivative into cyclohexane. The samples should be shaken thoroughly as described to insure that the extraction is complete.

No deleterious effects on the tritium cell were observed even after its use for the determination of these samples over a period of several months.

Results

Typical chromatograms are shown in Fig. 1. Parts **a** and **b** show a reagent blank and a 0.1 ppm (0.5 μg) ethopabate standard after conversion to *m*-phenetidine-DNP. Chromatograms **c** and **d** are liver control and 0.1 ppm recovery samples. Note that although there is some sample background, both the reagent blank and control are clear at the retention time of *m*-phenetidine-DNP. Standard curves of ethopabate converted to *m*-phenetidine-DNP for both process standards and recoveries from liver are presented in Fig. 2. Table 1 shows the extraction efficiency and conversion to the DNP derivative. No tissue was present in these samples. As can be seen from the recovery values, the partition steps are nearly quantitative, while the conversion is 80% or better.

Table 2 lists results from control liver samples fortified with ethopabate at various levels. These data represent a composite of several sets of samples assayed on several days and are indicative of the overall performance of the procedure. These data demonstrate that the recovery of standard added to liver tissue is approximately 75% at the recommended fortification level of 0.50 ppm. Recoveries are above 50% at levels of 0.1 ppm; ethopabate can be detected at a level of 0.05 ppm. Recoveries at 0.1 ppm for kidney, lean, skin, and fat averaged 65, 66, 98, and 89%, respectively, for a total of 16 determinations on the 4 tissues.

Initially, it was intended that the recommended fortification level for tissues would be 0.1 ppm. However, in view of the publication of ethopabate tolerances (6) of 1.5, 1.5, and 0.5 ppm for kidney, liver, and lean, respectively, it is recommended that the fortification level for controlling the procedure be 0.5 ppm.

Table 3 demonstrates day-to-day and within-day variability of the assay procedure in the hands of 1 analyst. These data demonstrate that the precision of the procedure is good at the 0.5 ppm fortification level.

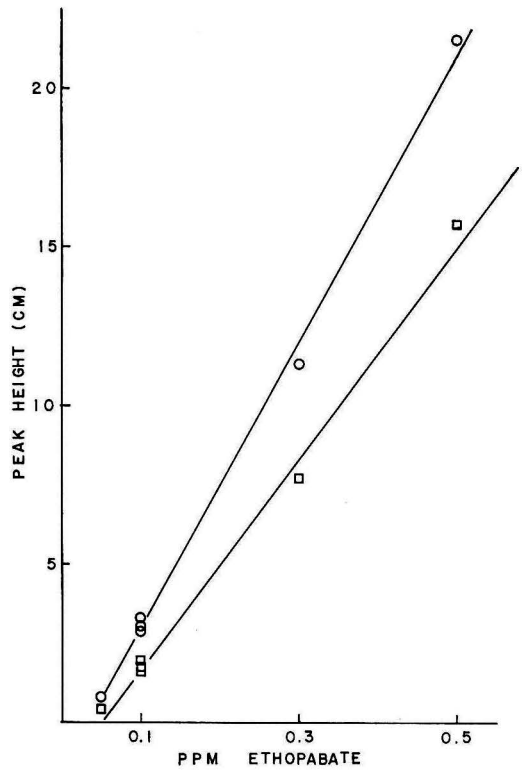

FIG. 2—Ethopabate standard curves: ○, process standards; □, recovery standards from liver.

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Table 2. Ethopabate recovery from fortified control liver

Fortification Level, ppm				
0.05, %	0.1, %	0.3, %	0.5, %	
50	56	68	73	70
36	52	71	97	68
59	58	72	78	74
	85		86	76
			66	85

Acknowledgment

The authors wish to thank L. G. Turner for his excellent technical assistance during the final stages of the assay development.

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Table 3. Precision of procedure for recoveries at 0.5 ppm fortification of chicken liver

Sample	Day 1 Peak Height, cm	Day 1 Rec., %	Day 2 Peak Height, cm	Day 2 Rec., %
Control 1	—		—	
Control 2	—		—	
Control 3	—		—	
Control + std 1	5.45	66	5.45	74
Control + std 2	5.80	70	5.65	76
Control + std 3	5.65	68	6.30	85
Process std	8.30		7.40	
Mean, all values		73.2		
Std dev.	6.9			
Coeff. of var., %	9.43			

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Nalidixic Acid in Chicken Tissue

By ROBERT S. BROWNING and EDWARD L. PRATT (Sterling Winthrop Research Institute, Rensselaer, N.Y. 12144)

Nalidixic acid in chicken muscle and liver homogenates can be estimated by spectrophotofluorimetric measurement. The method, effective for determining nalidixic acid in tissue at levels of 100 ppb, involves extraction, cleanup, and measurement of fluorescence of the isolated drug in 60% H_2SO_4. In a collaborative study the average recovery was 93% of actual values and the overall estimated standard deviation was 26 ppb. The method is recommended for adoption as official first action.

Nalidixic acid is the nonproprietary name for the antibacterial agent, 1-ethyl-1,4-dihydro-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid, first described by Lesher *et al.* (1). This now established chemotherapeutic agent has high anti-infective efficacy against a wide spectrum of gram-negative bacteria. It has been used for a number of years in the treatment of human infections. It is also an effective aid in the control of gram-negative infections in chickens. When used for the latter purpose it is added to drinking water as the sodium salt at a concentration of 0.5 g/gallon.

Methodology was needed to monitor drug residue levels in chicken tissue. McChesney *et al.* (2) developed analytical methodology for estimating absorption, excretion, and metabolism of nalidixic acid in man. The methodology described in this report is a refinement of that presented by McChesney. It provides improvements in selectivity of drug isolation and sensitivity of drug detection when applied to chicken liver and chicken muscle homogenates. Intralaboratory studies indicated that the proposed method was capable of measuring 100 ppb nalidixic acid in chicken muscle and chicken liver with coefficients of variation of 6 and 10%, respectively. Recov-

Azolix TM is the Sterwin Laboratories, Inc. brand name for sodium nalidixate.

eries of drug added to homogenates were quantitative. The blank response for muscle was 9 ppb; for liver, 21 ppb. The following method was collaboratively studied.

METHOD[1]

(Applicable to chicken liver and muscle contg ≥100 ppb nalidixic acid)

37.014 *Principle*

Nalidixic acid is extd from aq. tissue homogenate with EtOAc. EtOAc is collected, concd, and passed thru Al_2O_3 column which retains nalidixic acid. Nalidixic acid is removed from column with borate buffer, acidified, and re-extd with $CHCl_3$. After $CHCl_3$ removal, residual nalidixic acid is made to fluoresce with H_2SO_4 and resultant fluorescence is measured with spectrophotofluorimeter.

37.015 *Apparatus*

(a) *Spectrophotofluorimeter.* — (*Caution: See* **46.008.**) Aminco-Bowman 4-8202, or equiv., with Xe lamp, IP 28 photomultiplier tube, and operated with manufacturer's slit arrangement No. 3. Precise wavelength settings for excitation and emission may vary slightly between instruments. Det. optimum wavelengths after evapn of 2 ml working std soln (1 μg nalidixic acid) and soln of residue in 10 ml 21.5*N* H_2SO_4.

(b) *Chromatographic tubes.* — 11.5 × 160 mm (Kontes Glass Co. No. K-420000, or equiv.).

(c) *Shaker.*—Reciprocating (Sargent-Welch Scientific Co. No. S-74060, or equiv.).

37.016 *Reagents*

(a) *Phosphate buffer soln.*—pH 6.0. Weigh 28 g $NaH_2PO_4.H_2O$ into 1 L beaker, add ca 600 ml H_2O, and adjust pH electrometrically with aq. NaOH. Dil. to 1 L.

(b) *Borate buffer soln.*—pH 10.0. Dissolve 30 g H_3BO_3 in ca 600 ml H_2O and adjust pH electrometrically with aq. NaOH. Dil. to 1 L.

(c) *Dilute sulfuric acid.*—(*Caution: See* **46.030.**) (*1*) *21.5N.*—Measure 200 ml H_2O into 1 L flask and add gradually, with cooling, 300 ml H_2SO_4. Use soln at room temp. (*2*) *7N.*—Dil. 1 vol. (*1*) with 2 vols H_2O.

(d) *Alumina.*—Neutral (Fisher Scientific Co. No. A-950, or equiv.).

(e) *Nalidixic acid std solns.*—(*1*) *Stock soln.*—500 μg/ml. Dissolve 50.0 mg nalidixic acid (available from Sterling Winthrop Research Institute, Rensselaer, NY 12144) in 100 ml MeOH. (*2*) *Intermediate soln.*—5.0 μg/ml. Dil. 2.0 ml stock soln to 200 ml with MeOH. (*3*) *Working soln.*—0.5 μg/ml. Dil. 10.0 ml intermediate soln to 100 ml with MeOH.

37.017 *Determination*

(*Caution: See* **46.005, 46.039, 46.040, 46.056,** and **46.057.**)

Transfer 10 g chicken liver or muscle to high-speed blender. Add 100 ml phosphate buffer and blend 2–3 min. Transfer homogenate to 500 ml g-s extn bottle and add 300.0 ml EtOAc.

Add 100 ml phosphate buffer to each of five 500 ml g-s extn bottles. Transfer 0, 1.0, 2.0, 3.0, and 4.0 ml working soln contg 0.0, 0.50, 1.0, 1.5, and 2.0 μg nalidixic acid, resp. Add 300.0 ml EtOAc to each. Mech. shake all bottles contg sample and std 10–15 min and centrf. ca 5 min at 2500 rpm. Withdraw 250.0 ml supernatant EtOAc from each and transfer to sep. 600 ml beakers. Evap. each under air current on steam bath to ca 60 ml.

Prep. adsorption column for sample and each std as follows: Place glass wool plug at bottom of chromatgc tube and add Al_2O_3 to depth of 3 cm (ca 3 g). Place another glass wool plug at top of column. Wash each column with 25 ml EtOAc. Transfer tissue and std exts from beakers to respective columns. Rinse each beaker with 25 ml EtOAc followed by two 25 ml portions ether and two 25 ml portions MeOH. Transfer each solv. rinse to corresponding column and discard all eluates.

Add two 25 ml portions borate buffer and collect eluate in 50 ml graduate. Transfer eluate from graduate to 125 ml separator with Teflon stopcock. Ext with 25 ml ether and discard ether. Acidify aq. soln with 10 ml 7*N* H_2SO_4. Thoroly ext with 25 ml and 10 ml $CHCl_3$. Drain each $CHCl_3$ ext and combine in 100 ml beaker. (Do not introduce any aq. phase.) Evap. solv. just to dryness on steam bath.

Add 10.0 ml 21.5*N* H_2SO_4 to each beaker. Mix thoroly ≥10 min. Det. relative fluorescence (product of linear scale meter reading and meter multiplier setting) of processed blank, stds, and tissue sample in 1 cm cell at excitation 325 nm and emission 408 nm. Subtract relative fluorescence of reagent blank from relative fluorescence of all std and sample prepns. (*See* Fig. 1.)

Prep. std curve with reagent blank-corrected relative fluorescence values of processed stds as ordinate and corresponding μg nalidixic acid as abscissa. From std curve, det. amt nalidixic acid (X) which corresponds to reagent blank-corrected relative fluorescence of processed tissue sample.

ppb Nalidixic acid = $(X \times 1000)/10$ g (tissue wt).

[1] The section numbers within the method are those for the 11th ed. of *Official Methods of Analysis*, 1970. Cautionary notes, if present, refer to the new chapter on safety, Chapter 46.

This report of the Associate Referee, E. L. Pratt, was presented at the 83rd Annual Meeting of the AOAC, Oct. 13–16, 1969, at Washington, D.C.

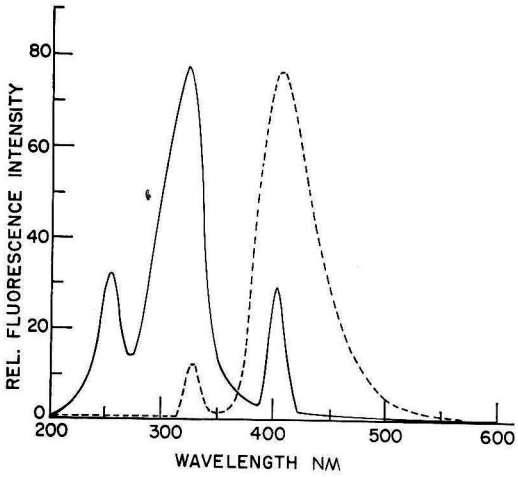

FIG. 1—Excitation (solid line) and fluorescence (dashed line) spectra of nalidixic acid in 60% sulfuric acid.

Collaborative Study

Collaborators were supplied with packets of chicken liver and chicken muscle, reference standard drug, and coded and randomized spiking solutions containing known but unspecified quantities of nalidixic acid. No attempt was made to homogenize or to otherwise control uniformity of tissue. Prescribed methodology was applied in each collaborator's laboratory. Levels of drug to be isolated and measured by induced fluorescence were approximately 100 ppb nalidixic acid.

Data from 7 collaborating laboratories reporting a total of 64 separate determinations have been evaluated using the guidelines set forth by Youden (3).

Results and Discussion

Analysis of the study data returned from collaborators indicates that the average recovery of nalidixic acid from chicken tissue homogenates was 93% of theoretical values. The standard deviation was 26%, with a random error of 21% and a systematic error of 15%.

Collaborative data are presented in Table 1. Of the 64 determinations reported, 3 were judged aberrant by the Associate Referee. These values are identified; their removal from subsequent computation is based on criteria put forth by Dixon (4) and carries a 5% (or less) risk of unjust rejection.

The ranking of all data is found in Table 2. In the tabulation of rank, all laboratories fall within prescribed 5% limits, indicating the absence of a pronounced systematic error in any particular laboratory.

An estimate of systematic and random variation in the collaborative study follows the unit block approach likewise proposed by Youden. Returned data have been assembled into 4 sets of pairs. These sets consist of results reported by each laboratory for the 2 lowest and the 2 highest concentration levels of drug added to each type of tissue. Without exclusion, all pairs of data are illustrated in Fig. 2. The 2-sample (X, Y) plots illustrate quite well the random and systematic contributions to the overall variation. The graphic display indicates that neither the random components nor the systematic (bias) components predominate in the contributions to overall variation. Pairs are circled which contain a value judged aberrant.

Table 1. Results obtained in collaborative method for nalidixic acid in tissue

	Recovered, ppb[a]									
Coll.	10M 130.5	14M 112.0	12M 101.0	13L 90.3	11M 0	20L 65.1	22L 99.5	21L 125.4	23L 112.8	24L 0
1	100.0	110.0	62.0	59.0	N.R.[b]	55.0	105.0	55.0	105.0	25.0
2	118.0	130.0	120.0	74.0	15	106.0	110.0	134.0	130.0	0.0
3	110.0	101.0	86.0	60.0	48.0	39.0	80.0	101.0	84.0	0.0
4[c]	167.0	31.0[d]	55.0	55.0	0.0	—	—	—	—	—
5	248.0[e]	129.0	109.0	104.0	14.0	46.0	107.0	31.0	117.0	54.0
6	166.0	98.0	86.0	94.0	11.0	155.0	N.R.	138.0	113.0	104.0
7	132.0	91.0	114.0	86.0	45.0	29.0	23.0[d]	103.0	97.0	12.0
Av., ppb	132.2	109.8	90.3	76.0	17.0	71.7	100.5	93.7	107.7	33.0
Av., %	101.3	98.0	89.4	84.2	—	110.1	101.0	74.7	95.4	—

[a] Sample identification (M = muscle and L = liver), followed by amount added.
[b] No result.
[c] Collaborator 4 did not report results for liver.
[d] Less than 1% risk of unjust rejection.
[e] Less than 5% risk of unjust rejection.

Table 2. Nalidixic acid in tissue, ranked results (except Sample 22L)

Coll.	Samples, Ranked Results							Coll. Score[a]		
	10M	12M	13M	14M	20L	21L	23L	A	B	C
1	7	6	6	3	3	5	4	22	12	34
2	5	1	4	1	2	2	1	11	5	16
3	6	4.5	5	4	5	4	6	19.5	15	34.5
4	2	7	7	7	–	–	–	23	—	—
5	1	3	1	2	4	6	2	7	12	19
6	3	4.5	2	5	1	1	3	14.5	5	19.5
7	4	2	3	6	6	3	5	15	14	29

[a] A = muscle determinations only (7 labs, 4 materials) (5% limits, 5–27).
B = liver determinations only (6 labs, 3 materials) (5% limits, 3–18).
C = all determinations except by Coll. 4 (6 labs, 7 materials) (5% limits, 12–37).

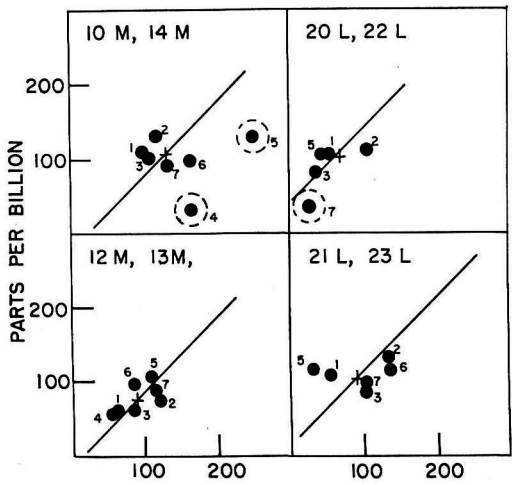

FIG. 2—Two-sample charts of results reported in collaborative study of determination of nalidixic acid in muscle (M) and liver (L). Numbers adjacent to plotted points identify collaborators.

In the determination of drug in muscle, the (X, Y) plot, as well as the ranking of results, indicates Laboratory 4 had the greatest bias on the low side of theoretical and Laboratory 5 had the greatest bias on the high side. Unfortunately, Laboratory 4 did not complete the analyses for drug in liver. For nalidixic acid in liver the greatest bias appears in data reported by Laboratory 2.

Recoveries of the actual quantity of drug added to each tissue are presented in Table 3. The estimated standard deviation associated with analyses from each laboratory is also tabulated here. As generally is the case, variations within a laboratory were greater in determinations of drug in liver than in determinations in muscle. With the exception of Laboratories 4 and 5, all collaborators reported data for drug in muscle with similar variances. The muscle data from Laboratory 4 exhibited the greatest variation even after removal of 1 result judged aberrant. The good reproducibility indicated for muscle determinations reported by Laboratory 5 followed removal of 1 result judged aberrant. The high estimated variances associated with the data of Laboratories 5 and 6 are caused by 1 extreme value in each set of data, which the Associate Referee could not justifiably discard. The standard deviations reported in Table 3, at best, are rough estimates based on a few determinations from each laboratory. It appears reasonable, however, to conclude that Laboratory 3 has demonstrated the greatest ability to reproduce recoveries and that Laboratory 4 experienced the greatest difficulty in reproducing recoveries.

The results of analysis of paired data are presented in Table 4. The random and systematic standard deviations of the method have been calculated. As indicated in Fig. 2 the precision and systematic errors are approximately of the same magnitude: 21 and 15 ppb, respectively, for average tissue.

The average blank effect reported for determination of drug in muscle and liver was 22 and 33 ppb, respectively.

Summary and Recommendation

The described method effectively measures 100 ppb nalidixic acid in chicken muscle and chicken liver homogenates. Collaborative study has shown that recoveries of drug from tissue were good and associated variances are judged acceptable for stressed methodology being collaboratively applied. The method has been used to monitor drug residue levels in the tissues of chickens which have ingested nalidixic acid and

Table 3. Nalidixic acid recovery

	Nalidixic Acid Recovered, %			Estimated Std Dev.		
Coll.	Muscle	Liver	Overall	Muscle	Liver	Overall
1	75.4	81.7	78.6	16.6	26.7	22.2
2	101.8	123.9	112.9	19.0	26.2	22.9
3	81.5	73.9	77.7	10.4	9.6	10.0
4	81.1	—	—	40.7	—	—
5	112.8	76.7	92.1	4.2	38.4	27.4
6	101.0	149.3	121.7	19.5	76.8	55.0
7	97.6	71.0	86.2	13.1	22.9	18.7

Table 4. Analytical recovery and error in nalidixic acid collaborative study data

Parameter	Muscle	Liver
Av. drug concn added, ppb	107	101
Av. recovery of added drug, %	92.7	94.8
Precision error (S_r), ppb	17.9	25.4
Systematic error (S_b), ppb	15.4	15.9
Standard deviation (S_d), ppb	23.6	30.0

the results of these measurements have permitted the establishment of drug depletion curves. The method is recommended for adoption as official first action.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee B and was adopted by the Association. See *JAOAC* 53, 380 (1970).

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EGGS AND EGG PRODUCTS

Note on Determination of Free Succinic Acid in Eggs by Gas-Liquid Chromatography

By SAMMIE BETHEA[1] (Division of Food Chemistry and Technology, Food and Drug Administration, Washington, D.C. 20204)

Free succinic acid can be determined in eggs by gas-liquid chromatography, using a flame ionization detector, after prior extraction of the acid from the sample by ether. Decomposed or incubator reject eggs all exhibited sample peaks with the same retention time as succinic acid, while undecomposed eggs showed no peaks.

Succinic acid, an important chemical index of decomposition in eggs (1,2), is generally determined by column partition chromatography (3). Both succinic and lactic acids can be extracted from decomposed eggs (4) with ether in the AOAC method (5).

In an investigation of acids in eggs, using both AOAC silicic acid columns (3, 6), some previously unreported and unidentified acid bands were observed (7). No report could be found in the literature concerning gas-liquid chromatography (GLC) of either succinic or lactic acid as the free acid in ether extract from decomposed egg samples. In the pre-

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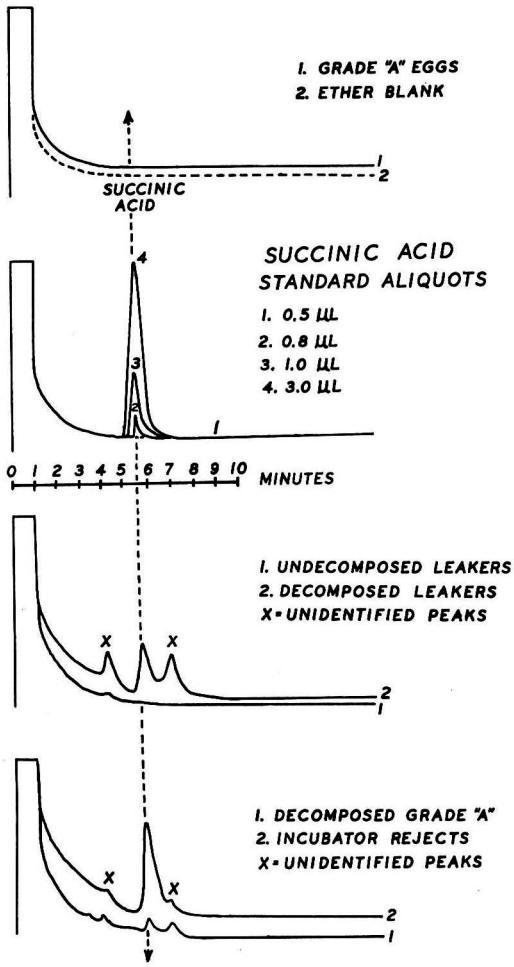

FIG. 1—Gas chromatograms of ether blanks, succinic acid standards, and four classes of eggs.

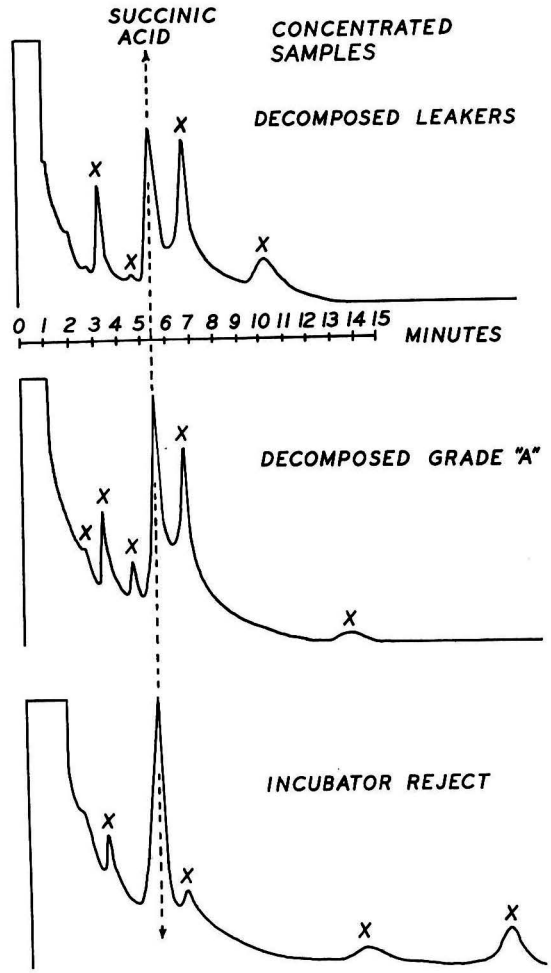

FIG. 2—Gas chromatograms of sample concentrates and decomposed incubator reject eggs; X = unidentified acid peaks.

vious work (7), succinic acid did not chromatograph under the conditions used. Subsequently, the GLC conditions were adjusted so as to determine succinic acid. Thus lactic and succinic acids can be gas chromatographed from the same ether extract of eggs, and several determinations can be made of each acid from a single AOAC ether extraction. The procedure is rapid, simple, and reproducible.

Experimental

Egg samples used in this investigation were taken from experimental lots of eggs described in the previous publication (7). Aliquots of a solution of analytical grade succinic acid in reagent grade ether were used in the gas chromatography as reference standards. A Barber-Colman 5000 instrument was used, with the following conditions: temperatures—column, flame ionization detector (FID), and injector, 200, 225, and 225°C, respectively; sensitivity setting 3×10^{-10} mv; attenuation 1×; 6′ × 4 mm id glass column (presilanized) packed with 15% DEGS coated with 2% H_3PO_4 on Anakrom ABS, conditioned at 210°C for 12 hr, with helium carrier gas at 60 ml/min.

A standard solution containing 5 mg succinic acid/ml ether was prepared, and aliquots of 0.5, 0.8, 1.0, and 3.0 µl were injected into the gas chromatograph, with an ether blank. Aliquots of ether extracts of the following classes of eggs were gas chromatographed directly: Grade "A", "leakers," Grade "A" that had been allowed to decompose, and incubator rejects. Five ml portions of the ether extract of each class of eggs were evaporated to dryness

on the steam bath, the residues were dissolved in 1.0 ml ether, and 1 μl of each concentrate was injected into the gas chromatograph.

Results and Discussion

The ether blank exhibited only the chromatographic solvent front, as shown in Fig. 1. The succinic acid standard solutions each showed a nearly symmetrical peak with identical retention times (about 5½ min) except the 0.5 μl aliquot, which was below the sensitivity of the GLC conditions used (Fig. 1). All of the egg samples showed several peaks except Grade "A" eggs (not decomposed), which showed no peaks. The peak from the standard succinic acid solution matched one of the peaks of each decomposed egg sample that showed several peaks. The retention times of the unidentified peaks for unconcentrated samples were about 3, 4, and 7 min, respectively (Fig. 1), compared to 5½ min for succinic acid. The peaks for the sample concentrates were more pronounced and showed some extra peaks not observed on the corresponding unconcentrated samples (Fig. 2).

These results indicate that free succinic acid in ether extracts of decomposed eggs can be gas chromatographed in the presence of other unidentified compounds assumed to be acids by analogy with previously reported results (7).

Succinic acid in quantities as low as 0.004 mg in aliquots of standard solutions was detectable by GLC under the conditions used; in an aliquot containing between 0.002 and 0.003 mg, succinic acid could not be detected (Fig. 1).

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FORENSIC CHEMISTRY

Comparison of Elemental Composition of Pistol Bullets by Atomic Absorption: Preliminary Study

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A method is presented for the determination of antimony, copper, bismuth, and silver in bullet leads by atomic absorption. Interference in the determination of these elements from high lead concentrations was studied. Bullets of the same caliber, produced by 3 different manufacturers, can be distinguished based on the antimony concentration alone. Measurable variations in the concentrations of antimony, copper, bismuth, and silver exist and can be used to estimate batch differences in lead produced by the same manufacturer.

Forensic laboratories are often required to compare bullets or bullet fragments to determine the relationship between the questioned and known specimens. In some cases, adequate comparisons by standard microscopic techniques are not possible because the questioned bullet is badly mutilated. When this situation exists, a chemical analysis of the bullet lead can provide information about the similarity of the specimens to be compared.

Although the elemental composition of bullet

leads can be determined by spectrographic and wet chemical methods, techniques such as neutron activation analysis (NAA) and atomic absorption (AA) can also be used. However, NAA employing (n, γ) type reactions is not ideally suited to the multielemental examination of bullet leads when a purely instrumental approach (i.e., no radiochemical separations) is used. This is primarily due to relatively large concentrations of antimony which obscure other elements that may be present. Atomic absorption is not subject to this type of interference and the technique offers the potential for detecting and measuring a large number of chemical elements rapidly and rather inexpensively.

In this paper, a procedure for the examination of bullet leads by AA is described and the results of the analyses of over 300 bullets are discussed. Although the study was primarily limited to .38 special cartridges produced by the 3 major domestic manufacturers (Winchester, Federal, and Remington), some information from other caliber cartridges was obtained and used for comparison with the .38 special results. The objectives of this study were to determine whether the same caliber bullets produced by different manufacturers can be distinguished and whether measurable batch-to-batch variations exist in cartridges produced by a single manufacturer.

Experimental

Method

The lead bullets examined were prepared and solubilized in the following manner: Portions weighing ca 0.5 g were removed from the lead bullets, then accurately weighed, and placed into 100 ml beakers containing 30 ml 3*N* HNO_3. The lead was dissolved by gentle heating on a hot plate and the cooled solution was transferred to a 50 ml volumetric flask and diluted to volume with 3*N* HNO_3. Copper-clad bullets were washed with acetone to remove organic lubricants and the copper was removed with concentrated HNO_3 before the lead core was solubilized. A Perkin-Elmer Model 303 atomic absorption spectrophotometer with a triple slot laminar flow burner (acetylene-air mixture) and DCR readout was used for all measurements. The standard solutions, to which 1000 ppm lead was added, and the unknown solutions were measured alternately to monitor possible instrumental variations. The quantitative determination of each element was made by comparing the absorptions of the unknown solutions with the standards.

Interferences

All bullets examined in this study contained antimony as the hardening agent. Mostyn and Cunningham *et al.* (1) reported that high concentrations of lead enhance the antimony absorption at the principle wave length, 2175.8Å. Since lead is the major constituent of the bullet matrix, the absorption of lead at 2175.8Å was studied. Solutions containing 0–10,000 ppm lead measured at 2175.8Å shown no appreciable absorbance at this wavelength. Dilute HNO_3 solutions containing 80 ppm antimony and various concentrations of lead were prepared and their absorbances were measured. The results presented in Table 1 show that when lead is present in solutions containing antimony, the absorption by antimony at this wavelength is enhanced.

The effect of lead on the absorption of copper, silver, and bismuth in bullets was also studied. Dilute HNO_3 solutions containing these elements were measured in the presence of 0–10,000 ppm lead at 3247, 3281, and 2231Å, respectively. Table 2 shows that high lead concentrations enhance the absorption of these elements. Although this effect was not studied in detail, it may be the result of a change in flame temperature due to the high lead concentrations.

Compensation for the enhancement of antimony, copper, silver, and bismuth produced by the lead in the bullet matrix was effected by preparing standard solutions for these elements which contained approximately the same lead concentrations found in the sample.

Table 1. Effect of lead on antimony determination at 2176Å

Antimony, ppm	Lead, ppm	Observed Antimony, ppm
80	0	80
80	400	97
80	1000	99
80	3000	95
80	8000	96
80	10,000	98

Table 2. Effect of lead on determination of copper, bismuth, and silver by AA[a]

Lead Present, ppm	Copper (3247Å), ppm		Silver (3281Å), ppm		Bismuth (2231Å), ppm	
	Added	Observed	Added	Observed	Added	Observed
0	10.0	10.0	10.0	10.0	100	101
10	10.0	10.0	10.0	9.9	100	100
100	10.0	10.0	10.0	10.1	100	103
10,000	10.0	11.2	10.0	11.2	100	112

[a] In 3*N* HNO_3.

Table 3. Variations in bullet lead from a single box of Federal (F), Remington (R), and Winchester (W) .38 special ammunition[a]

Bullet	Antimony, %			Copper, ppm			Bismuth, ppm			Silver, ppm		
	F	R	W	F	R	W	F	R	W	F	R	W
1	1.71±0.30	0.90±0.02	2.07±0.24	199±29	637±15	223±2	143±25	353±52	200±48	25±6	32±3	48±5
2	1.62±0.07	0.91±0.01	2.21±0.14	203±4	605±26	196±4	197±38	510±70	240±28	25±4	46±8	40±5
3	1.63±0.10	0.87±0.08	2.31±0.12	207±15	616±45	220±7	207±82	470±62	210±16	23±7	43±6	39±1

[a] 2 Sigma deviation shown.

Homogeneity of Bullet Leads

A single box of .38 special ammunition from each of the 3 manufacturers was selected and 3 bullets from each box were randomly removed. Lead from the tip, middle, and base of each bullet was analyzed in triplicate by AA.

The results obtained from these analyses were used to determine the standard deviation for the measurement of the elements antimony, copper, silver, and bismuth, as well as the in-box variations in bullet compositions. The data in Table 3 show the elemental variations found in bullets from single boxes of Federal, Remington, and Winchester .38 special ammunition.

Comparison of NAA and AA for Determination of Antimony

Since the use of neutron activation analysis for determining the concentration of antimony in bullet leads has been reported (2), a comparison between this technique and the AA method for measuring antimony was conducted. Fragments of lead from single .38 special bullets produced by the 3 manufacturers were irradiated with appropriate antimony standards 1 min in a thermal neutron flux of 10^{13} n/sq. cm/sec. The samples and standards were subsequently counted by gamma scintillation spectrometry and the amount of antimony in the bullet fragments was calculated by the standard peak height comparison method.

The same bullet leads were examined by AA for antimony and a comparison of the results of the 2 methods is shown in Table 4.

Examination by AA of 10 lots of .38 special bullets produced by these manufacturers over an extended period of time shows that batch-to-batch differences exist. Variations in the concentrations of antimony, copper, and bismuth are shown in Figs. 1, 2, and 3, respectively. The lot numbers were arbitrarily assigned and are not necessarily sequential. It was established that a correlation between the concentrations of silver and copper exists and for this reason the variation in silver concentration from lot-to-lot is not shown. A limited number of bullets from other caliber cartridges produced by Remington, Federal, and Winchester were also examined and these findings are shown in Table 5.

Table 4. Antimony concentrations in .38 special lead bullets determined by NAA and AA[a]

Manufacturer	Antimony, %	
	NAA	AA
Remington	0.82±0.18	0.89±0.10
Federal	1.57±0.20	1.63±0.18
Winchester	2.45±0.28	2.20±0.26

[a] Standard deviation values are 2 sigma.

Discussion

The elements tin, arsenic, sodium, molybdenum, potassium, chromium, and zinc were not detected in the bullet leads studied. Since arsenic has been reported as a major constituent in lead shot (3), it was surprising that this element was not detected in lead from pistol bullets. For this reason, the specimens obtained for this study were also examined by NAA for the presence of arsenic, using spectrum stripping techniques, but this element was not detected.

It appears that for a specific caliber, bullets produced by the 3 major domestic manufacturers can be distinguished based on the concentration of antimony alone. However, due to the overlap in antimony concentrations for the various calibers, it is doubtful that this criterion could be used to identify the manufacturer unless the caliber of the bullet is known.

The homogeneity of bullets from a single box and the variations observed from lot-to-lot indicate that it is possible to distinguish between different batches of bullet leads. Further work is necessary, however, to establish the nature and extent of these variations. It would be highly desirable to detect and measure additional elements in these bullet leads to have a larger number of identifying characteristics on which to base the comparison of 2 or more specimens.

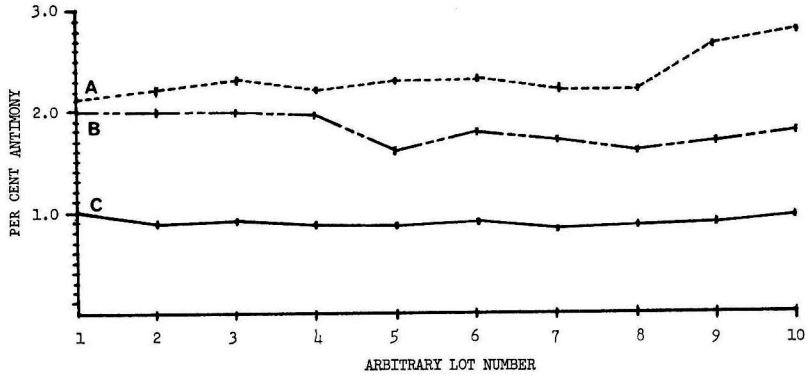

FIG. 1—Concentrations of antimony in various lots of .38 special bullets produced by the 3 major United States manufacturers: A, Winchester; B, Federal; and C, Remington.

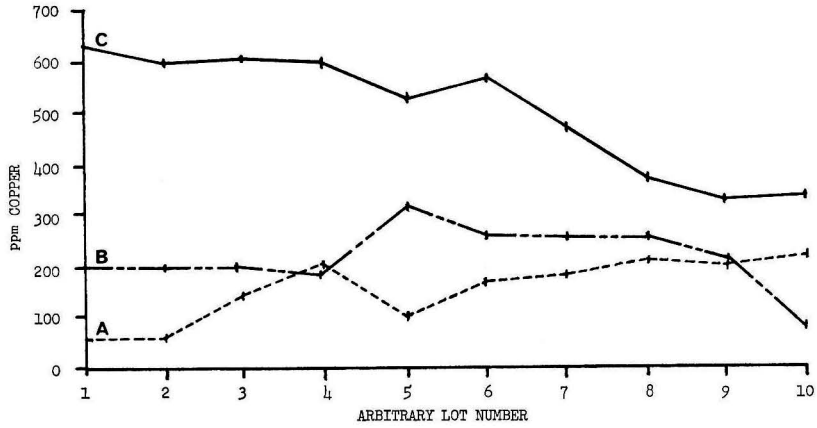

FIG. 2—Concentrations of copper in various lots of .38 special bullets produced by the 3 major United States manufacturers: A, Winchester; B, Federal; and C, Remington.

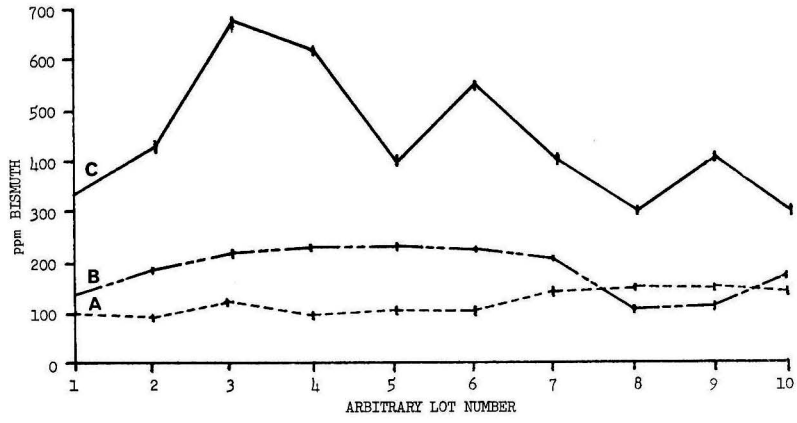

FIG. 3—Concentrations of bismuth in various lots of .38 special bullets produced by the 3 major United States manufacturers: A, Winchester; B, Federal; and C, Remington.

Table 5. Antimony, copper, bismuth, and silver concentrations in bullets of various calibers

Manufacturer	Caliber	Type	Antimony, %	Copper, ppm	Bismuth, ppm	Silver, ppm
Winchester	.22	LR-std vel.	1.40	180	150	25
	.32	S & W long	3.20	550	150	65
	.32[a]	Lubaloy	3.20	655	160	80
	.38[a]	158 grain lubaloy	2.70	380	180	65
	.38 Spec.	158 grain round nose	2.70	51	130	15
	.45	230 round nose	0.50	trace	trace	10
Remington	.22	158 grain round nose	0.91	700	370	35
	.32	S & W long	0.92	590	270	40
	.38 Spec.	158 grain round nose	0.96	370	360	55
	.45	230 grain round nose	1.59	645	230	65
Federal	.22	LR-std vel.	1.26	220	220	40
	.38 Spec.	158 grain	2.01	80	590	15

[a] Copper cladding removed.

Since this study only involves pistol bullets, these findings may not be applicable to rifle bullets because of compositional differences in the 2 types of projectiles. For example, tin is often substituted for antimony as the hardening agent in rifle bullets.

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This paper was presented at the 83rd Annual Meeting of the AOAC, Oct. 13–16, 1969, at Washington, D.C.

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CACAO PRODUCTS

Collaborative Study of an Electrometric Method for Determination of pH of Cacao Products

By JOHN L. IVERSON (Division of Food Chemistry and Technology, Food and Drug Administration, Washington, D.C. 20204)

The sample is dispersed in hot water, filtered, and cooled and the pH of the filtrate is measured with a pH meter standardized against standard buffers. Results of a collaborative study show good agreement between samples and laboratories. It is recommended that the method be adopted as official first action.

This report of the Associate Referee was presented at the 83rd Annual Meeting of the AOAC, Oct. 13–16, 1969, at Washington, D.C.

A measure of the acidity of a solution is its pH, defined as the logarithm of the reciprocal of the molar hydrogen ion concentration. Colorimetric methods such as **13.022–13.026** (1) use a series of reference solutions and pH is estimated by visual comparison. Electrometric methods similar to **13.027** are now in common use. However, such methods have not been adopted for cacao products.

A joint effort by the AOAC and the OICC (International Office of Cocoa and Chocolate) has

recently resulted in an electrometric method for determining the pH of cacao products. This method combines features of both the OICC method (2) and **13.027**. It was considered for adoption at the June 1969 Codex meeting and further action was deferred pending collaborative study.

The proposed method uses standard acid potassium phthalate buffer at pH 4.00 at 20°C (**42.007(c)**) and a phosphate buffer at pH 6.88 at 20°C (**42.007(d)**). The method is applicable to cacao products and to cacao butter and similar fats with special treatment.

METHOD[1]

(a) *For products other than cacao butter.*—Weigh 10 g sample into 150 ml beaker and slowly add, with stirring, 90 ml boiling H_2O. Suspension must be free from lumps. Filter, cool filtrate to 20–25°, and immediately det. pH, using electrodes and potentiometer stdzd with buffers at pH 4.00, **45.007(c)**, and 6.88, **45.007(d)**. Report to nearest 0.1 pH unit.

(b) *For cacao butter.*—Melt sample and mech. stir 5 min with equal wt of H_2O. Sep. aq. layer, cool to 20–25°, filter, and det. pH as in (a).

Results and Recommendation

Seven samples were sent to each of 6 collaborators. To test the precision of this method, Samples 3 and 5 were identical. The results are given in Table 1. Collaborators were asked to report results to the nearest 0.1 pH unit. The values reported are in satisfactory agreement.

[1] The section numbers within the method are those for the 11th ed., of *Official Methods of Analysis*, 1970; secs. **45.007(c)** and (d) (11th ed.) = **42.007(c)** and (d) (10th ed.), respectively. Cautionary notes, if present, refer to the new chapter on safety, Chapter 46.

Table 1. Results of collaborative study of electrometric determination of pH of cacao products[a]

	pH of Samples:						
Coll.	1	2	3	4	5	6	7
1	6.9	5.4	7.5	6.2	7.5	5.4	5.4
2	6.8	5.3	7.5	6.2	7.6	5.3	5.4
3	6.9	5.4	7.4	6.1	7.6	5.2	5.3
4	6.9	5.5	7.5	6.2	7.4	5.4	5.5
5	7.0	5.5	7.6	6.3	7.6	5.3	5.4
6	6.9	5.4	7.4	6.3	7.4	5.3	5.3
7	6.9	5.4	7.5	6.2	7.5	5.3	5.4
Av.	6.9	5.4	7.5	6.2	7.5	5.3	5.4

[a] Samples: 1, alkali-treated (normal); 2, natural cacao; 3, alkali-treated (maximum); 4, alkali-treated (minimum); 5, alkali-treated (maximum); 6, breakfast cocoa; 7, chocolate liquor.

It is recommended that the electrometric method for the determination of pH of cacao products be adopted as official first action.

Acknowledgments

The author wishes to thank the following collaborators for participation in the study: A. Y. Taira, Chicago; F. G. McNerney, Buffalo; J. A. Jones, Boston; G. Lester, Dallas (all of the Food and Drug Administration); K. E. McCloskey, Wilbur Chocolate Co., Inc., Lititz, Pa., and W. Kreiser, Hershey Chocolate Co., Hershey, Pa.

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The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee D and was adopted by the Association. See *JAOAC* **53**, 388–389 (1970).

Stone Cell Count Method and Stone Cell Group Method for Estimating Shell in Chocolate Products

By MANION M. JACKSON[1] (Food and Drug Administration, 2nd and Chestnut Sts., Philadelphia, Pa. 19106)

Per cent shell in the chocolate component of cocoa and similar products is determined on the basis of an average stone cell content of 9340 stone cells/mg dry fat-free 250 mesh shell. The sample is weighed and diluted to a weighed suspension in 60% (v/v) glycerine. The total number of stone cells in a weighed drop on a slide are counted microscopically at 100–200X. The per cent shell chocolate component is then calculated. Seven cocoa samples were analyzed and their results were compared to previous results obtained by the spiral vessel count and pectic acid methods. Average results for shell in the chocolate component were 6.5% for the stone cell count method, 6.6% for the spiral vessel count method, and 6.4% for the pectic acid method. Six collaborators studied 4 cocoa samples and compared 3 methods: the stone cell count method, the calculation procedure of Van Brederode and Reeskamp, and the stone cell group method. Five varied less than 1 standard deviation from the average for 3 samples and 4 collaborators were within 1 standard deviation in the stone cell count method for the remaining sample. The stone cell count method is recommended for adoption as official first action for the analysis of shell for cocoa, cocoa press cake, chocolate liquor, and expeller cake; the group method is recommended for other chocolate products.

Stone cells are constituents of cocoa beans that are found in the shell portion (1); this feature has been used to develop methods for the determination of shell in chocolate products. Some of these methods were reviewed by Stas and Nieuwenhuys (2). A method is presented here for determination of per cent shell chocolate component (c.c.) of cocoa and similar products based on the stone cell content of the product. From the data of this method, a simpler stone cell group procedure evolved. These 2 methods and the calculation procedure of Van Brederode and Reeskamp (3) were compared in a collaborative study.

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Shell fragments present in cocoa nibs will break up when ground. Groups of stone cells may be found in these fragments; groups and a few single stone cells may be found free of other shell material. In stone cell groups, some cells may be so fractured that only a portion of the cells remains. In this method, those portions larger than one-half stone cell are counted as complete cells and the smaller portions are not counted. Such fragmented stone cells are generally on the perimeter of the group and only occasionally in the interior. On 2 samples involving over 19,000 stone cells, 4% of the total stone cell count was due to broken stone cells larger than a one-half stone cell fragment.

Stone cells are generally tough and are resistant to being pulverized, but some can be destroyed by grinding. Commercial cocoa products, however, may contain some comparatively coarse shell fragments, a few of which may contain as many as 200 or more stone cells. Such fragments can lead to erratic results, high when present and low when absent in the drop counted. For this study, all products were ground to 250 mesh (U.S. No. 230 sieve openings = about 63.5 μm), defatted, and dried before analysis to avoid dissimilarities. An average value of 9340 stone cells/mg dry fat-free (d.f.f.) 250 mesh shell was obtained on a similarly treated authentic 5% shell c.c. sample. This value is used to estimate the amount of shell present in samples analyzed.

Experimental

A standard sample containing 5% shell in nibs (roasted basis), without defatting or drying, was made from a roasted 18-sample mixture of cocoa beans from 8 countries (4). It was reduced to 250 mesh, using initially hand grinding and defatting with ethyl ether, followed by a series of electric mortar grindings and sievings. It was again defatted with ether, dried, and gently reground. This is referred to as 5% shell c.c.

The composite cocoa sample consisted of a mixture of equal amounts of 15 cocoas and powdered cocoa press cakes; none were the black- or highly

Table 1. Stone cell count data and per cent shell c.c. on cocoa samples[a]

Sample	Duplicate Set 1						Duplicate Set 2						Av. % Shell c.c.
	mg	SC	%S	mg	SC	%S	mg	SC	%S	mg	SC	%S	
2	0.936	601	3.3	0.745	508	3.5	0.86	652	3.9	0.90	749	4.3	3.8
4	0.923	752	4.2	0.935	809	4.5	1.343	943	3.6	1.15	1097	4.9	4.3
5	0.985	477	2.4	1.008	571	2.9	0.931	557	3.0	0.917	606	3.4	2.9
12	1.175	1444	6.5	0.913	1302	7.6	0.921	931	5.2	0.975	1223	6.6	6.5
14	1.365	1840	7.1	1.278	2162	9.2	0.815	1446	9.7	0.918	1719	10.3	9.1
15	1.03	2015	10.8	1.085	2559	13.4	0.925	2209	13.6	0.958	2519	15.2	13.3

[a] mg = milligrams d.f.f. product counted; SC = stone cell count; and %S = per cent shell c.c.

Dutched-types. The shell c.c. content of this mixture (5.6%) was the same as that calculated from the average of the 15 cocoas, both by pectic acid determination. This mixture, referred to as a composite cocoa, and 6 of its component samples (Nos. 2, 4, 5, 12, 14, and 15) were hand-ground in a glass mortar to 250 mesh, defatted, and dried by the procedure described for the spiral vessel count (4). The samples and the prepared shell standard were analyzed by the stone cell count method to determine per cent shell c.c.; *see* Tables 1–3. Table 4 gives a comparison of results obtained from the stone cell count method, the spiral vessel count method, and the pectic acid method.

Table 2. Stone cell count data on 5% shell c.c. sample

Sample	Stone Cells/mg d.f.f., 250 Mesh Sample						
	1	2	3	4	5	6	Av.
1	1106	1135	1116	1162	950		1094
2	1174	991	1439	1322	1323		1250
3	876	1019	974	728			899
4	719	752	654	914	856		779
5	896	741	1186	1031			964
6	686	726	717	1057	835	839	812
Grand av.							966

METHOD[2]

13.023 *Apparatus and Reagents*

(a) *Slide and cover glass.*—75 × 38 mm slide with lines 0.5 mm apart, nearly across slide, parallel to 75 mm side, and ruled from top to bottom; 33 × 33 × 0.2 mm cover glass.

(b) *Scoop.*—Thin (ca 0.01–0.02 mm thick) stainless steel strip ca 4.8 mm wide with 90° bend extending outward 3 mm.

(c) *Magnetic stirrer.*—With stirring bar ca 5/8″ long × ca 1/4″ diam. Stirring bar 1/2″ × 5/16″ with (ca 36 mm diam. × 40 mm high internal measure-ridge in center will circle walls of 1 oz ointment jar ments) with distinct convex bottom, giving both vortex mixing and stirring.

(d) *Bellucci's reagent.*—HOAc-H_2O-HNO_3 (36 + 9 + 5).

13.024 *Defatting and Grinding*

See **13.019**, Spiral Vessel Count, *JAOAC* **51**, 457 (1968).

13.025 *Determination*

Mix dried product (1 hr at 100°) by tumbling in covered dish. Make duplicate detns. Accurately weigh 0.500 g extd and dried material and transfer to 150 ml beaker. Gradually stir in portion of 20 ml Bellucci's reagent until smooth; rinse walls of beaker and stirring rod with remainder. Stir gently. Fill short-neck, 100 ml, r-b flask with cold H_2O to neck and place on top of beaker; let rod rest in spout of beaker. Bring soln to initial boil, using asbestos mat over small flame. Immediately reduce to very weak flame and boil gently 10 min with frequent gentle swirling, keeping beaker and flask together. Cool ca 5 min.

Accurately weigh 25 × 100 mm Pyrex, rimless culture tube in 30 ml beaker (holder). Quant. transfer sample to culture tube with small portions H_2O, scrubbing beaker and rod with rubber policeman. Centrf. ≥3 min at full speed in International Clinical centrf., using IEC No. 571 curved rubber cushion in IEC No. 320 shield, or equiv. Decant carefully and discard supernatant (some flocculent material may be present). Add H_2O to tube to ca 3/4 full, stopper, and shake until residue is well dispersed. Remove stopper, rinse, centrf., and decant as before.

Add aq. glycerine (3 + 2) to culture tube until tube and holder weigh 20 ±0.03 g more than original wt. Stopper, shake vigorously until well mixed, and transfer immediately to 1 oz ointment jar contg small magnetic bar. Stopper jar and let stand until bubbles disappear (ca 5–10 min).

Accurately weigh together ruled glass slide and

[2] The section numbers within the method are those for the 11th ed. of *Official Methods of Analysis*, 1970. Cautionary notes, if present, refer to the new chapter on safety, Chapter 46.

Table 3. Stone cell data on composite cocoa[a]

Detn	Analysis 1			Analysis 2			Analysis 3			Analysis 4			Av. % Shell
	mg	SC	%S	mg	SC	%S	mg	SC	%S	mg	SC	%S	c.c.
1	1.018	1168	6.0	0.933	766	4.2	1.065	1277	6.3				5.5
2	1.11	1221	5.7	1.038	939	4.7	0.985	1095	5.8	0.813	907	5.8	5.5
3	1.003	838	4.3	1.048	879	4.3	0.835	875	5.5	0.923	990	5.6	4.9
4	1.03	1281	6.6	0.955	1201	6.6	0.813	920	5.9				6.4
5	1.09	950	4.5	1.108	1112	5.2	1.103	1167	5.5	1.168	1184	5.3	5.1
Av.													5.5

[a] See Table 1.

Table 4. Estimated per cent shell in the chocolate component by 3 methods

Sample	Stone Cell Count	Spiral Vessel Counts	Pectic Acid
2	3.8	3.3	3.9
4	4.3	3.7	3.9
5	2.9	4.1	4.2
Composite cocoa	5.5	6.4	5.6
12	6.5	8.3	6.5
14	9.1	10.6	7.8
15	13.3	9.7	13.0
Av.	6.5	6.6	6.4

cover glass. Stir liq. in jar 1 min on magnetic stirrer at max. speed at which small bubbles do not form. Stop. In rapid sequence, push jar (to put magnetic bar next to wall of jar) and, using scoop, immediately transfer drop liq. (ca 0.04 ±0.01 g) to center of tared slide, rulings up. Tap slide gently with scoop several times to remove as much liq. as possible. Place cover slip so that one edge rests just above and parallel to lower edge of slide. Lower cover slip carefully until it touches liq. and then let it drop. Liq. will ooze to edges. Do not press cover slip. Weigh prepd slide to 4 decimal places. Place rubber stopper in jar to prevent evapn.

Place slide on compd microscope with or without upper half of condenser and with transmitted daylight-type filtered and diffused light. Count 2 slides from each of 2 detns as in (a) or (b):

(a) *Stone cell count.*—For cocoa, cocoa press cake, chocolate liquor, and expeller cake. Scan slide at 100× and count stone cells at ≥200×. Count whole stone cells, both single and in groups, and all broken stone cells. Consider ≥0.5 stone cell as complete cell and do not count smaller cell fragments.

(b) *Stone cell group count.*—For chocolate products not included in (a). Proceed as in (a), counting all stone cell groups contg ≥2 stone cells.

13.026 *Description of Stone Cells*

Stone cells vary considerably in size, shape, and general appearance. Some are very distinct and others are relatively indistinct. Their size varies from ca 10 to 38 μm; the longest are very slender. Some very coarse stone cells up to ca 40 μm with thick, beaded-appearing outside wall ca 7 μm wide are occasionally found. Stone cell shapes are polygonal, generally irregular, and may contain curved areas. On well developed stone cells outside walls are 2–3.5 μm wide. On less distinct stone cells, outside walls are narrower and thinner; such cells are not fully developed or immature. Several near-parallel thin walls or lines, viewed microscopically, are easily visible in many stone cells. They are generally more distinct in those where outside wall is thin. *See* Fig. 1 for microphotographs of stone cells. Stone cells usually are in group formation, consisting of ≥2 stone cells.

13.027 *Calculations*

For either method, average four S values from one of formulas below and report as % shell in chocolate component:

(a) *Stone cell count.*—$S_1 = 84C/(17200M - C)$.

(b) *Stone cell group count.*—$S_2 = 84G/(1700M - G)$.

where W = g sample; L = g dild sample; D = g of drop counted; C = stone cell count of drop; M = mg dry fat-free sample in drop counted (= 1000 WD/L); S = % shell in chocolate component; G = stone cell groups in drop; and 9340 = number stone cells in 1 mg dry, fat-free, 250 mesh shell.

(*Example:* For 0.5 g sample dild to 20 g, $S_1 = 84C/(430000D - C)$ and $S_2 = 84G/(42500D - G)$.)

Discussion

Derivation of Formulas

In deriving the formulas for the per cent shell in the chocolate component, 4 other definitions in addition to those described in the *Calculations* will be used: B = per cent shell on d.f.f. basis; R = 92% d.f.f. solids in shell; T = 42% d.f.f. solids in nibs (4); and N = 9340, number of stone cells per mg d.f.f. 250 mesh shell. Then, in 100 g roasted cacao nibs before defatting or drying, referred to

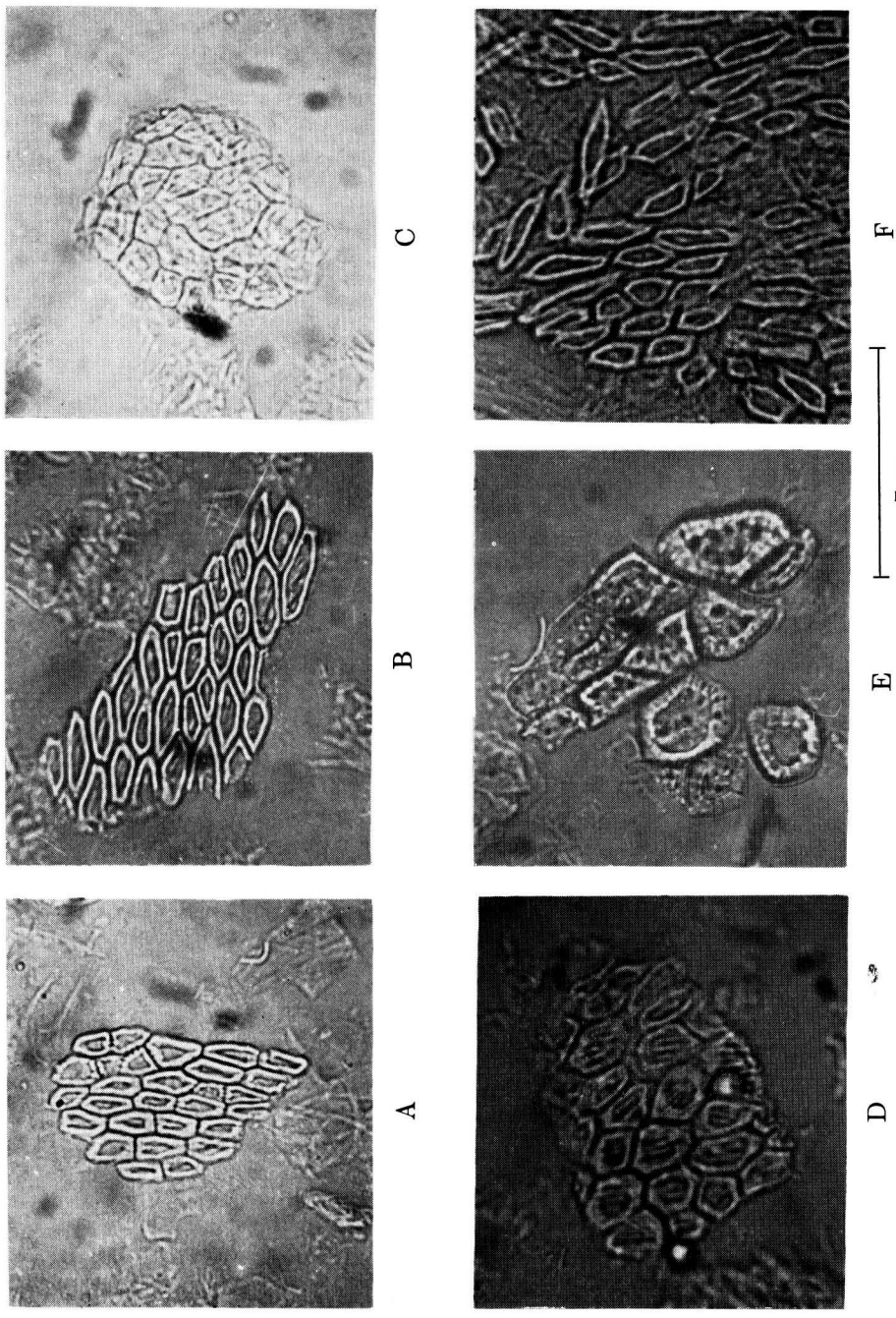

FIG. 1—Stone cells ca 330X: A and B, distinct stone cells; C, indistinct stone cells; D, stone cells showing distinct parallel lines in central area; E, very large stone cells infrequently found; F, long stone cells attached to a large piece of shell, showing separations between stone cells on shell.

as the chocolate component (c.c.), the amount of shell present in a dry fat-free state is as follows:

g d.f.f. shell
$$= 100 \times (S/100) \times (R/100) = RS/100 \quad (1)$$

Since the amount of nibs is equal to 100% of the product minus the per cent shell $(100 - S)$

$$\text{g d.f.f. nibs} = 100 \times [(100 - S)/100] \times (T/100) = (100T - TS)/100 \quad (2)$$

Then, stating the formula for per cent shell on a d.f.f. basis in its simplest form

$$B = 100 \times \text{g d.f.f. shell}/(\text{g d.f.f. shell} + \text{g d.f.f. nibs}) \quad (3)$$

or

$$B = 100(RS/100)/[(RS/100) + (100T - TS)/100] \quad (4)$$

Solving for S, we obtain

$$S = 100BT/[100R - B(R - T)] \quad (5)$$

By substituting known values

$$S = 4200B/(9200 - 50B) \quad (6)$$

This formula may be so stated that the per cent shell d.f.f. (B) may be computed from S, or

$$B = 9200S/(4200 + 50S) \quad (7)$$

Thus, for the prepared 5% shell c.c. sample, if 5 is substituted for S we find that B, the per cent shell d.f.f., is equal to 10.34%. In this sample 966 stone cells were found in 1 mg d.f.f. sample; this was an average of 6 determinations totaling 29 counts (Table 2). Since stone cells are a component of the shell and not of the nibs, the per cent of shell on a d.f.f. basis would be proportional to the number of stone cells present in a d.f.f. sample as compared to the number present in an equivalent amount of d.f.f. shell where both have been treated in the same manner. This is expressed by a second formula for B:

$$B = 100 \times (\text{no. stone cells per mg d.f.f. sample})/(\text{no. stone cells per mg d.f.f. shell}) \quad (8)$$

Then, for the 5% shell c.c. sample, by substituting its 10.34% shell d.f.f. value for B, its 966 stone cells per mg d.f.f. value for the sample value, and N for the number of stone cells per mg d.f.f. shell:

$$B = 10.34 = (966 \times 100)/N$$

or

$$N = 9342 \text{ stone cells per mg d.f.f. shell} \quad (9)$$

For calculations, 9342 is rounded off to 9340.

Stone cells per mg d.f.f. sample may be expressed by the fraction C/M. Substituting this and the 9340 stone cells per mg d.f.f. shell value in the second formula for B we obtain

$$B = [(C/M) \times 100]/9340 = C/93.4M \quad (10)$$

Substituting this value for B in the formula for S, we obtain

$$S = (4200 \times C/93.4M)/[9200 - (50C/93.4M)] = 84C/(17200M - C) \quad (11)$$

Since, for a 0.5 g sample diluted to 20 g

$$M = (WD/L) \times 100 = (0.5 \times 1000D)/20 = 25D \quad (12)$$

the formula for S becomes

$$S = 84C/(430000D - C) \quad (13)$$

On a d.f.f. basis the per cent shell d.f.f.

$$= B = C/(93.4 \times 25D) = C/2335D \quad (14)$$

In further discussions in this paper the word "group," unqualified, shall mean a stone cell group of 2 or more stone cells. The term "group method" shall mean a method based on such groups.

In the group method the per cent shell d.f.f. is determined by dividing the groups per mg d.f.f. cacao material by 9.24. This was derived by dividing the average of the number of groups per mg d.f.f. cacao material by the per cent shell d.f.f. obtained by the stone cell count method, on 6 separate cocoa samples ground to 250 mesh, and by averaging the results; *see* Table 5. Thus, the per cent shell d.f.f. is

$$B = \text{groups per mg d.f.f. cacao material}/9.24 = (G/M)/9.24 = G/9.24M \quad (15)$$

Since $S = 4200B/(9200 - 50B)$ and by substituting the above value for B in the formula for S, we obtain

$$S = 4200G/(85008M - 50G) = 84G/(1700M - G) \quad (16)$$

For a 0.5 g sample made up to 20 g

$$S = 84G/(42500D - G) \quad (17)$$

In theory, the 9.24 value indicates that, on an average, each 9.24 stone cell groups in 1 mg d.f.f. 250 mesh cacao material represents 1% shell d.f.f.

Stone Cell Value

The use of 9340 stone cells per mg d.f.f. 250 mesh (U.S. No. 230 sieve) shell is supported by

Table 5. Data for the determination of per cent shell d.f.f. by different calculation procedures

Sample, Series B	Av. mg	Av. Stone Cell Count		Av. Group Count	Groups/ mg	Stone Cells/ Group	Groups,[a] 1% Shell d.f.f.	% Shell[b] d.f.f.	Factor[c]
		Total	In Groups						
4	0.881	1244	1236	128.8	146.2	9.6	10.22	15.15	9.65
1	1.030	437	431	44.8	43.4	9.7	10.16	4.54	9.58
5	1.002	1183	1176	119.7	119.5	9.8	10.08	12.60	9.48
6[d]	1.111	924	921	92.7	83.3	10.0	9.95	8.90	9.37
2	1.154	796	790	78.3	68.2	10.1	9.88	7.38	9.20
3	1.041	309	306	26.8	25.7	11.4	8.93	3.15	8.16
Av.									9.24
Authentic 5% shell c.c.	0.881	853	849	75.9	86.9	11.2	8.85		

[a] Values taken from Van Brederode and Reeskamp's type of curve denoting groups of d.f.f. cacao material containing 1% shell d.f.f.
[b] By stone cell count method.
[c] Quotient obtained by dividing groups/mg by % shell d.f.f.
[d] Three drops analyzed to each of 2 determinations.

data from 2 different sources. An average of 5.5% shell c.c. was found by the stone cell count method for the analysis of the composite cocoa sample (5 determinations, 17 slides total). This value is very close to the value (5.6%) obtained by the chemical pectic acid determination. The values for per cent shell c.c. in the composite cocoa and 6 of its components, using the stone cell count, the spiral vessel count, and the pectic acid methods, were 6.5, 6.6, and 6.4, respectively. In commercial cocoas that were laboratory-ground to 250 mesh, the individual group sizes varied up to about 40 stone cells per group. The authentic 5% shell sample contained several groups about twice this size. In those analyses in which the number of groups, as well as the number of stone cells were counted, the average group of the composite cocoa contained 10.8 stone cells and the average group of the 5% shell sample contained 11.2 stone cells.

In developing this method the initial samples were cocoa and cocoa press cake. Such products generally are not as finely ground as the chocolate materials used in confectionery, which are rolled and conched to reduce the particle size of any cacao shell present. The size of such particles may be expressed as stone cells per group. On the average, the smaller the value the smaller the particle size.

Other analysts have reported on the particle size of chocolate products. Schetty (5) states that the number of sclerous cells in one group often varies between 4 and 8 for chocolates and between 9 and 20 for cacao pastes. Van Brederode and Reeskamp (3) found that the group sizes of cacao paste and cocoa powder commonly were between 9 and 20, but that for most of the commercial cacao products, the mean group size was between 5 and 12. The average group size of six 250 mesh cocoa samples varied from 9.6 to 11.4 stone cells per group; *see* Table 5. A limited survey was made on 4 brands of bittersweet chocolate bars to ascertain their average group size; these were not ground but were examined as is. The minimum and maximum average group sizes were 8.5 and 14.3, respectively, both foreign brands. The other two were 9.2 (foreign) and 14.2 (domestic). Thus 2 of these 4 chocolate bars contained shell coarser than the 250 mesh cocoa samples. The other 2 could probably be brushed through a 250 mesh screen after defatting. All 4 would be within the scope of the stone cell count method after defatting, grinding to 250 mesh, and removing the sugar. Further data to provide a better background in the areas of fine chocolate analysis would be very helpful.

Study of Three Calculation Procedures

The stone cell count method was compared with the calculation procedure of Van Brederode and Reeskamp (3) and the group method. The number of stone cell groups of 2 or more stone cells and the number of stone cells in these were counted in addition to the total stone cell count.

All the data for the comparison of these 3 methods were obtained by the stone cell count method; *see* Tables 5 and 6. The group procedure gave slightly higher per cent shell d.f.f. values than

Table 6. Comparative per cent shell in cocoa powder by different calculation procedures

Sample	Stone Cells/Group	% Shell d.f.f. from Calcn:[a] 1	2	3
4	9.6	15.2	14.3	15.8
1	9.7	4.5	4.3	4.7
5	9.8	12.6	11.9	12.9
6	10.0	8.9	8.4	9.0
2	10.1	7.4	6.9	7.4
3	11.4	3.2	2.9	2.8
Av.	10.1	8.6	8.1	8.8
Authentic 5% shell c.c.	11.2	10.4	9.8	9.4

[a] Per cent shell d.f.f. obtained using data from stone cell count method and the following calculation procedures: 1, stone cell count method described in this report; 2, calculation procedure by Van Brederode and Reeskamp; 3, dividing stone cell groups per mg d.f.f. cacao material by 9.24 (group method).

the stone cell count method when the stone cells per group values were low, and vice versa. This indicates that as the stone cells per group value becomes smaller, there are fewer stone cells present. Also, when a chunk of shell is ground, some of the stone cells on the perimeter of the new smaller fragments and even occasionally one in the interior could be destroyed. Thus, even though the differences between the shell values obtained by the stone cell count and the stone cell group count are small on these samples, the stone cell count method is more accurate when the stone cell per group value is near 11 and the group method may give a preferable evaluation of the per cent shell present for samples of much lower stone cells per group values.

All results obtained by the calculation procedure of Van Brederode and Reeskamp were lower than those obtained by the stone cell count method and generally lower than those by the group procedure. The Van Brederode and Reeskamp procedure appears to be theoretically sound and allows the calculation of shell at different fineness of grind. However, irregularities in the original data are not sufficiently corrected in the adjusted data used to prepare the final curve for the calculation of per cent shell d.f.f. From the corrected data in Table 6 of their report (3), about 9729 stone cells per mg d.f.f. shell in the stone cell groups were calculated for the 1.5 hr interval, and about 9900 for the 2.0 hr interval. These values were obtained by multiplying the value in the 8-fold column by its respective stone cell per group value and then multiplying the product by 100 to get the 100% shell d.f.f. value (i.e., $6.9 \times 14.1 \times 100 = 9729$ and $9 \times 11.0 \times 100 = 9900$). The increase in the number of stone cells in the 2 hr value over the 1.5 hr value (an additional ½ hr of grinding) appears irrational. The 2 hr value should have been definitely lower as some stone cells are destroyed during any grinding period.

Since the slope of their final curve is about the same for 14.1 to 7 and 14.1 to 11 stone cells per group (1.5 and 2.0 hr intervals, respectively) instead of curving earlier to a milder slope, evaluation of shell by this method would give low values on many samples. Shell values obtained by this method were lower than by the stone cell count method in the laboratory study of the 3 calculation procedures.

Since the authors (3) worked initially with the group count, apparently without separately considering the stone cell count values at each interval, a procedure is presented here to obtain a curve in which stone cell values are used. From the initial data of their table (5), average values of 19020, 10043, 9384, 9406, 7522, 4292, 2904, 1213, and 518 stone cells per mg d.f.f. shell in groups with 2 or more stone cells were calculated for periods of 0.5, 1.5, 2.1, 2.7, 3.2, 4.3, 5.3, 7.4, and 10.05 hr (Table 7). The fourth value calculated was greater, not smaller, than the third, implying that more than the 4 counts taken

Table 7. Data from which Figs. 2 and 3 were drawn

Hours	Calcd Stone Cells in Groups/mg d.f.f. Shell	Stone Cells/Group	Groups/mg d.f.f. Cacao Material Contg 1% Shell d.f.f.
0.0	20250		
0.5	16884	38.4	4.4
1.0	14074		
1.5	11731	14.1	8.3
2.0	9778	10.6	9.2
2.5	8151	8.5	9.6
3.0	6794	7.1	9.6
3.5	5663	6.1	9.3
4.0	4720	5.4	8.7
4.5	3934	4.85	8.1
5.0	3280	4.4	7.5
5.5	2734		
6.0	2279	3.7	6.2
6.5	1899		
7.0	1583	3.3	4.8
7.5	1320		
8.0	1100	3.0	3.7
8.5	917		
9.0	764	2.8	2.7
9.5	637		
10	531[a]	2.7	2.0

[a] Increase 19.97% every ½ hr back to 0 hr.

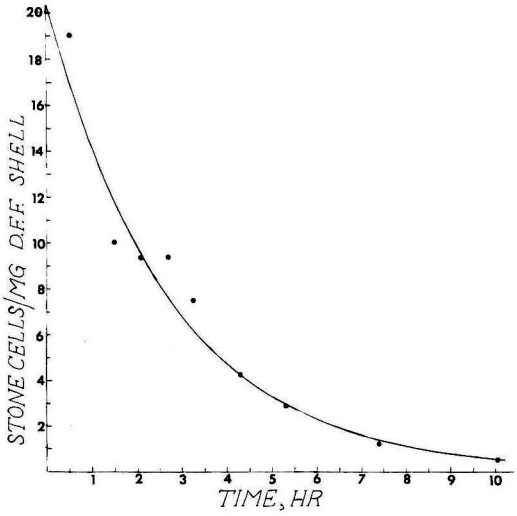

FIG. 2—Calculated curve from some data of Van Brederode and Reeskamp (3) showing relationship of stone cells/mg d.f.f. shell to grinding time in ball mill used.

per period would have been preferable, particularly in the earlier periods where the product was coarser. The values between 1.5 and 2.7 hr were equally spaced at 0.6 hr; using a combination of 3 sets of 4 counts each would have the effect of taking 12 individual values for one period, producing greater accuracy. To do this, the stone cell values are plotted versus time intervals. The curve is started at the tenth hour value of 531, interpolated between 10.05 and 7.04 hr, and, increasing the 531 value by 19.97% every half hour, a curve is formed which passes through the average values obtained between 1.5 and 2.7 hr (Fig. 2). The values obtained from the calculated data at these intervals were 10043, 9384, and 9406, averaging 9611. The values taken from the curve at the same time intervals were 11731, 9500, and 7620, averaging 9617. This close check shows that the curve passes through the average of the 3 values.

All values except those at 0.5, 1.5, 2.7, and 3.2 hr follow the curve closely, indicating that the curve is a proper one for the values used. The original value at 3.2 hr is high compared to that of the new curve, but the percentage difference is approximately the same as that for the original value at 7.4 hr compared to the curve value. This value is on the opposite side. If all such percentage differences (1.5 − 10.05 values) are averaged, the result is 1.00, which also adds credence to the curve. At the 0.5 hr time interval, not used by the authors, the curve passes as closely as it does between 1.5 and 3.2 hr; this indicates that in the ball mill used, stone cells were destroyed at a constant rate. Groups per mg d.f.f. sample containing 1% shell d.f.f. may be obtained by dividing the stone cell count values, mathematically calculated, by the stone cell per group value at the same time interval and then by 100 (*see* Table 7). The stone cells per group values were obtained in the manner of Van Brederode and Reeskamp, except that the curve was extended to contain the value at 0.5 hr and finer graph paper may have been used (*see* Table 7 for values). Several of our values differed a little from theirs. The final curve is then prepared in which groups per mg d.f.f. cacao material containing 1% shell d.f.f. are plotted versus stone cells per group values (*see* Fig. 3). It is much flatter than the one obtained by Van Brederode and Reeskamp and has a comparatively flat peak at 8 stone cells per group at which the group value

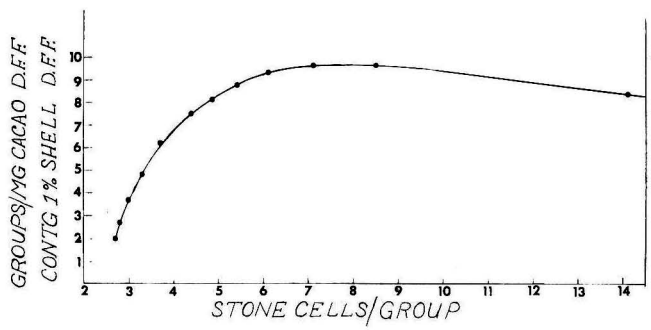

FIG. 3—Revised curve for determining groups/mg d.f.f. cacao containing 1% shell d.f.f. from stone cells/group.

Table 8. Collaborative stone cell data on a dry fat-free basis

Detn	Drop	Collaborator, Sample 1						
		1	2	3	4	5	6	7
		Sample Weight, mg						
1	1	1.293	1.084	0.90	0.602	1.008	0.89	0.57
	2	0.945	0.844	1.17	0.419	0.89	0.938	0.573
2	1	1.148	0.677	1.125	0.465	0.803	0.856	0.525
	2	1.228	1.260	0.923	0.558	1.083	1.104	0.555
		Total Stone Cells						
1	1	509	354	392	177	349	424	375
	2	399	251	553	163	455	467	312
2	1	565	228	441	128	627	407	307
	2	357	570	363	234	428	624	395
		Groups						
1	1	53	47	39	21	50	53	35
	2	42	35	57	18	44	58	35
2	1	66	26	43	21	78	58	36
	2	41	59	40	38	58	63	40
		Stone Cells in Groups						
1	1	505	346	389	177	326	412	365
	2	391	246	545	147	425	449	301
2	1	555	202	433	109	576	381	298
	2	349	570	357	227	376	611	387
		Stone Cells per Group						
1	1	9.5	7.4	10	8.4	6.5	7.8	10.4
	2	9.3	7.0	9.6	8.2	9.7	7.7	8.6
2	1	8.4	7.8	10.1	5.2	7.4	6.6	8.3
	2	8.5	9.7	8.9	6.0	6.5	9.7	9.7
Av.		8.9	8.0	9.7	7.0	7.5	8.0	9.3
		Groups of Stone Cells per mg						
1	1	41.0	43.4	43.3	34.9	49.6	59.6	61.4
	2	44.4	41.5	48.7	43.0	49.4	61.8	61.1
2	1	57.5	38.4	38.2	45.2	97.1	67.8	68.6
	2	33.4	46.8	43.3	68.1	53.6	57.1	72.1
		B and R Group Factor						
1	1	10.3	11.5	9.95	11.0	11.7	11.3	9.7
	2	10.4	11.7	10.2	11.1	10.3	11.4	10.9
2	1	11.0	11.3	9.9	9.55	11.5	11.75	11.05
	2	10.9	10.15	10.7	11.4	11.7	10.15	10.2

(*Continued*)

to indicate 1% shell is 9.62. At both 5.7 and 11.4 stone cells per group it has a value of 9. A median value of 9.31 groups per mg d.f.f. cacao material containing 1% shell d.f.f, used to obtain the per cent shell d.f.f., would introduce a maximum error of only 3.4%. This would be for all samples between 5.7 and 11.4 stone cells per group and could cover all chocolate products ground to 250 mesh. The 9.31 median value is a very close check on the 9.24 value used in the group method and found by the stone cell count method.

Collaborative Study

Six collaborators each received a check sample, 4 unknown samples, a copy of the stone cell count method, and photographs of stone cells found in cacao shell. The Associate Referee's results, obtained previously, are listed as Collaborator 3 for comparison and evaluation purposes. His analyses of the check sample gave a count of 1177 stone cells per mg d.f.f. sample. The collaborators were instructed to analyze the check sample first to familiarize themselves with counting stone cells

Table 8. *(Continued)*

Detn	Drop	Collaborator, Sample 2						
		1	2	3	4	5	6	7
		Sample Weight, mg						
1	1	0.95	1.076	1.228	1.082	0.97	1.092	0.625
	2	0.775	1.009	1.245	0.967	0.893	1.012	0.575
2	1	1.073	0.909	1.10	0.971	0.898	0.945	0.55
	2	1.025	0.822	1.035	0.596	0.97	0.977	0.518
		Total Stone Cells						
1	1	559	679	884	600	515	945	559
	2	403	713	875	509	542	806	549
2	1	578	533	656	558	736	768	496
	2	642	585	769	283	716	767	443
		Groups						
1	1	61	69	88	79	56	106	55
	2	51	67	79	71	58	86	55
2	1	50	51	63	72	74	77	41
	2	48	57	83	42	68	99	48
		Stone Cells in Groups						
1	1	547	673	875	599	446	932	543
	2	391	708	870	509	477	794	537
2	1	569	533	652	558	673	745	485
	2	638	582	764	283	671	749	434
		Stone Cells per Group						
1	1	9.0	9.8	9.9	7.6	8.0	8.8	9.9
	2	7.7	10.6	11.0	7.2	8.2	9.2	9.8
2	1	11.4	10.5	10.3	7.8	9.1	9.7	11.8
	2	13.3	10.2	9.2	6.7	9.9	7.6	9.0
Av.		10.4	10.3	10.1	7.3	8.8	8.8	10.1
		Groups of Stone Cells per mg						
1	1	64.2	64.1	71.7	73.0	57.7	97.1	88.0
	2	65.8	66.4	63.5	73.4	64.9	85.0	95.7
2	1	46.6	56.1	57.3	74.2	82.4	81.5	74.5
	2	46.8	69.3	80.2	70.5	70.1	101.3	92.7
		B and R Group Factors						
1	1	10.6	10.1	10.0	11.5	11.3	10.7	10.0
	2	11.4	9.5	9.2	11.6	11.1	10.5	10.1
2	1	8.9	9.6	9.7	11.3	10.55	10.15	8.6
	2	7.5	9.8	10.5	11.8	10.0	11.4	10.6

(Continued)

and to provide a counting guide. They were instructed to count the total number of stone cell groups containing 2 or more stone cells, the total number of stone cells in these, and the total number of single stone cells present. The total stone cell count was to be obtained by adding the single stone cell count to that found in the groups. The additional data were used to compare 2 other methods.

The check sample and Samples 1–4 consisted of 10–12 g hand-ground, 250 mesh, defatted and dried cocoa material. Each sample was well mixed and carefully quartered to 10–12 g portions. These samples were the same as Samples 1–4 in Table 6. The collaborators were instructed to dry these samples 1 hr at 100°C before weighing a sample for analysis.

Results

The collaborative results are given in Tables 8, 9, and 10 (data obtained, per cent shell d.f.f. values, and per cent shell c.c. values, respec-

Table 8. *(Continued)*

Detn	Drop	Collaborator, Sample 3						
		1	2	3	4	5	6	7
		Sample Weight, mg						
1	1	1.378	0.949	1.01	0.894	0.833	1.214	0.51
	2	1.648	1.008	1.118	0.907	0.945	1.119	0.523
2	1	1.383	0.889	1.00	1.01	1.06	0.923	0.625
	2	0.73	1.046	1.035	0.513	1.07	0.886	0.653
		Total Stone Cells						
1	1	383	284	215	179	264	608	322
	2	418	239	435	213	214	437	267
2	1	475	319	320	226	350	451	276
	2	254	237	264	185	384	438	438
		Groups						
1	1	36	32	22	20	41	60	31
	2	28	29	35	25	38	58	34
2	1	46	37	27	23	27	49	38
	2	22	31	23	21	43	39	48
		Stone Cells in Groups						
1	1	379	283	212	176	232	598	315
	2	417	238	433	212	191	431	261
2	1	472	317	318	224	331	449	266
	2	250	235	262	181	336	428	428
		Stone Cells per Group						
1	1	10.5	8.8	9.8	8.8	5.7	10	10.2
	2	14.9	8.2	12.4	8.5	5.0	7.4	7.7
2	1	10.3	8.6	11.9	9.7	12.3	9.2	7.0
	2	11.4	7.6	11.5	8.6	7.8	11.0	8.9
Av.		11.8	8.3	11.4	8.9	7.7	9.4	8.5
		Groups of Stone Cells per mg						
1	1	26.1	33.7	21.8	22.4	49.2	49.4	60.8
	2	17.0	28.8	31.3	27.6	40.2	51.8	65.0
2	1	33.3	41.6	27.0	22.8	25.5	53.1	60.8
	2	30.1	29.6	22.2	40.9	40.2	44.0	73.5
		B and R Group Factor						
1	1	9.6	10.7	10.1	10.75	10.8	9.95	9.8
	2	6	11.1	8.2	10.9	9.0	11.5	11.4
2	1	9.7	10.9	8.6	10.15	8.2	10.5	11.7
	2	8.9	11.4	8.85	10.85	11.3	9.2	10.7

(Continued)

tively). Three calculation procedures were compared: the stone cell count method, Van Brederode and Reeskamp method, and the group method. As the Van Brederode and Reeskamp method calculates shell only to a d.f.f. basis, the other 2 methods were calculated to this same basis for comparison.

Four of the collaborators selected had experience in the Howard mold count technique, but not in counting stone cells. The other 2 collaborators had previous experience. As 1 collaborator used a phase contrast microscope, which was not specified in the method and is not common to most laboratories, his results were not used in calculating the standard deviation.

In the per cent shell c.c. values of the stone cell count method listed in Table 10, the values of 5 collaborators fall within 1 standard deviation unit of the average value for 3 samples. Four of 6 fall within 1 unit for the other sample. Since the standard deviation units are generally a little lower than those obtained for the spiral vessel

Table 8. *(Continued)*

Detn	Drop	Collaborator, Sample 4						
		1	2	3	4	5	6	7
				Sample Weight, mg				
1	1	1.078	1.047	0.833	0.769	0.92	1.192	0.613
	2	1.26	1.359	0.923	0.602	1.18	0.997	0.608
2	1	1.138	1.310	0.913	0.494	0.863	0.937	0.625
	2	0.638	1.465	0.853	0.520	1.108	1.273	0.705
				Total Stone Cells				
1	1	1629	1323	1177	1291	1496	1892	1248
	2	1557	2190	1215	750	1783	1722	1322
2	1	1487	1936	1275	624	1561	1488	1345
	2	920	1723	1307	588	1802	1823	1354
				Groups				
1	1	154	123	119	140	165	205	124
	2	139	197	129	102	172	182	128
2	1	140	198	130	79	160	156	130
	2	100	192	137	80	188	176	139
				Stone Cells in Groups				
1	1	1613	1319	1170	1289	1434	1864	1227
	2	1532	2187	1209	749	1727	1698	1299
2	1	1461	1926	1267	619	1504	1474	1322
	2	907	1718	1299	588	1720	1817	1335
				Stone Cells per Group				
1	1	10.5	10.7	9.8	9.2	8.7	9.1	9.9
	2	11.0	11.1	9.4	7.3	10.0	9.3	10.1
2	1	10.4	9.7	8.7	7.8	9.4	9.4	10.2
	2	9.1	8.9	9.5	7.4	9.1	10.3	9.6
Av.		10.3	10.1	9.6	7.9	9.3	9.5	10.0
				Groups of Stone Cells per mg				
1	1	142.9	117.5	142.9	182.1	179.3	172.0	202.3
	2	110.3	145.0	139.8	169.4	145.8	182.5	210.5
2	1	123.0	151.1	142.4	159.9	185.4	166.5	208.0
	2	156.7	131.1	160.6	153.8	169.7	138.3	197.2
				B and R Group Factor				
1	1	9.6	9.45	10.1	10.5	10.8	10.65	10.0
	2	9.2	9.15	10.4	11.6	9.95	10.4	9.9
2	1	9.7	10.15	10.2	11.3	10.4	10.4	9.8
	2	10.55	10.7	10.3	11.5	10.55	9.7	10.2

count method (4), though the samples and their per cent shell c.c. values were different, this stone cell count method is considered more satisfactory from this viewpoint than the spiral vessel count method.

In the group method, 5 collaborators' values were within 1 standard deviation unit of the average on Samples 1 and 3, and 4 of 6 on Samples 2 and 4. The standard deviations were slightly higher than in the stone cell count method. The average of the per cent shell c.c. value on the 4 samples was 4.08, compared to 3.73 for the stone cell count method, or 9.4% higher for the group method. The Associate Referee's average results were slightly higher. The average of the per cent shell d.f.f. values (Table 9) were the same for the Van Brederode and Reeskamp calculation procedure and the stone cell count method on Samples 1 and 3, a little lower on Sample 2, and lower still on Sample 4, 14.8 compared to 15.7. This is an overall difference of 3.5% between the 2 calculation procedures. The standard deviation was a littler lower in the Van Brederode and Reeskamp procedure than in the stone cell count method. It

Table 9. Collaborative values for per cent shell d.f.f. by 3 methods[a]

Coll.	Sample 1			Sample 2			Sample 3			Sample 4			Stone Cells/ Group, Av.
	A	B	C	A	B	C	A	B	C	A	B	C	
1	4.3	4.2	4.8	6.1	5.8	6.0	3.2	3.1	2.9	15.1	13.6	14.4	10.4
2	3.8	3.8	4.6	7.1	6.6	6.9	3.0	3.0	3.6	14.8	13.9	14.8	9.2
3	4.5	4.3	4.7	7.4	6.9	7.4	3.2	2.9	2.8	15.2	14.3	15.8	10.2
4	3.7	4.5	5.2	5.7	6.3	7.9	2.7	2.7	3.1	14.2	14.9	18.0	7.8
5	5.6	5.5	6.8	6.5	6.4	7.4	3.3	4.0	4.2	17.6	16.3	18.4	8.3
6	5.4	5.5	6.7	8.6	8.5	9.9	5.0	4.9	5.4	17.0	16.0	17.8	8.9
7[b]	6.7	6.3	7.1	9.7	8.9	9.5	6.1	6.0	7.1	22.2	20.5	22.1	9.5
Av.	4.6	4.6	5.5	6.9	6.7	7.6	3.4	3.4	3.7	15.7	14.8	16.5	
Std dev.	0.8	0.7	1.3	1.0	0.8	1.1	0.8	0.8	1.0	1.3	1.1	1.8	
Range	3.7	3.8	4.6	5.7	5.8	6.0	2.7	2.7	2.8	14.2	13.6	14.4	
	5.6	5.5	6.8	8.6	8.5	9.9	5.0	4.9	5.4	17.6	16.3	18.4	

[a] A = stone cell count method, B = calculation procedure of Van Brederode and Reeskamp, C = group method
[b] Phase contrast microscope used; results were not included in average.

should not be concluded from these results that the 2 complete methods would produce similar results. They differ particularly in the initial treatment of the sample; in the stone cell count method the product is ground to 250 mesh, but in the other method no grinding is specified.

The average value of stone cells per group found on the 4 samples by each collaborator varied from 7.8 to 10.4 (last column, Table 9). There can be some variation in analysis in a single sample but such variation is narrowed by averaging the 4 samples. A low stone cell per group value could indicate that the analyst had trouble recognizing some immature stone cells. Confidence in this technique comes with training, when the analyst sees such immature cells in groups containing some distinct stone cells. In chocolate material, there is some cell structure which the analyst, new to this counting technique, may mistake for immature stone cells. Counting some of such groups would increase the per cent shell values in the easier-to-count group method and the Van Brederode and Reeskamp calculation procedure. Counting the cells in such groups would increase the per cent shell values in the stone cell count method. It would also counterbalance some low counting of immature stone cells in the stone cell count method.

The stone cell count method was modified to include several suggestions by the collaborators. One was to scrub the beaker and rod with a policeman in transferring the digested material from the beaker to the test tube. Another was to allow using the microscope with or without the upper half of the condenser removed. The method was also modified to include the group method. As the type of light used with a microscope may have some effect in recognition of stone cells, the statement "transmitted light" was modified to "transmitted daylight-type filtered and diffused light." Of the 3 calculation procedures studied, that of Van Brederode and Reeskamp appears best in theory as it covers the analysis of chocolate material for shell over all ranges of grind. However, it should be supported by better data. A restudy in which more counts are taken at regular intervals would be desirable. Inasmuch as it appeared necessary to strongly correct some of the initial data in a range applicable to cocoa and some finer chocolate materials, a method using more median values would be as practical as the corrected curve to analyze for shell. The group

Table 10. Collaborative values for per cent shell c.c. by 2 methods[a]

Coll.	Sample 1		Sample 2		Sample 3		Sample 4	
	A	C	A	C	A	C	A	C
1	2.0	2.3	2.9	2.9	1.5	1.3	7.3	7.2
2	1.8	2.2	3.4	3.3	1.4	1.7	7.4	7.3
3	2.2	2.2	3.5	3.5	1.5	1.3	7.5	7.9
4	1.7	2.5	2.7	3.8	1.3	1.4	7.1	9.1
5	2.5	3.2	3.5	3.6	1.5	2.0	8.9	9.4
6	2.6	3.2	4.2	4.8	2.4	2.5	8.5	9.0
7[b]	3.2	3.4	4.6	4.6	2.9	3.3	11.5	11.5
Av.	2.1	2.6	3.4	3.7	1.6	1.7	7.8	8.3
Std dev.	0.4	0.5	0.5	0.6	0.4	0.5	0.7	1.0
Range	1.7	2.2	2.7	2.9	1.3	1.3	7.1	7.2
	2.6	3.2	4.2	4.8	2.4	2.5	8.9	9.4

[a] A = stone cell count method, C = group method.
[b] Phase contrast microscope used; results were not included in average.

method is such a method. The median value used is 9.24, which is comparable with the value of 9.31, obtained from the revised curve. For analyzing coarser chocolate products such as cocoa, cocoa press cake, chocolate liquor, and expeller cake, the stone cell count method is preferable. Bittersweet chocolate may be done by this method but, due to the lack of information on the fineness of grind of chocolates used in confectionery, the group method is preferable for these products.

Recommendations

It is recommended—

(1) That the stone cell count method be adopted as official first action for the analysis of per cent shell c.c. in coarser chocolate products such as cocoa, cocoa press cake, chocolate liquor, and expeller cake.

(2) That the group method be adopted as official first action for the analysis of per cent shell c.c. of other chocolate products.

(3) That the method of Van Brederode and Reeskamp or a similar method be studied and later compared with the stone cell count method and the group method.

(4) That the study of the group sizes of finely ground chocolate products be continued.

(5) That a procedure be studied to extract milk solids and/or sugar from chocolate products before analyses by a microscopic counting method to determine the per cent shell c.c.

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The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee D and were adopted by the Association. See *JAOAC* **53**, 388–389 (1970).

Collaborative Study of Methods for the Determination of Fat in Cacao Products

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A collaborative study was conducted comparing the AOAC methods (12.022 Method I and 12.023 Method II) for the determination of fat in cacao products with the OICC Soxhlet method and a rapid refractometric method. Six samples of cacao products (breakfast cocoa, vegetable fat coating, cacao nibs, milk chocolate, sweet chocolate, and chocolate liquor) were analyzed by 7 collaborators. Results indicate that the OICC method gives better precision and somewhat lower results than the AOAC method. It is recommended (*1*) that the OICC method as presented, using a final HCl concentration of 4*N* in the digestion step, be adopted as official first action to replace 12.023 Method II, which will be deleted official final action by suspension of the rules; (*2*) that study be continued to accumulate data needed to correlate the results of the analysis of cacao products by the OICC method and 12.022 Method I; and (*3*) that 12.022 be qualified by a statement "(Not applicable to cacao nibs unless finely ground)".

One of the most valuable vegetable fats in commerce is cacao fat, which occurs naturally as a component in cacao nibs obtained from cacao beans. The United States Standard for chocolate liquor (1), obtained by finely grinding cacao nibs, requires not less than 50% and not more than 58% cacao butter by weight. Chocolate liquor is a mandatory ingredient in various other standardized cacao products such as sweet chocolate and milk chocolate. Also, cacao butter may be added to these various products to provide needed plasticity for ease of molding. In several of the standardized cacao products, butter fat may also be added in the form of optional dairy ingredients.

Various analytical methods have been published for the determination of the fat content of cacao products. Two AOAC methods (2) are presently official final action for this determination: Method I (3, 4), which is not applicable to cacao products containing milk ingredients or to products prepared by cooking with sugar and water and drying, and Method II (5–7), which is applicable to the products excluded in Method I.

Method I was collaboratively studied and adopted in 1926; it involves a series of petroleum ether extractions of the sample in a Knorr-type extraction tube. Solvent is removed from the combined petroleum ether extract and the fat is dried to constant weight. Method II, adopted in 1950, introduces an initial HCl digestion step to hydrolyze cellulose and milk proteins which would otherwise bind fat and make it unavailable to extraction by solvents.

The OICC (International Office of Cocoa and Chocolate, Zurich, Switzerland) method (8) is similar in principle to AOAC Method II in that it uses an initial HCl digestion followed by a petroleum ether extraction. It differs, however, in that the acid digest is filtered directly through fluted paper and washed free of chloride ion. The filter paper and sample are transferred directly to an extraction thimble, thus eliminating the additional sample handling required by AOAC Method II. The sample is then extracted in a Soxhlet or similar extractor.

A rapid refractometric method (8) frequently used in industry for quality control purposes involves the admixture of a known weight of cacao sample to a known weight of Halowax (monochloronaphthalene). The sample mixture is filtered, the refractive index is determined, and the per cent cacao butter in the Halowax is obtained from a standard chart. The per cent cacao butter in the original sample is calculated from an equation relating per cent cacao butter in the Halowax, weight of Halowax, and sample weight. This method is applicable to the analysis of cacao products except cacao nibs and products containing fats other than cacao butter and milk fat.

Collaborative Study

Samples of breakfast cocoa, vegetable fat coating, cacao nibs, milk chocolate, sweet chocolate, and chocolate liquor were sent to each of seven collaborating laboratories. Collaborators were asked to analyze each sample in duplicate by

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each of three methods: the applicable AOAC method, **12.022** or **12.023**; the OICC Soxhlet method; and the Halowax method. The OICC and Halowax methods follow.

OICC METHOD

(Applicable to cacao products with or without milk ingredients or to products prepd by cooking with sugar and H_2O and drying)

Apparatus and Reagents

(a) *Soxhlet apparatus.*—With $\overline{\$}$ joints, siphon capacity ca 100 ml (33 × 80 mm thimble), 250 ml erlenmeyer, and regulated heating mantle.

(b) *Petroleum ether.*—Distd in glass, bp 30–60°.

Determination

If necessary, chill product until hard and grate or shave to fine, granular condition. Accurately weigh 2–4 g chocolate liquor, 4–5 g cocoas, 4–5 g sweet chocolate, or 9–10 g milk chocolate into 300–500 ml beaker. Add slowly, while stirring, 45 ml boiling H_2O to give homogeneous suspension. Add 55 ml ca 8*N* HCl (2 + 1) and few defatted SiC chips or other antibumping agent, and stir. Cover with watch glass, bring slowly to boil, and boil gently 15 min. Rinse watch glass with 100 ml H_2O. Filter digest thru 15 cm S&S No. 589 medium fluted paper, or equiv., rinsing beaker 3 times with H_2O. Continue washing until last portion of filtrate is Cl-free as detd by addn of 0.1*N* $AgNO_3$. Transfer wet paper and sample to defatted extn thimble and dry 6–18 hr in small beaker at 100°. Place glass wool plug over paper.

Add few defatted antibumping chips to 250 ml erlenmeyer and dry 1 hr at 100°. Cool to room temp. in desiccator and weigh. Place thimble contg dried sample in soxhlet, supporting it with spiral or glass beads. Rinse digestion beaker, drying beaker, and watch glass with three 50 ml portions pet ether, and add washings to thimble. Reflux digested sample 4 hr, adjusting heat so that extractor siphons at least 30 times. Remove flask and evap. solv. on steam bath. Dry flask at 100–101° to constant wt (1.5–2 hr). Cool in desiccator to room temp. and weigh. Constant wt is attained when successive 1 hr drying periods show addnl loss of <0.05% fat. % Fat = g fat × 100/g sample. Duplicate detns should agree within 0.1% fat.

HALOWAX METHOD

(Applicable for rapid fat determination in cacao products (except nibs and products containing other than cacao fat and milk fat))

Apparatus

(a) *Abbe-type refractometer.*—With scale range of 1.300–1.740 and precision of ±0.0002 units.

(b) *Constant temperature recirculating water bath.*—Capable of pumping water at 25°C through refractomer jacket.

(c) *Heating oven.*—Set at 70°C.

Reagents

(a) *Halowax 1007.*—Monochloronapthalene, commercial grade (E. H. Sargent & Co., or equivalent).

(b) *Filter paper.*—Folded Whatman No. 2V, 12.5 cm diameter, or equivalent.

Preparation of Sample

Grind sample so that particles pass through US sieve No. 35. Mix well. For high fat content product, like liquor and chocolate, refrigerate sample before grinding to prevent melting.

Determination

Accurately weigh ca 10 g product into tared beaker and record weight (*W*). Add ca 10 g Halowax and record exact weight (*H*). Stir contents with glass rod, making sure Halowax dissolves all small lumps. Place beaker in 70°C oven for 5±1 min. Remove sample from oven and again mix well with glass rod. Filter sample, using folded filter paper placed directly into 50 ml beaker, in warm place.

Place 2 drops of clear filtrate on prism of refractometer; close and check temperature. Adjust shadow to cross-hair and take reading. (Temperature correction may be applied to reading by adding or subtracting 0.0045 for every °C above or below 25°C, respectively.)

Calculation

Find per cent cacao fat in Halowax (*C*) corresponding to refractive index reading on Table 1 and use in following formula:

$$\% \text{ Cacao fat} = (C \times H \times 100)/[(100 - C) \times W]$$

Results and Discussion

Table 2 gives the results of duplicate determinations on samples of the six cacao products studied by the seven collaborators, using the AOAC, OICC, and Halowax methods.

One collaborator used a Goldfisch extraction apparatus in the OICC method. All others used a Soxhlet extraction apparatus. Five of the seven collaborators indicated their choice between AOAC **12.022** and **12.023** for each sample. All collaborators used **12.023** for the analysis of milk chocolate. All collaborators used **12.022** for the analysis of breakfast cocoa, cacao nibs, and choc-

Table 1. Per cent cacao butter (CB) in Halowax by refractive index[a]

CB, %	n	CB, %	n	CB, %	n	CB, %	n
0.0	1.63389	11.0	1.60961	22.0	1.58695	33.0	1.56606
0.2	1.63340	11.2	1.60922	22.2	1.58655	33.2	1.56570
0.4	1.63292	11.4	1.60880	22.4	1.58615	33.4	1.56533
0.6	1.63246	11.6	1.60839	22.6	1.58575	33.6	1.56497
0.8	1.63198	11.8	1.60795	22.8	1.58535	33.8	1.56463
1.0	1.63151	12.0	1.60751	23.0	1.58495	34.0	1.56428
1.2	1.63107	12.2	1.60708	23.2	1.58455	34.2	1.56392
1.4	1.63060	12.4	1.60667	23.4	1.58416	34.4	1.56358
1.6	1.63014	12.6	1.60623	23.6	1.58376	34.6	1.56322
1.8	1.62970	12.8	1.60580	23.8	1.58336	34.8	1.56284
2.0	1.62923	13.0	1.60535	24.0	1.58294	35.0	1.56250
2.2	1.62880	13.2	1.60492	24.2	1.58252	35.2	1.56210
2.4	1.62833	13.4	1.60450	24.4	1.58212	35.4	1.56173
2.6	1.62788	13.6	1.60408	24.6	1.58173	35.6	1.56136
2.8	1.62742	13.8	1.60367	24.8	1.58135	35.8	1.56100
3.0	1.62700	14.0	1.60323	25.0	1.58096	36.0	1.56061
3.2	1.62652	14.2	1.60281	25.2	1.58057	36.2	1.56028
3.4	1.62610	14.4	1.60238	25.4	1.58017	36.4	1.55991
3.6	1.62563	14.6	1.60195	25.6	1.57976	36.6	1.55956
3.8	1.62516	14.8	1.60152	25.8	1.57936	36.8	1.55920
4.0	1.62471	15.0	1.60111	26.0	1.57896	37.0	1.55885
4.2	1.62428	15.2	1.60068	26.2	1.57860	37.2	1.55850
4.4	1.62380	15.4	1.60026	26.4	1.57822	37.4	1.55815
4.6	1.62335	15.6	1.59984	26.6	1.57786	37.6	1.55780
4.8	1.62290	15.8	1.59942	26.8	1.57750	37.8	1.55747
5.0	1.62247	16.0	1.59902	27.0	1.57710	38.0	1.55712
5.2	1.62200	16.2	1.59862	27.2	1.57675	38.2	1.55680
5.4	1.62155	16.4	1.59822	27.4	1.57640	38.4	1.55645
5.6	1.62110	16.6	1.59780	27.6	1.57603	38.6	1.55612
5.8	1.62066	16.8	1.59740	27.8	1.57566	38.8	1.55578
6.0	1.62020	17.0	1.59700	28.0	1.57530	39.0	1.55545
6.2	1.61976	17.2	1.59660	28.2	1.57495	39.2	1.55512
6.4	1.61931	17.4	1.59621	28.4	1.57457	39.4	1.55480
6.6	1.61891	17.6	1.59581	28.6	1.57421	39.6	1.55446
6.8	1.61850	17.8	1.59541	28.8	1.57385	39.8	1.55412
7.0	1.61808	18.0	1.59500	29.0	1.57350	40.0	1.55379
7.2	1.61765	18.2	1.59460	29.2	1.57312	40.2	1.55346
7.4	1.61724	18.4	1.59421	29.4	1.57277	40.4	1.55312
7.6	1.61682	18.6	1.59381	29.6	1.57240	40.6	1.55278
7.8	1.61640	18.8	1.59341	29.8	1.57204	40.8	1.55234
8.0	1.61596	19.0	1.59300	30.0	1.57170		
8.2	1.61553	19.2	1.59261	30.2	1.57131		
8.4	1.61511	19.4	1.59221	30.4	1.57095		
8.6	1.61470	19.6	1.59182	30.6	1.57056		
8.8	1.61428	19.8	1.59142	30.8	1.57020		
9.0	1.61384	20.0	1.59100	31.0	1.56982		
9.2	1.61341	20.2	1.59056	31.2	1.56945		
9.4	1.61300	20.4	1.59014	31.4	1.56908		
9.6	1.61257	20.6	1.58976	31.6	1.56870		
9.8	1.61215	20.8	1.58936	31.8	1.56832		
10.0	1.61171	21.0	1.58896	32.0	1.56795		
10.2	1.61130	21.2	1.58855	32.2	1.56757		
10.4	1.61088	21.4	1.58815	32.4	1.56720		
10.6	1.61047	21.6	1.58775	32.6	1.56682		
10.8	1.61003	21.8	1.58735	32.8	1.56645		

[a] For every degree above or below 25°, add or subtract 0.00045.

olate liquor. However, two collaborators used **12.022** and three used **12.023** for the analysis of vegetable fat coating, while three selected **12.022** and two selected **12.023** for the analysis of sweet chocolate.

It should be noted that the results obtained for Sample 3, cacao nibs, vary considerably between both methods used and collaborators. The values obtained by the OICC method are consistent with the amount of cacao fat normally present in cacao nibs. The lower results obtained by Collaborators 1–5 reflect incomplete extraction of fat under the conditions used for sample preparation. This is confirmed by the results obtained by Collaborators 6 and 7, together with the comment by Collaborator 7 that AOAC **12.022** is applicable only to finely milled cacao products. Similar results were obtained by the Halowax method for Sample 3, indicating incomplete extraction of cacao fat into the Halowax from the coarsely ground nibs. It is apparent that cacao nibs must be finely ground to permit quantitative fat extraction, using either AOAC **12.022** or the Halowax method.

Standard deviations between duplicate determinations for each method and sample are given in Table 3. These data indicate the greater precision of the OICC method in comparison with the other two methods. The method means by sample are tabulated in Table 4. With the exception of cacao nibs and milk chocolate, the OICC method gave slightly lower results than the AOAC method.

Subsequent to this collaborative study, it was noted that the amount of hydrochloric acid specified for the digestion step in the OICC method resulted in a final acid concentration of 2.2*N* rather than 4*N* as used by the OICC. Accordingly a

Table 2. Collaborative results of the determination of fat in cacao products by three methods

	Collaborator 1			Collaborator 2			Collaborator 3		
Product	OICC	AOAC	Halo-wax	OICC	AOAC	Halo-wax	OICC	AOAC	Halo-wax
1 Breakfast cocoa	20.19	20.93	22.21	20.52	20.15	20.92	20.75	21.53	21.9
	20.19	21.23	22.18	20.40	20.29	21.01	20.78	21.55	21.4
2 Vegetable fat coating	58.01	59.62	64.84	54.77	60.85	63.92	56.66	57.41	62.3
	59.95	59.85	64.69	54.63	60.62	64.62	56.72	57.51	61.3
3 Cacao nibs	53.61	28.83	28.12	57.50	45.33	40.66	56.05	37.77	36.9
	53.00	28.74	27.95	58.90	48.16	41.05	55.61	39.32	36.9
4 Milk chocolate	33.04	33.13	35.11	34.69	34.06	34.66	34.29	33.22	35.4
	32.73	33.95	35.14	34.76	33.42	34.69	34.53	33.67	35.7
5 Sweet chocolate	36.90	37.35	38.07	37.07	36.27	36.56	36.64	37.03	37.4
	36.84	37.87	38.12	36.89	35.73	35.74	36.73	37.17	38.5
6 Chocolate liquor	53.94	55.59	57.28	55.20	55.72	56.83	55.54	55.91	56.8
	54.41	55.82	57.28	55.79	55.35	56.35	55.55	55.68	56.5

	Collaborator 4			Collaborator 5			Collaborator 6			Collaborator 7		
Prod.	OICC	AOAC	Halo-wax	OICC	AOAC	Halo-wax	OICC	AOAC	Halo-wax	OICC	AOAC	Halo-wax
1	21.20	22.67	20.95	20.08	22.97	21.38	21.0	21.5	21.1	20.71	20.27	21.3
	21.18	21.25	23.76	20.11	21.60	21.20	20.9	20.9	20.9	20.75	20.15	21.3
2	60.16	60.33	63.72	58.77	62.14	63.58	56.9	56.1	60.4	58.71	58.43	64.2
	59.93	59.45	63.26	59.02	61.91	63.70	56.9	53.0	59.7	58.37	59.13	63.4
3	54.81	23.92	—	55.89	48.70	48.26	56.9	57.5	56.5	56.87	52.84	54.1
	54.80	23.86	—	55.81	49.11	48.16	57.0	57.0	56.7	56.97	52.41	53.1
4	34.76	35.26	34.27	34.49	35.20	34.87	34.3	34.4	34.1	34.72	34.46	34.6
	34.71	36.34	34.22	34.64	35.47	34.65	34.2	34.2	34.2	35.10	34.10	34.8
5	37.17	37.80	37.03	36.73	37.52	37.42	36.9	37.8	36.8	37.02	36.09	37.3
	37.14	39.55	36.91	36.68	38.11	37.72	36.8	38.0	36.6	37.56	36.65	37.0
6	55.61	58.23	55.84	55.60	57.14	56.65	55.5	56.6	55.9	56.25	55.51	56.6
	55.47	56.13	55.79	55.38	57.03	56.50	55.2	56.9	55.8	56.08	55.74	56.0

Table 3. Standard deviations between duplicate determinations of fat in cacao products

Product	OICC	AOAC	Halowax
Breakfast cocoa	0.04	0.56	0.77
Vegetable fat coating	0.53	0.89	0.45
Cacao nibs	0.43	0.89	0.32
Milk chocolate	0.16	0.44	0.12
Sweet chocolate	0.16	0.56	0.39
Chocolate liquor	0.23	0.59	0.23

limited study was made in which two laboratories compared these two acid concentrations using the OICC method for duplicate analyses of three samples of cacao products. The results are shown in Table 5. An analysis of variance showed no significant difference between acid concentrations or laboratories.

In the method as originally published by OICC, the following note appears: "If sample is known to contain milk, low temperature filtration is easier. After hydrolysis, place acid digest in refrigerator for 3–4 hrs and wash filter paper residue with ice cold water". Since experience in our laboratories has shown no significant advantage in the use of low temperature filtration, the note has been omitted.

Table 4. Method means by sample

Product	OICC	AOAC	Halowax
Breakfast cocoa	20.63	21.21	21.54
Vegetable fat coating	57.84	59.02	63.12
Cacao nibs	55.98	42.39	44.03[a]
Milk chocolate	34.35	34.35	34.74
Sweet chocolate	36.93	37.35	37.23
Chocolate liquor	55.39	56.24	56.44

[a] Based on N = 12; Collaborator 4 did not report results for this method.

Table 5. Comparison of acid concentrations[a]

	4N HCl		2.2N HCl	
Product	Lab. 1	Lab. 2	Lab. 1	Lab. 2
Chocolate liquor	55.59	55.32	55.78[b]	55.59
Medium fat cocoa	10.75	10.51	10.73	10.83
Milk chocolate	30.39[c]	30.26	30.22[c]	30.56

[a] Average of duplicate determinations.
[b] Single determination.
[c] Average of four determinations.

Recommendations

It is recommended—

(1) That the OICC method as presented, using a final HCl concentration of 4*N* in the digestion step, be adopted as official first action to replace **12.023** Method II, which will be deleted official final action by suspension of the rules.

(2) That study be continued to accumulate data needed to correlate the results of the analysis of cacao products by the OICC method and **12.022** Method I.

(3) That AOAC **12.022** be qualified by the statement "(Not applicable to cacao nibs unless finely ground)".

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The recommendations in this paper were approved by the General Referee and by Subcommittee D and were adopted by the Association. See *JAOAC* **53,** 388–389 (1970).

PESTICIDE RESIDUES

Specific GLC Method for Determining Residues of Carbaryl by Electron Capture Detection after Derivative Formation

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Residues of carbaryl were determined by a specific procedure based on the preparation of two derivatives. Samples of bees, bee bread, bee pollen, and cows' milk were extracted with chloroform and cleaned up by acetonitrile-petroleum ether partitioning and then by liquid chromatography. Subsequently the residues were hydrolyzed to 1-naphthol, part of which was trichloroacetylated and part of which was brominated and acetylated. Both derivatives were then determined by electron capture gas chromatography on the same column and at the same temperature. This procedure required only slightly more time than analysis by preparation of a single derivative. Recoveries from samples fortified at levels of 0.005–16.0 ppm ranged from 70 to 112%.

The determination of residues of chlorinated pesticides by electron capture gas chromatography (ECGLC) is a widely accepted procedure that is convenient and highly sensitive but lacks specificity. Beroza and Bowman (1) improved specificity considerably by introducing the use of extraction p-values. This method of verifying analytical results is employed routinely in our laboratory for samples of known history. However, when samples of uncertain history are to be analyzed, the results are checked by another method. Semiquantitative thin layer chromatography is often employed for this purpose (2).

The determination of residues of carbaryl by ECGLC was introduced by Gutenmann and Lisk (3), who reported a method based on hydrolysis, bromination, and acetylation to produce an electron-capturing derivative. Subsequently, Butler and McDonough (4) reported a method of determining residues of carbaryl, carbofuran, and Mobam® (benzo[b]-thien-4-yl methylcarbamate) based on hydrolysis and trichloroacetylation. These two methods could be used independently to verify residues of carbaryl quantitatively. However, a single cleanup procedure that would be satisfactory for both derivatives would be desirable, and it would be helpful to find gas chromatographic conditions which would permit analysis on the same column at the same temperature. In the present paper, we report cleanup procedures and gas chromatographic conditions that meet both of these requirements. The result is a method which is highly specific and requires only slightly more time than analysis by preparation of a single derivative.

METHOD

Apparatus and Reagents

(a) *Gas chromatograph.*—A Research Specialties gas chromatograph (now HCL, Inc., Rockford, Ill.) containing a ^{90}Sr electron capture detector was equipped with 183 × 0.4 cm id glass column packed with 7% DC-200 on 80–100 mesh Gas Chrom Q (Applied Science Laboratories, State College, Pa.). Operating conditions: injection port 220°C; column 190°C; detector 235°C; nitrogen carrier gas flow 75–100 ml/min.

(b) *Aluminum oxide.*—Baker's Analyzed Reagent (J. T. Baker Chemical Co., Phillipsburg, N.J.), acidic, Brockman activity grade 1.

(c) *Florisil.*—60–100 mesh, activated at 1200°F (Floridin Co., 2 Gateway Center, Pittsburgh, Pa.). Add enough water to make the total 5% by weight and tumble Florisil 1 hr.

(d) *Solvents.*—Distill $CHCl_3$, CH_2Cl_2, acetonitrile, pentane, and hexane in glass.

(e) *Keeper solution.*—Dilute 1 g Shell Ondina Oil (Shell Oil Co.) to 100 ml with CH_2Cl_2.

(f) *Pyridine solution.*—Pass 50 ml reagent grade pyridine (J. T. Baker) through 10 mm id liquid chromatographic column, packed from bottom to top with glass wool, thin layer of anhydrous Na_2SO_4, 8 g Florisil, and ca 0.5″ anhydrous Na_2SO_4. Discard first eluate used to wet column; dilute 0.10 ml chromatographed pyridine to 100 ml with CH_2Cl_2 and store solution in dark bottle.

(g) *Trichloroacetyl chloride solution.*—Distill trichloroacetyl chloride (Columbia Organic Chemical Co., Columbia, S. C.) under vacuum through 18″ Vigreux column and collect fraction with bp 31°C/32 mm. Dilute 0.1 ml trichloroacetyl chloride to 10 ml with CH_2Cl_2 in volumetric flask.

Sample Extraction

Bees and pollen.—Blend 25 g sample in Waring Blendor 5 min with 250 ml $CHCl_3$. Pour mixture into 400 ml beaker while stirring vigorously, add 100 g anhydrous Na_2SO_4, and let mixture stand ≥ 10 min. Filter mixture through cotton plug covered with anhydrous Na_2SO_4 into glass bottles and refrigerate.

Bee bread.—Use same procedure as for bees, except blend 15 g sample and 150 ml $CHCl_3$.

Milk.—Add 100 g milk and 50 ml pentane to 500 ml separatory funnel and shake briefly. Add 200 ml ether and shake gently 2 min. After removing aqueous layer, dry ether-pentane layer with anhydrous Na_2SO_4 and filter through paper into graduated cylinder. Determine volume of solution and refrigerate.

Cleanup Procedures

Bees.—Evaporate portion of extract representing 5–20 g bees to dryness in 40°C water bath with gentle stream of air. (Do all evaporations in this manner.) Add 10 ml hexane to residue; then stir and transfer solution to 250 ml separatory funnel with 25 ml acetonitrile. Shake 1 min, transfer acetonitrile layer to second separatory funnel, and re-extract hexane with 5 ml acetonitrile; combine acetonitrile extracts. Filter any emulsion through glass wool into acetonitrile and wash glass wool with 1 ml acetonitrile. Extract combined extracts with 10 ml hexane. Separate and evaporate acetonitrile solution to dryness, and dissolve residue in 10 ml CH_2Cl_2 which has been equilibrated with water. Pack 20 mm id liquid chromatographic column from bottom to top with cotton plug, 15 g Florisil, and cotton plug. Add sample solution and rinse sample container 3 times with 10 ml water-equilibrated CH_2Cl_2. Elute with 100 ml water-equilibrated CH_2Cl_2 and dry entire eluted solution with anhydrous Na_2SO_4; filter eluate and three 10 ml CH_2Cl_2 rinses through paper into flask.

If 1-naphthol is not to be separated, add 0.1 ml keeper solution to filtrate and evaporate to small volume; transfer solution to 10–50 ml volumetric flask and dilute to volume with CH_2Cl_2. Evaporate appropriate aliquot to small volume and proceed with *Derivatization.*

If separation of 1-naphthol from carbaryl is desired, evaporate filtrate to small volume and transfer to 250 ml separatory funnel with enough rinses to make volume ca 50 ml. Extract with 20 ml 0.5*N* aqueous NaOH to remove free 1-naphthol. Allow complete separation and drain CH_2Cl_2 layer into 250 ml Erlenmeyer flask. Add 10 ml 6*N* HCl to base layer. Extract this layer 3 times with 20 ml CH_2Cl_2 combine extracts, add 0.1 ml keeper solution, and proceed as above.

Pollen and bee bread.—Evaporate portion of extract representing 5–20 g pollen or 5–10 g bee bread to dryness. Dissolve residue in 10 ml hexane and proceed as with bees through evaporation of acetonitrile fraction. Dissolve residue in 10 ml chloroform. Pack liquid chromatographic column from bottom to top with glass wool, anhydrous Na_2SO_4, 15 g Al_2O_3, and anhydrous Na_2SO_4. Add $CHCl_3$ solution, three 10 ml rinses, and additional 35 ml $CHCl_3$. As last liquid enters column, change receiver, and elute insecticide with 75 ml $CHCl_3$. Add 0.1 ml keeper solution to eluate and evaporate to small volume. Transfer solution and 3 rinses to 10–50 ml volumetric flask and dilute to volume with CH_2Cl_2. If 1-naphthol is not to be separated, evaporate appropriate portion to small volume for each derivatization reaction. If 1-naphthol is to be separated, proceed as for bees except use $CHCl_3$ solution in place of CH_2Cl_2 solution. The acidified solution of 1-naphthol, however, is still extracted with CH_2Cl_2.

Milk.—Add 0.1 ml keeper solution to 50–100 ml pentane-ether extract of milk and evaporate to dryness. Dissolve residue in 10 ml hexane and transfer to 250 ml separatory funnel along with 25 ml acetonitrile rinse. Shake funnel and discard hexane layer. Extract acetonitrile layer 3 more times with 10 ml portions of hexane. Add 0.1 ml keeper solution to acetonitrile and evaporate to dryness. Dissolve residue in CH_2Cl_2 and proceed with *Derivatization.*

Derivatization

(a) Place 0.1–50 μg insecticide dissolved in $CHCl_3$ or CH_2Cl_2 in test tube, add 0.1 ml keeper solution, and evaporate solution to dryness. Add 0.2 ml 0.1*N* NaOH in methanol, rotate test tube to wet its sides with mixture, and shake 5 min on shaker.

Evaporate methanol, add 1 ml pyridine solution, and heat tube in oil bath at 100–105°C until CH_2Cl_2 has evaporated (ca 1 min). Remove test tube, cool in ice water, add 1 ml trichloroacetyl chloride solution, shake briefly, and again heat in oil bath until CH_2Cl_2 has evaporated. Remove test tube from oil bath, add 10 ml hexane, shake briefly, add 5 ml water, and transfer contents to 60 ml separatory funnel. Rinse tube with 5 ml hexane. Shake separatory funnel 30 sec, draw off water phase, add 5 ml saturated $NaHCO_3$ solution, and shake 15 sec. Draw off $NaHCO_3$ layer and wash hexane once with 5 ml water. Draw off water layer, transfer hexane layer to 50 ml Erlenmeyer flask, and add anhydrous Na_2SO_4; let hexane solution stand 15 min before filtering into volumetric flask through folded paper containing

Mention of a pesticide or a proprietary product in this paper does not constitute a recommendation or an endorsement of this product by the U.S. Department of Agriculture.

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anhydrous Na_2SO_4. Wash flask and filter paper with enough hexane to give 50 ml hexane solution. Inject 1–5 μl hexane solution into gas chromatograph.

(b) Place 0.5–50 μg insecticide in $CHCl_3$ or CH_2Cl_2 in 40 ml test tube. Add 0.1 ml keeper solution and evaporate to dryness. Add 1 ml glacial acetic acid and 10 drops concentrated H_2SO_4, mix contents, and let test tube stand at room temperature 5 min. Add 0.2 ml solution of acetic acid saturated with I_2 crystals that contain 5% (by volume) liquid Br_2. Place small funnel for an air condenser on each tube (stem in test tube). Heat test tube 10 min at 130°C, rinse funnel with 5 ml water, and transfer solution to 60 ml separatory funnel. Rinse test tube with 10 ml hexane and 5 ml water. Shake separatory funnel 1 min and draw off water layer. Transfer hexane layer and three 5 ml hexane rinses to Erlenmeyer flask. Dry hexane solution with anhydrous Na_2SO_4, filter, and wash Na_2SO_4 3 times with 5 ml hexane. Add 0.1 ml keeper solution to hexane and evaporate to dryness. Dissolve residue in 2 ml hexane and repeat evaporation until all iodine has been removed (2–3 evaporations are required). Finally dissolve residue in 1–10 ml hexane and inject 1–5 μl into gas chromatograph.

Discussion

Standard curves for the trichloroacetylated and the brominated derivatives of carbaryl are shown in Fig. 1. Since the trichloroacetylated derivative gives a peak about 4 times as intense as the brominated derivative, the sample size injected should be adjusted accordingly. The retention times of the trichloroacetylated and brominated derivatives were 6.4 and 7.7 min, respectively.

The recoveries of carbaryl from samples of bees, pollen, and milk fortified before extraction are given in Table 1. There is generally good agreement between the brominated and the trichloroacetylated derivatives. The milk samples were fortified at lower levels than the bee or pollen samples because the tolerance for carbaryl in milk is zero; consequently, an analytical method must have high sensitivity for milk.

The bromination method of Gutenmann and Lisk (3) was followed in the preparation of derivatives except that hexane was substituted for benzene. The method of Butler and McDonough (4) was followed in the trichloroacetylation procedures except that commercial trichloroacetyl chloride was used instead of material synthesized in the laboratory. However, it was still necessary to distill this material to remove electron-capturing materials with long gas chromatographic

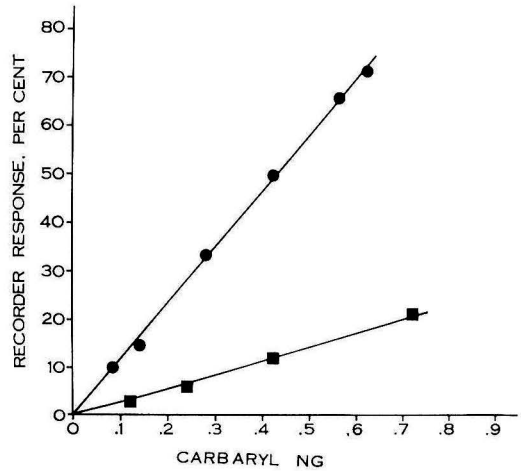

FIG. 1—Standard curves for the derivatives of carbaryl: ●, trichloroacetylated derivative; ■, brominated derivative.

retention times. One material that produced a strong peak with a retention time of 13 min could not be removed by distillation. This material did not interfere with the analysis, but it did extend the time between injections of samples by about 7 min.

Carbaryl could not be recovered from PR grade Florisil, which is activated at 1400°F. The standard grade, which is activated at 1200°F, was usually satisfactory, but some variability was

Table 1. Per cent recovery of carbaryl from samples fortified before extraction and cleanup

Sample	Added, ppm	Recovery, % TCA[a]	Recovery, % BR[b]
Bees	0.4	93	88
	0.8	92	106
	2.0	80	95
	2.0	90	100
	0.0	ND[c]	ND
Pollen	0.4	95	110
	4.0	83	80
	16.0	89	77
	0.0	ND	ND
Milk	0.005	88	112
	0.01	100	90
	0.03	70	110
	0.05	70	84
	0.05	94	110
	0.10	110	79
	0.0	ND	ND

[a] Trichloroacetylated derivative.
[b] Brominated derivative.
[c] None detected.

Table 2. Determination of residues of carbaryl in field-collected samples of bees, pollen, and bee bread with and without separation of 1-naphthol

Sample	TCA[a]			BR[b]		
	1-Naphthol Separated		1-Naphthol Not Separated,	1-Naphthol Separated		1-Naphthol Not Separated,
	Carbaryl, ppm	1-Naphthol, ppm	Carbaryl, ppm	Carbaryl, ppm	1-Naphthol, ppm	Carbaryl, ppm
Bees, 1	0.74	ND[c]	0.79	0.77	0.11	0.86
2	0.66	0.03	—	—	ND	0.72
Bee bread	9.0	ND	9.5	10.3	ND	10.4
Pollen, 1	5.7	ND	5.7	5.0	0.07	5.4
2	14.9	0.11	15.2	15.4	0.22	17.7

[a] Trichloroacetylated derivative.
[b] Brominated derivative.
[c] None detected.

found. It was necessary to add water to make the water content 5% before carbaryl could be eluted. Also, with some batches of Florisil, carbaryl could not be recovered even when water was added.

No attempt was made to develop the method to determine 1-naphthol, the non-toxic metabolite of carbaryl. Gutenmann and Lisk (3) pointed out that 1-naphthol is not a significant residue in samples treated with carbaryl, apparently because of its reactivity in biological systems. Otherwise, determination of 1-naphthol in bees would be worthwhile since it would demonstrate exposure to carbaryl. Recoveries of 1-naphthol in the carbaryl fraction were determined from bee and pollen samples. In pollen, 1-naphthol was not recovered when the samples were fortified with 0.2–4.0 ppm. In bees, the recoveries were about 90% after fortification at 4.0 ppm but decreased to zero for fortification at 0.4 ppm. An optional step has therefore been included in the cleanup procedures for the separation of 1-naphthol and carbaryl. This is the procedure developed by Johnson and Stansbury (5) to separate 1-naphthol from carbaryl. The cleanup procedure for pollen is the one developed by Morse *et al.* (6).

Residues in field-collected samples of bees, bee bread, and pollen are given in Table 2. The residues, as determined by each derivative, are in satisfactory agreement. Some samples were analyzed for 1-naphthol and carbaryl separately as well as together. As expected, negligible residues of 1-naphthol were found. Bee bread suitable for check samples and for the determination of percentage recovery was not available.

Since our previous paper (4), recent publications on the preparation of derivatives for the determination of residues of carbamates by electron capture gas chromatography have included the preparation of substituted aniline derivatives (7,8) and the preparation of a monochloroacetate (9).

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GLC Retention Times of Pesticides and Metabolites Containing Phosphorus and Sulfur on Four Thermally Stable Columns

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The relative retention times of 138 pesticides and metabolites containing phosphorus and sulfur were determined on packings containing liquid phases OV-101, OV-17, OV-210, and OV-225, using a flame photometric detector. These packings have excellent thermal stability and are likely to find widespread use in pesticide residue analyses, especially for the identification of multicomponent residues in environmental samples or in samples of unknown or indefinite history. Relative retention times were determined isothermally and by temperature programming; the latter procedure was more reproducible and also more useful for analyzing mixtures with widely differing retention times.

The GLC retention times (t_R) of 138 pesticides and their metabolites containing phosphorus and/or sulfur were determined on packings prepared from 4 recently introduced liquid phases that have excellent thermal stability. Attractive features of these packings, compared with others used previously, are shorter conditioning periods, decreased bleeding of liquid phase, longer column life, and the capability of operating at higher temperatures. The low bleed rate of the liquid phases also avoids excessive rise in baseline in temperature programming to high temperatures.

The t_R data will facilitate detection of phosphorus (P) and sulfur (S) pesticides in multicomponent analyses, in environmental samples, and in materials containing unknown pesticides. A complete listing of t_R data will also alert residue chemists to those compounds that may interfere in methods devised for use with the new liquid phases; with this knowledge, the chemist can take appropriate measures to either recognize or exclude such interference. For example, if a certain pesticide is known to interfere with the compound of interest on one of the liquid phases, another liquid phase may be chosen for the separation.

In this study, the analyses were made with the flame photometric detector of Brody and Chaney (1), which responds to either P or S compounds. A dual version of the detector monitors both P and S compounds simultaneously (2) and can be used to determine the ratio of P and S atoms in a molecule. Because the detector responds with high specificity to these compounds (3), little or no cleanup of extracts is normally required for analysis. As little as a fraction of a nanogram (ng) P compound and several ng S compound can be detected. The detector was used in isothermal and temperature-programmed analyses; the latter allow extracts containing multicomponent residues of pesticides with a wide range of retention times to be analyzed with a single injection of sample (4).

References to earlier multicomponent and temperature-programmed analyses were cited as part of a previous study of 20 P compounds (4) and will therefore not be repeated. More recently Watts and Storherr (5) have presented data for a large number of organophosphorus pesticides on 3 columns using a thermionic detector, and Wright and Riner (6) have listed relative retention times for some of these compounds obtained using Dow Corning 11 substrate and a flame ionization detector. A general multicomponent method for the determination of organophosphorus pesticide residues in water has been described (7).

METHOD

Apparatus and Reagents

(a) *Gas chromatographic equipment and operating conditions.*—A Hewlett-Packard Model 5750 instrument equipped with the flame photometric detector (Tracor, Inc., Austin, Texas) operated in either the phosphorus (526 nm filter) or sulfur (394 nm filter) mode was used with 240 cm glass columns (4 mm id, 6 mm od) packed with 5% (w/w) of the following liquid phases on 80–100 mesh Gas Chrom Q (Applied Science Laboratories, State College, Pa.): OV-101 (dimethyl silicone), OV-17 (phenyl methyl silicone-50% phenyl), OV-210 (trifluoropropyl silicone), and OV-225 (cyanopropyl phenyl silicone). Condition columns overnight at 320°C before use. Set flow rates of gases in ml/min as follows: nitrogen (carrier) 160, oxygen 40, hydrogen 200. Maintain injection port temperature at 225°C except in iso-

thermal analyses when it is held 20°C above oven temperature. Hold detector and transfer line from column to detector at 280°C. (Detector was equipped with a water cooling coil to avoid overheating photomultiplier tube (8).)

Isothermal analyses.—The oven was operated isothermally at several temperatures between 150 and 300°C to obtain convenient ratios of t_R compound to t_R parathion, the t_R standard in this study.

Temperature-programmed analyses.—Let oven come to temperature at 150°C, inject sample, immediately program oven temperature upward at 10°C/min for 15 min, and then hold it at 300°C until last peak emerges.

(b) *Solvents.*—Acetone and benzene (chemically pure redistilled solvents) and absolute methanol (use as received).

(c) *Pesticide standards.*—Most of the compounds were analytical grade supplied by A. R. Glasgow, Jr. and J. R. May of the Food and Drug Administration (Washington, D.C. and Perrine, Fla., respectively) and by the manufacturers. Except for the chemicals listed in Table 1, the test compounds are identified in references given in Table 2; metabolites (phenols, oxygen analogs or oxons, sulfoxides, and sulfones) are also identified in the references in Table 2 or are obviously the stated derivative of the parent compound.

(d) *Liquid phases.*—OV-101, OV-17, OV-210, and OV-225 (Supelco, Inc., Bellefonte, Pa.)

GLC Procedure

Five μl solution containing the compound was injected into the gas chromatograph operated isothermally or with temperature programming.

Pesticides were injected in benzene solution except for a few that were incompletely soluble, in which case acetone was added to attain complete dissolution. Chemosterilants were injected in methanol. Each compound was evaluated on each column both isothermally and with temperature programming. If a compound failed to produce a peak, several injections of 5 μg compound were made.

Results

Table 2 lists the test compounds alphabetically and gives the retention time of each relative to that of parathion on the 4 columns in isothermal and temperature-programmed analyses. The last entries are the actual retention times of parathion at 200°C and in the temperature-programmed runs; these values may be used to estimate the retention times of the individual compounds (discussed later). Retention times of parathion were readily reproduced (e.g., within 0.1–0.2 min). The P and S content of each compound is included to indicate which detector (P or S or both) will respond. When more than one significant peak for a compound appeared, the t_R of each peak is given. Footnote *d* in Table 2 shows when several injections of 5 μg amounts of compound did not give a significant peak under the conditions used.

Figures 1–4 are typical chromatograms obtained with 25 ng amounts of compound (except when otherwise noted) in temperature-programmed analyses with the detector in the phosphorus mode.

Table 1. Chemical identification of pesticide names not referenced in Table 2

Pesticide	Chemical Identification
Amidithion	O,O-dimethyl phosphorodithioate S-ester with 2-mercapto-N-(2-methoxyethyl)acetamide
Bay 37342	O,O-dimethyl O-[4-(methylthio)-3,5-xylyl] phosphorothioate
Chipman RP-11783	O,O-dimethyl phosphorodithioate S-ester with 3-(mercaptomethyl)-2-benzoxazolinone
Ciba C-7019	2-azido-4-(isopropylamino)-6-(methylthio)-*s*-triazine
Ciba C-8874	O-(2,5-dichloro-4-iodophenyl) O,O-diethyl phosphorothioate
Ciba C-14421	2,4-dinitrophenyl dipropyldithiocarbamate
DEF®	S,S,S-tributyl phosphorotrithioate
Difolatan®	N-[(1,1,2,2-tetrachloroethyl)thio]-4-cyclohexene-1,2-dicarboximide
Geigy G-28029	S-[[(2,5-dichlorophenyl)thio]methyl] O,O-diethyl phosphorodithioate
Merphos	S,S,S-tributyl phosphorotrithioite
Phosfon®	tributyl(2,4-dichlorobenzyl)phosphonium chloride
Plantvax®	5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide 4,4-dioxide
Shell SD-8280	2-chloro-1-(2,4-dichlorophenyl)vinyl dimethyl phosphate
Shell SD-8436	2-chloro-1-(2,4-dibromophenyl)vinyl dimethyl phosphate
Shell SD-8448	2-chloro-1-(2,4,5-trichlorophenyl)vinyl diethyl phosphate
Union Carbide UC-8305	cyclic O,O-(1,2-cyclohexyleneethylidene) phosphorochloridothioate
Vitavax®	5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide

Table 2. Relative retention times of P and S compounds on 4 columns operated isothermally and with temperature programming (t_R parathion = 1.00)[a]

Number	Compound[b]	PS in molecule	Ratio of t_R Compound to t_R Parathion (1.00) for Conditions Indicated: OV-101 Isoth	OV-101 Progr	OV-17 Isoth	OV-17 Progr	OV-210 Isoth	OV-210 Progr	OV-225 Isoth	OV-225 Progr	Ref.[c]
1	Abate® (9)	P_2S_3	20.9	2.67	29.6	3.06	12.5	2.43	[d]	[d]	8
2	Accothion® (9)	PS	0.84	0.93	0.98	1.00	0.86	0.93	0.98	1.00	10
3	Accothion O-analog	P	0.69	0.81	0.81	0.91	1.18	1.08	1.01	1.00	10
4	Aldicarb (9)	S	0.03	0.07	0.03	0.06	0.03	0.07	0.02	0.06	11
5	Aldicarb sulfoxide	S	0.05	0.11	0.06	0.12	0.06	0.14	0.06	0.18	11
6	Aldicarb sulfone	S	0.14	0.27	0.08	0.17	0.11	0.24	0.10	0.27	11
7	Amidithion	PS_2	0.90	0.95	1.15	1.04	1.03	1.01	1.48	1.15	
8	Apholate (12)	P_3	4.60	1.83	6.94	1.83	2.14	1.41	[d]	[d]	13
9	Azinphosethyl (9)	PS_2	4.77	1.85	7.11	1.85	3.74	1.75	4.93	1.72	
10	Azinphosmethyl (9)	PS_2	4.00	1.75	6.39	1.79	3.52	1.70	[d]	[d]	4
11	Azodrin® (9)	P	0.37	0.55	0.52	0.73	0.67	0.82	0.84	0.95	14
12	Bay 30911	PS	0.47	0.65	0.48	0.67	0.23	0.42	0.30	0.61	4
13	Bay 37289 (9)	PS	1.16	1.08	0.93	0.96	0.48	0.68	0.56	0.80	
14	Bay 37342	PS_2	1.04	1.01	1.17	1.05	0.53	0.73	0.78	0.92	
15	Bay 77488 (9)	PS	1.43	1.19	1.74	1.22	1.90	1.33	[d]	[d]	
16	Bidrin® (9)	P	0.37	0.55	0.48	0.67	0.64	0.81	0.51	0.78	14
17	Captan (12)	S	1.16	1.08	1.56	1.18	1.07	1.02	1.59	1.17	
18	Carbophenothion (9)	PS_3	2.28	1.48	2.64	1.41	1.18	1.08	1.84	1.22	4
19	Carbophenothion O-analog	PS_2	1.85	1.35	2.23	1.33	1.44	1.18	1.82	1.21	
20	Chipman RP-11783	PS_2	2.10	1.42	3.25	1.49	2.37	1.45	3.18	1.45	
21	Chlorbenside (9)	S	1.34	1.15	1.38	1.13	0.51	0.71	0.89	0.96	
22	2-Chlorovinyl diethyl phosphate	P	0.08	0.18	0.08	0.17	0.08	0.18	0.06	0.18	
23	Chlorthion® (12)	PS	1.02	1.00	1.16	1.05	1.08	1.03	1.25	1.08	
24	Ciba C-2307 (9)	P	0.34	0.53	0.42	0.64	0.50	0.69	0.40	0.70	
25	Ciba C-7019	S	0.58	0.74	0.58	0.77	0.36	0.53	0.45	0.73	
26	Ciba C-8874	PS	1.97	1.39	2.05	1.30	0.87	0.93	1.26	1.08	
27	Ciba C-9491 (9)	PS	1.57	1.25	1.80	1.23	0.74	0.86	1.20	1.06	15
28	Ciba C-9491 O-analog	P	1.32	1.15	1.58	1.18	1.04	1.01	1.30	1.09	15
29	Ciba C-14421	S_2	2.14	1.45	1.17	1.05	1.33	1.14	1.31	1.09	
30	Ciodrin® (9)	P	1.27	1.14	1.44	1.16	1.33	1.14	1.23	1.07	
31	Compound 4072 (9)	P	1.25	1.13	1.29	1.10	0.94	0.98	0.97	1.00	16
32	Coumaphos (9)	PS	6.02	1.97	7.88	1.88	7.66	2.10	6.62	1.81	17
33	Coumaphos O-analog	P	5.07	1.90	6.91	1.83	9.85	2.29	7.02	1.86	17
34	Dasanit® (9)	PS_2	1.92	1.36	2.77	1.43	2.84	1.56	2.91	1.42	
35	Dasanit O-analog	PS	1.59	1.27	2.39	1.36	3.63	1.72	3.03	1.43	
36	Dazomet (18)	S_2	0.40	0.58	0.69	0.83	0.63	0.80	[d]	[d]	
37	DEF®	PS_3	1.64	1.32	1.54	1.16	0.78	0.89	0.87	0.95	
38	Demeton (9)	PS_2	0.31	0.48	0.29	0.50	0.16	0.31			
			0.43	0.62	0.43	0.67	0.38	0.55	0.31	0.63	
39	Diazinon (9)	PS	0.57	0.73	0.50	0.71	0.24	0.41	0.26	0.58	19
40	Diazoxon	P	0.54	0.69	0.49	0.70	0.42	0.60	0.31	0.63	19
41	Dicapthon (9)	PS	1.04	1.01	1.10	1.03	0.95	0.98	1.11	1.03	
42	Dichlorvos (9)	P	0.08	0.17	0.08	0.18	0.08	0.17	0.06	0.21	20
43	Difolatan®	S	2.58	1.53	3.92	1.55	2.18	1.42	[d]	[d]	
44	Dimethoate (9)	PS_2	0.43	0.61	0.61	0.78	0.52	0.72	0.88	0.96	21
45	Dioxathion (9)	P_2S_4	0.14	0.23	0.12	0.23	0.04	0.16	0.06	0.20	
			0.49	0.67	0.57	0.76	0.21	0.51	0.42	0.71	
			6.10	2.01			3.32	1.67			
46	Disulfoton (9)	PS_3	0.59	0.75	0.55	0.74	0.30	0.47	0.34	0.66	22
47	Disulfoton sulfoxide	PS_3	1.38	1.18	1.86	1.25	2.15	1.42	2.43	1.36	22
48	Disulfoton sulfone	PS_3	1.38	1.18	1.87	1.25	2.23	1.43	2.43	1.36	22
49	Disulfoton O-analog	PS_2	0.46	0.63	0.47	0.66	0.39	0.55	0.33	0.65	22
50	Disulfoton O-analog sulfoxide	PS_2	1.06	1.02	[d]	[d]	[d]	[d]	[d]	[d]	22
51	Disulfoton O-analog sulfone	PS_2	1.04	1.01	1.48	1.16	2.41	1.46	[d]	[d]	22
52	Dition® (9)	PS	10.9	2.34	14.6	2.23	11.6	2.40	11.2	2.16	
53	Dursban® (9)	PS	0.98	1.00	0.94	0.98	0.45	0.65	0.58	0.82	23
54	Dursban O-analog	P	0.94	0.97	0.97	1.00	0.88	0.95	0.79	0.93	23
55	Dyfonate® (9)	PS_2	0.55	0.72	0.56	0.75	0.28	0.46	0.34	0.66	

(Continued)

Table 2. (*Continued*)

Number	Compound[b]	PS in molecule	Ratio of t_R Compound to t_R Parathion (1.00) for Conditions Indicated: OV-101 Isoth	OV-101 Progr	OV-17 Isoth	OV-17 Progr	OV-210 Isoth	OV-210 Progr	OV-225 Isoth	OV-225 Progr	Ref.[c]
56	Dyfonate O-analog	PS	0.42	0.60	0.46	0.65	0.38	0.54	0.32	0.64	
57	Endosulfan I (12)	S	1.52	1.22	1.43	1.14	0.68	0.83	0.87	0.95	
58	Endosulfan II	S	1.85	1.35	2.21	1.33	1.23	1.09	1.93	1.24	
59	Endosulfan sulfate	S	2.30	1.48	3.03	1.45	2.63	1.50	3.20	1.46	
60	EPN (9)	PS	3.43	1.66	4.18	1.59	2.93	1.58	3.21	1.46	4
61	Ethion (9)	P_2S_4	2.05	1.41	2.42	1.36	1.27	1.12	1.65	1.19	4
62	EXD (18)	S_4	0.46	0.62	0.49	0.69	0.20	0.37	0.25	0.57	
63	Famphur (9)	PS_2	2.20	1.46	3.33	1.50	3.83	1.75	3.60	1.55	
64	Fenson (9)	S	0.95	0.98	1.15	1.04	0.82	0.91	1.00	1.00	
65	Fenthion (9)	PS_2	1.00	1.00	1.08	1.02	0.52	0.72	0.79	0.93	24
66	Fenthion sulfoxide	PS_2	1.92	1.36	3.12	1.47	3.00	1.60	3.06	1.44	24
67	Fenthion sulfone	PS_2	1.94	1.36	3.14	1.47	3.29	1.66	3.42	1.50	24
68	Fenthion O-analog	PS	0.80	0.89	0.95	0.99	0.78	0.88	0.85	0.95	24
69	Fenthion O-analog sulfoxide	PS	1.60	1.27	2.71	1.42	3.87	1.76	3.16	1.45	24
70	Fenthion O-analog sulfone	PS	1.60	1.27	2.68	1.42	4.26	1.80	3.55	1.54	24
71	Folpet (12)	S	1.24	1.12	1.66	1.20	0.99	1.00	1.44	1.14	
72	Gardona® (9)	P	1.49	1.21	1.62	1.19	1.12	1.05	1.29	1.09	16
73	Geigy G-28029	PS_3	3.64	1.69	4.11	1.58	1.76	1.27	2.50	1.37	
74	Genite 923® (9)	S	1.47	1.20	1.77	1.23	1.23	1.09	1.49	1.15	
75	Hempa (9)	P	0.10	0.23	0.11	0.22	0.16	0.31	0.09	0.25	13
76	Imidan® (9)	PS_2	3.30	1.64	4.97	1.68	3.01	1.60	4.17	1.60	25
77	Imidoxon	PS	2.44	1.51	4.19	1.59	3.54	1.70	[d]	[d]	25
78	Lethane A-70® (12)	S_2	0.36	0.55	0.59	0.77	0.75	0.87	1.06	1.02	
79	Lethane 384® (9)	S	0.27	0.40	0.20	0.41	0.14	0.30	0.17	0.41	
80	Malathion (9)	PS_2	0.94	0.98	0.93	0.97	0.75	0.87	0.78	0.92	19
81	Malaoxon	PS	0.74	0.85	0.77	0.88	0.95	0.99	0.78	0.92	19
82	Menaxon (9)	PS_2	3.28	1.63	5.84	1.75	2.05	1.39	[d]	[d]	
83	Merphos	PS_3	1.25	1.13	0.93	0.97	0.33	0.51	0.36	0.68	
			1.64	1.29	1.54	1.17	0.77	0.88	0.86	0.95	
84	Mesurol®, phenol (9)	S	0.28	0.42	0.24	0.44	0.09	0.21	0.26	0.58	26
85	Mesurol sulfoxide	S	0.73	0.85	1.02	1.00	0.92	0.96	[d]	[d]	26
86	Mesurol sulfone	S	0.80	0.90	1.14	1.04	1.07	1.02	[d]	[d]	26
87	Metepa (9)	P	0.27	0.41	0.24	0.44	0.27	0.44	0.24	0.54	13
88	Methiotepa (12)	PS	0.28	0.43	0.23	0.43	0.13	0.28	0.16	0.39	13
89	Methyl parathion (9)	PS	0.74	0.85	0.84	0.93	0.79	0.90	0.92	0.97	4
90	Methyl Trithion® (9)	PS_3	1.88	1.36	2.40	1.36	1.00	1.00	1.80	1.21	4
91	Mevinphos (9)	P	0.15	0.29	0.17	0.34	0.18	0.34	0.15	0.38	
92	Mobam®, phenol (27)	S	0.26	0.38	0.26	0.45	0.11	0.23	0.34	0.66	28
93	Molinate (18)	S	0.27	0.42	0.23	0.43	0.10	0.22	0.13	0.35	
94	Morestan® (9)	S_2	1.40	1.18	1.60	1.18	0.61	0.78	1.05	1.01	
95	Naled (9)	P	0.35	0.55	0.39	0.61	0.25	0.43	0.25	0.57	
96	Nemacide® (12)	PS	0.72	0.84	0.65	0.80	0.38	0.54	0.37	0.69	
97	Ovex (9)	S	1.50	1.21	1.82	1.24	1.34	1.15	1.59	1.17	
98	Oxydemetonmethyl sulfone (12)	PS_2	0.77	0.88	1.23	1.08	2.00	1.38	[d]	[d]	22
99	Parathion (9)	PS	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	4
100	Paraoxon	P	0.81	0.90	0.87	0.95	1.31	1.14	1.02	1.00	
101	Pebulate (18)	S	0.24	0.36	0.16	0.28	0.08	0.17	0.07	0.21	
102	Phenothiazine	S	1.18	1.08	1.53	1.17	0.70	0.84	1.74	1.20	
103	Phorate (9)	PS_3	0.41	0.60	0.36	0.60	0.19	0.35	0.24	0.53	29
104	Phorate sulfoxide	PS_3	0.91	0.96	1.17	1.05	1.15	1.05	1.52	1.16	29
105	Phorate sulfone	PS_3	0.93	0.97	1.17	1.05	1.33	1.14	1.52	1.16	29
106	Phorate O-analog	PS_2	0.33	0.50	0.31	0.54	0.25	0.43	0.23	0.51	29
107	Phorate O-analog sulfoxide	PS_2	0.71	0.83	0.93	0.97	1.43	1.17	1.41	1.14	29
108	Phorate O-analog sulfone	PS_2	0.71	0.83	0.93	0.97	1.46	1.18	1.42	1.14	29
109	Phosalone (9)	PS_2	4.07	1.77	4.94	1.68	3.64	1.72	3.95	1.58	
110	Phosfon® (9)	P	0.58	0.75	0.37	0.60	0.64	0.81	0.32	0.64	
111	Phosphamidon (9)	P	0.75	0.85	0.78	0.89	1.26	1.12	0.92	0.97	

(*Continued*)

Table 2. *(Continued)*

Number	Compound[b]	PS in molecule	Ratio of t_R Compound to t_R Parathion (1.00) for Conditions Indicated: OV-101 Isoth	OV-101 Progr	OV-17 Isoth	OV-17 Progr	OV-210 Isoth	OV-210 Progr	OV-225 Isoth	OV-225 Progr	Ref.[c]
112	Pirazinon® (12)	PS	0.32	0.50	0.32	0.56	0.35	0.52	0.25	0.57	
113	Plantvax®	S	2.64	1.55	4.71	1.65	[d]	[d]	[d]	[d]	
114	Potasan® (12)	PS	3.94	1.73	5.23	1.70	5.58	1.98	4.79	1.70	17
115	Prometryne (18)	S	0.80	0.89	0.78	0.90	0.40	0.57	0.53	0.79	
116	Propyl thiophrophosphate (9)	P_2S_2	0.98	1.00	0.79	0.90	0.48	0.68	0.49	0.77	
117	Ronnel (9)	PS	0.83	0.93	0.77	0.88	0.42	0.60	0.48	0.76	
118	Ruelene® (9)	P	1.05	1.02	1.14	1.04	1.01	1.00	1.09	1.03	4
119	Schradan (12)	P_2	0.56	0.70	0.53	0.73	0.16	0.31	0.57	0.81	
120	Shell SD-8280	P	0.98	1.00	1.14	1.04	0.78	0.89	0.92	0.97	
121	Shell SD-8436	P	1.57	1.24	2.00	1.29	1.21	1.09	1.62	1.18	
122	Shell SD-8448	P	1.70	1.33	1.85	1.25	1.31	1.14	1.33	1.11	
123	Stauffer N-2788	PS_2	0.76	0.86	0.74	0.86	0.40	0.57	0.46	0.75	4
124	Sulfoxide (9)	S	0.14	0.24	0.07	0.13	0.05	0.12	0.02	0.06	
125	Sulphenone® (12)	S	1.23	1.11	1.60	1.19	1.38	1.16	1.69	1.19	
126	Tepa (12)	P	0.21	0.33	0.27	0.46	0.24	0.40	0.26	0.58	13
127	Tepp (9)	P_2	0.05	0.12	0.05	0.12	0.06	0.14	0.04	0.12	
128	Tetrachlorothiophene	S	0.09	0.21	0.08	0.17	0.04	0.09	0.04	0.12	
129	Tetradifon (9)	S	3.90	1.73	4.75	1.66	3.25	1.65	3.38	1.49	
130	Thanite® (9)	S	0.66	0.78	0.60	0.78	0.58	0.76	0.56	0.79	
131	Thiotepa	PS	0.28	0.41	0.28	0.48	0.14	0.29	0.21	0.48	13
132	Thiram (12)	S_4	0.30	0.47	0.43	0.64	0.38	0.54	0.43	0.72	
133	Union Carbide UC-8305	PS	0.54	0.68	0.64	0.79	0.50	0.70	0.67	0.87	
134	Velsicol VCS-506	PS	4.27	1.79	4.76	1.66	1.86	1.32	2.79	1.40	30
135	Velsicol VCS-506 O-analog	P	3.38	1.66	4.18	1.59	2.10	1.40	2.75	1.39	30
136	Vitavax®	S	1.66	1.33	2.20	1.33	1.24	1.10	2.16	1.31	
137	Zinophos® (9)	PS	0.30	0.48	0.31	0.54	0.17	0.33	0.22	0.51	4
138	Zytron® (9)	PS	0.94	0.97	0.86	0.94	0.49	0.68	0.67	0.88	
	Parathion – t_R (min) isothermally, 200°C		4.75		7.25		3.65		10.3		
	temp. programmed			7.50		10.3		7.10		10.8	

[a] P = phosphorus, S = sulfur, t_R = retention time, isoth = isothermal, progr = temperature programmed. Gas holdup (time for nonadsorbed substance to pass from injection port to detector) was not subtracted in determining relative retention times. Gas holdup was 0.30 min in temperature-programmed analyses. Temperature programming: started at 150°C, 10°/min increase for 15 min, held at 300°C until last peak emerged.

[b] Compounds are identified in reference given beside name, in last column, or in Table 1.

[c] Literature reference to quantitative procedure with flame photometric detector.

[d] No peak appeared despite several 5 μg injections of compound.

Discussion

The retention times in Table 2 may be used to identify the listed compounds in samples of unknown history, especially when retention times on several columns coincide, but it should not be inferred that the conditions set forth here are optimum for quantitative analysis. Many P and S compounds—especially the more polar ones—are irreversibly adsorbed by GLC packings or are decomposed at the high temperatures used in GLC. Good quantitative data for many of these polar compounds have been obtained (3) by using a nonadsorptive support, performing the GLC at as low a temperature as possible and as quickly as possible, and conditioning the column packing just before use by repeated injections of relatively large amounts of the polar compound in a control extract (if available). Thus, short glass columns (30–60 cm) with nonpolar packings (5–10% liquid phase by weight) or with lightly loaded polar ones (1–2% liquid phase by weight) are first conditioned in the usual way (20–30°C above operating temperature) and then by repeated injections of μg amounts of compound in the crop or other extract. Periodically, 5 ng P compound or 25 ng S compound are injected; the response to these trial injections grows until it becomes constant, at which time quantitative analyses may begin. The more polar the compound, the more conditioning is required to secure the constant response.

Since the short columns (30–60 cm) needed for quantitative analysis do not provide sufficient resolution to identify the many compounds of this qualitative study, 240 cm long columns were used. As an aid to the analyst who has obtained evidence of the presence of compounds, references to available quantitative procedures for determining the compounds with the flame photometric detector are given in Table 2. Many of these procedures utilize packings with the DC-200 liquid phase. The use of OV-101 in place of DC-200 (both are methyl silicones) may be a worthwhile change in these analyses. The order of elution of 20 phosphorus compounds (4) was the same on the 2 column packings. Similar substitutions for the other packings may also be worthwhile, e.g., the combination of DC-200 and QF-1 suggested by others (31) might be replaced by a mixture of phases OV-101 and OV-210, which have similar compositions but lower bleed rates and would be

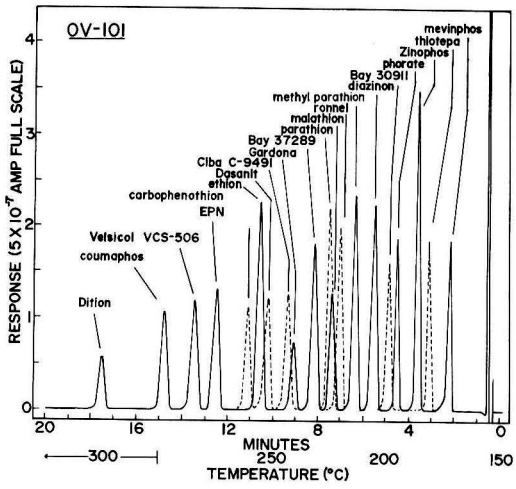

FIG. 1—Composite chromatograms of 25 ng pesticides and metabolites (exception, 10 ng thiotepa) from temperature-programmed analyses on OV-101 with the phosphorus flame photometric detector.

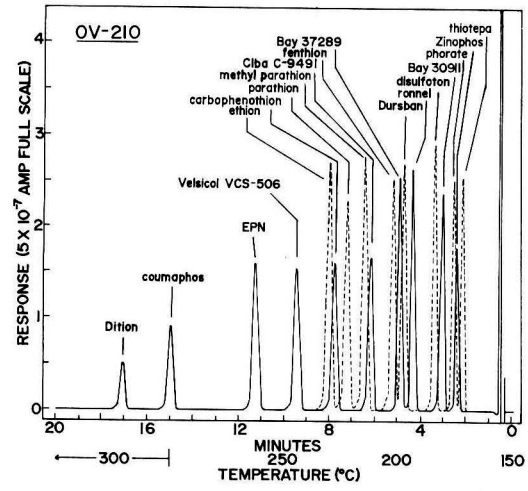

FIG. 3—Composite chromatograms of 25 ng pesticides and metabolites (exception, 10 ng each thiotepa and Zinophos) from temperature-programmed analyses on OV-210 with the phosphorus flame photometric detector.

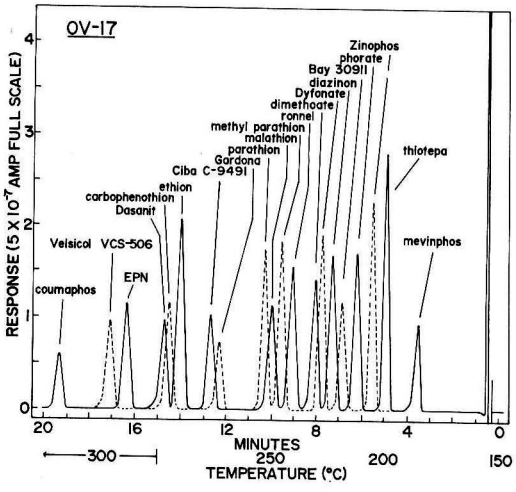

FIG. 2—Composite chromatograms of 25 ng pesticides and metabolites from temperature-programmed analyses on OV-17 with the phosphorus flame photometric detector.

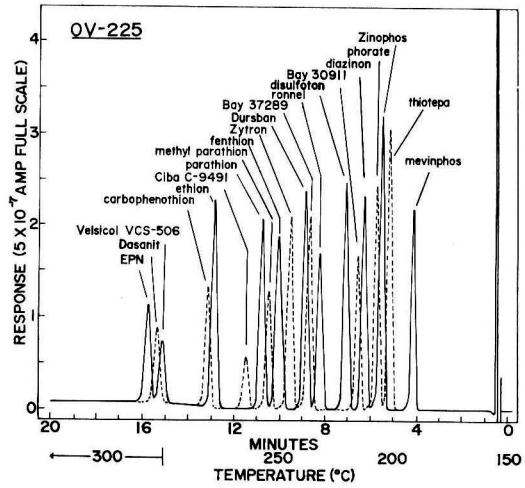

FIG. 4—Composite chromatograms of 25 ng pesticides and metabolites from temperature-programmed analyses on OV-225 with the phosphorus flame photometric detector.

potentially useful for high temperature operation with the ^{63}Ni electron capture detector. (The order of elution of the same 20 phosphorus compounds was generally similar on QF-1 and OV-210.)

Although relative t_R data are given for both isothermal and temperature-programmed analyses, the latter data are more reliable and reproducible for several reasons: The temperature-programmed analysis subjects the compounds to a single procedure, while the isothermal analyses had to be conducted at different temperatures to elute the compounds of interest in a reasonable time. In the isothermal analyses, it was not possible to secure direct t_R relationships between parathion and those compounds having comparatively long or short retention times. Accordingly, compounds with intermediate retention times for which the relative t_R with respect to parathion was known (e.g., Zinophos, tetrachlorothiophene, carbophenothion, Imidan) were used as reference compounds to estimate the relative retention times of compounds with retention times much longer or shorter than that of parathion. Since relative retention times of compounds vary at different temperatures (32, 33), especially with polar columns, the estimation of retention times relative to parathion via an intermediate compound cannot be considered highly reproducible. Identifications should therefore be checked by comparing the t_R of the unknown and known on several columns operated under a given set of conditions or by using some other procedure.

For convenience in locating compounds with similar retention times, the compounds of Table 2 (identified by their numbers) are listed in Table 3 in the order of their elution from each column in the temperature-programmed analysis.

Several injections of 5 μg amounts of compound were required to obtain a peak for some compounds. The need for these injections normally diminishes as the column is conditioned to substrates and pesticides, but with a few compounds no useful peak was obtained on some of the columns despite these repeated injections (typically, a tailing peak with humps on the baseline appeared), and no peak was obtained on any column with oxydemetonmethyl (a P=O compound with a sulfoxide group) or with the carbamates Mesurol (and its sulfoxide and sulfone) and Mobam. Hydrolysis and/or decomposition in these instances was virtually complete under our conditions of analysis. This result was not unexpected, since reports that organophosphates, aryl carbamates, and sulfoxides decompose in passage through gas chromatographic packings at elevated temperatures are not uncommon (22, 28, 29, 34, 35). In any event, Mesurol and Mobam will not be detected with the present procedure, and other methods will have to be used for their detection (22, 26). (Although the GLC of the aforementioned carbamates was not successful, the retention times of their phenolic derivatives were readily obtained.) Also, analyses for the following compounds were definitely substandard: captan, Ciba C-14421, folpet, Lethane A-70, dazomet, all sulfoxides and especially their oxygen analogs (P=O), and oxydemetonmethyl sulfone.

No effort was made to determine whether the emerging compounds were identical with those injected, and there is little doubt that some of the compounds were decomposed in the GLC system, even though a single peak was obtained, e.g., aldicarb and the synergist sulfoxide (compound 124) are known to decompose (11, 34, 36). However, the data show the expected results and, despite the decomposition, the t_R of the peaks still serve to identify the injected compounds.

The OV-101 and OV-17 liquid phases are rated for use at temperatures as high as 350°C and the OV-210 and OV-225 at above 275°C. (One supplier lists 350°C as maximum temperature for the latter 2 phases.) The OV-210 and OV-225 phases did bleed at temperatures exceeding 275°C and, after long periods of use (e.g., programming to 300°C and holding), the glass window in the detector became coated (reducing sensitivity) and had to be replaced. Although replacement of the window is easily made, the use of temperatures above 275°C with the OV-210 and OV-225 phases probably should be held to a minimum to prolong column life.

The OV phases are especially attractive for use in GLC-mass spectrometer systems, which are becoming increasingly available. The mass spectrometer alone is not suitable for analyzing a pesticide in a crop extract because it responds to all ingredients of the extract. However, after an extract is cleaned up and passed through a gas chromatograph, the GLC effluent may be free enough of impurities to allow positive identification of the pesticide residues to be made with the mass spectrometer (37). Less than certain identification may also be made by observing several

Table 3. Order of elution and relative retention times of P and S compounds in temperature-programmed analyses

OV-101		OV-17		OV-210		OV-225	
Rel t_R	Compd[a]	Rel t_R	Compd[a]	Rel t_R	Compd[a]	Rel t_R	Compd[a]
0.07	4	0.06	4	0.07	4	0.06	4,124
0.11	5	0.12	5,127	0.09	128	0.12	127,128
0.12	127	0.13	124	0.12	124	0.18	5,22
0.17	42	0.17	128,22,6	0.14	5,127	0.20	45
0.18	22	0.18	42	0.16	45	0.21	42,101
0.21	128	0.22	75	0.17	42,101	0.25	75
0.23	45,75	0.23	45	0.18	22	0.27	6
0.24	124	0.28	101	0.21	84	0.35	93
0.27	6	0.34	91	0.22	93	0.38	91
0.29	91	0.41	79	0.23	92	0.39	88
0.33	126	0.43	93,88	0.24	6	0.41	79
0.36	101	0.44	84,87	0.28	88	0.48	131
0.38	92	0.45	92	0.29	131	0.51	106,137
0.40	79	0.46	126	0.30	79	0.53	103
0.41	87,131	0.48	131	0.31	38,75,119	0.54	87
0.42	84,93	0.50	38	0.33	137	0.57	62,95,112
0.43	88	0.54	106,137	0.34	91	0.58	39,84,126
0.47	132	0.55	55	0.35	103	0.61	12
0.48	38,137	0.56	112	0.37	62	0.63	38,40
0.50	106,112	0.60	110,103	0.40	126	0.64	56,110
0.53	24	0.61	95	0.41	39	0.65	49
0.55	11,16,78,95	0.64	24,132	0.42	12	0.66	46,55,92
0.58	36	0.65	56	0.43	95,106	0.68	83
0.60	56,103	0.66	49	0.44	87	0.69	96
0.61	44	0.67	12,16,38	0.46	55	0.70	24
0.62	38,62	0.69	62	0.47	46	0.71	45
0.63	49	0.70	40	0.51	45,83	0.72	132
0.65	12	0.71	39	0.52	112	0.73	25
0.67	45	0.73	11,119	0.53	25	0.75	123
0.68	133	0.74	46	0.54	56,96,132	0.76	117
0.69	40	0.75	55	0.55	38,49	0.77	116
0.70	119	0.76	45	0.57	115,123	0.78	16
0.72	55	0.77	25,78	0.60	40,117	0.79	115,130
0.73	39	0.78	44,130	0.65	53	0.80	13
0.74	25	0.79	133	0.68	13,116,138	0.81	119
0.75	46,110	0.80	96	0.69	24	0.82	53
0.78	130	0.83	36	0.70	133	0.87	133
0.81	3	0.86	123	0.71	21	0.88	138
0.83	107,108	0.88	81,117	0.72	44,65	0.92	14,80,81
0.84	96	0.89	111	0.73	14	0.93	54,65
0.85	81,85,89,111	0.90	115,116	0.76	130	0.95	11,37,57,68,83
0.86	123	0.91	3	0.78	94	0.96	21,44
0.88	98	0.93	89	0.80	36	0.97	89,111,120
0.89	68,115	0.94	138	0.81	16,110	1.00	2,3,31,64, 99,100
0.90	86,100	0.95	100	0.82	11	1.01	94
0.93	2,117	0.96	13	0.83	57	1.02	78
0.95	7	0.97	80,83,107, 108	0.84	102	1.03	41,118
0.96	104	0.98	53	0.86	27	1.06	27
0.97	54,105,138	0.99	68	0.87	78,80	1.07	30
0.98	64,80	1.00	2,54,85,99	0.88	68,83	1.08	23,26
1.00	23,53,65, 99,116,120	1.02	65	0.89	37,120	1.09	28,29,72

(Continued)

Table 3. *(Continued)*

OV-101		OV-17		OV-210		OV-225	
Rel t_R	Compd[a]	Rel t_R	Compd[a]	Rel t_R	Compd[a]	Rel t_R	Compd[a]
1.01	14,41,51	1.03	41	0.90	89	1.11	122
1.02	50,118	1.04	7,64,86, 118,120	0.91	64	1.14	71,107,108
1.08	13,17,102	1.05	14,23,29, 105,104	0.93	2,26	1.15	7,74
1.11	125	1.08	98	0.95	54	1.16	104,105
1.12	71	1.10	31	0.96	85	1.17	17,97
1.13	31,83	1.13	21	0.98	31,41	1.18	121
1.14	30	1.14	57	0.99	81	1.19	61,125
1.15	21,28	1.16	30,37,51	1.00	71,90,99, 118	1.20	102
1.18	47,48,94	1.17	83,102	1.01	7,28	1.21	19,90
1.19	15	1.18	17,28,94	1.02	17,86	1.22	18
1.20	74	1.19	72,125	1.03	23	1.24	58
1.21	72,97	1.20	71	1.05	72,104	1.31	136
1.22	57	1.22	15	1.08	3,18	1.36	47,48
1.24	121	1.23	27,74	1.09	58,74,121	1.37	73
1.25	27	1.24	97	1.10	136	1.39	135
1.27	35,69,70	1.25	47,48,122	1.12	61,111	1.40	134
1.29	83	1.29	121	1.14	122,29,30, 100,105	1.42	34
1.32	37	1.30	26	1.15	97	1.43	35
1.33	122,136	1.33	19,58,136	1.16	125	1.44	66
1.35	19,58	1.36	35,61,90	1.17	107	1.45	20,69
1.36	34,66,67,90	1.41	18	1.18	19,108	1.46	59,60
1.39	26	1.42	69,70	1.27	73	1.49	129
1.41	61	1.43	34	1.32	134	1.50	67
1.42	20	1.45	59	1.33	15	1.54	70
1.45	29	1.47	66,67	1.38	98	1.55	63
1.46	63	1.49	20	1.39	82	1.58	109
1.48	18,59	1.50	63	1.40	135	1.60	76
1.51	77	1.55	43	1.41	8	1.70	114
1.53	43	1.58	73	1.42	43,47	1.72	9
1.55	113	1.59	60,77,135	1.43	48	1.81	32
1.63	82	1.65	113	1.45	20	1.86	33
1.64	76	1.66	129,134	1.46	51	2.16	52
1.66	60,135	1.68	76,109	1.50	59		
1.69	73	1.70	114	1.56	34		
1.73	114,129	1.75	82	1.58	60		
1.75	10	1.79	10	1.60	66,76		
1.77	109	1.83	8,33	1.65	129		
1.79	134	1.85	9	1.66	67		
1.83	8	1.88	32	1.67	45		
1.85	9	2.23	52	1.70	10,77		
1.90	33	3.06	1	1.72	35,109		
1.97	32			1.75	9,63		
2.01	45			1.76	69		
2.34	52			1.80	70		
2.67	1			1.85	9		
				1.98	114		
				2.10	32		
				2.29	33		
				2.40	52		
				2.43	1		

[a] See Table 2 for identification.

of the strongest peaks in the spectrum of a pesticide. In this regard, only those compounds with similar retention times would be expected to interfere (*see* Table 3). Since the low bleeding rate of the OV liquid phases minimizes interference and avoids contaminating the spectrometer, these phases are apt to be preferred in GLC-mass spectral systems; the retention times in Table 2 can be useful to those employing these systems.

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Investigation on Determination and Confirmation of Dyrene Added to Plant Extracts: GLC and TLC of Dyrene and Products of Its Reaction in Methanolic Sodium Hydroxide

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As determined by electron capture GLC, recoveries of Dyrene from strawberries, potatoes, tomatoes, and cucumber samples, using an acetonitrile extraction-hexane partition procedure, were more than 80%. TLC was used for qualitative confirmation. The possible interference in the GLC and TLC analyses from selected pesticides was investigated. Treatment with methanolic sodium hydroxide converted Dyrene to 2 major products with longer GLC retention times and slower TLC migration rates than the parent compound. Dyrene and the products were visualized on thin layer plates by using a silver nitrate spray reagent or by viewing induced fluorescence under UV light. The products were also detected by an enzyme inhibition procedure.

Determination of Dyrene (2,4-dichloro-6-(*o*-chloroaniline)-*s*-triazine) has been based on detection of its reaction products: potentiometric titration of chlorine released from the cyanuric chloride ring (1), diazotization of *o*-chloroaniline produced by acid hydrolysis (2), and formation of pyridine condensation products by the Zincke reaction (3). These methods are tedious and subject to interference from hydrolyzable chlorine, some organochlorine and organophosphorus pesticides, and *s*-triazine herbicides. Recently, the detection of Dyrene and Dyrene derivatives, by gas-liquid and thin layer chromatography (GLC and TLC) has been reported (4, 5). GLC and TLC facilitate the separation and identification of compounds which might be present with Dyrene, thus making the procedures rapid and specific for the determination of Dyrene.

This paper reports evaluation of a modified acetonitrile extraction-hexane partition procedure (6) for the recovery of Dyrene added to blended samples of strawberry, tomato, cucumber, and potato. GLC with electron capture (EC) detection was used to quantitate Dyrene in the extracts without further cleanup. For confirmation, Dyrene in the presence of the plant extractives was reacted with methanolic sodium hydroxide before analysis by TLC or GLC.

This report of the former Associate Referee, P. J. Wales, was presented at the 83rd Annual Meeting of the AOAC, Oct. 13–16, 1969, at Washington, D.C.

METHOD

Apparatus and Reagents

(a) *Gas chromatograph.*—Aerograph Hy-Fi Model 550, equipped with Wilkins electrometer Model 500, tritium foil electron capture detector, and 4.5′ × ¼″ id coiled Pyrex glass column. Column packings (*see* Table 1), were prepared according to procedure of Mendoza *et al.* (7); column temperatures used are listed in Table 1. Other conditions are as follows: nitrogen flow rate 50 ml/min, oven temperature 205–215°C, inlet temperature 190°C, and detector temperature 190°C. Attenuator setting at 4 gave ½ FSD for 5 ng Dyrene after column was loaded. Columns 1 and 2 (Table 1) were used for quantitative analyses of Dyrene in plant extracts.

(b) *Thin layer plates.*—20 × 20 cm; coat plates with 250 μm thick layer of MN kieselgel G-HR (activated 1 hr at 110°C) and store them over desiccant.

(c) *Phosphate buffer pH 6.*—Mix 5.6 ml 0.1*N* NaOH with 50 ml 0.1*M* KH_2PO_4.

(d) *Silver nitrate solutions (8)*—*(1) Stock solution.*—Dissolve 20 g $AgNO_3$ (reagent grade) in 100 ml distilled water and store in brown bottle in dark. *(2) Spray solution.*—Mix 4 ml stock solution, 100 ml acetone, 10 ml distilled water, 6 ml concentrated NH_4OH, and 8 drops 2-phenoxyethanol; store solutions in brown bottle in dark and discard if precipitate forms.

(e) *Methanolic sodium hydroxide.*—0.01 or 0.02*N* NaOH in methanol.

(f) *Solvents.*—Glass-distilled acetone, acetonitrile, benzene, and hexane.

Sample Extraction

Chop 500 g sample and divide into 100 g subsamples. Fortify subsamples (treated) with appropriate standard solution of Dyrene (99.6% pure, obtainable from Chemagro Corp., Kansas City, Mo.). Blend subsample 5 min with 200 ml acetonitrile in Waring Blendor with Variac setting 50V. Filter homogenate under vacuum through coarse

Table 1. GLC column packing materials evaluated for Dyrene determination as 4.5′ column at 195°C

No.	Stationary Phase mg/g Support	Name	Support, 80–100 Mesh
1	40	SE-30	Chromosorb W
	60	QF-1	
2	20	OV-17	
	40	SE-30	Chromosorb W
	60	QF-1	
3	40	SE-30	Gas Chrom Q
	60	QF-1	
4	40	SE-30	Gas Chrom P
	60	QF-1	
5	40	DC-200	Chromosorb W
	60	QF-1	
6	40	SE-30	Chromosorb W
7	60	QF-1	Gas Chrom P
8	100	DC-200	Gas Chrom Q
	150	QF-1	
9	5	DEGS	Chromosorb W

porosity sintered glass funnel. Blend residue with 150 ml acetonitrile 5 min and filter homogenate as before. Combine filtrates and make to 500 ml with acetonitrile.

Transfer 125 ml acetonitrile extract to 1 L separatory funnel. Add 10 ml reagent grade dichloromethane, 20 ml 0.1*M* pH 6 phosphate buffer, and 200 ml hexane; shake mixture vigorously 1 min. Add 500 ml water and 50 ml saturated Na_2SO_4 solution, shake mixture vigorously 2 min, and let layers separate completely. Discard lower acetonitrile-water layer. Carefully wash hexane portion twice, with 100 ml water each time, by gently rocking solution to minimize emulsion formation; discard water portions. Make hexane fraction to 250 ml, i.e., 100 mg/ml plant equivalent.

Reaction in Methanolic Sodium Hydroxide

For GLC, mix 0.5 ml 0.02*N* methanolic NaOH with 1 ml hexane extract with or without Dyrene 1 min with test tube vibrator. Analyze upper hexane layer and compare it with control solution.

For TLC, pipet aliquots of Dyrene standard or hexane extract with or without Dyrene into duplicate 15 ml vials and evaporate to dryness under stream of nitrogen in 35°C water bath. Thoroughly dissolve residue in 1 vial with 100 or 200 μl 0.01 or 0.02*N* NaOH in methanol; stopper vial. Just before spotting, add equal volume of acetone to second (control) vial. (Dyrene reacted with methanol to form Dyrene II (monomethoxy Dyrene) and Dyrene III (dimethoxy Dyrene), Mendoza *et al.* (5).) Spot appropriate volumes of treated and control extracts, using 5 μl disposable micropipets (Drummond Scientific Co., Broomall, Pa.).

TLC Analysis

Develop plates either in 10 ml benzene made to 100 ml with hexane for separation of organochlorine compounds or in 20 ml acetone made to 100 ml with hexane for separation of Dyrene and its reaction products. For visualization, either spray plates with $AgNO_3$ solution and expose to UV light (5) 1–2 min to produce black spots or expose plates to UV light ≥15 min to induce compounds to fluoresce. Observe plates under UV viewer (obtainable from Fisher Scientific Co., Ltd.).

Results and Discussion

Standardization of GLC Response

Among various column packing materials tried for GLC analysis of Dyrene (Table 1), 4% SE-30 and 6% QF-1 on Chromosorb W (Column No. 1) gave the most symmetrical peak. However, at 195°C column temperature, the response increased with repeated injections of the same amount of Dyrene. Conditioning the column with 1–2 μg Dyrene produced a nonlinear increase in the GLC response. Quantitative response was consistently obtained only at 208–212°C. Packing material 2 (Table 1) with the same initial response as material 1 retained sensitivity throughout the day, without additional conditioning. Both columns were used in the quantitative work reported here.

Before each quantitative analysis of Dyrene in the plant extracts, the column was conditioned with 1–2 μg Dyrene and the linearity of the GLC response was checked by injecting 1–10 ng Dyrene. Solvent flush technique gave accurate and reproducible injections (1–2 μl hexane, 1 μl air, and the sample aliquot were drawn into the syringe with the exact volume of the aliquot being noted before injection).

Chromosorb W (HMDS) coated with 5% diethylene glycol succinate (DEGS) was tried but was not used for quantitation of Dyrene in the plant extracts. At 185°C with a nitrogen flow rate of 60 ml/min for 60–80 mesh or 150 ml/min for 80–100 mesh, a 2′ column gave reproducible responses for repeated Dyrene injections. The log-log plot of peak height vs. ng injected was linear from 2 to 20 ng and was nonlinear above 20 ng. This column, however, was not satisfactory for simultaneous analysis of Dyrene and Dyrene-methanol products.

Evaluation of Extraction Procedure by GLC

Extractions of 100 g fortified subsamples in triplicate were replicated on different days. Before extractions, some subsamples were fortified with 1 ml appropriate Dyrene standard in acetone to simulate levels of 20 ppm in strawberry, 10 ppm in tomato and potato, 5 ppm in cucumber, and 1 ppm in potato. As controls, fractions from the unfortified subsamples in hexane were fortified with standard solutions. Equal volumes of samples and control solutions were analyzed alternately by GLC using column material 1 or 2 (Table 1); the percentage recoveries were calculated against the average of the 2 adjacent control peaks.

Recoveries were more than 80% for strawberry fortified at 20 ppm, potato and tomato at 10 ppm, and cucumber at 5 ppm, with a standard error of the mean ranging from 1.0 to 3.6% (Table 2). Comparison of GLC analyses for strawberry extracts immediately and 1 day after extraction showed only a 4% difference, confirming the stability of extracted Dyrene in the final hexane solution.

At attenuation 2, only 57% mean recovery was obtained by analyzing 15 μl injections of the hexane extracts of potato fortified at 1 ppm; the GLC background noise interfered with quantitation. When the extract was dried and redissolved in hexane to obtain more concentrated solutions appropriate for analysis at attenuation 4, no Dyrene was detected.

To account for this loss, extracts were fortified with Dyrene at amounts simulating its presence in the plant material at 1, 2, and 10 ppm. Aliquots (1, 5, and 10 ml) fortified with equal amounts of Dyrene were evaporated and left dry overnight. The residues were redissolved in hexane the next day and compared with residues redissolved in hexane immediately after drying. Dyrene left overnight with the dry potato residues dropped to 52% at a 10 ppm level and was lost completely at 1 ppm, while the Dyrene standard with and without the dry residues of strawberry, tomato, and cucumber showed comparatively small losses (8–22%), even at the 1 ppm level. Heat-labile enzymes were eliminated as a major degradative factor because heating the dry potato residues 1 hr at 100°C before addition of Dyrene did not prevent the loss. Burchfield and Storrs (9) attributed loss of Dyrene during extraction of plant samples to reaction with compounds containing amino, sulfhydryl, and other nucleophilic groups and observed that losses increased as the pH increased.

Table 2. Recoveries of Dyrene from fortified plant samples as analyzed by electron capture GLC (5–10 μl injections at attenuation 4)

Sample	Dyrene Added, ppm	Extraction Replication	% Recovery[a] x	% Recovery[a] $s_{\bar{x}}$
Strawberry	20	A	83.7	2.2
		B	92.0[b]	2.2
Tomato	10	A	87.7	3.4
		B	86.8	1.8
Potato	10	A	85.7	2.8
		B	86.8	3.6
Cucumber	5	A	89.7	1.8
		B	83.5	1.0
Potato	1[c]	A	57.5	1.2

[a] Mean of 3 simultaneous extractions; x = mean and $s_{\bar{x}}$ = standard error of the mean.
[b] GLC analysis of B solutions next day gave 87.9±2.1%.
[c] 15 μl injections at attenuation 2.

Sensitivity to Detection on TLC

The limit of detection was 0.1 μg Dyrene with the silver nitrate spray and 1 μg with induced fluorescence by 15 min UV exposure. Dyrene, Dyrene II, and Dyrene III, each equivalent to 5 μg original Dyrene, were detected by the silver nitrate spray and by induction of fluorescence under UV. After treatment of 10–80 μg Dyrene with 200 μl 0.01*N* NaOH in methanol for 30 min, Dyrene II, corresponding to 1, 2, and 9 μg original Dyrene, was detected as very faint, faint, and definite spots, respectively. The intensity of the Dyrene II spot decreased after 60 min reaction. With 0.02*N* NaOH in methanol, the Dyrene II produced was detected as a very faint spot after 30 min but not after 60 min. Under the conditions just mentioned, Dyrene III equivalent to more than 1 μg original Dyrene showed as a definite spot. To ensure complete conversion of Dyrene to Dyrene III, reaction with 0.02*N* NaOH in methanol for 1 hr is, therefore, recommended. If Dyrene II is to be detected, an aliquot equivalent to 4 μg or more original Dyrene must be spotted after reaction with 0.01*N* NaOH in methanol for 30 min.

TLC Analysis of Plant Extracts

Evaporation of the hexane extracts was necessary to give sufficient concentrations of Dyrene

Table 3. GLC retention times relative to Dyrene (R_D) and sensitivities to electron capture detection for some pesticides separated on Chromosorb W coated with SE-30 + QF-1 or with DEGS

Compound	R_D 4% SE-30+6% QF-1	R_D 5% DEGS	ng for ½ FSD[a]
Atrazine	0.4 (b,c)[b]	0.4 (d)	350
Propazine	0.4 (b,c)	0.4 (d)	350
Simazine	0.4 (b,c)	0.5 (d)	700
Aldrin	0.7 (a,b,c)	0.1 (d)	0.6
Heptachlor epoxide	1.0 (a,b,c)	0.3 (d)	0.6
Dyrene	1.0 (a,b,c)	1.0 (d,e)	5
Malathion	1.1 (a,b)	0.5 (d,e)	10
Parathion	1.3 (b)	0.6 (d,e)	10
p,p'-DDE	1.3 (a,c)	0.4 (d)	1
Dyrene II	1.4 (a)	—	—
Captan	1.5 (a,c)	1.1 (d,e)	2
Dieldrin	1.6 (a,b)	0.5 (d)	1
Dyrene III	1.8 (a)	—	—
o,p'-DDT	1.8 (a,b)	0.4 (d)	3
p,p'-DDT	2.4 (a,b)	1.0 (d)	3

[a] Full scale deflection.
[b] See Table 4 for description of columns and conditions.

for TLC analysis. As GLC analysis had shown loss of Dyrene in the presence of dried potato extracts, TLC analysis of potato was omitted.

Extracts of fortified and blank strawberry, tomato, and cucumber samples were evaporated to dryness and redissolved in acetone for detection of Dyrene or in 0.02*N* NaOH for formation and detection of Dyrene III. Volumes equivalent to 1 μg Dyrene/spot were analyzed.

Plant pigments that were resolved on the plates did not interfere with spots produced by Dyrene or Dyrene III, even with cucumber green pigments that remained after spraying with silver nitrate.

Possible Interferences

Table 3 shows the GLC retention times and detectable amounts of selected pesticides separated by Chromosorb W coated with SE-30 and QF-1 or with diethylene glycol succinate (DEGS). The GLC parameters for the columns are given in Table 4. Retention times were calculated relative to Dyrene instead of aldrin, because aldrin was eluted very quickly from the DEGS column. With both columns, Dyrene was completely separated from and was detectable at much lower amounts than atrazine, propazine, and simazine. These 3 herbicides were insoluble in hexane and were not expected to be recovered from plant samples by the extraction procedure used. The

Table 4. GLC columns and conditions for Dyrene analysis

Column	Length, ft	Mesh	Flow Rate, ml/min	Oven Temp., °C	Dyrene Elution Time, min
a	4.5	80–100	110	191	3.2
b	4.5	80–100	50	191	5.1
c	4.5	80–100	50	212	2.5
d	2.0	80–100	150	185	3.6
e	2.0	60–80	60	185	3.6

other pesticides listed in Table 3 could be extracted if present in plant samples and could be detected as readily as Dyrene. Captan, *p,p'*-DDT, and Dyrene had the same retention times on the DEGS column but different ones on the SE-30/QF-1 column; heptachlor epoxide and malathion coincided with Dyrene on the SE-30/QF-1 column but were separated on the DEGS column. Treatment with methanolic sodium hydroxide destroyed malathion and captan and converted Dyrene to Dyrene II and III (5).

Figure 1 illustrates TLC separation for Dyrene, captan, and some organochlorine pesticides in 2 solvent systems. With 10% benzene in hexane, the organochlorine compounds were distinctly separated, while captan and Dyrene remained at the origin. With 20% acetone in hexane, Dyrene and captan separated completely at the middle of the plate, while the organochlorine compounds moved to just behind the solvent front.

Although malathion also moved to the same

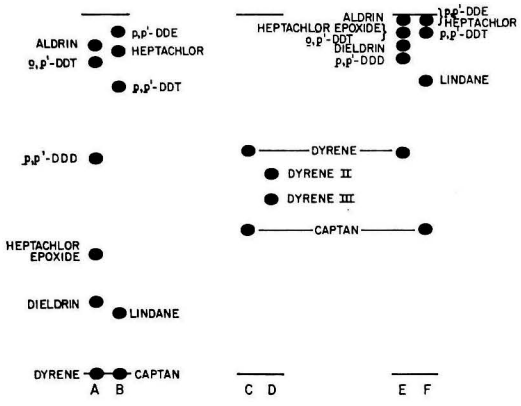

FIG.1—Typical TLC separation of Dyrene, captan, and 9 organochlorine pesticides with 10% benzene in hexane (A, B) and with 20% acetone in hexane (C, D, E, and F). D is a mixture of Dyrene and captan reacted with methanolic sodium hydroxide before spotting.

position as Dyrene after resolution with 20% acetone in hexane, it did not interfere with the silver nitrate detection of Dyrene. Only malathion was detected by the TLC-enzyme inhibition technique after exposure to bromine (10), because Dyrene was not an enzyme inhibitor. Mendoza, Wales, and Hatina (5) demonstrated inhibition of liver esterases by Dyrene II and III; the latter was detectable at amounts equivalent to 0.1 μg original Dyrene. Dyrene II and III that had been detected by silver nitrate can then be detected again by the TLC-enzyme inhibition technique after exposure of the plate to the bromine vapor. The inhibitor produced by exposing malathion to bromine had a migration rate similar to that of Dyrene III. However, the methanolic sodium hydroxide treatment used to produce Dyrene III destroyed malathion.

Recommendations

For quantitation of Dyrene by GLC, the column containing SE-30/QF-1 on Chromosorb W must be conditioned before analysis and must be used at approximately 212°C column temperature.

It is also recommended that 5% DEGS on Chromosorb W (60–80 mesh) packed as a 2′ column be evaluated for quantitative determination of Dyrene in extracts of plant samples. If, in the presence of plant extracts, Dyrene elutes quantitatively from this DEGS column as observed with the standard alone, a collaborative study should be undertaken to evaluate the extraction and determination reported here for Dyrene.

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The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee E and were accepted by the Association. See *JAOAC* **53**, 393 (1970).

Thin Layer Chromatographic Separation and Chromogenic Detection of Diazinon and Some of Its Known or Suspected Metabolites and/or Degradation Products

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Diazinon and 5 of its known or suspected transformation products were chromatographed on thin layer sheets and thin layer silica gel plates, using a variety of solvents. Ten chromogenic sprays with sensitivity ranges from 0.05 to 1 μg were used to identify the migrated compounds on the thin layer chromatogram. Two-dimensional TLC separated all the compounds in a single procedure. Several crops fortified at levels of 0.5 to 2.0 ppm were successfully analyzed for these compounds.

The continuing necessity to determine the ultimate fate of pesticides added to our environment requires methods which will separate and detect not only the original compound, but its degradation or metabolic products as well.

Diazinon is of major concern because it is used extensively for soil and foliar applications to protect a wide variety of crops. Various investigators have studied the fate of diazinon in soil and plants. The disappearance rate of diazinon in soil was determined by Bro-Rasmussen *et al.* (1). Gunner *et al.* (2) studied the distribution and persistence of diazinon-C^{14} when applied to plants and soil and its influence on soil microflora. These studies showed that diazinon is absorbed and translocated in plants. In determining the metabolism of radioactive diazinon in soil, Getzin and Rosefield (3) isolated an unidentified compound in addition to diazinon.

In further studies, Getzin (4) found 2-isopropyl-4-methyl-6-pyrimidinol 3 weeks after soil was treated with radioactive diazinon. J. G. Konrad *et al.* (5) confirmed the formation of this product and proposed possible hydrolysis pathways of diazinon. Studying the metabolites formed after spraying diazinon-S^{35} on spinach, tomato, and bean plants, Ralls *et al.* (6) found the same pyrimidinol compound and small amounts of diazinon. Kansouh and Hopkins (7) studied the root absorption, translocation, and metabolism of diazinon-C^{14} in bean plants. They found diazinon, the pyrimidinol compound, a small amount of $C^{14}O_2$, and unidentified radioactive metabolites.

Since thin layer chromatography (TLC) is a relatively simple and rapid technique for the separation and identification of small amounts of compounds, it has been widely used in initial investigations of pesticide residues. Numerous papers have been published on the TLC analysis of organophosphorus compounds. Walker and Beroza (8), El-Refai and Hopkins (9), Bunyan (10), and Getz and Wheeler (11) developed TLC methods for the analysis of mixtures of organophosphorus insecticides. Kovacs (12) adapted a tetrabromophenolphthalein ethyl ester-silver nitrate-citric acid spray reagent system for the visualization of organic thiophosphate residues in various crops. Watts (13) and Ragab (14) employed 4-(*p*-nitrobenzyl)pyridine spray reagent in their TLC studies of thio- and nonthioorganophosphorus compounds. Diazinon had been included in some of these studies but the suspected degradation products were not, since these studies pertained primarily to organic phosphorus insecticides. The investigation of an important compound such as diazinon requires a system which will separate and detect the parent compound and its breakdown products. This paper details the use of a variety of solvent systems and spray reagents to detect diazinon and 5 of its known or possible transformation products.

Experimental

Apparatus and Reagents

(a) *Diazinon and transformation products.*—(*1*) Diazinon: O,O-diethyl O-(2-isopropyl-4-methyl-6-pyrimidinyl) phosphorothioate; (*2*) diazoxon: diethyl (2-isopropyl-4-methyl-6-pyrimidinyl) phosphate; (*3*) dithiono TEPP (sulfotepp): O,O,O′,O′-tetraethyl dithionopyrophosphate; (*4*) monothiono TEPP: O,O,O′,O′-tetraethyl monothionopyrophos-

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phate; (*5*) pyrimidinol: 2-isopropyl-4-methyl-6-pyrimidinol; (*6*) pyrimidinethiol: 2-isopropyl-4-methyl-6-pyrimidinethiol. The common names are used throughout the text.

(b) *Extraction apparatus.*—Lourdes Volu-Mix homogenizer with pint mason jars (Lourdes Instrument Corp., Brooklyn, N.Y.); flash evaporator (Buchler Instruments, Fort Lee, N.J.).

(c) *Thin layer equipment.*—Automatic plate leveller and spreader (Reeve Angel, Quickfit, Inc., Fairfield, N.J.); 20 × 20 cm glass plates; developing tanks, lined with filter paper; sample streaker (Applied Science Laboratories, State College, Pa.); disposable micropipets, 1 μl (Drummond Scientific Co., Broomall, Pa.).

(d) *Adsorbent layers.* — Eastman Chromatographic Sheets 6061 Silica Gel (Rochester, N.Y.); SilicAR® TLC-7G (Mallinckrodt, New York, N.Y.). To prepare 5 glass plates (20 × 20 cm) for (*1*) 0.25 mm thickness, spread slurry of 30 g silica gel + 60 ml distilled H_2O; (*2*) 1.0 mm thickness, spread slurry of 100 g silica gel + 160 ml water. Air-dry 1 hr, dry in oven at 130° 1 hr, cool, and store in desiccator.

(e) *Ultraviolet light sources.*—Uvi-Arc Model 421-Ul (George W. Gates and Co., Franklin Square, N.Y.); Mineralight long wave UV-B 50 (Ultraviolet Products, Inc., South Pasadena, Calif.).

(f) *Solvents.*—Acetonitrile, ethyl acetate, acetone, hexane, methanol, chloroform, carbon tetrachloride, ether, benzene, all nanograde or equivalent.

(g) *Chromogenic reagents.*—(*1*) *Congo red-bromine* (15): 200 mg congo red in 100 ml ethanol + 30 ml water. Spray plate lightly, air-dry, and expose to bromine vapor until spots appear. (*2*) *Palladium chloride* (16): 0.5% solution of palladium chloride in 1*N* HCl. (*3*) *Ninhydrin* (17): Mix 95 ml 0.2% ninhydrin in butanol with 5 ml 10% aqueous acetic acid. Spray plate and heat at 120°C 10–15 min. (*4*) *Silver nitrate* (18): Mix solution of 1.7 g $AgNO_3$ in 5 ml water with 20 ml 2-phenoxyethanol and dilute to 200 ml with acetone. Add 1 drop 30% H_2O_2. Spray plate and activate under UV light until brown spots appear. (*5*) *Ferric chloride–potassium ferrocyanide* (19): Mix 10 ml 5% aqueous $K_4Fe(CN)_6$, 20 ml 10% aqueous $FeCl_3$, and 80 ml water. (*6*) *4-(p-Nitrobenzyl) pyridine* (14): (*a*) 2% 4-(*p*-nitrobenzyl)-pyridine in acetone; (*b*) 20% $(NH_4)_2CO_3.H_2O$ in water (dilute 1:1 with acetone before use); (*c*) 10% NaOH in ethanol-water (1 + 1). Spray plate heavily with solution (*a*), lightly with solution (*b*), heat in oven at 130°C 10 min, cool, and spray with solution (*c*). (*7*) *Bromine-ferric chloride–2-(o-hydroxyphenyl) benzoxazole* (15): (*a*) Dissolve 0.1 g $FeCl_3.6H_2O$ in 1 ml 1*N* HCl and dilute to 100 ml with 80% (v/v) ethyl alcohol; (*b*) 0.05% 2-(*o*-hydroxyphenyl) benzoxazole in 2% (v/v) dimethyl sulfoxide in methyl ethyl ketone. Expose plate to bromine vapor until spots appear, spray lightly with ferric chloride solution, dry, and spray with solution (*b*). Examine plate under longwave UV light source. (*8*) *Tetrabromophenolphthalein ethyl ester–silver nitrate–citric acid* (12): (*a*) 0.2% tetrabromophenolphthalein ethyl ester in acetone; (*b*) dissolve 0.5 g $AgNO_3$ in 25 ml water and dilute to 100 ml with acetone; (*c*) dissolve 5 g citric acid in 50 ml water and dilute to 100 ml with acetone. Spray plate moderately heavily with solution (*a*), spray lightly with solution (*b*) and, after 2 min, spray with solution (*c*). (*9*) *Brilliant green–bromine* (20): 5% brilliant green in acetone. Spray plate with solution and expose to bromine vapor until spots appear.

Procedure

Extract crop samples using acetonitrile extraction method of Storherr *et al.* (21). Spot chromatogram with standard mixture and aliquots of crop extract containing 0.1–5 μg pesticide. If all compounds in an extract are to be identified on 1 plate, use 2-dimensional TLC system. Develop to solvent front of 10 cm in tank lined with filter paper. Air-dry chromatogram and use suitable reagent for detection.

For preliminary cleanup of highly concentrated samples and isolation of products, streak 15 cm across a 1 mm preparative thin layer chromatogram. Spot standard mixture in unstreaked area. Develop plate. Apply chromogenic reagent as directed to portion of plate on which standard was spotted; protect other portion of adsorbent during visualization procedure. Scrape sample areas corresponding to standards and elute spots from silica gel with acetone. Concentrate extract with flash evaporator.

Results and Discussion

Diazinon and 5 of its possible metabolites were separated by TLC on silica gel G-coated glass plates and on Eastman sheets, using a variety of solvents (Table 1). The classic eluotropic series of solvents, developed by Trappe (22), was applied to the TLC analysis. Solvents ranging from nonpolar to very polar and mixtures of these solvents were employed. Fresh solvent systems should be used for each experiment because evaporation can change the concentrations in mixed solvent systems. Small changes in composition can result in different R_f values. For the most part, the best separations with the least amount of tailing of spots was observed on the silica gel G glass plates; *see* Table 1A. There was no movement with nonpolar solvent systems.

Table 1. TLC R_f values of diazinon and some of its possible metabolites or degradation products on silica gel G

Solvent System	Diazinon	Diazoxon	Dithiono TEPP	Monothiono TEPP	Pyrim-idinol	Pyrim-idinethiol
A. Silica Gel G Glass Plates						
Hexane	0[a]	0	0	0	0	0
Carbon tetrachloride	0	0	0.12	0	0	0
Toluene	0.08	0	0.70	0	0	0
Chloroform	0.69	sf[b]	0.89	0.34	0.02	0.21
Ethyl ether	sf	0.88	sf	sf	sf	sf
Ethyl acetate	sf	sf	sf	0.17	0.30	sf
Acetone	sf	0.75	sf	sf	sf	sf
Ethyl acetate-hexane (1 + 3)	0.50	0.45	0.77	0.77	0.03	0.60
Ether-hexane (1 + 3)	0.40	0	0.56	0.05	0	0.25
Acetone-carbon tetrachloride (1 + 4)	0.70	0.05	0.70	0.56	0.13	0.71
B. Silica Gel Sheets						
Hexane	0	0	0	0	0	0
Carbon tetrachloride	0	0	0.27	0	0	0
Toluene	0.18	0	0.74	0.02	0	0.04
Benzene	0	0	0	0.60	0.60	0
Chloroform	0.94	sf	sf	0.89	0.15	0.82
Ethyl ether	sf	sf	sf	0.92	0.55	0.47
Ethyl acetate	sf	sf	sf	sf	sf	sf
Methanol	0.84	0.84	0.86	0.85	0.81	0.85
Ethyl acetate-hexane (1 + 3)	0.55	0.48	0.90	0.90	0.10	0.65
Ethyl acetate-hexane (1 + 1)	0.70	0.68	0.80	0.40	0	0
Acetone-hexane (1 + 4)	0.93	0.88	0.95	0.95	0.15	0.52
Acetone-carbon tetrachloride (3 + 7)	0.92	0.80	0.82	0	0.28	0.82
Ether-hexane (1 + 3)	0.65	0.50	0.81	0.80	0	0.30
Carbon tetrachloride-benzene-acetone (1 + 1 + 1)	0.95	0.90	0.95	0.90	0.95	0.95
Acetone-ether-carbon tetra-chloride (1 + 1 + 18)	0.75	0.23	0.79	0.33	0.07	0.36

[a] 0—compound did not migrate.
[b] sf—compound migrated with solvent front.

The R_f values increased as the polarity of the solvent system increased. No single solvent system clearly separated all the components. It was necessary to use 2 plates to determine all the compounds in the mixture; however, the mixture was completely separated on 1 plate by a 2-dimensional TLC system. Ether-hexane (1 + 3) was used as the first solvent system and acetone-chloroform (1 + 4) as the second solvent system.

By using preparative TLC silica gel plates, the compounds were isolated and their presence was confirmed by IR analysis. In addition, the separated compounds may be determined quantitatively by other methods, such as gas-liquid chromatography.

Crop samples of lettuce, blueberry, and potato were fortified to contain 0.5, 1.0, and 2.0 ppm of diazinon and each of its metabolites. The crop extracts were developed (Table 1A) and sprayed with chromogenic reagents (Table 2). Very little interference was observed at these levels of fortification.

When greater sensitivity is needed, the Eastman thin layer sheets are recommended (Table 1B). Because of the thinness of the layer (100 μm), smaller samples can be analyzed. In addition, for radioactive studies these sheets can be conveniently and directly analyzed in paper radiochromatographic scanner equipment (23), whereas the fragility of the silica gel layers and the thickness of the glass plates require special handling and special attachments (24).

The response of diazinon and metabolites to various spray reagents was analyzed (Table 2). These reagents included general sprays, which detect all types of compounds, and specific sprays, which detect functional groups and the type of compound.

Table 2. Response of diazinon and some of its possible metabolites or degradation products (0.1 μg of each spotted) to various chromogenic reagents

Reagent[a]	Diazinon	Diazoxon	Dithiono TEPP	Monothiono TEPP	Pyrimidinol	Pyrimidinethiol
1: Congo red-bromine	blue	blue	dark blue	dark blue	faint blue	blue
2: 0.5% Palladium chloride in HCl	yellow, turns brown	white	yellow, turns dark brown	faint yellow	faint white	yellow, turns brown
3: Ninhydrin, heat 10 min at 120°C	white	white	deep purple	faint white	negative	faint white
4: Silver nitrate, UV light	light brown	light brown	brown	brown	brown	brown
5: Ferric chloride-potassium ferrocyanide	blue	blue	dark blue	dark blue	faint blue	dark blue
6: 4-(*p*-Nitrobenzyl)pyridine, ammonium carbonate, heat 10 min at 130°C, sodium hydroxide in ethanol	deep blue-purple	deep blue-purple	light blue	light blue	negative	light blue
7: Bromine, ferric chloride, o-(hydroxyphenyl)benzoxazole, UV light	blue	negative	blue	blue	negative	blue
8: Tetrabromophenolphthalein ethyl ester	faint yellow	bright yellow	negative	bright yellow	negative	blue with bright yellow center
Silver nitrate	bright blue	yellow	yellow with blue center	yellow	blue	blue
Citric acid	bright blue	yellow	yellow with blue center	bright yellow	fades	blue
9: Brilliant green-bromine	yellow	bright green	yellow	yellow	blue-green	yellow-green

[a] *See* (g) under Apparatus and Reagents for complete descriptions.

Iodine vapor, a universal reagent, yielded brown spots for all the compounds. Since iodine produces brown spots for most organic compounds, interferences from concentrated crop materials may obscure the organophosphorus compounds.

Congo red spray and exposure to bromine vapor produced blue spots against a pink background for all compounds studied.

Palladium chloride reagent, which formed a light brown background, yielded a faint yellow spot for monothiono TEPP and white spots for diazoxon and pyrimidinol. Diazinon, dithiono TEPP, and pyrimidinethiol appeared as yellow spots which turned brown on standing.

When sprayed with ninhydrin reagent and heated, diazinon and diazoxon appeared as white spots against a light purple background. Monothiono TEPP and pyrimidinethiol formed faint white spots. The compound most sensitive to the reagent was dithiono TEPP, which appeared as a deep purple spot. Pyrimidinol did not react.

Silver nitrate spray reagent and exposure to ultraviolet light produced brown spots for all the compounds.

Ferric chloride-potassium ferrocyanide spray reagent has been used to detect aromatic amines (25) and phenolic compounds (26). All the compounds appeared as blue spots against a yellowish green background; however, dithiono TEPP, monothiono TEPP, and pyrimidinethiol formed darker blue spots than the remaining compounds.

The 4-(*p*-nitrobenzyl)pyridine spray reagent did not detect pyrimidinol. The remaining compounds appeared as blue spots, with the exceptions of diazinon and diazoxon, which appeared as bluish purple spots. Pyrimidinol was visualized by overspraying with congo red which produced red spots for all compounds against a pink background.

The ferric chloride-benzoxazole spray reagent has been reported to be relatively specific for thiophosphorus compounds (15). Since oxygen analogs and nonthiophosphorus compounds do not respond, this reagent can distinguish between diazinon and diazoxon. Thus, diazoxon and pyrimidinol, which do not contain any sulfur, were not visualized. The remaining sulfur-containing compounds appeared under long wave UV light as a fluorescent blue against a dark blue background.

The Kovacs' (12) spray combination of tetra-

bromophenolphthalein ethyl ester, silver nitrate, and citric acid detected all the compounds. The dye spray produced yellow spots against a blue background for all the compounds except monothiono TEPP and pyrimidinol. After the silver nitrate spray, blue spots formed for diazinon, pyrimidinol, and pyrimidinethiol. Diazoxon and monothiono TEPP were yellow spots. The dithiono TEPP appeared as a yellow spot with a blue center. Overspraying with citric acid reagent produced more intense spots against a bluish yellow background for all compounds except the hydroxy pyrimidine which faded.

Brilliant green spray reagent has been used to detect organophosphorus compounds (20) and triazine herbicides (27). The reagent produced a bright green spot for diazoxon, a blue-green spot for pyridinol, and a yellow-green spot for pyrimidinethiol. Diazinon, dithiono TEPP, and monothiono TEPP appeared as yellow spots against an off-white background.

The sensitivity for the various spray reagents ranged from 0.05 to 1 μg. The lower limit of detectability may vary according to the technique used.

Conclusion

This thin layer chromatographic procedure can be used as a rapid method to isolate and identify diazinon and 5 of its possible metabolites from crop materials.

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Rapid Colorimetric Method for Estimation of Thiram Residues on Grains

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A rapid and relatively simple method has been developed for the colorimetric determination of thiram residues on grains. Cuprous chloride is used to form a yellow complex with thiram, which is measured at 420 nm. The method is satisfactory for analyses of 4–200 μg thiram.

Since thiram (tetramethylthiuram disulfide) has been increasingly used as an antifungal agent in seeds and during storage of moist grains, a rapid method for routine analysis was needed for estimation of thiram residues on grains. Lowen (1) used an electrometric method for estimating thiram present as an impurity in ferbam by reducing thiram with sodium sulfide. A paper chromatographic method, using 5% formamide-chloroform mixture as the solvent system, has also been reported (2). The commonly employed method (3) consists of hydrolysis of thiram with boiling dilute sulfuric acid (1.1*N*) and absorption of carbon disulfide, the product of hydrolysis, in the Viles reagent; a yellow complex suitable for photometric measurement at 430 nm is formed. Heuermann (4) has also reported a similar method in which dimethylamine is trapped in a solution of copper salt containing carbon disulfide; the color developed is measured at 435 nm. Hine's method (5) is based on the characteristic UV absorption of thiram at 250–285 nm. Essentially, the colorimetric method described by Keppel (6) consists of mixing cuprous chloride with thiram solution in chloroform (4–42 μg thiram/ml) to form a yellow cuprous complex. The absorbance of the filtrate (420 nm) should be read within 15 min after filtration.

The procedure described below is an improvement over the method of Keppel (6) because it is simpler, more rapid, and quantitative.

METHOD

Reagents

(a) *Thiram solutions.*—Prepare solutions containing 4–100 μg/ml chloroform.

(b) *Cuprous chloride reagent (0.1%).*—Dissolve 100 mg chemically pure cuprous chloride in 20 ml 0.1*N* HCl; dilute clear solution to 100 ml with 95% ethanol. Prepare fresh before use.

Preparation of Standard Curve

Pipet 0.1–2.0 ml aliquots of chloroform solution of thiram into separate clean dry test tubes. Pipet 2 ml freshly prepared cuprous chloride reagent into each test tube. (The solutions should turn yellow immediately.) Using 95% alcohol, dilute contents of each test tube to 5 ml and immediately read absorbance at 385 nm in spectrophotometer against cuprous chloride reagent as blank. (Amount of chloroform aliquot should not be more than 2 ml of the total 5 ml so emulsions will not form. Make additional dilutions with 95% alcohol, if necessary.) Plot absorbances at 385 nm vs. concentration of thiram (4–200 μg). Figure 1 shows the straight line relationships obtained under different conditions.

Extraction of Sample

Extract 1–5 g grain 5–6 times with 1–2 ml chloroform in 100 ml conical flask by shaking 1–2 min. Combine extracts and dilute to 25 ml with chloroform and filter if solution is turbid. Use known amount of extract (2 ml or less for color development). Proceed as described under *Preparation of Standard Curve.*

Recovery Experiments

Known quantities of a 75% thiram formulation were used for recovery experiments by both the method described and the acid hydrolysis method (3). The results are given in Table 1. Grains (wheat, sorghum, and rice) sprayed with thiram dispersion were used for residue analysis of thiram, both by the acid hydrolysis and the cuprous chloride methods; *see* Table 2. The stability of the yellow color was assessed by measuring the absorbance at 30 min

Table 1. Comparison of recoveries (μg) of thiram from fortified grain samples

Thiram Added, μg	Present Method, μg	Acid Hydrolysis (3), μg
10	9.67±0.47	9.57
20	18.66±0.48	19.14
40	37.66±0.72	38.28
70	65.33±0.41	64.15
100	91.67±0.71	93.07

Table 2. Comparison of recoveries of thiram residues

Strength of Spray Used, g/L	Present Method, ppm	Acid Hydrolysis (3), ppm
	Wheat	
4.0	192.7	193.22
2.0	106.1	96.61
0.5	30.05	31.81
0.25	8.67	9.66
	Sorghum	
10	300.64	315.20
8.0	294.89	292.70
6.0	290.89	292.20
4.0	158.52	157.55
2.0	93.89	94.76
0.5	28.92	29.24
0.25	6.10	6.34
	Rice	
8.0	186.9	184.7
4.0	96.74	94.5
2.0	48.07	50.01
1.0	18.49	18.50

intervals. Percentages of loss in recoveries are given in Table 3. The amount of cuprous chloride reagent needed for complete development of color was decided by adding from 0.5 to 3.0 ml reagent to 1 ml chloroform solution containing 200 μg thiram and calculating recoveries. The stability of the cuprous chloride reagent was assessed by using both fresh and 2-day-old reagent for color development with 1 ml chloroform solution containing 200 μg thiram; *see* Fig. 1.

Results and Discussion

While several alternative procedures are available, the commonly employed method for estimation of thiram residues is the acid hydrolysis method of Pease (3). Although the recoveries in this method are good (93–96%), it is time-consuming (about 1½ hr for each analysis) and requires boiling sulfuric acid. In addition the hydrolysis of thiurams is not quantitative under the experimental conditions described (3) due to the difficulty of dispersing the water-insoluble thiurams in the acid medium. Hence the analysis must be repeated several times to obtain consistent results. In view of these disadvantages, the rapid method described in this paper was developed to estimate thiram residues on grains.

Table 3. Loss in thiram recoveries (%) with time

Thiram, μg	Absorbance Read after (min): 0	15	30	60	120
5.25	0.0	0.0	11.6	21.06	34.6
42.0	0.0	0.0	11.0	21.08	28.05
200.0	0.0	0.0	11.47	14.75	24.60

Characteristics of the Method

The method described can be used to estimate as little as 4 μg thiram and the relation between absorbance and concentration is linear up to 200 μg. The reaction is also quantitative because of the high solubility of thiurams in chloroform.

Of the many organosulfur compounds tested, only thiocyanates gave a yellow color with cuprous chloride reagent. The yellow color formed with potassium thiocyanate did not show an absorption peak at 385 nm, when examined in an automatic recording spectrophotometer. Other chloroform-soluble dithiocarbamates (e.g., ferbam and ziram) form yellow cuprous complexes with this reagent and also have a maximum absorption at 385 nm. However, we are currently studying ways to remove these potential interferences.

No interfering colored substances were encountered in the chloroform extracts of sound grains. If the chloroform extracts are turbid, due to dust washed from the grains, they should be diluted to 25 ml with chloroform and then filtered. Usually the chloroform of the fungus-infected grains was yellow; the absorbance of this color (after dilution to 5 ml) was subtracted from the absorbance of the copper complex.

Cuprous Chloride Reagent

The maximum absorption of the yellow cuprous complex in alcohol-acid-chloroform is at 385 nm, as shown by an automatic recording spectrophotometer DK-2 (Fig. 2). Although Keppel (6) used cuprous chloride for color development with thiram, he measured the absorbance at 420 nm, probably because of the yellow color in the visible region. The differences in absorbance at 385 and 420 nm for a given concentration of thiram are quite considerable; the losses in recoveries at 420 nm range from 25 to 71% and are maximal at higher concentrations (Fig. 1). Thus it is advantageous to read the absorbance at 385 nm, instead of depending upon the yellow color in the visible region.

Although Janssen reported that cuprous complexes of dialkyl dithiocarbamates are stable in 75% alcohol (7), based on their stability con-

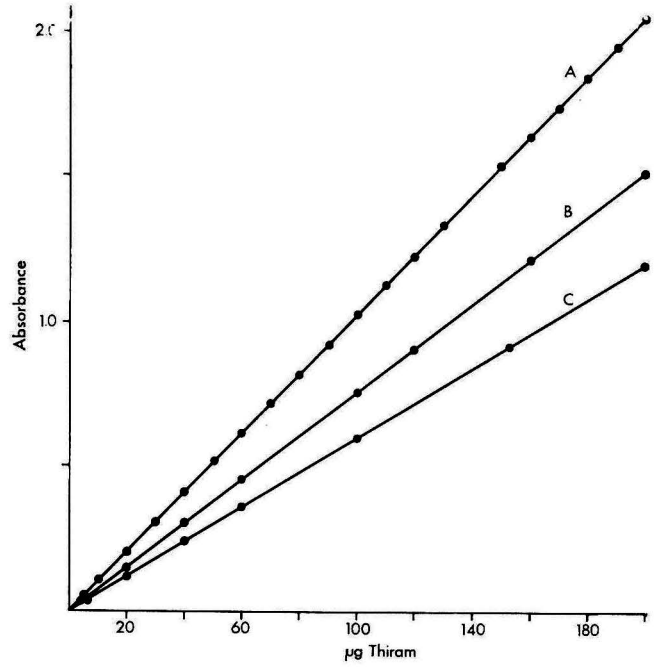

FIG. 1—Calibration curves of the thiram-cuprous chloride complex, using freshly prepared reagent, at 385 (A) and 420 (B) nm; C is the curve obtained at 385 nm when a 2-day-old reagent was used.

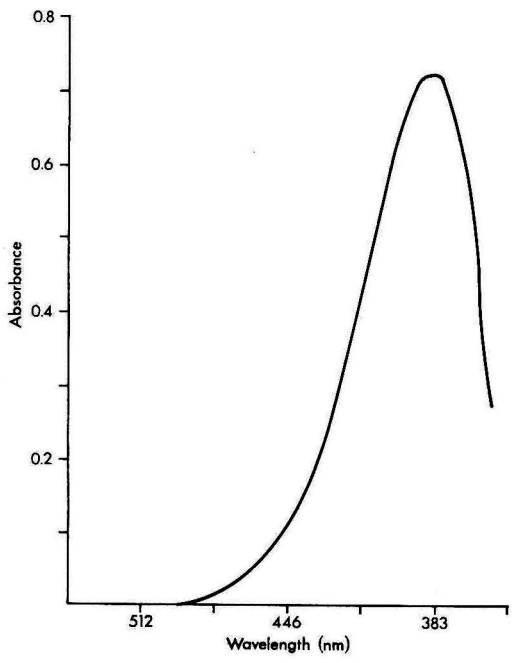

FIG. 2—Absorption spectra of thiram-cuprous chloride in chloroform-alcohol solutions (2 + 3, v/v).

stants, we found that the color faded rapidly after 15 min (Table 3). Irrespective of thiram concentration, there is about 11% loss in the recovery after 30 min; the loss is more at lower concentration as more time elapses before the absorbance is measured. The method specifies measurement of color within 15 min of development.

We found significant differences in recoveries (25–38% loss) when cuprous chloride reagent stored for 2–3 days was used. Thus cuprous chloride reagent must be freshly prepared before use.

Although both 0.01 and 0.1% cuprous chloride reagents gave 94.4% recoveries, a 0.1% solution was employed as the reaction rate is increased by an excess of cuprous ions in the reaction mixture. With concentrations greater than 0.1% both the reagent and the reaction mixture become turbid. Recoveries of thiram were 74 and 79.5%, respectively, when 0.5 and 1.0 ml cuprous chloride was used for the reaction. Recoveries were increased to 94.9% when 2 ml reagent was used. Since the recovery remained constant for 2–3 ml reagent, 2 ml reagent was selected as the minimum amount for maximum recovery.

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GLC Determination of Hexachlorophene in Several Agricultural Samples

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A method is described for analysis of the fungicide hexachlorophene in agricultural samples after cleanup on a Celite-H_2SO_4 column. It is based on electron affinity gas chromatography of the methyl ether and is sensitive to about 0.02 ppm of the fungicide. The procedure has been applied to analysis of hexachlorophene in tomatoes, sweet corn, cucumbers, and milk.

Hexachlorophene (2,2′-methylene-bis-(3,4,6-trichlorophenol)) has long been used as an antibacterial additive to soaps. The toxicological properties of the compound have been reviewed by Gump (1). Electron affinity gas chromatographic detection for hexachlorophene in skin (2), body tissues and fluids (3), and blood (4) have been reported.

More recently, hexachlorophene has shown broad spectrum activity for control of fungus and bacterial diseases of plants. It has exhibited outstanding control of bacterial spot of tomatoes and Helminthisporium blight of corn. A spectrophotometric method for analysis of the compound in agricultural samples has been published (5). In the work reported, hexachlorophene is extracted from samples with acetone, interferences are removed by Celite-H_2SO_4, and hexachlorophene is chromatographed as the methyl ether (2,2′-methylene - bis - (1 - methoxy - 3,4,6 - trichlorobenzene)), using an electron affinity detector.

Experimental

Gas Chromatography

A Barber-Colman Model 10 gas chromatograph was used with battery-operated, No. A-4071, 6 ml electron affinity detector containing 56 μCi radium-226, and Wheelco recorder, 0 to 50 mv, equipped with 10″ chart paper, running 10″/hr. The U-shaped, borosilicate glass column, 6 mm id × 2′, was packed with 10% DC-200 on 80–100 mesh Gas Chrom Q. Operating conditions: column, flash heater, and detector, 200, 250, and 235°C, respectively; electrometer gain setting, 10,000; nitrogen carrier gas, 75 ml/min. Retention time of methyl ether of hexachlorophene was 16.5 min.

Procedure

Thoroughly blend 25 g sample with 65 ml acetone. Filter mixture and rinse filter with acetone until a total volume of 100 ml is collected. Partition entire filtrate successively with 50, 20, and 10 ml portions of $CHCl_3$. Each time drain $CHCl_3$ solution through layer of anhydrous Na_2SO_4 and combine $CHCl_3$ extracts in 300 ml round-bottom flask. Evaporate $CHCl_3$ with rotating evaporator and 40°C water bath.

Dissolve residue in 25 ml CCl_4. Add 18 g mixed Celite 545-H_2SO_4 (6 ml H_2SO_4 + 7 g Celite 545) (6), swirl, and let stand 20 min. Filter solution through 0.25″ layer of Celite 545 supported on sintered glass funnel. Rinse funnel with 50 ml CCl_4. Partially evaporate solution with air. Transfer solution to 50 ml volumetric flask, dilute to volume with CCl_4,

Table 1. Recovery of hexachlorophene from agricultural samples

Sample	Hexachlorophene Added, ppm	Recovery, %
Cucumbers	0.1	71, 70, 65, 71
Tomatoes	0.02	92
	0.04	88, 82
	0.06	79
	0.08	75
	0.1	85
	0.2	67
Corn	0.02	137
	0.04	109
	0.06	98
	0.08	89
	0.1	91, 84, 58, 69
Milk	0.02	142
	0.04	116
	0.06	116
	0.08	105
	0.1	130, 91, 89

and mix. Evaporate 2 ml of solution in test tube with air. Dissolve residue in 1 ml diethyl ether and methylate sample by bubbling diazomethane gas into solution for 10 min as described by Schlenk and Gellerman (7). Evaporate solution with air, dissolve residue in 1 ml ethyl acetate, and inject an appropriate aliquot (up to 10 μl representing 0.01 g equivalent sample) onto gas chromatographic column. An injection representing 1 ng methylated hexachlorophene gave approximately one-half scale recorder response at a sensitivity of 1×10^{-10} AFS.

Results

The method was applied to analysis of tomatoes, sweet corn, cucumbers, and milk. Table 1 lists the recoveries of hexachlorophene added at various levels to samples before extraction. Figure 1 shows typical chromatograms of the compound recovered from tomatoes at 0.08 ppm and the corresponding control. Peak height was calculated by measuring the vertical distance from the peak apex to a line forming the base line of the peak. It is apparent from inspection of the control chromatogram that a small contribution to peak height was obtained and this was estimated as equivalent to about 1 cm. The method was sensitive to about 0.02 ppm hexachlorophene in the samples.

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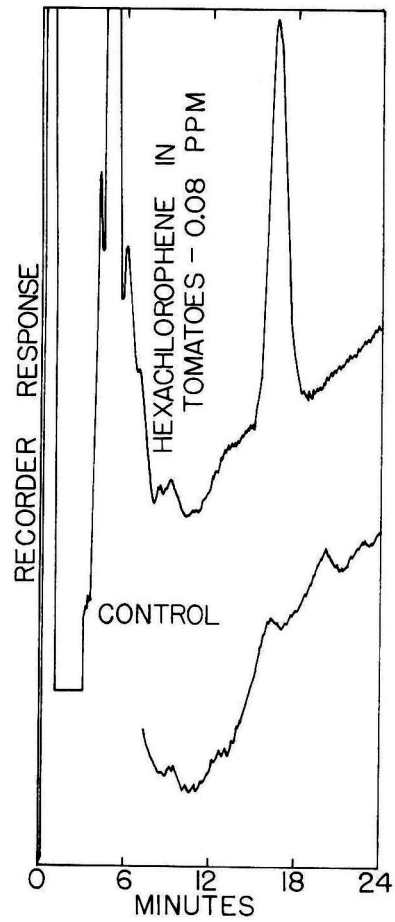

FIG. 1—Chromatograms of 0.08 ppm hexachlorophene added to tomatoes and the control.

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Elimination of Interferences in the Determination of Toxaphene Residues

By A. K. KLEIN and J. D. LINK (Division of Pesticides, Food and Drug Administration, Washington, D.C. 20204)

The presence of DDT interferes in the GLC method for toxaphene. The interference is overcome by treating the sample extract with cold sulfuric-fuming nitric acids (1 + 1). The acid treatment converts DDT to compounds which do not register on the GLC. Toxaphene is scarcely affected and responds in the usual manner. The interference of various other pesticides is also prevented. The acid treatment also aids the toxaphene colorimetric method by destroying sample organic background. Toxaphene levels below 1.0 ppm can now be determined by the colorimetric method. Chlordane is not removed by the acid treatment and interferes in both the GLC and colorimetric toxaphene methods.

This report describes work in 2 essentially separate areas of research: (*a*) removal of interferences in the gas-liquid chromatographic (GLC) and colorimetric determinations of toxaphene residues and (*b*) improvements in the colorimetric procedure for determination of toxaphene residues. Procedures for achieving these objectives are described and, finally, results obtained by using combinations of these recommended procedures are presented and compared.

Elimination of Interference in Toxaphene Analyses

The Problem

In the 1967 Associate Referee's Report (1), the interference of DDT in the GLC determination of toxaphene was stressed. It was explained that electron capture GLC detector response to DDT is several times greater than to an equal amount of toxaphene. Consequently, the degree of interference caused by DDT in a GLC analysis of toxaphene is disproportionate to the amount of DDT present. It interferes by overlapping toxaphene peaks which are needed for the correct evaluation of the pesticide.

The interference of DDT is illustrated impressively in Fig. 1. Two tracings are shown of the GLC analysis of a sample extract of experimental kale to which 8.0 ppm toxaphene and 8.0 ppm DDT had been added. The tracings show the marked difference in response of the electron capture detector to DDT and toxaphene. If the analyst adjusts his sample injection to measure DDT accurately, toxaphene is barely detectable (Curve B). It might even be overlooked, although 8.0 ppm is present. If the analyst were interested only in the DDT content, no further sample treatment would be necessary. The data listed in Table 1 support this statement. Although DDT and toxaphene were present in a 1:1 ratio in the the 10 levels cited, the average DDT recovery, 95.6%, indicates that toxaphene did not interfere in the determination of DDT.

Curve A of Fig. 1 is equally meaningful. It shows that if the analyst adjusts his injection to measure toxaphene in the sample under discus-

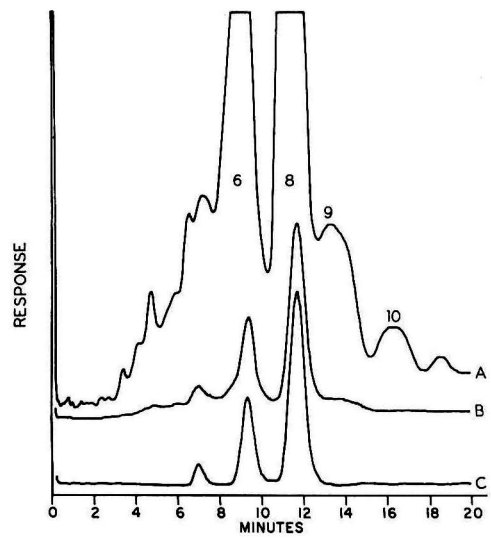

FIG. 1—Gas chromatograms of A, acid-treated kale sample, 8.0 ppm toxaphene and 8.0 ppm DDT (technical) added to 100 g kale (final volume 100 ml, 5 μl injected); B, sample solution diluted 8-fold (8 μl injected); and C, standard DDT (technical) (1 μg/ml, 8 μl injected).

Table 1. Recovery of DDT by electron capture GLC in the presence of toxaphene

DDT Added,[a] ppm	DDT Found, ppm	Rec., %
1.0	0.95	95.4
2.0	1.95	97.7
3.0	2.86	95.4
4.0	3.94	98.5
5.0	4.77	95.4
5.0	4.81	96.2
5.0	4.85	97.0
6.0	5.77	96.2
7.0	6.73	96.2
8.0	7.52	94.0
9.0	8.25	91.7
10.0	9.32	93.2
		Av. 95.6

[a] DDT added with toxaphene 1:1.

sion, DDT goes off the chart and overlaps toxaphene peaks up to peak 10. Peaks 6 and 9, which we prefer to use in the toxaphene calculations, are obviously unusable. Peaks 10 and others with later retention times can be used to alert the analyst to the presence of toxaphene and to give an approximate value of its concentration. However, the 1967 report (1) demonstrated that the composition of toxaphene applied as a spray residue changes during weathering. A complete GLC scan of the pesticide seemed necessary not only for an accurate analysis but also for an appraisal of changes in composition of the pesticide. The removal of DDT when present in amounts that hinder the determination of toxaphene seemed imperative.

Approach to Solution

Erro and coworkers (2) and later Kawano and coworkers (3) contributed an important advance in this direction. They observed that when sulfuric-fuming nitric acids (1 + 1), the mixture employed by Schechter *et al.* (4) for the colorimetric determination of DDT, are added as an ice-cold reagent to the test samples, toxaphene is scarcely affected. However, DDT, DDD (TDE), methoxychlor, Perthane, dicofol (Kelthane), aldrin, dieldrin, heptachlor, tetradifon (Tedion), and carbophenothion (Trithion) are either decomposed or form compounds which cause no response on the GLC with the electron capture detector.

The success of the removal of DDT by the acid treatment is shown clearly in Fig. 2. A sample aliquot containing 8.0 ppm toxaphene and 8.0 ppm DDT (*see* Fig. 1, Curve A) was treated with the acid mixture and processed in a manner that will be described later. The GLC curve of the resulting solution is shown in Curve C. No DDT is indicated. Of equal importance is the fact that the solution yields a characteristic continuum curve which is almost identical with that of standard toxaphene (Curve A).

Kawano and coworkers (3) reported that some loss of toxaphene occurs during the acid treatment. In a series of 14 determinations they obtained an average recovery of 92%, with a range from 79 to 96%. The writers also noted a slight loss (Table 2). They compensated for the loss by using a toxaphene standard which was prepared by putting known amounts of toxaphene through the acid treatment and all the determinative steps of the method to be described.

Experimental

Kale was chosen for the recovery studies because it contains a relatively large amount of waxes. Vegetable waxes are known to resist nitration and might prevent the normal nitration and

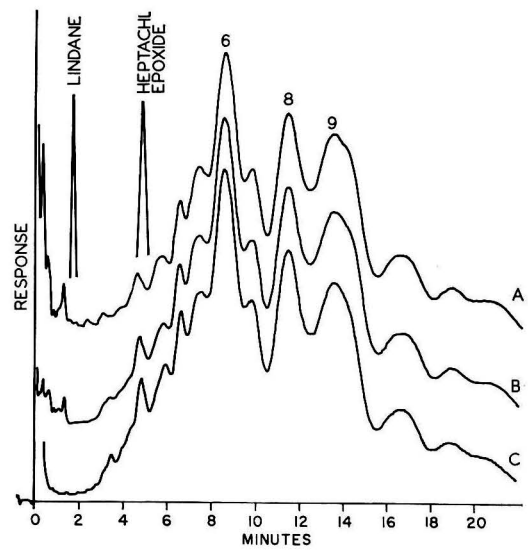

FIG. 2—Gas chromatograms of A, normal toxaphene standard without acid treatment (10.0 μg/ml, 8 μl injected); B, acid-treated toxaphene standard (1.0 mg toxaphene treated with acids and diluted to 100.0 ml, 8 μl injected); and C, acid-treated kale sample (8.0 ppm toxaphene and 8.0 ppm DDT added to 100 g kale, final volume 80.0 ml, 8 μl injected).

Table 2. Comparison of electron capture and colorimetric methods for toxaphene recovery in presence of DDT, after sulfuric-fuming nitric acid treatment

Toxaphene Added,[a] ppm	Toxaphene Found: Electron Capture GLC ppm	Electron Capture GLC %	Colorimetry ppm	Colorimetry %
1.0	0.83	83.0	1.00 1.20	110.0
2.0	1.82	91.0	2.36 3.04	135.0
3.0	2.66	88.6	3.96 3.66	127.0
4.0	3.63	90.7	4.64 4.16	110.0
5.0	4.48	89.6	4.30 4.00	83.0
5.0	4.40	88.1	4.10 3.90	80.0
5.0	4.33	86.5	5.10 5.60	107.0
6.0	5.44	90.7	6.12 6.12	102.0
7.0	6.52	93.2	7.00 6.44	96.0
8.0	7.62	95.3	7.68 6.08	86.0
9.0	8.12	90.2	8.64 6.66	85.0
10.0	8.87	88.7	10.30 8.60	94.5
		Av. 89.6		Av. 101.2

[a] DDT added with toxaphene 1:1.

removal of DDT. Kale would serve as a test of the method.

To eliminate aliquot calculations the writers varied the blending procedure described by Mills, Onley, and Gaither (5). The sample, 100 g kale, was blended with 200 ml acetonitrile and filtered as outlined in (5). The blender and lid were rinsed with an additional 50 ml acetonitrile. The rinse was transferred to the filter cake and filtered. The filtrate was diluted with 600 ml water and extracted twice with 100 ml petroleum ether. Entrained water in the separated petroleum ether layer was removed by filtration through a glass tube containing 10 g anhydrous sodium sulfate. The filtrate was concentrated to 10 ml and eluted through a column of activated Florisil with 200 ml ethyl ether-petroleum ether (6 + 94), as outlined in (5). The eluate was then concentrated and made to a suitable volume.

Samples fortified with both toxaphene and DDT and samples containing field-incurred residues of toxaphene (1), processed as described above, were then put through the following procedure to remove DDT and other interferences.

METHOD

Transfer suitable aliquot of ethyl ether-petroleum ether (6 + 94) Florisil eluate (5) to 50 ml glass-stoppered Erlenmeyer flask and concentrate *just* to dryness. Add 5 ml cold (<0°C) H_2SO_4-fuming HNO_3 (1 + 1) to residue. Shake flask 30 sec and let mixture react 15 min at room temperature. Carefully add 25 ml ice water and shake 30 sec. Let flask stand until solution reaches room temperature. Transfer solution to 125 ml separatory funnel. (We prefer to use CH_2Cl_2 as extraction solvent rather than petroleum ether, the solvent employed by Kawano and coworkers (3).) Add 25 ml CH_2Cl_2 to flask, shake 1 min, and transfer solvent to separatory funnel. Repeat 25 ml CH_2Cl_2 treatment. Shake separatory funnel vigorously 2 min. Drain CH_2Cl_2 (lower layer) into second 125 ml separatory funnel. Add 100 ml water to CH_2Cl_2 and shake funnel 30 sec. Drain CH_2Cl_2 layer into third 125 ml separatory funnel containing 10 ml 5% $NaHCO_3$ and shake 30 sec. Filter CH_2Cl_2 layer through tube containing 10 g anhydrous Na_2SO_4 into 125 ml flask. Concentrate solution *just* to dryness. Add 2.5 ml petroleum ether-acetone (80 + 20) to residue. Remove interfering substances contributed by acid treatment by eluting solution through column of activated Florisil with 200 ml ethyl ether-petroleum ether (6 + 94) as described before.

Complete analyses as described in (5). Use a standard made by carrying 1.0 mg toxaphene through the H_2SO_4-fuming HNO_3 and all subsequent steps. Make final volume to 100 ml. Label concentration as 10.0 μg/ml.

Discussion

In quantitating toxaphene by GLC we prefer to use area measurements of peaks 6 and 9 (*see* Fig. 2). Recovery data for 1–10 ppm toxaphene, the range in which we are interested, are listed in Table 2. The average recovery is 89.6%, ranging from 83.0 to 95.3%. The average deviation from the mean value, 89.6%, is ±2.1%. Based on these data the analyst could expect a recovery from 87.5 to 91.7%. In obtaining the results listed in Table 2 all the steps of the method were completed, which included sample blending, removal of DDT, and the 2 Florisil elutions. The

toxaphene values were computed by comparing sample GLC response to that of the acid-treated toxaphene standard.

The success of the sulfuric-fuming nitric acid reaction in removing DDT was amply demonstrated during the analyses of the 12 samples listed in Table 2. In none of the final determinative sample solutions was DDT observed by GLC. The removal of DDT from toxaphene is important, not only to the spray residue chemist but also to workers interested in the study of toxaphene storage in animal fatty tissues where DDT is likely to be present in amounts which could obscure the GLC evaluation of toxaphene.

The samples chosen for the recovery study were subsamples of the experimental kale described in the 1967 report (1). Part of the sample aliquots were treated as if they contained excessive amounts of DDT and, accordingly, were reacted with the sulfuric-fuming nitric acid reagent. There is no significant difference in toxaphene recovery data among results for these samples using either an untreated toxaphene standard or an acid-treated standard, which would indicate that the small losses noted in Table 2 were not due to the acid treatment. This statement is supported by the data shown in Table 3. Up through the 14th day of weathering, in those samples in which the acid mixture was not used in any of the methods, the toxaphene residue results agree with those shown for the acid-treated samples. (In the analysis without the acid treatment, the kale data for days 21 and 28 of weathering could not be used because of residual DDT interference.)

Erro and coworkers (2) reported that the pesticides lindane, heptachlor epoxide, and chlordane are not removed by the sulfuric-fuming nitric acid treatment. Figure 2 shows that lindane and heptachlor epoxide would not greatly change the toxaphene GLC curve because they peak close to the origin, a region of minor interest to the toxaphene analyst. Moreover, both compounds contribute comparatively little blue color in the toxaphene colorimetric method. For these reasons lindane and heptachlor epoxide are not regarded as interfering compounds.

However, the curves in Fig. 3 show that chlordane offers a serious interference. The components of chlordane that peak between 5 and 10.5 min are barely affected by the acid treatment (Curve B). The components which peak between

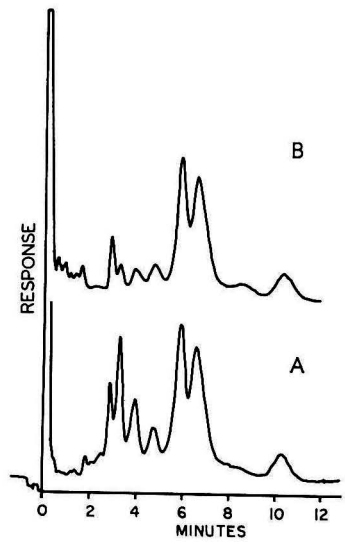

FIG. 3—Gas chromatograms of A, chlordane standard (1.0 μg/ml, 5 μl injected) and B, 1.0 mg chlordane treated with acid and diluted to 1 L (5 μl injected).

2 to 4 min are only partially removed. When both pesticides are present in equal quantity chlordane overlaps the toxaphene GLC curve up to and including peak 7. In this instance the analyst would have to rely on peaks 8 and 9 to compute the amount of toxaphene present. As said before the analyst is hesitant about basing his judgment of the composition of weathered toxaphene residues on these late-eluting peaks.

Improvements in Colorimetry of Toxaphene Residues

The writers continued to study the colorimetric method which was described in (1). In the method a blue substance is formed when toxaphene is heated with *p*-diphenylamine sulfonic acid sodium salt at 205–210°C in the presence of anhydrous zinc chloride for 10 min. A methanol solution of the blue product is evaluated in the spectrophotometer at 625 nm.

The method is an empirical one and, for that reason, we made a detailed study of the conditions that control it. It was observed that to obtain the blue colored complex the reaction must be carried out with dry solid reagents. When organic solvents were added to the reagents very little or no color was noted, although the reaction

medium was liquid at 205–210°C. Mineral oils and fruit waxes inhibit the reaction; in some instances no color was observed. The condensing agent zinc chloride is essential to the reaction. Anhydrous aluminum chloride, which was also studied, was not as efficient. However, when too much zinc chloride was used, the yield of the blue substance decreased. The lowering of the yield also occurred when an abnormal excess of diphenylamine sulfonic acid reagent was used. These low yields were probably due to poor heat penetration. The amount of heat supplied by the oil bath is also a very important factor. When the temperature of the bath was lowered below 200°C, the amount of the toxaphene reaction dropped quickly. Conversely, when the bath temperature was raised above 210°C or when the reaction mixture was heated longer than 10 min at 205–210°C (e.g., 15 to 30 min), the absorbance of the blue solution increased greatly. However, the standard curves were very erratic. It was noted in these experiments that some of the toxaphene reaction product peeled off from the wall of the reaction tube as small black particles which would not totally dissolve in methanol or in the common organic solvents. This variation in solubility would account for the erratic standard curves. As a result of this study, we developed the colorimetric method which is described below. This procedure may be used on the ethyl ether-petroleum ether (6 + 94) eluate of the Mills, Onley, Gaither method (5), either before or (if DDT is present) after the sulfuric-fuming nitric acid treatment.

METHOD

Apparatus and Reagents

Prepare agents (a) and (b) on day of use.

(a) *p-Diphenylamine sulfonic acid sodium salt.*—0.5% in methanol. (The amine salt should have a pH of 7.0–7.1 in water.)

(b) *Zinc chloride.*—0.5% in acetone. The solid reagent must be anhydrous.

(c) *Toxaphene standard.*—100 μg/ml in acetone. Prepare weekly.

Determination

Concentrate volume of sample solution containing preferably ca 20 μg toxaphene to ca 1 ml. Transfer solution to 25 ml culture tube with glass stopper, using ≤5 ml petroleum ether for transfer, and concentrate *just* to dryness. Add 0.2 ml each of reagents (a) and (b). Put culture tube in warm water and remove solvent with air jet. Stopper tube, hold stopper in place with wire, and suspend tube exactly 2 min in well stirred 210°C oil bath (USP white paraffin oil, Saybolt viscosity, 340–355 at 100°F). (Tubes must be securely stoppered to prevent oil fumes from entering them. The oils would inhibit the color formation.) Cool tube immediately in ice or cold water and wash inner walls of tube with ca 0.5 ml methanol. Remove solvent with air jet, stopper tube securely, suspend it in oil bath exactly 8 min, and cool immediately. Add 10.0 ml methanol. Shake tube 1.0 min and read absorbance at 625 nm after 15 min. Compare with absorbance of closest toxaphene standard.

Prepare standard curve in identical manner, using 0, 10, 20, 30, 40, and 50 μg toxaphene. Prepare curve on same day samples are analyzed. (We obtained a straight line when these amounts of toxaphene were plotted vs. absorbance.)

It was our practice to work with 5 and 6 tubes at one time. The bath temperature would decrease from 214 to 210–205°C, the optimum temperature for our work. Although the final colors are stable, the authors standardized the procedure by reading the absorbance 15 min after addition of 10 ml methanol.

Results and General Discussion

Results by the colorimetric method are given in Table 2. Although the average recovery is 101.2%, the average deviation from this mean value is 14.0%. Based on these data the analyst could expect to obtain recoveries ranging from 87.2 to 115.2%, a much wider range than that obtained by the GLC method. This variation is not due to sampling errors, because the color method was applied to aliquots of the same solutions used to collect the GLC data listed in Table 2. The average recovery, 101.2%, obtained by the colorimetric method is about 10.0% higher than that of the GLC method, 89.6%. We have no explanation for this difference.

Additional colorimetric data are listed in Table 3. Except for the results on the sample designated as 0, the agreement between check analyses is satisfactory. The check analyses were performed 4 days after the first analyses, using aliquots of the same solutions. If the results derived from the sample designated 0 are excluded, the agreement between the recovery values obtained from the colorimetric and GLC methods may be regarded as satisfactory, although the re-

Table 3. Recovery of toxaphene (ppm) from kale samples by electron capture GLC and colorimetry

Days after Spraying	Electron Capture GLC: No Acid Treatment of Sample and Regular Std[a] Peak 6	Peak 9	Av.	Sulfuric-Fuming Nitric Acid Treatment of Sample: Regular Std[a] Peak 6	Peak 9	Av.	Acid-treated Std[b] Peak 6	Peak 9	Av.	Colorimetry Found	Av.
0	120.5	134.1	127.3	119.5	130.1	124.8	122.5	131.7	127.1	175.0,126.0	150.5
3	62.4	73.2	67.8	62.5	73.5	68.0	64.1	74.4	69.2	48.7,47.5	48.1
7	10.8	11.5	11.2	10.0	11.4	10.7	10.3	11.6	10.9	7.80,8.60	8.20
14	1.30	1.23	1.27	1.07	1.22	1.15	1.10	1.23	1.17	0.77,0.73	0.75
21	——	0.32	0.32	0.46	0.43	0.44	0.47	0.44	0.45	0.29,0.31	0.30
28	——	0.14	0.14	0.23	0.15	0.19	0.23	0.15	0.19	0.22,——	0.22

[a] Normal untreated toxaphene standard.
[b] Toxaphene standard treated with sulfuric-fuming nitric acids.

sults by the colorimetric method tend to be 25% lower. In the 1967 report it was stated that, due to background interfering material, toxaphene could not be determined on the 14, 21, and 28 day-weathered kale samples. Apparently, the sulfuric-fuming nitric acid reagent removes this interference, because Table 3 lists credible toxaphene values determined on these samples by the colorimetric method. Based on microcoulometric GLC analysis of the 14, 21, and 24 day-weathered kale samples, the 1967 report concluded that the toxaphene residues are organic chlorine compounds. The data in Table 3 confirm this statement and show that these residues contain compounds that yield the typical blue complex that is considered to be and equated as toxaphene.

Chlordane also gives a blue color in the toxaphene colorimetric method. Although the absorbance is much less than that which the GLC result would anticipate, the color could be mistaken for toxaphene.

Recommendations

It is recommended that (*1*) work be done to overcome chlordane interference; (*2*) a concerted effort be made to isolate and identify the components of toxaphene; (*3*) studies dealing with changes of composition of toxaphene ingested by rats be resumed; and (*4*) the method be studied collaboratively.

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This report of the Associate Referee, A. K. Klein, was presented at the 83rd Annual Meeting of the AOAC, Oct. 13–16, 1969, at Washington, D.C.

The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee E and were accepted by the Association. See *JAOAC* **53**, 392–393 (1970).

Note on the Determination of Mercury in Soil by Oxygen Flask Combustion

By MERRIE N. WHITE and D. J. LISK (Pesticide Residue Laboratory, Cornell University, Ithaca, N.Y. 14850)

Losses of mercury from biological samples subjected to ashing in open systems can be obviated by oxygen flask combustion. The sample must support combustion, however. A method is described for the determination of mercury in mineral soil which is mixed with cellulose acetate powder to allow combustion. Mercury is then determined spectrophotometrically as the dithizonate. The sensitivity of the method is about 0.1 ppm mercury. The recovery of mercury from soil is reported.

Mercurial fungicides have long been used for control of fungus diseases of fruit and vegetables. Because of the toxicity of mercury and its extensive use in the past, much effort has been devoted to developing routine analytical methods for mercury residues.

This laboratory had experienced much difficulty in attempts to recover mercury from samples when numerous wet ashing procedures were used. Loss of mercury by vaporization was serious and often complete. The application of oxygen flask combustion in the determination of mercury in apples was therefore first made and reported in 1960 (1).

There has been much interest in studying the behavior of mercury in soil because of the possibility of its translocation into plants. Although organic soil (2) can be effectively combusted in an oxygen-filled flask, most mineral soils contain too little organic matter to support combustion. In the work reported, a method was developed for combustion of mineral soil prior to the determination of mercury.

METHOD

Thoroughly mix 1 g well mixed, dry soil with 0.4 g cellulose acetate powder (obtainable as pulverized cellulose acetate flake HLFS-85 from Celanese Plastics Co., Box 1000, Belvidere, N.J. 07823). Transfer mixture to Parr pellet press with 0.5″ diameter bore and pelletize sample. Prepare 6 pellets of each soil sample in this manner. Using forceps, place 3 pellets into platinum holder of 5–1 combustion flask (1). Insert paper fuse and combust sample in oxygen as described (1), absorbing gases in 200 ml 0.1*N* HCl contained in flask. Remove platinum holder and dry it in Bunsen flame. Place remaining 3 pellets in holder and combust as above, absorbing gases in same HCl solution. Filter absorbing solution through cotton plug into 500 ml separatory funnel. Rinse flask and platinum holder with two 50 ml portions of 0.1*N* HCl. Filter rinsings through cotton plug, combining them with filtrate of absorbing solution. Proceed with method, including extraction of mercury as dithizonate, spectrophotometric analysis, and development of the standard curve, as described earlier (1).

Results

The procedure was applied to the analysis of soil (Honeyoye sandy loam) to which mercury was added. The soil was fortified by adding up to 30 μl of an aqueous standard solution of mercuric chloride before pelletizing. Recoveries of 0.25, 0.25, 0.25, 0.50, 0.50, 0.50, and 0.50 ppm added mercury were, respectively, 90, 113, 90, 77, 110, 77, and 90%. The value for apparent mercury in control soil was less than 0.05 ppm. The sensitivity of the method was about 0.1 ppm mercury.

The check value did not increase appreciably when the analysis was based on combustion of 6 pellets as compared to 3. Thus the sensitivity of the method could presumably be increased by burning more than 6 pellets, with absorption of gases in the same absorbing solution. The time for analysis would be approximately 30 min longer per sample if 9 pellets were combusted instead of 6.

During combustion the sample was completely engulfed in flame internally as well as externally. Organic matter in the soil was completely oxidized and only charred clay particles were left, which were filtered out before analysis. The temperature attained during combustion of a sample in an oxygen-filled flask is usually above 1000°C. Mercury present in soil in organic matter or in exchangeable form on clay surfaces should be readily volatilized at temperatures below 300°C. The method described should thus provide an accurate measure of the levels of residual mercury in soils resulting from past fungicide applications.

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METALS AND OTHER ELEMENTS

Atomic Absorption Spectrometric Determination of Copper and Nickel in Tea

By KEITH E. BURKE and C. H. ALBRIGHT (The International Nickel Co., Inc., Paul D. Merica Research Laboratory, Sterling Forest, Suffern, N.Y. 10901)

Atomic absorption spectroscopy is one of several analytical tools which can be applied to the determination of less than 20 ppm quantities of copper and nickel in tea. A rapid and accurate atomic absorption method is described which is sensitive to these elements at the levels of interest. After the decomposition of organic matter by oxidation with nitric and perchloric acids, or by dry ashing, a solution is obtained which contains only a few major elements. An average matrix composition is determined and synthetic standards for copper and nickel which are free of potential interferences are prepared for a calibration curve. Tea leaves have been analyzed chemically and by X-ray and optical emission spectroscopy in order to evaluate the accuracy of the atomic absorption method. The average of 50 determinations gives a mean of 11 ppm nickel and 32 ppm copper with standard deviations of ±1.6 σ and 1.8 σ, respectively. The method should be applicable to most organic material.

From our work (1) and that of others (2–4) the level of the metallic constituents of tea leaves is found to vary according to the type of tea and its geographical source, as shown by the ranges cited in Table 1. Tea is subject to a fungus disorder known as blister blight. Copper salts are commonly used to spray tea plants in certain areas of the world; therefore, copper would be higher when the plants are sprayed with the copper protectant. Nickel salts are also useful as a protectant and eradicant against blister blight. An accurate method for the determination of copper and nickel in teas is required for quality control.

Atomic absorption spectroscopy is an ideal tool for the determination of ppm quantities of copper and nickel in tea leaves because it is a rapid method that is sensitive to these elements at the levels of interest. After the decomposition of organic matter by oxidation with nitric and perchloric acids, or by dry ashing, a solution is obtained which contains only a few major elements. An average matrix composition is determined and synthetic standards are prepared for a calibration curve. We have analyzed teas by a number of techniques and prepared standards in order to evaluate the precision and accuracy of the atomic absorption method.

Table 1. General range of metals present in teas by atomic absorption and flame emission analysis

Metal	Range, %	
	Made Tea	Instant Tea
Potassium	1.6–2.0	3.0–8.0
Calcium	0.3–0.4	0.1–0.2
Magnesium	0.1–0.2	0.3–0.8
Iron	0.05–0.2	0.003–0.005
Manganese	0.02–0.1	0.04–0.1
Aluminum	0.05–0.1	0.005–0.1
Sodium	0.01–0.03	0.1–0.5
Zinc	0.0002–0.003	<0.0002
Copper	0.002–0.007	0.0005–0.001
Cobalt	0.0001–0.0005	0.0003–0.001
Nickel	0.0003–0.0015	0.0005–0.002
Bismuth, tin, lead, chromium, titanium, vanadium	<0.001	<0.001

Experimental

Apparatus

A Perkin-Elmer Model 303 atomic absorption spectrophotometer equipped with a premix chamber and a triple-slot Boling-type burner head was used in this work. Perkin-Elmer hollow cathodes served as source lamps and a Sargent Model SRG recorder as a readout device. Instrument conditions were: aspiration rate for water, 9.5 ml/min; air pressure, 30 psi with flow of 24 L/min; and acetylene pressure, 8 psi with flow of ca 4 L/min. The copper 324.7 nm and nickel 232.0 nm resonance lines were used.

Reagent

Nickel standard solution,—1000 ppm (w/v). Dissolve 1.0000 g high purity, 99.999%, nickel (Gallard-Schlesinger Chemical Corp., Carle Place, N.Y. 11514) in 20 ml nitric acid, cool, and dilute to 1 L with water.

Use reagent grade chemicals for the preparation of other solutions.

Calibration Curve

Prepare solutions with nickel and copper levels of 0, 0.2, 0.4, 0.8, 1.6, 2, 4, 8, and 10 μg/ml and which also contain the major matrix metal components as follows: (*1*) For 3 g sample of made tea, the standards should contain 180 μg calcium/ml, 100 μg magnesium/ml, and 40 μg aluminum/ml; (*2*) for 6 g sample of instant tea, the matrix should contain 7000 μg potassium/ml, 70 μg sodium/ml, 700 μg magnesium/ml, and 130 μg calcium/ml. Use 50 ml volume for both types of teas.

Standard Sample

Analyze one sample by several methods to establish the nickel level. Use this standard reference material as a control for each set of subsequent analyses.

Procedure

(Procedure is written for determination of nickel, but is applicable to copper and other elements at a similar level.)

Select sample weight to give solution of 0.05–20 μg nickel/ml (i.e., dilute 10 g tea containing 100 μg nickel to 50 ml to give 2 μg nickel/ml). Use ca 3 g sample for dried tea leaves and stems and 6 g sample for instant teas. Always wet ash instant teas. Do not add perchloric acid in analyzing instant teas because decomposition proceeds rapidly with nitric acid and precipitation of potassium perchlorate can thus be avoided.

(a) *Wet ash.*—Accurately weigh sample into 400 ml beaker, add 100 ml nitric acid, and swirl contents. Let acid react with tea 10 min; then place on hot plate. Evaporate acid to near dryness, cool, and add 50 ml nitric acid and 10 ml perchloric acid. Evaporate solution until strong fumes of perchloric acid are obtained. (Omit perchloric acid for instant teas.) Check blank for each bottle of nitric and perchloric acids.

(b) *Dry ash.*—Accurately weigh sample (made teas only) into 50 ml Vycor crucible. Place crucible in muffle furnace at ca 300°C for ½ hr until moisture is removed. Increase temperature to 550°C and ignite with furnace door partly open for 3–4 hr until oxidation is complete. Remove crucible from furnace and cool. Add 1 ml perchloric acid and 2 ml nitric acid, cover crucible with watch glass, and heat on hot plate until solution clears. Rinse down cover glass and slowly evaporate acid to fumes of perchloric acid; then continue heating until all perchloric acid is volatilized. Cool, add 2 ml hydrochloric acid, and evaporate to dryness. Avoid baking residue. Add 2 ml 10% hydrochloric acid and warm to dissolve salts.

Transfer solutions from wet or dry ash procedure to 50 ml volumetric flask. Insoluble potassium perchlorate is likely to be present for whole teas, but it does not interfere during aspiration of sample in atomic absorption process because it settles to bottom of volumetric flask. Measure absorbance and calculate metal concentration by reference to calibration curve. Use 3× scale expansion for levels less than 2 μg/ml.

Table 2. Effect of decomposition procedure on the determination of nickel in a tea

Nickel Added, μg	Nickel Found, μg[a]	
	Wet Ash	Dry Ash
5	4	5
10	9,8	9,11
20	17	21
50	49	50

[a] Data corrected for nickel present in original sample: 7.2±0.5 ppm by wet ashing and 5.7±0.4 ppm by dry ashing.

Results and Discussion

The decomposition of an organic material in the preparation for analysis presents certain problems (4). We have tested 2 procedures and found both to be satisfactory (Table 2). In wet ashing with acids the oxidants are at low temperatures. In dry ashing the sample is heated to a high temperature and the organic matter is oxidized by the air. Dry ashing is reported to cause loss by volatilization, adsorption on unburned carbon, or formation of insoluble silicates. Nickel and copper are reported to be among the elements lost (5). Volatilization losses associated with dry ashing can be minimized by using the wet ashing method. However, the advantages of wet ashing, particularly at the microgram level, are offset by almost certain contamination from the ashing reagents and their leaching action on the walls of the container.

The accuracy of the proposed method is established by analyzing one tea by several techniques.

Table 3. Verification of method accuracy and preparation of standard sample

Method	Found, ppm	
	Nickel	Copper
AAS, standard addition	10	31
AAS, calibration curve	8	35
Chemical	10	35
X-ray[a]	8	35
Emission spectrography	8	—

[a] The details of this method will be the subject of a future communication.

Table 4. Comparison of atomic absorption values for nickel and copper in teas by the proposed method with an independent laboratory[a]

	Nickel, ppm		Copper, ppm	
Tea	Prop.	Indep.	Prop.	Indep.
A	6,5,6	6,7	33,35	34,34
B	6	6,6	37	31,35
C	8	8,8	35,30	30,31

[a] The calibration curve technique was used; all samples and standards were made to contain 400 ppm (w/v) aluminum.

Table 5. Precision study in the analysis of tea (n = 50)

		Nickel		Copper	
Tea	Method	ppm, Av.±σ	% σ	ppm, Av.±σ	% σ
D	1 weighed portion	5.3±0.3	6	8.1±0.9	11
E	50 weighed portions	10.7±1.6	15	31.8±1.8	6
F[a]	50 weighed portions	16.7±1.1	7	15.7±1.7	11

[a] Synthetic tea spiked with nickel.

Table 6. Effect of several metals on the determination of 10 ppm nickel by atomic absorption

Foreign Metal: Nickel	ppm Ni Found in Presence of:						
	K	Ca	Mg	Fe	Al	Mn	Na
0.1	9.5	10.2	10.3	10.1	10.4	10.2	9.2
1	8.2	10.1	10.2	9.6	10.4	9.6	8.3
10	6.9	10.2	10.4	10.4	10.0	10.4	8.2

The data in Table 3 show that the agreement between the 2 atomic absorption methods, the chemical method, and the spectrographic methods is within 2 ppm for nickel and within 4 ppm for copper. Table 4 gives the results for a few teas which were analyzed by the proposed method as well as by an independent laboratory which also used atomic absorption. The agreement is within 2 ppm for nickel and within 6 ppm for copper. Precision of the method is illustrated by the data in Table 5.

To compensate for possible matrix effects, calibration solutions are made to contain the same general matrix composition as the sample. The synthetic tea standards are prepared to contain a matrix composition which approaches that of a variety of teas. The average matrix composition is determined by analysis of the sample by optical emission spectrography, atomic absorption (standard addition), and flame emission (Table 1). Once the calibration curve is prepared and values are obtained, they are easily checked against other methods. This approach yields a standard reference material which is useful in subsequent analyses as a control sample applicable to most teas. It eliminates the checking of potential interferences and allows the analysis of samples in a relatively short time. The alternative approach is to prepare a calibration curve and check for potential interferences by the addition of 1 matrix element at a time. The data in Table 6 show that potassium and sodium decrease the response due to nickel. Even when the calcium or magnesium concentrations exceeded the nickel or copper levels by 500 times, there was no appreciable change in their recovery.

The flame noise due to the weak signal of the nickel hollow cathode gave some problems in accurately reading the absorbance when the scale was expanded 10 times. The noise level due to the acetylene-air flame can be reduced by using the hydrogen-argon-entrained air flame for the determination. Ammonium perchlorate must be present in order to atomize nickel or copper with this low temperature flame (1).

Acknowledgments

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COLOR ADDITIVES

TLC Separation and Spectrophotometric Determination of 1-(2-Hydroxy-1-naphthylazo)-2-naphthalene Sulfonic Acid in D&C Red No. 12

By CHARLES STEIN (Division of Colors and Cosmetics, Food and Drug Administration, Washington, D.C. 20204)

A thin layer chromatographic procedure is presented by which 1-(2-hydroxy-1-naphthylazo)-2-naphthalene sulfonic acid (ortho color) is separated from D&C Red No. 12. The ortho color is dissolved from the adsorbent and determined spectrophotometrically. Recoveries of 1.6–11.3% added color averaged 90%. The content of commercial samples of colors analyzed by this procedure ranged from 0 to 0.8%.

The certifiable color D&C Red No. 12 is the barium salt of the color prepared by coupling diazotized 2-aminonaphthalene-1-sulfonic acid with β-naphthol. The color prepared from 1-aminonaphthalene-2-sulfonic acid and β-naphthol is an isomer of D&C Red No. 12 and is found in commercial samples of this color.

A search of the literature revealed no reports of previous work on the analysis of D&C Red No. 12 for 1-(2-hydroxy-1-naphthylazo)-2-naphthalene sulfonic acid.

METHOD

Apparatus and Reagents

(a) *Chromatographic accessories.*—(*1*) Chromatographic tank: suitable for development of 20 × 20 cm TLC plates. (*2*) TLC plates: 20 × 20 cm coated with silica gel G, ca 0.375 mm thick, and dried overnight at room temperature.

(b) *Spectrophotometer.*—Cary Model 15, or equivalent.

(c) *Developing solvent.*—Reagent grade dioxane-benzene-dimethyl formamide-NH_4OH-water (20 + 10 + 4 + 1 + 1, by volume).

(d) *Standard solution.*—Diazotize purified 1-aminonaphthalene-2-sulfonic acid and couple with pure β-naphthol. Recrystallize from water and dry at 130°C under vacuum. Prepare standard solution of 20–25 mg/L dimethyl formamide. The sodium salt of the color is a suitable standard.

Procedure

Transfer weighed sample estimated to contain 0.2–0.3 mg straight color to 5 ml beaker. Add 0.1 ml dimethyl formamide (DMF). Using capillary tube as stirring rod, break up sample until no dark specks remain and sample is in solution.

Lightly draw line across thin layer plate ca 1″ from bottom. With capillary tube, apply solution to this line as a streak starting about ½″ from edge. Wash beaker twice with 2–3 drops DMF each time and transfer washings to plate. Let dry at room temperature ≥4 hr (preferable overnight).

Develop plate in chromatographic tank until solvent has moved to ca ½″ from upper edge. Remove plate and let dry thoroughly. Replace plate in tank and re-chromatograph as before. Remove plate and let dry.

The ortho color will appear in a band just below D&C Red No. 12. Scrape band of silica gel containing ortho color from plate, transfer scrapings to 5 ml beaker, and pulverize any lumps. Add 3–4 ml DMF and stir. Filter by suction through fine sintered glass filter into vacuum flask. Wash beaker and filter with three 1 ml portions of DMF. Transfer filtrate to 10 ml volumetric flask, wash vacuum flask with small portions of DMF, and add washings to volumetric flask. Dilute to volume with DMF and determine absorbance of solution at 434 nm in 1 cm cell. For solutions having absorbances much below 0.1, use 0.0–0.1 absorbance scale, if available.

$$\% \text{ Ortho color} = A/(A' \times \text{mg sample})$$

where A = absorbance of sample and A' = absorbance of standard, mg/L/cm cell.

Results and Discussion

The series of colors D&C Red Nos. 10, 11, 12, and 13 consist primarily of the sodium, calcium, barium, and strontium salts, respectively, of the color formed by coupling diazotized Tobias acid with β-naphthol. Various isomers of Tobias acid, if present, will yield subsidiary colors in the final color. One subsidiary color results from the presence of Bronner's acid (2-aminonaphthalene-6-sulfonic acid) in Tobias acid. A method for its determination has been described (*JAOAC* **53**, 240–241 (1970)). The presence of 1-aminonaphthalene-1-sulfonic acid in Tobias acid will result

Table 1. Recoveries of 1-(2-hydroxy-1-naphthylazo)-2-naphthalene sulfonic acid added to D&C Red No. 12 as determined by TLC and spectrophotometry (2 determinations each of 5 samples)

Added, μg	Found, μg	Recovered, %
40.3[a]	32.1	80
41.9[a]	34.2	82
67.7	54.8	81
47.4	38.2	81
27.1	28.6	105
47.4	36.6	77
27.1	24.2	89
67.7	57.2	85
6.8	7.6	112
13.5	15.3	113

[a] Test sample contained 0.76% (average of 3 determinations) ortho color, as the sodium salt. The values are the totals after 33.8 μg was added.

in another subsidiary color (ortho color) in the final color.

Two recoveries were run on each of 5 different samples of D&C Red No. 12. Only one of these contained the ortho color. Table 1 shows the results of 10 recoveries. Recoveries averaged about 90%. Qualitative chromatographic tests indicate that the method is equally applicable to D&C Red Nos. 10, 11, 12, and 13. These tests also indicate that the present procedure could be used for the simultaneous determination of both the ortho isomer and Bronner's color, if both were present in a given sample. None of the samples analyzed in this laboratory contained both subsidiary colors.

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DAIRY PRODUCTS

Collaborative Study of the Extraction of Plant Sterols from Adulterated Butter Oil Using a Digitonin-Impregnated Celite Column

By DENIS E. LACROIX (Dairy Products Laboratory, Eastern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture, Washington, D.C. 20250)

A rapid screening method for the analysis of the phytosterol, β-sitosterol, in butter oil adulterated with vegetable oil has been studied collaboratively. The sterols are removed from the adulterated butter oil by passing the sample through a digitonin-impregnated Celite 545 column, eluting the sterols with dimethyl sulfoxide, and analyzing the eluate for β-sitosterol by gas-liquid chromatography using a 3% JXR column. The average coefficient of variation for those samples containing more than 4 mg β-sitosterol/100 g adulterated butter oil is 12.6%. Therefore, β-sitosterol can be used as an index to qualitatively detect vegetable oil adulteration of butter oil.

At the present time there is no rapid AOAC method for the determination of vegetable oil adulteration of butter oil. The method described in this paper is designed as a rapid screening method for analysis of β-sitosterol in adulterated butter oil. The method was first reported by Katz and Keeney (1), based on the digitonin-impregnated column procedure of Schwartz *et al.* (2). LaCroix (3) demonstrated the suitability of this procedure and a collaborative study was recommended based on the results of the investigation.

Collaborative Study

Each of 8 collaborators was sent 10 samples of butter oil obtained from the USDA Dairy Products Laboratory, Beltsville, Md. 20705. Nine of the samples contained 4 vegetable oils at 2 levels of adulteration, and 1 sample contained no vegetable oil. One of the samples was designated as a practice sample of stated β-sitosterol composition. The collaborators were instructed to obtain

satisfactory results on the known sample, using the test method, before proceeding with the unknown sample. Some precautionary remarks were included in the instructions. Collaborators were also instructed to report any difficulties and commentary associated with the method.

METHOD[1]

(Applicable to samples contg ≥4 mg free β-sitosterol/100 mg butter oil)

28.075 *Principle*

Free 3-β-OH sterols are removed from butter oil by complexing with digitonin and sterols are then removed from digitonide-Celite column by elution with dimethyl sulfoxide (DMSO). Butter oil has range of 0–1 mg β-sitosterol/100 g and ice cream has apparent value of ca 4 mg/100 g fat from emulsifiers.

28.076 *Reagents*

(a) *Diatomaceous earth.*—Celite 545, or equiv.

(b) *Digitonin.*—(Mann Research Laboratories, Mountain View Ave, Orangeburg, NY 10963).

(c) *β-Sitosterol std soln.*—2 μg β-sitosterol/μl $CHCl_3$. Prep. from Aldrich Chemical Co., 2371 N. 30th St, Milwaukee, WI 53210, reagent (64% β-sitosterol, 36% campesterol) or Applied Science Laboratories, Inc. reagent (90% β-sitosterol, 10% campesterol).

(d) *n-Hexane.*—Distill pure grade over KOH. (*Caution: See* **46.011, 46.037, 46.039,** and **46.061.**)

28.077 *Apparatus*

(a) *Gas chromatograph.*—Operating conditions: temps, column 225–245° and injection port and flame ionization detector 265–285°. Adjust N carrier gas flow (ca 50–60 ml/min) to obtain following retention times: cholesterol 16–18 min, campesterol 22–24 min, and β-sitosterol 28–30 min. Use 6′ × 4 mm id column contg 3% JXR silicone on 100–120 mesh Gas Chrom Q, or equiv., prepd as in **28.073(b)**, and condition column 24 hr at 250° with 15–20 psi N.

(b) *Performance.*—Monitor performance of gas chromatograph by noting sepn of campesterol and sitosterol expressed as peak resolution = $2D/(C + B)$, where D = distance between the 2 peak maxima, C = campesterol peak base width, and B = β-sitosterol peak base width. Peak resolution should be ≥1.6.

(c) *Injection technic.*—With 10 μl Hamilton microsyringe, draw 1 μl air into barrel, insert needle into soln, and draw desired amt into barrel. Remove needle from soln and draw 1 μl air into barrel. Note vol. on scale and adjust to desired vol., if necessary.

(d) *Preparation of std curve.*—Prep. std soln of 2 μg β-sitosterol/μl $CHCl_3$. (Det. composition of std as in **28.072(e)**.) Obtain std curves daily covering range 1–10 μg β-sitosterol, using ≥3 points. Plot area of β-sitosterol peak against μg β-sitosterol.

28.078 *Preparation of Column*

Dissolve, with heating, 300 mg digitonin in 5 ml H_2O, add to mortar and pestle contg 10 g Celite, and mix thoroly. (Packing material can be kept several months if stored at 5° in tightly closed container.) Transfer 3 g Celite-digitonin mixt. to 2 × 12 cm column and pack firmly, using packing rod. (Flow rate of tightly packed column is 0.5–0.75 ml/min.) Sat. column with 5 ml *n*-hexane and let flow thru packing until *n*-hexane reaches top of packing material. Use column immediately. Do *not* let dry.

28.079 *Preparation of Sample*

Dissolve 900 mg butter oil in 3 ml *n*-hexane. Quant. transfer soln, using disposable pipet, to digitonin-Celite column and let pass thru column until soln has entered packing material. Wash sample beaker twice with 2 ml *n*-hexane and add each wash to column, rinsing column sides. Wash column with five 2 ml portions *n*-hexane. After all hexane has entered column, wash with five 2 ml portions benzene. After last portion benzene reaches top of packing material, stop flow and wash column tip thoroly with benzene to remove traces of fat. (Failure to wash column sides and column tip with solv. will result in poor chromatograms due to interference from triglycerides.) Discard hexane and benzene. Elute sterols with 10 ml DMSO and collect entire eluate in 15 ml screw-cap centrf. tube.

Add 3 ml *n*-hexane to eluate, shake, and centrf. Transfer upper layer contg sterols to second screw-cap centrf. tube. Repeat extn of DMSO layer in first tube with two 4 ml portions *n*-hexane-benzene (1 + 1), carefully transferring upper layer to second tube each time. Vigorously shake pooled upper layers with 3 ml H_2O and centrf. until clear. Remove upper layer and evap. under N or filtered air in 30 ml beaker on steam bath. Transfer residue to 0.5 dram screw-cap vial with two 0.8 ml portions $CHCl_3$. After evapg solv. with N or filtered air over steam bath, redissolve sterols in 0.1 ml $CHCl_3$ for GLC analysis.

28.080 *Determination*

Inject 2–8 μl extd sample and calc. β-sitosterol by converting peak area to wt, using daily std curve.

[1] The secton numbers within the method are those for the 11th ed. of *Official Methods of Analysis*, 1970 secs. **28.072(e)** and **28.073(b)**, see p. 627 (4). Cautionary notes, if present, refer to the new chapter on safety, Chapter 46.

Table 1. Collaborative results for β-sitosterol content of adulterated butter oil (mg β-sitosterol/100 g butter oil)

Lab.	5% Adulteration by: 1. Cottonseed Oil	2. Soybean Oil	3. Safflower Oil	4. Peanut Oil	2% Adulteration by: 5. Cottonseed Oil	6. Soybean Oil	7. Safflower Oil	8. Peanut Oil	9. Butter Oil Blank	10. Known Peanut Oil (5%)
A	13.9	6.7	3.1	8.2	5.9	3.3	2.6	4.4	trace	8.3
B	13.9	6.4	4.2	8.5	6.2	1.9	1.1	2.3	trace	8.2
C[a]	11.3	7.0	3.2	8.2	4.6	2.9	1.1	3.0	0	8.1
D[a]	13.9	6.1	5.1	7.9	7.0	4.2	3.2	4.0	0	7.4
E[a]	13.9	5.6	4.9	9.2	6.9	2.8	3.3	4.3	0	7.2
F[a]	15.9	5.7	3.9	6.1	5.7	2.5	1.9	2.6	trace	7.1
G[a, b]	20.0	8.7	8.3	10.9	8.8	3.7	3.5	4.6	trace	9.7
Mean	13.8±1.4	6.3±0.54	4.1±0.8	8.0±1.0	6.1±1.0	2.9±0.8	2.2±1.0	3.4±0.9	0	7.8±0.7
Coeff. of var.	10.2%	8.6%	19.6%	12.5%	16.2%	36.2%	45.4%	37.8%	0	8.8%

[a] Average of two determinations.
[b] Eliminated by Youden's ranking test.

Calc. mg β-sitosterol/100 g butter oil = (μg from curve/1000) × (100/μl injected) × (100/g sample).

Identify peaks from butter oil samples by comparing their retention time to retention time of known compds. Relative retention times are cholesterol 1.0, campesterol 1.4, and β-sitosterol 1.7.

Results and Discussion

The results obtained by the collaborators are summarized in Table 1. Those results which were reported in duplicate for each sample were averaged and 1 value was reported in the table. Seven of 8 laboratories submitted reports. However, Laboratory G reported consistently high values on all samples; those results were omitted on the basis of the ranking test (5).

All the collaborators reported difficulty with quantitation of samples containing less than 4 mg β-sitosterol/100 mg butter oil, although the β-sitosterol could be qualitatively observed. Since the level of β-sitosterol present in samples 6, 7, and 8 was near the operational sensitivity limits of the instruments used by the collaborators, the high coefficient of variation of these samples is not surprising in view of these comments. The overall coefficient of variation of those samples containing 4 mg or more β-sitosterol/100 mg butter oil is 12.6%.

All values in Table 1 which were not rejected by the ranking test were subjected to the Dixon outlier test (6). No values were eliminated.

Also, the β-sitosterol content of butter oil adulterated with safflower oil proved to be the most difficult to quantitate due to the low β-sitosterol content as well as the presence of other sterol peaks which are in close proximity to β-sitosterol (1, 7, 8).

Figure 1 shows the GLC pattern of the sterols of cottonseed oil and butter oil adulterated at

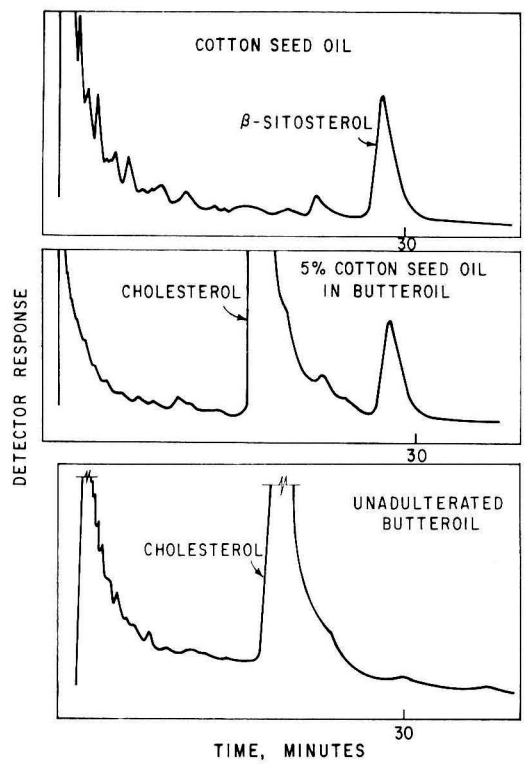

FIG. 1—GLC patterns of sterols of cottonseed oil, butter oil adulterated with cottonseed oil, and unadulterated butter oil.

5% with cottonseed oil and the GLC pattern of unadulterated butter oil. GLC analysis of 4 market brands of butter oil, as well as several preparations of butter oil prepared at the USDA Dairy Products Laboratory, showed that little or no β-sitosterol could be observed. Therefore, the presence of a sizable β-sitosterol peak would provide a suitable index of vegetable oil adulteration of butter oil.

The identification of some vegetable oils by their sterols has been published (1, 7, 8). Thus, in addition to detecting low levels of adulteration, correlation of the total sterol GLC pattern could be used as a means of identifying some types of oils used as adulterating agents.

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Regulatory Agency Acceptance of the Interpretation of the Freezing Point Value of Milk as Part of the Official Thermistor Cryoscopic Method

By R. W. HENNINGSON (Department of Dairy Science, Clemson University, Clemson, S.C. 29631)

A 1968 North American authentic milk sample survey determined that the mean freezing point value of milk is −0.5404°C. Statistical concepts permit the calculation of a value, −0.525°C, 2.326 standard deviations from a mean with 95% confidence that 99% of all subsequent observations will be below the value. Based on this survey, it was recommended that the *Interpretation* of the freezing point value of milk be made a part of the official final action thermistor cryoscopic method for the determination of the freezing point value of milk, and include the following: an upper limit for the freezing point value of milk, an official definition of an authentic milk sample, and a logical procedure for the confirmation of added water.

A copy of the report and an explanatory letter were sent to approximately 100 North American regulatory agencies. Reports were received from 49 regulatory agencies with 39 favoring the recommendations, 6 opposing the recommendations, and 4 having no opinion. It is recommended that the *Interpretation* of the freezing point of milk be included in both the thermistor cryoscopic and the Hortvet methods.

Specific directions for the thermistor cryoscopic method for the determination of the freezing point value of milk were adopted as official in 1967 (1). The Hortvet and thermistor official methods (2) have always included an interpretation of the freezing point value of milk obtained in the determination, but this interpretation is unofficial, incomplete, and based on inadequate information (3).

In 1968, a cooperative North American, authentic milk sample survey (3) determined that the mean freezing point value of milk is −0.5404°C. Accepted statistical techniques permitted the calculation of a value 2.326 standard deviations from the mean as an upper limit for the freezing point of "normal" milk. This value, −0.525°C, may be used as an upper limit with 95% confidence that 99% of all subsequent observations will be below the value. It was recommended (*1*) that the *Interpretation* of the freezing point value of milk be made a part of the official final action thermistor cryoscopic method for the determination of the freezing point value of milk; (*2*) that the *Interpretation* include a scientifically chosen upper limit for the freezing point value of milk above which milks will be designated "presumptive added water"; (*3*) that the *Interpretation* include an official definition of an authentic milk sample based on the criteria of maximum information, ease and economy of sampling, and recognition of modern milking procedures; (*4*) that the *Interpretation* include a logical procedure for the confirmation of "added water" or for the designation of a milk as "free of added water"; and (*5*) that the *Interpretation* be stated as in the 1968 Report of the Associate Referee (3). The Associate Referee, the Referee, Subcommittee C, and the Association, realizing that the recommendations would be of little value unless accepted by regulatory agencies, decided to submit the recommendations to regulatory agencies for a considered opinion before acting on them.

Survey

A copy of the Associate Referee's published report (3), a report form for indicating approval of, or opposition to, the adoption of the report recommendations, and a detailed letter of explanation were sent to approximately 100 North American regulatory agencies. The head of each regulatory agency, with jurisdiction, was requested to consult with the appropriate professional staff members and indicate whether the agency favored or opposed the recommendations of the Associate Referee.

Results

Reports were received from 49 regulatory agencies: 39 favored and 6 opposed the addition of the *Interpretation* as part of the official final action thermistor cryoscopic method for the determination of the freezing point value of milk. Four indicated no opinion. Of the 6 agencies expressing opposition, 3 indicated that they were

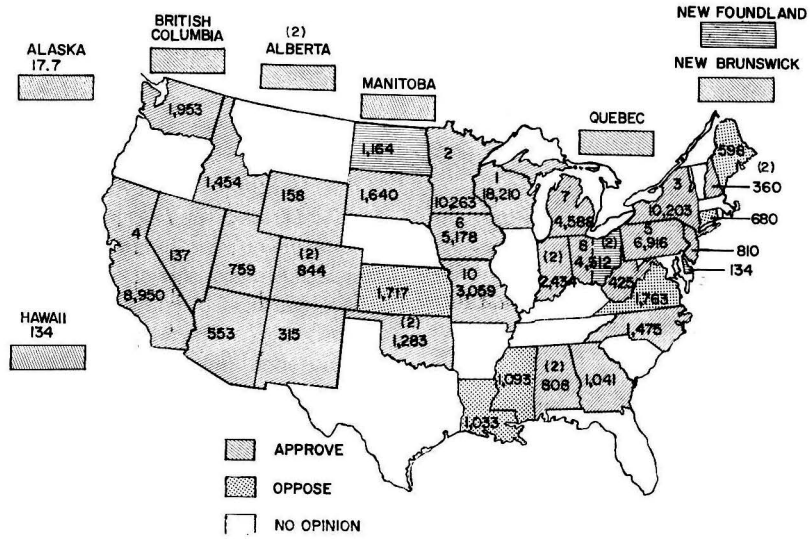

FIG. 1—States and Provinces responding to the regulatory agency survey on the addition of an interpretation of the freezing point value of milk as part of the official thermistor cryoscopic method.

Numbers 1–10 indicate 9 of the 10 leading States in milk production. Larger numbers indicate the milk production of the appropriate State in millions of pounds. The designation (2) indicates States from which replies were received from both regulatory agencies.

opposed only to a time limit for obtaining confirmatory milk samples from herds producing milk designated as "presumptive added water."

In terms of political subdivisions, 28 States and 5 Provinces favored the addition of the *Interpretation* as presented, while 6 States opposed the addition. Three States and 1 Province indicated no opinion. In 6 States where responsibility for determination of the freezing point value of milk was shared by 2 State regulatory agencies, both agencies favored the addition of the *Interpretation*. In no State where responsibility for the determination of the freezing point value of milk was shared did 2 regulatory agencies report a difference of opinion. Three of the 6 States opposed to the addition of the *Interpretation* did so only because of the confirmatory sample time limit; they favored the addition of the *Interpretation* in principle. The survey results are summarized in Table 1 and Fig. 1.

Discussion

Since the regulatory agencies of 36 of the 42 political subdivisions replying to the survey approved or did not oppose the addition of the proposed *Interpretation* to the thermistor cryoscopic method, it appears that this change is needed and would be of value to regulatory agencies. This belief is strengthened by consideration of the statements of 3 of the 6 regulatory agencies opposing the addition: Two opposing regulatory agencies stated that they considered 48 hr for securing a confirmatory herd milk sample to be too short a time to fit into their operating schedule. The third agency felt this was a problem but also was opposed to the 0.010°C confirmatory limit, feeling this was too lenient. One of the agencies favoring the *Interpretation* also felt that the 0.010°C confirmatory limit was too lenient.

Consideration of the fraction of the United

Table 1. Response of North American regulatory agencies to survey requesting an opinion regarding the addition of an interpretation as part of the thermistor cryoscopic method

Political Subdivision	Response		
	Favor	Oppose	No Opinion
State	28[a]	6[b]	3
Province	5	0	1

[a] Of the 6 States which have 2 regulatory agencies sharing responsibility for determining the freezing point value of milk, both agencies favored the addition.

[b] Three States opposed addition because of a short confirmatory sample time limit; 1 State opposed addition because of 0.010°C confirmatory limit.

Table 2. Fraction of United States milk supply produced in States favoring or opposing addition of an interpretation as part of the thermistor cryoscopic method

Response Category of States	Per Cent of U.S. Milk Supply Produced[a]
Favoring	75.5
Favoring (except for time element or confirmatory limit)	3.6
No opinion	1.1
Opposing	2.3
Fraction of U.S. milk supply covered or accounted for	82.5

[a] Based on April 1969 USDA Crop Reporting Board information (4).

States milk production (4) represented by the States approving or not opposing the addition of the proposed *Interpretation* also strengthens the belief that the change is needed and will be of value to regulatory agencies. This information is summarized in Table 2. It is also of interest to note that the regulatory agencies in 9 of the 10 leading milk-producing States (4) favor the addition of an interpretation as part of the method.

Some regulatory agencies included comments with the survey report form which they returned. All such comments are reported below, except those dealing with the time limit for confirmatory milk samples and the confirmatory limit of 0.010°C which were previously mentioned. No regulatory agency opposing the addition of an interpretation to the thermistor cryoscopic method included comments.

"The method of sampling, method of analysis, the geographical distribution of samples, and the statistical evaluation of the findings have been considered carefully. Because of the small standard error, confidence can be placed in the mean freezing point value and the calculated freezing point value. The uniform use of a specific, revised upper limit as part of the method will definitely be of benefit to both milk producers and milk consumers."

"After studying the proposed interpretation to be added to the official test for added water, I agree that it would increase the usefulness of the test."

"After consulting people involved in our laboratories (health), we are in favor of the proposed interpretation for the determination of the freezing point value of milk."

This report of the Associate Referee was presented at the 83rd Annual Meeting of the AOAC, Oct. 13–16, 1969, at Washington, D.C.

Recommendations

Although this report concerns the application of the *Interpretation* to the thermistor cryoscopic method only, the *Interpretation* will be equally applicable to the Hortvet method for the determination of freezing point of milk. Accordingly, it is recommended—

(1) That the interpretation of the freezing point value of milk be made a part of the official final action methods **15.037–15.041** (10th edition) for the determination of the freezing point value of milk and designated accordingly.

(2) That the *Interpretation* include a scientifically chosen upper limit for the freezing point value of milk above which milks will be designated "presumptive added water" and an official definition of an authentic sample based on the criteria of maximum information, ease and economy of sampling, and recognition of modern milking procedures.

(3) That the *Interpretation* include a logical procedure for the confirmation of added water and for the designation of a milk as water-free.

(4) That the *Interpretation* be printed as follows:

16.073[1] **Interpretation**

Official First Action.—If fp is −0.525° or below, milk *may* be presumed to be H_2O-free *or may* be confirmed as H_2O-free by tests specified below. If fp is above −0.525°, milk *will* be designated "presumptive added H_2O" and *will* be confirmed as "added H_2O" or "H_2O-free" by tests specified below. Evaluate extreme daily fluctuations in fp of herd, pooled herd, or processed milk for presence of added H_2O.

To confirm herd milk as "added H_2O" or "H_2O-free," det. fp of authentic sample of herd milk obtained $\leq$48 hr after sample to be "confirmed." Authentic sample is sample of milk from 1 complete, supervised herd milking (either AM or PM but beginning not <11 or >13 hr after beginning of previous milking) obtained from bulk tank after entire herd has been milked thru approved, properly sanitized, and thoroly drained milking system into empty

[1] The section numbers within the method are those for the 11th ed. of *Official Methods of Analysis*, 1970. Cautionary notes, if present, refer to the new chapter on safety, Chapter 46.

The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee C and were adopted by the Association. See *JAOAC* **53**, 384 (1970).

bulk tank but before rinsing or washing of system has begun. Compare fp of authentic sample and sample to be confirmed. If fps differ by $\leq 0.010°$, sample is confirmed as H_2O-free.

To confirm pooled herd milk as "added H_2O" or "H_2O-free," det. fp of authentic samples of all herd milks composing pooled herd milk, calc. weighted av. fp for the authentic samples of herd milk, and compare values as above.

To confirm processed milk as "added H_2O" or "H_2O-free," det. fp of samples of all pooled herd milk received by processing plant, calc. weighted av. fp for milk received, and compare with fp value to be confirmed. If fps differ by $\leq 0.010°$, processed sample is confirmed as H_2O-free during processing. If 1 or more samples of pooled herd milk are "presumptive added H_2O," proceed as above for pooled herd milk. Fp for pasteurized-homogenized milk should be same as that of pooled herd milk unless processing includes vac. pasteurization, which raises fp approx. 0.005°.

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VITAMINS AND OTHER NUTRIENTS

Collaborative Study of Extraction Methods for Fluorometric Assay of Riboflavin

By E. DE RITTER (Food and Agricultural Products Development Department, Hoffmann-La Roche Inc., Nutley, N.J. 07110)

The AOAC fluorometric method for riboflavin is not applicable in the presence of materials that adsorb riboflavin. Deutsch *et al.* described a modified extraction with methanol-pyridine-water-glacial acetic acid (30 + 10 + 10 + 1) which yields higher recovery of riboflavin from samples containing interfering adsorbents. A collaborative study of 4 samples confirmed the more efficient extraction of riboflavin by the modified method. The study also shows that the permanganate oxidation step is unnecessary for extracts of relatively high potency products such as concentrates, premixes, and multivitamin supplements. It is recommended that an alternative method be adopted as official first action, with the following modifications: extracting with the new solvent mixture, omitting the permanganate treatment of the extract, and measuring the fluorescence of the standard riboflavin solution separately rather than after addition to an aliquot of sample solution.

This report of the Associate Referee was presented at the 83rd Annual Meeting of the AOAC, Oct. 13–16, 1969, at Washington, D.C.

Deutsch *et al.* (1) reported on the extensive use of an efficient method for extracting riboflavin from samples containing adsorbents. The extracting solution consisted of methanol-pyridine-water-glacial acetic acid (30 + 10 + 10 + 1). Refluxing samples 1 hr in this solvent system yielded higher recoveries of riboflavin from a variety of pharmaceutical preparations, food supplements, and feed premixes and supplements than the normal hot extraction with dilute HCl.

Samples have been encountered frequently in this laboratory which require the modified extraction method to achieve complete recovery of riboflavin. Typical examples are the feed premixes listed in Table 1, which were assayed in duplicate in each of 3 different laboratories by both the AOAC method (2) and the modified extraction procedure described below. Recoveries of riboflavin by the AOAC method averaged 86–91% of the values obtained by the modified procedure.

For further evaluation of the modified extraction method, a collaborative study was prepared

Table 1. Comparative assays of riboflavin in feed premixes by AOAC and modified extraction methods

Premix	Method	g Riboflavin/lb			
		Lab 1	Lab 2	Lab 3	Av.
A	AOAC	1.43	1.19	1.18	1.27
	Modified	1.48	1.38	1.34	1.40
B	AOAC	1.41	1.31	1.24	1.32
	Modified	1.46	1.48	1.43	1.46
C	AOAC	1.32	1.40	1.34	1.35
	Modified	1.62	1.55	1.54	1.57
D	AOAC	1.35	1.37	1.34	1.36
	Modified	1.52	1.46	1.54	1.50

with 4 samples: a concentrate, 2 feed premixes, and a multivitamin tablet. Seven collaborators participated in the study; each was given a copy of the AOAC method and the modified method and asked to assay the 4 samples in duplicate. It was requested that the 2 assays be run on different days and by a different analyst, if possible. The approximate level of riboflavin in each sample was indicated.

As a second part of this study, the collaborators were asked to omit the permanganate oxidation step and to dilute the extracts prepared by both the AOAC and the modified methods directly to the desired potency for fluorescence reading (usually about 0.1 μg/ml) and to read samples and riboflavin standard solutions separately. Extensive experience with relatively high potency samples of this type had indicated that the permanganate oxidation step and the use of an internal standard procedure are unnecessary. Reading a separate standard solution (external standard) inherently gives more precision than reading a standard mixed with a separate aliquot of sample dilution (internal standard).

METHOD[1]

(Not applicable in presence of materials that adsorb riboflavin)

(*Caution: See* **46.008.**)

39.039 *Apparatus*

Photofluorometer.—Use fluorometer suitable for accurately measuring fluorescence of solns contg riboflavin in concns of 0.05–0.2 μg/ml. Input filter of narrow *T* range with max. ca 440 nm and output filter of narrow *T* range with max. ca 565 nm have been found satisfactory.

39.040 *Reagents*

(Do not shake std solns stored under toluene.)

(a) *Riboflavin std solns.*—(*1*) *Stock soln.*—100 μg/ml. Dissolve 50 mg USP Riboflavin Ref. Std, previously dried and stored in dark in desiccator over P_2O_5, in 0.02*N* HOAc to make 500 ml. (To facilitate soln, warm with ca 300 ml 0.02*N* HOAc on steam bath with constant stirring until dissolved, cool, and add 0.02*N* HOAc to make 500 ml.) Store under toluene at ca 10°.

(*2*) *Intermediate soln.*—10 μg/ml. Dil. 100 ml stock soln to 1 L with 0.02*N* HOAc. Store under toluene at ca 10°.

(*3*) *Working soln I.*—1 μg/ml. Dil. 10 ml intermediate soln to 100 ml with H_2O. Prep. fresh for each assay.

(*4*) *Working soln II.*—0.1 μg/ml. Dil. 10 ml intermediate soln to 1 L with H_2O. Prep. fresh for each assay.

(b) *Sodium hydrosulfite.*—High purity and stored to avoid undue exposure to light and air. Check suitability as follows: To each of ≥2 tubes add 10 ml H_2O and 1 ml std riboflavin soln contg 20 μg/ml, and proceed as in **39.042** with respect to addn of HOAc, $KMnO_4$ soln, and H_2O_2 soln. Then when 8 mg $Na_2S_2O_4$ is added with mixing, riboflavin should be completely reduced in ≤5 sec.

(c) *Extraction soln.*—Mix 300 ml MeOH, 100 ml pyridine, 100 ml H_2O, and 10 ml HOAc. (Proportionate quantities may be prepd.)

39.041 *Preparation of Sample Solution*

(Thruout all stages of method protect solns from undue exposure to light and keep at pH < 7.0. Where directed to filter thru paper, use paper known not to adsorb riboflavin (ash-free papers have been found satisfactory).)

Place measured amt of sample in suitable size flask and proceed by one of following methods:

(a) *For dry or semidry materials containing no appreciable amount of basic substances.*—Add vol. 0.1*N* HCl equal in ml to ≥10 times dry wt sample in g; resulting soln must contain ≤0.1 mg riboflavin/ml. If material is not readily sol., comminute so that it may be evenly dispersed in liq. Then agitate vigorously and wash down sides of flask with 0.1*N* HCl.

Heat mixt. in autoclave at 121–123° (1.1–1.2 kg/sq cm) 30 min and cool. If lumping occurs, agitate mixt. until particles are evenly dispersed. Adjust, with vigorous agitation, to pH 6.0–6.5 with NaOH soln; then immediately add dil. HCl until no further pptn occurs (usually ca pH 4.5, isoelec. point of many proteins).

[1] The section numbers within the method are those for the 11th ed. of *Official Methods of Analysis*, 1970. Cautionary notes, if present, refer to the new chapter on safety, Chapter 46.

Dil. mixt. to measured vol. contg >0.1 μg riboflavin/ml and filter thru paper. (In case of mixt. difficult to filter, centrfg and/or filtering thru fritted glass, using suitable analytical filter-aid, may often be substituted for, or may precede, filtering thru paper. Ash-free filter paper pulp and Celite Analytical Filter-Aid have been found satisfactory.) Take aliquot of clear filtrate and check for dissolved protein by adding dropwise, first dil. HCl, and if no ppt forms, then, with vigorous agitation, NaOH soln, and proceed as follows:

(*1*) If no further pptn occurs, add, with vigorous agitation, NaOH soln to pH 6.8, dil. soln to final measured vol. contg ca 0.1 μg riboflavin/ml, and if cloudiness occurs, filter again.

(*2*) If further pptn occurs, adjust soln again to point of max. pptn, dil. to measured vol. contg >0.1 μg riboflavin/ml, and then filter. Take aliquot of clear filtrate and proceed as in (*1*).

If riboflavin content of sample is so low that these requirements cannot be met, conc. clear filtrate obtained at ca pH 4.5 to suitable vol. with heat under reduced pressure. Filter if necessary and proceed as in (*1*).

(b) *For dry or semidry materials containing appreciable quantities of basic substances.*—Adjust mixt. to pH 5.0–6.0 with dil. HCl. Add such amt of H_2O that total vol. liq. is equal in ml to ≥10 times dry wt sample in g. (Resulting soln must contain ≤0.1 mg riboflavin/ml.) Then add equiv. of 1.0 ml 10*N* HCl/100 ml liq. and proceed as in (a), beginning with second sentence.

(c) *For liquid materials.*—Adjust pH to 5.0–6.0 with dil. HCl or, with vigorous agitation, NaOH soln, and proceed as in (b), beginning with second sentence.

(d) *For concentrates, premixes, and multivitamin supplements (Official First Action).*—Place measured amt sample in flask and add vol. extn soln equal in ml to ≥10 times dry wt sample in g; resulting soln must contain ≤0.1 mg riboflavin/ml. If sample is not readily sol., comminute so that it may be dispersed evenly in liq. Then agitate vigorously and wash down sides of flask with extn soln.

Reflux mixt. 1 hr and cool. If lumping occurs, agitate mixt. until particles are dispersed evenly. Dil. mixt. to measured vol. with extn soln and let any undissolved particles settle, or filter or centrf., if necessary. Take aliquot of clear soln and dil. with H_2O to measured vol. contg ca 0.1 μg riboflavin/ml and filter if soln is not clear. Proceed with detn, **39.043.**

39.042 ***Determination***

To each of ≥4 tubes (or reaction vessels) add 10 ml sample soln. (If fluorometer is type that requires tubular cuvets, all reactions may be carried out in matched set of these cuvets.) To each of ≥2 tubes add 1 ml std riboflavin working soln I and mix, and to each of ≥2 remaining tubes, add 1 ml H_2O and mix. To each tube add 1 ml HOAc and mix; add, with mixing, 0.5 ml 4.0% $KMnO_4$ soln (quantity may be increased for sample solns that contain excess of oxidizable material, but ≤0.5 ml in excess of that required to complete oxidn of foreign material should be added). Let stand 2 min; then to each tube add, with mixing, 0.5 ml 3.0% H_2O_2 soln; permanganate color must be destroyed within 10 sec. Shake vigorously until excess O is expelled. If gas bubbles remain on sides of tubes after foaming stops, remove by tipping tubes so that soln flows slowly from end to end.

In fluorometer, measure fluorescence of sample soln contg 1 ml added std riboflavin working soln I and call this reading *A*. Next, measure fluorescence of sample soln contg 1 ml added H_2O and call this reading *B*. Add, with mixing, 20 mg *powd* $Na_2S_2O_4$ to ≥2 tubes, measure fluorescence within 5 sec, and call reading *C*. Calc. on basis of aliquots taken as follows:

mg Riboflavin/ml final sample soln = $[(B - C)/(A - B)] \times 0.10 \times 0.001$. (Value of $(B - C)/(A - B)$ must be ≥0.66 and ≤1.5.)

Note: Quantity of $Na_2S_2O_4$ appreciably >20 mg may reduce foreign pigments and/or foreign fluorescing substances, thereby causing erroneous results.

39.043 ***Alternative Determination—Official First Action***

(Applicable to high potency samples)

Add 10 ml sample soln to ≥2 cuvets. Add 10 ml working std soln II to each of another set of ≥2 cuvets. Add 1 ml HOAc to each tube and mix. Measure fluorescence of sample solns and std solns in fluorometer. Add, with mixing, 20 mg *powd* $Na_2S_2O_4$ to 1 tube each of std and sample and measure fluorescence within 5 sec. Calc. on basis of aliquots taken as follows: mg riboflavin/ml final sample soln = $[(I_u - Q_u)/(I_s - Q_s)] \times (0.1 \times 0.001)$, where I_u and I_s = fluorescence intensities of sample and std, resp., and Q_u and Q_s = fluorescences of sample and std, resp., after $Na_2S_2O_4$ addn.

Results and Recommendations

The results of the 7 collaborators are summarized in Table 2. For samples I, II, and III, the average results obtained by the modified extraction method are higher than by the AOAC method. The difference is particularly great for the lower potency premix (Sample II), where the AOAC results averaged only about half the averages by the modified extraction method. Average

differences between the results by the 2 extraction methods for Sample IV were small.

The permanganate treatment of the extracts in both methods appears to be unnecessary for high potency samples of this type. There is a definite increase in the coefficient of variation in

Table 2. Collaborative study of fluorometric assay of riboflavin

Coll.	Assay	AOAC Method: Present	AOAC Method: No $KMNO_4$, Ext. Std	Modified Extraction: $KMNO_4$, Int. Std	Modified Extraction: No $KMNO_4$, Ext. Std
Sample I: Concentrate[a] (% by Weight)					
1	1	12.8	17.2	26.0	26.3
	2	16.8	18.7	25.0	25.8
	3	—	—	27.7	25.8
2	1	20.4	19.7	28.4	26.7
	2	20.4	21.2	25.7	26.3
3	1	24.8	25.0	23.8	30.0
	2	24.8	26.6	23.8	30.0
	3	23.8	27.4	23.8	28.4
	4	23.8	27.4	23.8	28.4
4	1	23.4	21.8	31.6	27.6
	2	24.9	18.6	26.1	25.8
5	1	25.0	26.0	24.0	26.0
	2	15.0	25.3	23.0	25.5
6	1	20.7	20.0	25.4	24.8
	2	20.3	20.8	25.1	23.9
7	1	28.2	24.4	—	25.7
	2	21.7	23.0	—	29.1
Av.		21.7	22.7	25.6	26.8
Std dev.		4.1	3.4	2.3	1.7
Coeff. of var., %		18.8	15.0	9.0	6.3
Sample II: Premix 1 (mg/lb)					
1	1	215	202	322	319
	2	187	197	336	322
	3	—	—	328	318
2	1	253	262	356	340
	2	246	245	294	321
3	1	176	132	484	359
	2	164	132	484	359
	3	151	148	455	370
	4	151	148	455	370
4	1	190	188	376	376
	2	300	173	326	307
5	1	35	166	426	300
	2	38	51	456	287
6	1	136	133	254	290
	2	156	147	233	238
7	1	251	200	—	334
	2	212	190	—	327
Av.		179	170	372	326
Std dev.		72	50	83	36
Coeff. of var., %		40	29	22	11

(*Continued*)

Table 2. (*Continued*)

Coll.	Assay	AOAC Method: Present	AOAC Method: No $KMNO_4$, Ext. Std	Modified Extraction: $KMNO_4$, Int. Std	Modified Extraction: No $KMNO_4$, Ext. Std
Sample III: Premix 2[b] (mg/lb)					
1	1	1120	1140	1300	1300
	2	1120	1130	1270	1280
	3	—	—	1350	1370
2	1	1150	1150	1380	1380
	2	1160	1180	1320	1320
3	1	1153	1208	1308	1208
	2	1144	1208	1362	1208
	3	1235	1144	1362	1480
	4	1235	1144	1416	1480
4	1	1238	1155	1341	1561
	2	1637	1177	1197	1362
5	1	1300	1320	1035	1340
	2	1200	1140	1500	1300
6	1	1210	1192	1169	1178
	2	1242	1180	1066	1099
7	1	1285	1185	—	1353
	2	1187	1210	—	1300
Av.		1226	1179	1292	1325
Std dev.		123	46	127	116
Coeff. of var., %		10.0	3.9	9.8	8.4
Sample IV: Tablets[c] (mg/Tablet)					
1	1	2.51	2.63	2.80	2.92
	2	2.49	2.62	2.59	2.89
	3	—	—	2.88	3.03
2	1	2.45	2.55	3.04	2.96
	2	2.67	2.62	3.04	2.98
3	1	2.60	3.70	2.89	3.30
	2	2.60	3.70	2.98	3.30
	3	2.42	3.90	2.87	3.60
	4	2.42	3.90	2.69	3.60
4	1	3.15	2.57	3.19	3.21
	2	3.60	2.58	2.57	2.73
5	1	2.00	2.50	1.40	2.43
	2	2.25	2.90	1.45	2.60
6	1	2.48	2.71	2.74	2.78
	2	2.68	2.57	2.32	2.51
7	1	3.30	2.84	—	3.20
	2	2.78	2.82	—	3.06
Av.		2.65	2.94	2.63	3.01
Std dev.		0.40	0.52	0.54	0.34
Coeff. of var., %		15.1	17.7	20.5	11.3

[a] 26% riboflavin added.
[b] 1333 mg riboflavin/lb added.
[c] 2.88 mg riboflavin/tablet added.

most cases when the permanganate oxidation is employed. Wide differences between duplicate assays in a given laboratory are observed more frequently when permanganate treatment is used.

The modified extraction method, omitting the permanganate oxidation step, has the following advantages: (*1*) It yields best agreement with the added levels of riboflavin, which were known for all samples except Sample II; (*2*) riboflavin recoveries are higher than those obtained with the present AOAC extraction method for Samples I, II, and III; (*3*) standard deviations are lower when the permanganate oxidation is omitted; and (*4*) it is more convenient. It is recommended that an alternative method for fluorometric assay of riboflavin be adopted as official first action for concentrates, premixes, and multivitamin supplements in which the modified extraction procedure is utilized, the permanganate treatment of the extract is omitted, and the fluorescence of the standard riboflavin solution is measured separately rather than after addition to an aliquot of sample solution.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee D and was adopted by the Association. See *JAOAC* **53**, 391 (1970).

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Microbiological Assay of Vitamin B_6 and Its Components

By EDWARD W. TOEPFER and MARILYN M. POLANSKY (Human Nutrition Research Division, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md. 20705)

Nine collaborators studied a method for determining vitamin B_6 and pyridoxine, pyridoxal, and pyridoxamine in foods and like materials. The 3 vitamin B_6 components in food extracts were eluted in separate fractions from an ion exchange column and determined separately by microbiological assay, using the yeast *S. carlsbergensis* as the test organism. It is recommended that the method be adopted as official first action.

The AOAC collaborative studies on the microbiological assay procedure for total vitamin B_6 and for the vitamin B_6 components pyridoxine, pyridoxal, and pyridoxamine were initiated in 1968. The procedure is based on the method given in detail by Toepfer and Lehmann (1) with slight modifications as given by Toepfer *et al.* (2) and Polansky *et al.* (3). *Saccharomyces carlsbergensis* is the test organism for the microbiological assays.

For total vitamin B_6, the extract is diluted to a known volume containing about 1 ng pyridoxine/ml equivalent; pyridoxine is the standard. Results are expressed as μg pyridoxine/g sample. For the individual forms of vitamin B_6, the extract is separated into pyridoxine, pyridoxal, and pyridoxamine fractions on a Dowex AG 50W-X8 ion exchange resin column.

Collaborators were furnished with the detailed procedure, 4 samples, 3 vitamin B_6 standards, a culture of the test organism, ion exchange resin, and data sheets. Collaborators were instructed to make 2 determinations at 2 different times. Among 17 laboratories invited to participate in the study, 9 laboratories returned completed assays.

METHOD[1]

39.142 *Reagents*

(Work in subdued light with all solns contg vitamin B_6.)

(a) *Potassium acetate buffers.*—(*1*) *0.01M, pH 4.5.*—Dissolve 0.981 g KOAc in H_2O and dil. to 1 L. Adjust pH with HOAc. (*2*) *0.02M, pH 5.5.*—Dissolve 1.96 g KOAc in H_2O and dil. to 1 L. Adjust pH with HOAc. (*3*) *0.04M, pH 6.0.*—Dissolve 3.92 g KOAc in H_2O and dil. to 1 L. Adjust pH with HOAc. (*4*) *0.1M, pH 7.0.*—Dissolve 9.815 g KOAc in H_2O and dil. to 1 L. Adjust pH with HOAc or KOH soln.

(b) *Potassium chloride-phosphate buffer.*—pH 8.0. Dissolve 74.6 g KCl and 17.4 g K_2HPO_4 in 800 ml H_2O and adjust pH with HOAc. Dil. to 1 L.

(c) *Ion exchange resin.*—Dowex AG 50W-X8, 100–200 mesh.

(d) *Acid-hydrolyzed casein soln.* — 100 mg/ml. (*Caution: See* **46.011** and **46.015.**) Mix 100 g vitamin-free casein with 500 ml constant-boiling HCl (ca 5*N* HCl, 208 ml HCl dild to 500 ml with H_2O) and reflux 8 hr. Remove HCl from mixt. by distn under vac. until very thick sirup remains, keeping H_2O bath temp. <80°. Dissolve sirup in H_2O and conc. again in same manner. Redissolve sirup in H_2O.

Adjust to pH 4 with 40% NaOH, add H_2O to ca 600 ml, add 40 g activated C, stir 4 hr, and filter with vac. thru buchner with thin pad of HCl-washed Filter-Cel. Continue following activated C treatments only if soln is not clear and colorless: Add 20 g activated C to filtrate, stir 1 hr, and refilter. Repeat with fresh 10 g portion activated C and filter. When soln is clear and colorless, dil. to 1 L with H_2O. (Before soln is dild to vol., 2–3 ml 6*N* HCl may be added to extend time before microbial growth occurs.) Store in refrigerator.

(e) *Vitamin soln I.*—Dissolve 10 mg thiamine and 1 g inositol in ca 200 ml H_2O and dil. to 1 L. Store in refrigerator. (1 ml = 10 μg thiamine and 1 mg inositol.)

(f) *Vitamin soln II.*—Dissolve 10 mg biotin in 100 ml 50% alcohol. Store in refrigerator. (1 ml = 100 μg biotin.) Dissolve 200 mg Ca pantothenate and 200 mg niacin in ca 200 ml H_2O; add 8 ml biotin soln and dil. to 1 L with H_2O. Store in refrigerator. (1 ml = 200 μg each Ca pantothenate and niacin and 0.8 μg biotin.)

(g) *Salt soln I.*—Dissolve 17 g KCl, 10.3 g $MgSO_4.7H_2O$, 100 mg $FeCl_3.6H_2O$, and 100 mg $MnSO_4.H_2O$ in ca 800 ml H_2O. Add 2 ml HCl. Dissolve 5 g $CaCl_2.2H_2O$ in ca 100 ml H_2O, add to first soln, and dil. to 1 L with H_2O. Store in refrigerator. (1 ml = 17 mg KCl, 10.3 mg $MgSO_4.7H_2O$, 100 μg $FeCl_3.6H_2O$, 100 μg $MnSO_4.H_2O$, and 5 mg $CaCl_2.2H_2O$.)

(h) *Salt soln II.*—Dissolve 22 g KH_2PO_4 and 40 g $(NH_4)_2HPO_4$ in H_2O and dil. to 1 L. Store in refrigerator. (1 ml = 22 mg KH_2PO_4 and 40 mg $(NH_4)_2HPO_4$.)

(i) *Polysorbate 80 soln.*—Weigh 2.5 g polysorbate 80 (Tween 80) in small beaker. Transfer with warm (45°) H_2O and dil. to 500 ml. Store in refrigerator. (1 ml = 5 mg polysorbate 80.)

(j) *Citric acid soln.*—(1 + 1). Dissolve 50 g citric acid in 50 ml H_2O. Store at room temp. in bottle with plastic stopper.

(k) *Ammonium phosphate soln.*—(1 + 2). Dissolve 25 g $(NH_4)_2HPO_4$ in 50 ml H_2O. Store at room temp. in bottle with plastic stopper.

(l) *Pyridoxine, pyridoxal, and pyridoxamine std solns.*—Prep. sep. solns for each as follows: (*1*) *Stock soln.*—10.0 μg/ml. Dissolve 12.16 mg pyridoxine .HCl, 12.18 mg pyridoxal.HCl, and 14.34 mg pyridoxamine.HCl, resp., in 1*N* HCl and dil. to 1 L with 1*N* HCl. Store in g-s bottles in refrigerator.

(*2*) *Intermediate soln.*—1.0 μg/ml. Dil. 10 ml stock soln to 100 ml with H_2O.

(*3*) *Working soln.*—1.0 ng/ml. Dil. 5 ml intermediate soln to 500 ml with H_2O and mix. Dil. 10 ml to 100 ml with H_2O. Prep. fresh for each assay.

(m) *Mixed pyridoxine, pyridoxal, pyridoxamine solns (for liquid broth culture.).*—Pipet 2 ml of each intermediate soln (1.0 μg/ml) into 1 L vol. flask and dil. to vol. with H_2O.

(n) *Citrate buffer soln.*—Dissolve 100 g K citrate and 20 g citric acid in H_2O and dil. to 1 L. Store in refrigerator. (1 ml = 100 mg K citrate and 20 mg citric acid.)

(o) *Basal medium stock soln (for 200 tubes).*—To make 1 L medium, add to ca 400 ml H_2O: 100 ml citrate buffer, 100 ml hydrolyzed casein soln, 50 ml vitamin soln I, 25 ml vitamin soln II, 50 ml salt soln I, and 50 ml salt soln II. Dissolve 100 g glucose in this soln. Dissolve 22 mg DL-tryptophan, 27 mg L-histidine.HCl, 100 mg DL-methionine, 216 mg DL-isoleucine, and 256 mg DL-valine in 10 ml 10% HCl in small beaker and add to above. Add 20 ml polysorbate 80 soln. Adjust to pH 4.5 with citric acid (1 + 1) or $(NH_4)_2HPO_4$ (1 + 2) solns. Dil. to 1 L with H_2O. Store in Pyrex bottle plugged with cotton in refrigerator. Prep. ≤24 hr before use. When ready, steam 10 min and cool.

(p) *Test organism.*—*Saccharomyces carlsbergensis* (ATCC No. 9080). Maintain by weekly transfers on wort agar slants (q). Incubate these freshly seeded agar slants 24 hr at 30° and refrigerate.

[1] The section numbers within the method are those for the 11th ed. of *Official Methods of Analysis*, 1970. Cautionary notes, if present, refer to the new chapter on safety, Chapter 46.

(q) *Agar culture medium.*—Suspend 25 g Bactowort agar in ca 400 ml H_2O in marked 500 ml wide-mouth erlenmeyer. Plug with cotton, steam ca 10 min to dissolve agar, and adjust vol. to 500 ml. Pipet hot agar in ca 10 ml amts into 20 × 150 mm test tubes, plug with absorbent cotton, and autoclave 15 min at 121°. Since this medium has acid reaction, avoid overheating which results in softer medium. Tilt hot agar tubes to form slants and cool in this position.

(r) *Liquid culture medium.*—Pipet 5 ml mixed soln, (m), into 16 × 150 mm test tubes contg two 4 mm glass beads, plug with absorbent cotton, and autoclave 10 min at 121°. Add 5 ml steamed vitamin B_6-free basal medium, (o), under aseptic conditions. Store tubes in refrigerator.

(s) *Inoculum rinse.*—Pipet 5 ml H_2O into test tubes, plug with absorbent cotton, and autoclave 10 min at 121°. Add 5 ml steamed vitamin B_6-free basal medium, (o), under aseptic conditions. Store tubes in refrigerator.

39.143 *Assay Inoculum*

(*Caution: See* **46.005**.)

Incubate cells for inoculum on agar 24 hr at 30° before use. Transfer these cells under aseptic condition to liq. broth culture tubes. Plug with absorbent cotton held on with masking tape and place tubes on shaker 20 hr in 30° room. Replace cotton plugs aseptically with sterile rubber stoppers; centrf. 1.5 min at 2500 rpm. Decant liq. and resuspend in 10 ml inoculum rinse. Sep. by centrfg 1.5 min at 2500 rpm. Decant liq., resuspend in second 10 ml sterile inoculum rinse, centrf. 1.5 min, and decant. Cells suspended in third 10 ml inoculum rinse are assay inoculum.

39.144 *Preparation of Exchange Resin and Column*

To 250 g Dowex AG 50W-X8 (100–200 mesh) in H form add excess 6*N* KOH until supernatant is blue to litmus. Let settle, decant, and rinse resin with H_2O until supernatant is clear. Add ca 600 ml 3*N* HCl, stir, and heat 0.5 hr in boiling H_2O bath. Decant and repeat treatment with 3*N* HCl twice. Rinse resin until rinse H_2O is neutral. Add 6*N* KOH until pH is strongly basic and stir 1 hr. Rinse with H_2O until rinse H_2O is neutral. Suspend in 2*M* KOAc and store in refrigerator until needed. Just before use, wash resin with H_2O until H_2O is green to bromothymol blue. Resin can be regenerated, beginning with 3*N* HCl treatment.

Prep. tubes by sealing capillary stopcock, 1.5 mm bore and 5 cm side arms, to 19 mm id glass tube ≥40 cm long. Pour 5–10 ml H_2O onto tube. Place glass wool plug in bottom of tube and remove bubbles from capillary and glass wool. Rinse measured 30 ml prepd resin, settled out of H_2O suspension, into tube with H_2O. After resin settles in tube, place glass wool plug on top of resin. Rinse column with 50 ml hot H_2O followed by two 50 ml portions hot 0.01*M* KOAc (pH 4.5). pH of last buffer rinse from column should be 4.5; otherwise, more rinsing with buffer is required. Do not permit liq. level on column to fall below top glass wool plug at any time.

39.145 *Preparation of Sample*

Weigh 1–2 g dry product into 500 ml erlenmeyer. For plant products, add 200 ml 0.44*N* HCl and for animal products, add 200 ml 0.055*N* HCl. Autoclave plant soln 2 hr at 121°, and animal soln 5 hr at 121°. Cool to room temp., adjust to pH 4.5 with 6*N* or satd KOH, and dil. to 250 ml with H_2O in vol. flask. Filter thru Whatman No. 40 paper. Take 40–200 ml filtered aliquot for chromatgy.

39.146 *Chromatography*

Place desired amt filtered ext on ion exchange column in ca 50 ml portions and let pass completely thru with no flow regulation. Wash beaker and column 3 times with ca 5 ml portions hot 0.02*M* KOAc (pH 5.5), followed by similar washing to column sides. Wash column with same soln until total of 100 ml 0.02*M* KOAc (pH 5.5) soln is used. Elute pyridoxal with two 50 ml portions boiling 0.04*M* KOAc (pH 6.0), using 100 ml vol. flask as receiver. Elute pyridoxine with two 50 ml portions boiling 0.1*M* KOAc (pH 7.0), using 100 ml vol. flask as receiver. Elute pyridoxamine with two 50 ml portions boiling KCl-K_2HPO_4 (pH 8.0) soln, using 250 ml beaker as receiver. Adjust pH to 4.5. Dil. pyridoxine and pyridoxal eluates to 100 ml and pyridoxamine eluate to 200 ml with H_2O unless otherwise desired.

For std pyridoxine, pyridoxal, and pyridoxamine, mix 10 ml each intermediate soln, neutze with KOH, and adjust to pH 4.5 with HOAc. Put this soln on column, wash, and elute fractions as above. Dil. eluted pyridoxine and pyridoxal stds to 100 ml and dil. eluted pyridoxamine, after pH is adjusted to 4.5, to 200 ml with H_2O. Dil. eluted stds to 1.0 ng/ml with H_2O.

39.147 *Assay*

Heat clean tubes and glass beads 2 hr at 260°. Place two 4 mm glass beads in each 16 × 150 mm screw-cap glass culture tube. For std curve, pipet into triplicate tubes appropriate freshly prepd std working solns to give 0.0, 0.0, 1.0, 2.0, 3.0, 4.0, and 5.0 ng pyridoxine, pyridoxal, or pyridoxamine/tube. Similarly prep. set of tubes for eluted stds, omitting blanks. Dil. sample eluates from chromatgc column to contain ca 1 ng vitamin B_6 component/ml. Pipet 1, 2. 3, 4, and 5 ml dild eluates into triplicate tubes.

Pipet H_2O into all tubes to bring vol. to 5 ml/tube. Cap tubes with plastic caps with 1/8" hole thru top. Autoclave entire set 10 min at 121°. Cool tubes to room temp. Using automatic pipet with sterilized delivery attachments, pipet 5 ml steamed medium, (o), thru hole in cap. Cover tubes with sterile cheesecloth and place in refrigerator. Remove from refrigerator 1 hr before inoculation. Aseptically inoculate thru cap of each tube, except first set of 0.0 level for std curves, with 1 drop assay inoculum of *S. carlsbergensis* suspended cells. Take care to maintain uniform cell suspension, since they may settle out during inoculation step. Incubate tubes on constant rotary shaker 22 hr in temp.-regulated room (30°). Steam tubes in autoclave 5 min, cool, and remove caps. Read % T at 550 nm on spectrophtr. Set 100% T with H_2O to read uninoculated blank. Set 100% T with uninoculated blank to read inoculated blank. Mix 9 inoculated blank tubes, and with this mixt. set at 100% T on instrument, read all other tubes.

Average readings of triplicate tubes and plot % T against ng eluted std pyridoxine, pyridoxal, or pyridoxamine/tube on semilog paper. Det. amt pyridoxine, pyridoxal, or pyridoxamine/sample tube by interpolation. Report μg pyridoxine, pyridoxal, and pyridoxamine/g sample.

Results

The data submitted by the collaborators are given in Table 1. The means and the standard deviations are also shown. There has been no attempt to show the within-laboratory ranges or laboratory error terms. Overall, the coefficients of variation were: total vitamin B_6, 18.9%; total vitamin B_6 as the sum of the 3 components, 15.9%; pyridoxine, 22.9%; pyridoxal, 24.8%; and pyridoxamine, 27.8%. These values were computed from all the data reported, including data which the collaborators said they would not use without repeating the assay. The mean values changed little when these questionable data were excluded. However, the coefficients of variation changed as follows: total vitamin B_6, 9.0%; total vitamin B_6 as the sum of the 3 components, 7.6%; pyridoxine, 13.1%; pyridoxal, 12.4%; and pyridoxamine, 14.3%.

The mean values for total vitamin B_6 and the sum of 3 components were significantly different for beef liver only. Products high in pyridoxamine are subject to this difference, because the test organism responds to a lesser degree to pyridoxamine than to pyridoxine or pyridoxal (1, 4).

The microbiological assay described uses standard curves showing the growth response of the yeast test organism over a range of 1–5 ng standard/10 ml. Measurement by interpolation means that 0.1 ppb is within the analytical determina-

Table 1. Values for vitamin B_6 determined by collaborating laboratories

Coll.	Total, μg/g	Components: Sum, μg/g	Pyridoxine, μg/g	Pyridoxal, μg/g	Pyridoxamine, μg/g
		Lima Beans			
1	6.61	6.21	4.32	1.17	0.72
2	4.94	6.15	4.47	1.06	0.62
3	5.28	4.45	3.28	0.80	0.37
4	6.58	5.63	3.75	1.21	0.68
5	7.18	6.90	5.20	0.98	0.72
6	8.55	6.86	3.97	1.92	0.97
7	5.72	5.31	3.40	1.35	0.56
8	6.13	6.70	4.50	1.36	0.84
9	6.02	6.26	4.20	1.42	0.64
Mean	6.33	6.05	4.12	1.25	0.68
Std dev.	1.08	0.80	0.60	0.32	0.17
		Whole Wheat Flour			
1	3.73	3.62	2.32	0.79	0.51
2	2.83	3.22	2.43	0.40	0.39
3	2.55	2.35	1.64	0.50	0.21
4	3.80	3.39	2.43	0.45	0.51
5	4.40	4.03	3.28	0.30	0.45
6	4.80	3.04	2.08	0.44	0.52
7	3.07	3.17	2.42	0.28	0.47
8	3.90	4.39	3.01	0.58	0.81
9	3.69	3.64	2.64	0.50	0.50
Mean	3.64	3.43	2.47	0.47	0.49
Std dev.	0.72	0.59	0.48	0.15	0.16
		Dried Beef Liver			
1	22.40	33.08	2.86	4.19	26.03
2	25.00	37.79	1.77	8.82	27.20
3	31.38	29.37	1.44	6.37	21.56
4	24.58	43.54	2.32	7.62	33.60
5	40.69	42.13	2.80	7.88	31.45
6	33.00	59.76	3.51	8.44	47.81
7	26.88	35.74	1.28	8.67	25.79
8	24.82	40.51	1.26	7.69	31.57
9	23.65	37.75	1.35	7.40	29.00
Mean	28.04	39.97	2.07	7.45	30.45
Std dev.	5.91	8.64	0.83	1.44	7.75
		Enriched Bread			
1	0.77	0.74	0.21	0.19	0.34
2	0.58	0.51	0.13	0.17	0.21
3	0.56	0.58	0.19	0.20	0.19
4	0.60	0.55	0.16	0.13	0.26
5	0.80	0.59	0.20	0.18	0.21
6	0.91	0.56	0.15	0.28	0.13
7	0.61	0.59	0.15	0.16	0.28
8	0.69	0.71	0.19	0.18	0.35
9	0.63	0.62	0.16	0.16	0.30
Mean	0.68	0.60	0.17	0.18	0.25
Std dev.	0.12	0.07	0.03	0.04	0.07

tion. At this low concentration some of the collaborators had high blanks from some source in the culture media. Experience and care in the preparation of the culture and the analytical medium are essential.

There was some indication that the technique involved with the ion exchange column improved with experience. Collaborators were furnished the ion exchange resin from a lot known and tested in the laboratory of the Associate Referee. Otherwise, the analyst would need to test the performance of the resin used. With a satisfactory resin and good column technique, the procedure of adding 100 ml buffer and collecting 100 ml eluate is simple and does not involve the collection and testing of fractions. There were some comments on the various instruments used to measure turbidity, but the excellent analytical experience of the collaborators and the members of their staffs was equal to this situation.

Recommendation

The Associate Referee recommends adoption as official first action of the microbiological methods for the determinations of total vitamin B_6 and the 3 components, i.e., pyridoxine, pyridoxal, and pyridoxamine, in foods and like materials.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee D and was adopted by the Association. See *JAOAC* **53**, 391 (1970).

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EXTRANEOUS MATERIALS

Rapid Method for Isolation of Pecan Curculio Larvae from Pecan Pieces

By JOHN S. GECAN and PARIS M. BRICKEY, Jr. (Division of Microbiology, Food and Drug Administration, Washington, D.C. 20204)

A rapid procedure for the isolation of curculio larvae from pecan pieces has been studied collaboratively. The larvae are separated from 115 g portions of nutmeats by flotation from isopropanol. The collaborative study showed that the method requires approximately 10 min analytical time/1 lb sample; average recovery of curculio larvae was 99%. The method is recommended for adoption as official first action.

Larvae of the pecan weevil, *Curculio caryae*, may be found in pecan pieces ranging in size from midget through medium; however, as a result of processing operations, they are concentrated primarily in the midget and small sizes.

Presently, no official AOAC method exists for the isolation of curculio from pecan pieces. The method commonly used is a macroscopic examination of the entire sample; however, recovery is

limited to the ability of the analyst to discern larvae from the similarly appearing pecan pieces. Another disadvantage is that analysis of a 1 lb sample may take up to 2 hr analytical time, depending on the size of the pecan pieces being examined.

Experimental

A study was initiated to develop a method that was less time-consuming and tedious than the macroscopic hand-pickout procedure. Macroscopic examination under ultraviolet light afforded a 50% reduction in analytical time, but required a preliminary overnight drying of the sample and resulted in slightly lower recoveries. Studies were also conducted on several staining procedures that involved macroscopic examination of dyed nutmeats under either natural or ultraviolet light. These procedures further reduced analytical time but resulted in lower recoveries of curculio larvae.

The proposed method utilizes the difference in density between pecan nutmeats and curculio larvae for their separation. It involves flotation of the larvae from 115 g nutmeats in an aqueous solution of isopropanol.

METHOD[1]

40.029 ***Curculio Larvae in Pecan Pieces***

Weigh 115 g (ca ¼ lb) sample into 1.5 L beaker and add magnetic stirring bar, **40.002(s)**. Add 300 ml undild isopropanol and stir on magnetic stirrer, **40.002(r)**, 5–10 sec. Add H_2O (200 ml for midget pieces and 300 ml for small, small medium, medium, and mixed pieces) and stir 5–10 sec on stirrer. After few sec, gently agitate settled nutmeats with stirring rod to release any entrapped curculio. Remove all floating material and examine for curculio larvae. Reclaim flotation soln by pouring thru No. 12 sieve and use for one addnl sample.

Results and Discussion

Collaborative Study

Four collaborators were each requested to analyze a total of 8 (115 g) spiked samples (4 midget and 4 small size pieces) by the proposed method. Before spiking, the sample material was examined twice macroscopically to insure removal of naturally contaminating curculio larvae. Each sample was then spiked with varying amounts of curculio larvae (8–21/sample) and sealed in tin cans.

Table 1. Collaborative results for recovery (%) of curculio larvae from spiked pecan samples

	Midget Pieces				Small Pieces				Av. %
Coll.	1	2	3	4	1	2	3	4	Rec.
1	100	100	94	100	89	100	100	100	98
2	100	100	100	100	100	100	100	100	100
3	100	100	100	100	100	100	100	100	100
4	100	100	100	100	89	100	100	100	99
Av.									99

Table 1 shows a comparison of collaborative recoveries. The average overall recovery for the collaborative study was 99%.

Conclusions and Recommendations

The proposed method for isolation of pecan curculio larvae from pecan pieces required approximately 10 min analytical time/1 lb sample and yielded excellent recoveries; negligible amounts of interfering nutmeats were extracted. The accuracy, speed, and minimal equipment and reagent requirements of this method make it ideal for both laboratory and field examinations. The method is recommended for adoption as official first action.

Acknowledgments

The authors wish to acknowledge the help of W. V. Eisenberg and the following collaborators: Diane J. Howarth, Division of Microbiology, Washington, D.C.; James D. Jones, Dallas; Don J. Vail, Atlanta; and Joe A. Weishaar, Minneapolis.

[1] The section numbers within the method are those for the 11th ed. of *Official Methods of Analysis*, 1970: sec. **40.002(r)** (11th ed.) =**36.007(d)** (10th ed.); **40.002(s)** =**36.007(e)**. Cautionary notes, if present, refer to the new chapter on safety, Chapter 46.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee F and was adopted by the Association. See *JAOAC* **53**, 396 (1970).

This report of the Associate Referee, J. S. Gecan, was presented at the 83rd Annual Meeting of the AOAC, Oct. 13–16, 1969, at Washington, D.C.

Extraction of Light Filth from Casein and Sodium Caseinate

By PARIS M. BRICKEY, JR. (Division of Microbiology, Food and Drug Administration, Washington, D.C. 20204)

Collaborative results are presented on a new method for filth in casein and sodium caseinate. The method consists of dispersing the product in a Versene solution, wet sieving on a No. 230 sieve, and transferring filth directly from sieve onto filter paper. The collaborative study resulted in an average recovery of 98 and 96% for insect fragments and rodent hairs, respectively. The method is recommended for adoption as official first action.

There is no existing method for the extraction of light filth from casein and sodium caseinate. Casein, a protein precipitate made from skim milk, is insoluble in water. Because of this property, a study was initiated to develop a method for dissolving casein in an aqueous phase to extract any extraneous materials present.

Experimental

A number of unpublished methods developed by the Food and Drug Administration utilized either alkali, acids, or alcohols in conjunction with pancreatin to disperse or dissolve casein. However, this author found that most of these harsh treatments resulted in damage or destruction of rodent hairs. A method was finally developed in which 20% Versene (tetrasodium ethylenediamine tetraacetate) was used to dissolve casein sufficiently for rapid sieving on a No. 230 sieve.

METHOD[1]

40.021 ***Casein***

Weigh 50 g sample into 1 L beaker. Slowly stir in 170 ml 20% Na_4EDTA soln until well mixed with sample. With constant stirring, bring vol. to 1 L with hot tap H_2O (55–70°). Wet sieve on No. 230 sieve, **40.002(q)**, with forcible spray of hot tap H_2O until foam subsides. Wash sieve retainings into beaker and pour onto ruled filter paper. Examine papers microscopically.

[1] The section numbers within the method are those for the 11th ed. of *Official Methods of Analysis*, 1970: sec. **40.002(q)** (11th ed.) =**36.007(b)** (10th ed.). Cautionary notes, if present, refer to the new chapter on safety, Chapter 46.

This report of the Associate Referee was presented at the 83rd Annual Meeting of the AOAC, Oct. 13–16, 1969, at Washington, D.C.

Results and Discussion

Collaborative Study

Eight collaborators each examined 4 subsamples of casein. Each subsample was spiked with 15 insect elytral squares (about 0.5 × 0.5 mm and 15 rat hairs (about 1 mm). Table 1 shows the average per cent recoveries of spiked light filth. Seven of the 8 collaborators reported that the method was easy to perform and required a total analytical time of 1 hr or less for 4 subsamples.

Sodium caseinate was not used in this study

Table 1. Collaborative results for recovery (%) of insect fragments and rodent hairs (15 each/50 g subsample) from casein

Coll.	Insect Fragments	Rodent Hairs
1	13	15
	15	15
	15	15
	14	14
2	14	14
	15	15
	15	15
	14	15
3	15	15
	15	13
	15	15
	15	13
4	15	15
	15	15
	15	15
	15	15
5	14	15
	15	12
	15	15
	15	12
6	15	15
	15	15
	15	15
	15	15
7	13	15
	14	14
	14	14
	15	15
8[a]	10	10
	10	9
	10	9
	9	10
Av. rec., %	97.6	96.3

[a] Ten insect fragments and rodent hairs added to samples for Collaborator 8.

because none was available, but no difficulty should be encountered with this soluble sodium salt.

Conclusions and Recommendation

The collaborative study on the proposed method for casein and sodium caseinate resulted in average recoveries of 98 and 96% for spiked insect fragments and rodent hairs, respectively. The method is very rapid and results in clean extraction papers.

The method is recommended for adoption as official first action.

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The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee F and was adopted by the Association. See *JAOAC* **53**, 396 (1970).

New Method for the Extraction of Light Filth from Whole and Granulated Nutmeats

By JOHN S. GECAN, DIANE J. HOWARTH, and PARIS M. BRICKEY, JR. (Division of Microbiology, Food and Drug Administration, Washington, D.C. 20204)

A new method has been developed which is applicable to all nutmeats, with or without testa, either raw, dry, or oil roasted. Nutmeats are defatted by a 10 min boiling in chloroform (pecans require 3 defattings), after which the solvent is removed by aspiration and an isopropanol rinse. Following a short boiling deaeration and acid hydrolysis, light filth elements are extracted with mineral oil from a mixture of 60% ethanol and calcium chloride. Collaborative trials by the proposed method for nutmeats (except pecans) yielded significantly cleaner extraction papers and better recoveries of all light filth spike elements than by the official method. In addition, the proposed method resulted in reductions of coefficients of variation ranging from 47 to 93% of that for the official method. The modification for pecans, included in this method, required more extensive defatting and also resulted in improved light filth recoveries and reductions in coefficients of variation. Not all varieties of nutmeats were collaboratively studied; however, intralaboratory recovery trials on these nutmeats proved the proposed method superior to the official methods. The method is recommended for adoption as official first action.

The AOAC *Official Methods of Analysis* contains 5 different methods for the isolation of light filth: (*1*) whole nutmeats and large pieces, (*2*) peanuts with adhering testa, (*3*) granulated nutmeats (except black walnuts and pecans), (*4*) granulated black walnuts, and (*5*) whole or granulated pecans. The first 4 methods involve gasoline flotations from 60% ethanol, or in conjunction with sodium oleate or tween 80-Versene, with or without a preliminary organic solvent defatting. The method for whole and granulated pecans consists of a preliminary organic solvent defatting, sieving, deaerating, and floating with light mineral oil from 60% ethanol-calcium chloride.

A new method has been developed that is applicable to all sizes and types of nutmeats, with or without testa, either raw, dry, or oil roasted. It involves a single short organic solvent defatting (triple defatting for pecans), rapid deaerating, and floating the light filth with light mineral oil from a solution of 60% ethanol and calcium chloride.

This report of the Associate Referee, J. S. Gecan, was presented at the 83rd Annual Meeting of the AOAC, Oct. 13–16, 1969, at Washington, D.C.

Table 1. Comparison of average per cent recoveries of light filth spike elements from nutmeats not collaboratively tested

Nutmeat	% Recovery of Light Filth Spikes (Av. of 5 Subsamples)[a]							
	Adult *Tribolium*		Larval *Tribolium*		Rodent Hairs		Insect Fragments	
	Off.	Prop.	Off.	Prop.	Off.	Prop.	Off.	Prop.
Peanuts, raw, with testa	100	100	0	100	0	90	80	100
Peanuts, raw, without testa	80	100	100	100	90	100	100	100
Peanuts, dry roasted, with testa	100	100	20	100	70	[b]	80	100
Peanuts, dry roasted, without testa	80	100	80	100	100	100	90	100
Peanuts, oil roasted, with testa	100	100	10	100	64	96	80	94
Peanuts, oil roasted, without testa	80	100	80	100	90	100	100	100
Almonds, granulated	36	100	4	100	24	100	52	98
English walnuts, halves and large pieces	68	100	92	100	88	95	73	96

[a] Off. = official method; Prop. = proposed method.
[b] Spike element not added.

Experimental

Preliminary tests using methods for whole and large pieces of nutmeats (**36.017(a)**) and peanuts with testa (**36.017(b)**) resulted in fair recoveries of light filth and relatively clean extraction papers; however, the methods for granulated black walnuts (**36.018(b)**(*2*)) and other granulated nutmeats (**36.018(b)**(*3*)) generally yielded poor recoveries and resulted in tissue-laden extraction papers.

Due to the variety of different methods and their variable performance in regard to recoveries and cleanliness of extraction papers, a study was initiated to develop a single method applicable to all nutmeats. Since the recently developed official first action method (Vazquez, A. W., and Gecan, J. S., *JAOAC* **51**, 527–530 (1968)) for whole and granulated pecans gave good recoveries and clean extraction papers, steps were taken to apply this method to other nutmeats. Subsequent tests with this method revealed that the petroleum ether defatting, carbon tetrachloride sedimentation, and No. 230 sieving operation could be entirely eliminated and that the 10 min boiling deaeration step could be reduced. This modified method, now applicable to the other nutmeats, was tested on whole and granulated pecans. It was found acceptable, but, because of the high oil content of pecans, more extensive defatting was required.

From the wide selection of nutmeats studied, only 3 were chosen for collaborative testing. English and black walnuts were selected because the official light filth method gave erratic recoveries and/or tissue-laden extraction papers. Pecans were chosen because it was necessary to demonstrate that the proposed method, a modification of the 1967 official first action pecan method, was applicable. Table 1 shows the results of experimental trials for recovery of light filth by the proposed and official methods for those nutmeats not chosen for collaborative testing.

METHOD[1]

40.025 *Apparatus and Reagents*

(a) *Buchner funnel.*—100 mm plate diam., Coors No. 3, or equiv.

(b) *Filter paper.*—S&S No. 588; 24 and 32 cm, folded, or equiv.

(c) *60% Alcohol-calcium chloride soln.*—To each 3 L 60% alcohol (amt for 1 analysis) add 200 g anhyd. $CaCl_2$. Stir well until salt dissolves. Cloudiness from traces of $CaCO_3$ will clear up during analysis when soln is acidified.

40.026 *Nutmeats, All Sizes, Except Pecans*

(*Caution: See* **46.039, 46.040, 46.049, 46.056,** and **46.073.**)

Weigh 100 g sample into 1.5 L beaker. Add 600 ml $CHCl_3$; boil 15 min. Prep. 24 cm paper in buchner by moistening with H_2O and forming around base of 1 L beaker. Place 7 cm disk of bolting cloth, **40.002(d)**, (mesh size not critical) in funnel, insert paper, apply vac., and press moistened paper until good seal is obtained. Rinse paper with isopropanol. Quant. transfer nutmeats and $CHCl_3$ onto previously prepd paper. Maintain suction on nutmeats in buchner 5

[1] The section numbers within the method are those for the 11th ed. of *Official Methods of Analysis*, 1970: sec. **40.002(d)** (11th ed.) = **36.001(b)** (10th ed.), **40.002(h)**(*3*) = **36.001(o)**, **40.002(r)** = **36.007(d)**, **40.002(s)** = **36.007(e)**, and **40.003(w)** = **36.002(s)**. Cautionary notes, if present, refer to the new chapter on safety, Chapter 46.

min after visible dripping ceases. Quant. transfer nutmeats on paper to 2 L trap flask, **40.002(h)**(*3*). Scrape all fines from paper with spatula and finally rinse paper clean with 60% alcohol-$CaCl_2$ soln. Bring vol. to 1 L with 60% alcohol-$CaCl_2$ soln and add 50 ml HCl. Add magnetic stirring bar, **40.002(s)**, to flask. Place flask on magnetic stirring hot plate, **40.002(r)**, and heat to *full boil* with gentle stirring. Immediately transfer flask to cool stirring unit and add 40 ml mineral oil, **40.003(w)**, by pouring down stirring rod. Stir magnetically, **40.004(b)**, 2 min.

Fill with 60% alcohol-$CaCl_2$ soln and gently stir 5–10 sec with stirring rod. Let stand 2 min and trap off. Add 25 ml mineral oil, hand stir gently 30 sec, and let stand 10 min. Repeat trapping. Wash flask neck thoroly with isopropanol, and transfer washings to beaker with trappings. Filter onto ruled paper and examine microscopically.

40.027 ***Pecans, All Sizes***

Prep. 32 cm filter paper by forming around base of 1 L beaker. Place paper into 1.5 L beaker and weigh 100 g sample into filter paper cup. Add 400 ml $CHCl_3$ and boil 5 min. After few min cooling, lift paper and drain. Repeat 5 min boil and drain with two addnl 400 ml portions $CHCl_3$. Proceed as in **40.026**, beginning "Place 7 cm disk of bolting cloth . . ."

Results and Discussion

Collaborative Study

Ten collaborators were requested to analyze a total of 14 spiked samples: 3 each by the official and proposed methods for granulated black walnuts and English walnuts and 2 by the proposed method for pecans. The collaborators were not requested to analyze pecans by the 1967 official first action method because the results of the 1967 collaborative study could be applied to the present collaborative study. The spike material for each sample consisted of 20 *Tribolium sp.* elytral fragments (0.3–0.5 mm), 20 rat hair fragments (3–4 mm), 5 *Tribolium sp.* larvae, and 5 *Tribolium sp.* adults.

Table 2 shows a comparison of recoveries obtained by collaborators for all 3 products. The average per cent recoveries of light filth elements from black walnuts and English walnuts and the respective coefficients of variation are distinctly in favor of the proposed method. Since the proposed method for whole and granulated pecans was only a slightly modified version of the 1967 official first action method, it was expected that the collaborative results would be comparable. However, the proposed method was shown to be superior, since it provided a cumulative improvement of 19% for average per cent recoveries of light filth and an average reduction in coefficient of variation of 30%.

Collaborators generally agreed that the official method for both English and black walnuts involved easy manipulations; however, both were considered time-consuming and yielded extraction papers containing moderate-to-excessive amounts of plant tissue. All collaborators commented that the proposed methods for the 3 nut products tested yielded clean extraction papers. Eight collaborators felt that the proposed methods were easy to perform, but the remaining 2 indicated that the manipulations were difficult for the following reasons: (*1*) problems with magnetic stirring during oil dispersion, (*2*) tearing of cupped filter paper during defatting, and (*3*) slow aspiration of solvents after defatting. The magnetic stirring problem probably resulted either from using a smaller stirring bar than is specified in **36.007(e)** or from a stirring unit with a weak drive magnet. Slow aspiration of solvents after defatting is usually a result of a poorly sealed filter paper. Tearing of the filter paper cup containing the nutmeats during defatting can be avoided by use of a rapid filtering, high wet-strength paper, such as S&S No. 588.

Conclusions and Recommendations

The proposed method for whole and granulated nutmeats (except pecans) yielded significantly cleaner extraction papers and better recoveries of all spiked light filth. In addition, the proposed method resulted in reductions of coefficients of variation ranging from 47 to 93% of that calculated for the official method. The proposed method for pecans, a modified version of the 1967 official first action pecan method, also resulted in better light filth recoveries and reductions in coefficients of variation. Light filth recovery trials on those nutmeats not collaboratively studied showed that the proposed method resulted in good recoveries and clean extraction papers.

The proposed methods for whole and granulated nutmeats are recommended for adoption as official first action to replace the method for granulated pecans, adopted as official first action in 1967 (see *JAOAC* **51**, 503–504, 527–530, 1968),

The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee F and were adopted by the Association. See *JAOAC* **53**, 396 (1970).

Table 2. Collaborative results[a] for recovery (%) of added filth elements from granulated black walnuts, English walnuts, and pecans

	Adult *Tribolium*						Larval *Tribolium*						Insect Fragments						Rodent Hairs					
	Off.			Prop.			Off.			Prop.			Off.			Prop.			Off.			Prop.		
Coll.	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Black Walnuts																								
1	60	80	80	100	100	100	0	0	0	100	100	100	20	15	25	90	95	90	10	45	85	90	100	95
2	100	100	100	100	80	80	0	20	0	100	100	80	10	50	45	80	90	75	50	50	55	80	95	100
3	100	100	NR[b]	100	100	100	0	0	NR	100	80	100	40	25	NR	95	100	95	60	80	NR	95	100	95
4	100	100	80	100	100	100	20	40	20	100	100	100	60	45	50	100	85	95	85	85	65	95	90	95
5	60	100	[c]	100	100	100	0	0	[c]	100	100	100	0	20	[c]	95	85	40	60	80	[c]	90	95	100
6	80	100	80	100	100	100	20	0	0	100	100	100	45	45	45	95	100	80	15	0	0	100	90	95
7	NR	NR	NR	100	80	100	NR	NR	NR	100	80	80	NR	NR	NR	85	85	80	[c]	[c]	[c]	80	80	80
8	[c]	[c]	[c]	80	80	100	[c]	[c]	[c]	80	80	100	[c]	[c]	[c]	75	100	100	[c]	[c]	[c]	50	35	60
9	80	[c]	[c]	100	80	NR	0	[c]	[c]	100	100	NR	25	[c]	[c]	90	85	NR	60	[c]	[c]	95	90	NR
10	[c]	[c]	[c]	100	100	80	[c]	[c]	[c]	80	60	100	[c]	[c]	[c]	40	80	80	[c]	[c]	[c]	85	85	100
Av. rec.		88			95			7			93			34			86			52			88	
Coeff. of var.		17			9			172			12			53			17			56			18	
English Walnuts																								
1	0	0	0	100	100	100	0	0	0	100	80	100	15	25	30	100	90	100	55	30	25	95	85	100
2	40	40	40	100	40	100	20	40	0	100	100	100	35	45	65	95	70	80	0	25	55	90	85	85
3	80	100	80	100	100	100	0	20	0	100	100	100	35	45	20	100	95	95	0	20	0	90	90	95
4	100	40	60	80	100	100	40	0	20	80	100	60	20	35	70	80	90	85	15	20	30	90	95	85
5	0	0	40	80	100	80	0	0	0	80	100	80	40	50	85	85	95	90	60	45	60	95	100	95
6	80	100	NR	100	100	100	40	0	NR	100	100	100	40	45	NR	100	90	85	85	65	NR	100	95	100
7	NR	NR	NR	NR	100	80	NR	NR	NR	NR	80	100	NR	NR	NR	NR	80	90	NR	NR	NR	NR	80	95
8	0	0	0	100	80	80	0	0	0	60	60	100	50	40	60	90	90	90	10	25	10	80	80	85
9	0	0	0	100	100	100	0	0	0	100	100	100	70	90	70	80	85	85	30	55	30	80	90	100
10	[c]	[c]	[c]	100	100	80	[c]	[c]	[c]	100	60	80	[c]	[c]	[c]	95	80	90		[c]	[c]	100	80	100
Av. rec.		35			93			8			90			47			89			35			91	
Coeff. of var.		154			14			185			16			44			8			73			8	

(Continued

Table 2. (*Continued*)

	Adult *Tribolium*					Larval *Tribolium*					Insect Fragments					Rodent Hairs				
	Off.			Prop.		Off.			Prop.		Off.			Prop.		Off.			Prop.	
Coll.	1	2	3	1	2	1	2	3	1	2	1	2	3	1	2	1	2	3	1	2
										Pecans										
1	100	100	90	100	100	100	100	100	100	100	75	100	75	100	95	65	70	60	95	100
2	100	60	80	100	100	100	100	80	100	100	80	95	90	65	95	60	75	85	70	50
3	100	70	80	100	100	100	80	60	80	100	60	75	50	80	80	55	55	65	85	90
4	80	100	90	80	100	80	80	100	80	100	90	95	85	80	85	90	70	90	100	100
5	100	100	100	80	100	100	100	100	80	100	85	65	70	90	85	70	55	40	100	95
6	100	100	80	100	100	100	100	100	100	100	100	100	100	95	95	100	90	95	80	95
7	40	90	90	100	80	60	100	100	80	80	45	65	100	90	80	65	75	35	90	90
8	100	60	100	80	100	100	100	80	80	100	100	100	85	90	75	95	100	80	70	15
9	60	100	90	80	80	80	100	100	60	100	85	80	95	90	75	60	70	100	80	85
10	90	NR	NR	80	100	100	NR	NR	80	80	100	NR	NR	80	90	100	NR	NR	95	100
Av. rec.		88			93		93			90		84			86		74			89
Coeff. of var.		19			11		13			13		19			10		25			15

[a] Off = official method, i.e., **36.018(b)**(*2*)—black walnuts; **36.018(b)**(*3*)—English walnuts; and *JAOAC* **51**, 503–504 (1968)—pecans; and Prop. = proposed method.
[b] NR = results not reported due to analytical error.
[c] Recoveries not determined because of excessive plant debris on the extraction paper.

and the following methods: (1) **36.017(a)**—whole nutmeats and large pieces (except peanuts with testa); (*2*) **36.017(b)**—peanuts with adhering testa; (*3*) **36.018(b)**(*2*)—granulated black walnuts; and (*4*) **36.018(b)**(*3*)—granulated nutmeats (except pecans and black walnuts).

It is also recommended that section **36.018(a)** for heavy filth be retained except that the sentence, "Reserve residue on paper for (**b**)," be deleted; and that the subtitle under shelled nuts be changed to read, "Nutmeats, whole, large pieces, and granulated."

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Extraction of Light Filth from Corn- and Rice-Based Ready-to-Eat Breakfast Cereals and Corn-Based Snack Foods

By RUSSELL G. DENT, ANDREE L. ROAF, and PARIS M. BRICKEY, JR. (Division of Microbiology, Food and Drug Administration, Washington, D.C. 20204)

Collaborative results are presented on a proposed method for light filth extraction from corn- and rice-based ready-to-eat breakfast cereals and corn-based snack foods. Samples are digested with hydrochloric acid, followed by wet sieving on a No. 230 mesh plain weave sieve. Sieve retainings are boiled in 40% isopropanol and filth is extracted in either a Kilborn funnel or a trap flask. Average per cent recoveries of spiked elytral fragments and rodent hairs were 91.0 and 80.0% (using trap flask) and 86.1 and 81.7 (using Kilborn funnel), respectively. The method is recommended for adoption as official first action.

There is no existing official AOAC method for the extraction of light filth from ready-to-eat breakfast cereals and snack foods. A method has been developed which consists of an acid digestion, with or without detergent, depending upon the fat content of the product. The dispersed product is sieved on a No. 230 plain weave sieve and deaerated by boiling in 40% isopropanol. Light filth is extracted with mineral oil from a hot lower phase contained in either a Wildman trap flask or a Kilborn separatory funnel.

This report of the Associate Referee, R. G. Dent, was presented at the 83rd Annual Meeting of the AOAC, Oct. 13–16, 1969, at Washington, D.C.

Experimental

The objective of this study was to develop a single procedure for isolating light filth from corn- and rice-based ready-to-eat cereals and corn-based snack foods. In order to establish the usefulness of the procedure on a variety of products, the following cereals and snacks were analyzed in this laboratory: (*A*) low fat—(*1*) rice products: Rice Honeys, Rice Krinkles, Rice Krispies, Special K, Puffa Puffa Rice, Puffed Rice; (*2*) corn products: Corn Flakes, Corn Chex, Quisp, Corn Bursts, Honeycomb; (*B*) high fat—corn snack foods: Fritos, Chee-tos, Corn Q's.

The proposed method and its modification for high fat content products proved effective for all products examined. The modification consisted of the addition of an emulsifier, DM-710, to promote emulsification of fat during the digestion stage, which results in cleaner extraction papers in the trapping stage.

The option of extraction vessels was made to facilitate the treatment of sieve residues. Heavy residues may be managed more efficiently in a Wildman trap flask, while light residues are quickly extracted with the Kilborn funnel. The vessels are interchangeable, however, and give comparable recoveries of filth elements. If extraction is carried out in the Kilborn funnel, it

should be noted that all refills after the initial drain are made with hot tap water. This does not affect the recovery of hairs and fragments. The option to use either 40% isopropanol or 55% ethanol was incorporated into the collaborative study; however, ethanol was found unsatisfactory.

METHOD[1]

40.042 ***Light Filth***

(a) *Cereals and food products containing no fats or oils.*—(Check ingredient label.) To 1–1.5 L beaker (depending on bulk of product), add 50 g sample, 500 ml hot (55–70°) tap H_2O, and 40 ml HCl. Bring mixt. to full boil on hot plate-magnetic stirrer, **40.002(r)**, using slow stirring speed. Boil 20 min and wet sieve immediately on No. 230 plain weave sieve, **40.002(q)**, with forceful hot (55–70°) H_2O spray until residue no longer passes thru sieve and H_2O is clear. Wash sieve retainings *either* into 2 L Wildman trap flask, **40.002(h)**(*3*), or back into original beaker if Kilborn separator, **40.002(h)**(*1*), is to be used, using 40% isopropanol.

(*1*) *Trap flask.*—Bring vol. to 800 ml with 40% isopropanol and add 30 ml HCl. Raise stirring rod plunger and secure above liq. with clamp. Add stirring bar, **40.002(s)**, and stir at slow speed while bringing mixt. to boil. Boil 5 min. Add 50 ml mineral oil, **40.003(w)**, and stir magnetically, **40.004(b)**, 3 min.

Remove from heat and fill with 40% isopropanol. Let stand 10 min and trap off, rinsing neck of flask and rod with isopropanol or alcohol. Filter trappings thru ruled paper.

(*2*) *Kilborn separator.*—Bring vol. in original beaker to 600 ml with 40% isopropanol and add 25 ml HCl. Bring to boil with slow stirring, boil 5 min, add 50 ml mineral oil, and stir magnetically 3 min.

Transfer from beaker to 1 L Kilborn separator, rinsing beaker into Kilborn with 40% isopropanol. If residue in Kilborn is heavy, resuspend with glass rod. Rinse rod into Kilborn.

Let stand 3 min and drain contents to within 1″ of bottom of oil layer. Refill with hot (55–70°) tap H_2O. Repeat drain and refill steps with 3 min intervals, until H_2O phase is free of plant material. Discard drainings. Drain oil layer into original beaker, rinsing sides of Kilborn alternately with isopropanol or hot H_2O and alcohol, using rubber policeman to clean sides. Filter contents of beaker thru ruled paper.

(b) *Cereals and food products containing natural and synthetic fats or vegetable oils.*—Proceed as in (a), beginning "500 ml hot (55–70°) tap H_2O . . ." but also add to this mixt. 20 ml DM-710, **40.003(t)**; then proceed as in (a) with no further changes.

[1] The section numbers within the method are those for the 11th ed. of *Official Methods of Analysis*, 1970: sec. **40.002(h)**(*1*) (11th ed.) =**36.028(a)** (10th ed.) **40.002(h)**(*3*) =**36.001(o)**, **40.002(q)** =**36.007(b)**, **40.002(r)** =**36.007(d)**, **40.002(s)** =**37.007(e)**, **43.003(w)** =**36.002(s)**, **40.004(b)** =**36.007(d)** and (e). Cautionary notes, if present, refer to the new chapter on safety, Chapter 46.

Results and Discussion

Collaborative Study

Six collaborators were requested to analyze 6 subsamples each of 2 types of breakfast cereals (Corn Flakes and Rice Krispies) and 1 type of cereal grain-based snack product (Corn Cheetos). Four subsamples were spiked with light filth and 2 subsamples were unspiked blanks to be used for familiarization and determination of intrinsic light filth levels. Of the 4 spiked subsamples, 1 each was to be analyzed by the proposed method according to the following scheme: (*1*) 40% isopropanol, Wildman trap flask; (*2*) 40% isopropanol, Kilborn funnel; (*3*) 55% ethanol, Wildman trap flask; and (*4*) 55% ethanol, Kilborn funnel. Spike material for each subsample consisted of 20 elytral squares (0.5 × 0.5 mm) and 20 rodent hairs (about 2 mm long).

The individual recoveries on the 3 collaborative samples, using 40% isopropanol and either the Wildman trap flask or the Kilborn separatory funnel, are given in Table 1. The use of 55% ethanol was discarded because it yielded lower recoveries for extraction of rodent hair in the Kilborn funnel. Collaborators average time per subsample analysis was 2 hr. Manipulations with the 2 vessels were described as "easy" by 5 out of 6 collaborators. There was some difficulty among the collaborators on obtaining debris-free papers. However, this difficulty is not encountered if wet sieving is done immediately after digestion.

Conclusions and Recommendation

The coefficients of variation for rodent hair recovery in the trap flask and Kilborn funnel were 14.65 and 24.87%, respectively. The coefficient of variation for insect fragment recovery in the trap flask and Kilborn funnel were 10.93 and 12.85%, respectively. Although there is a wider range between rodent hair recoveries with the 2 vessels this is thought to reflect the variations in manipulation technique among the collaborators. In the absence of an official method for the ex-

Table 1. Collaborative results for recovery of added fragments and rodent hairs from corn- and rice-based ready-to-eat breakfast cereals and corn-based snack foods[a]

	Insect Fragments (20 Added): 40% Isopropanol						Rodent Hairs (20 Added): 40% Isopropanol					
	Wildman Trap Flask			Kilborn Funnel			Wildman Trap Flask			Kilborn Funnel		
Coll.	A	B	C	A	B	C	A	B	C	A	B	C
A	19	18	18	20	20	15	18	16	15	20	20	6
B	4[b]	17	13	16	15	13	15	11	12	9	15	11
C	20	19	19	18	18	15	16	19	9[b]	17	19	15
D	19	15	17	18	17	15	16	17	13	20	20	12
E	19	18	19	15	18	19	15	20	19	18	19	17
F	20	18	19	20	20	18	20	15	16	17	19	20
Av. rec., %	97.0	87.5	87.5	89.2	90.0	79.2	83.3	81.7	75.0	84.2	93.3	67.5
Coeff. of var.,[c] %		10.93			12.85			14.65			24.87	

[a] A = corn-based ready-to-eat dry cereal; B = rice-based ready-to-eat cry cereal; and C = corn-based snack food.
[b] Eliminated as outliers.
[c] Values given are the average coefficients of variation for Samples A–C combined.

traction of light filth from breakfast cereals and snack foods, this proposed method is recommended for adoption as official first action.

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The authors wish to acknowledge the help of John S. Gecan and Joel J. Thrasher, Division of Microbiology, Washington, D.C., and the following collaborators: (Food and Drug Administration)—Louise Faherty, Baltimore; Don G. Vail, Atlanta; G. E. Russell, Detroit; M. T. Miller, Kansas City; and John A. Ross, Minneapolis; and E. Andrina, Kellogg Co., Battle Creek, Mich.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee F and was adopted by the Association. See *JAOAC* **53**, 396 (1970).

Extraction of Light Filth from Raw and Processed Wheat Germ

By ANDREE L. ROAF, PARIS M. BRICKEY, JR., and RUSSELL G. DENT (Division of Microbiology, Food and Drug Administration, Washington, D.C. 20204)

A method developed for the extraction of light filth from raw and processed wheat germ utilizes chloroform defatting and acid hydrolysis followed by wet sieving. Light filth is extracted with mineral oil from a hot ethanol or isopropanol solution. The collaborative study resulted in 90, 89, and 84% average recoveries of spike insect fragments, rodent hairs, and adult insects, respectively. The method is recommended for adoption as official first action.

This report of the Associate Referee, A. L. Roaf, was presented at the 83rd Annual Meeting of the AOAC, Oct. 13–16, 1969, at Washington, D.C.

There is no existing method for the extraction of extraneous materials from raw and processed wheat germ. A method has therefore been developed which involves defatting with an organic solvent, hydrolyzing with hydrochloric acid, sieving on a No. 230 sieve, deaerating by heating, and extracting the light filth with mineral oil from hot alcohol in a trap flask.

Experimental

One sample of processed wheat germ obtained from a retail outlet and 3 samples of raw wheat germ, each obtained from a different manufac-

turer, were used in the development of the method. Wheat germ contains up to 12% fat; therefore, extensive defatting was required to obtain clean extraction papers.

Since multiple defattings in chloroform were necessary, a filter paper cup was used to contain the sample and avoid loss of contaminants. In order to ensure clean separation and good light filth recoveries, vigorous wet sieving of the hydrolyzed sample was found necessary. Completeness of sieving can be determined by color change of residue from light tan to dark brown. The option to use either 55% ethanol or 40% isopropanol was included for the convenience of the analyzing laboratory.

METHOD[1]

40.043 *Apparatus and Reagent*

(a) *Filter paper.*—S&S No. 588, 32 cm folded, or equiv.

(b) *Antifoam A compound.*—Aerosol formula. Use as needed.

(c) *Alcohol.*—55% alcohol or 40% isopropanol. Use same alcohol thruout trapping.

40.044 *Determination*

Open folded 32 cm filter paper. Center paper over top of 400 ml beaker, place 250 ml beaker in middle of paper, and press into larger beaker. Remove 250 ml beaker. Tare 400 ml beaker and paper, weigh 50 g sample into filter paper in beaker, and add ca 150 ml $CHCl_3$. Boil on steam bath 5 min, occasionally rinsing down sides of filter paper with $CHCl_3$ to maintain original level. Remove sample from heat. Carefully lift paper contg sample from beaker so as to prevent any loss of sample. Let most of $CHCl_3$ drain into beaker; then discard drainings. Continue above operation 2 addnl times beginning "add ca 150 ml $CHCl_3$." After last $CHCl_3$ defatting, place filter paper contg sample in buchner. Apply vac. until draining slows to drip. Rinse sides of paper and sample with undild isopropanol and apply vac. until draining has ceased. Turn off vac. Add ca 50–60 ml undild isopropanol to sample. Let stand 2 min; then apply vac. until dripping ceases and sample appears dry. Transfer sample from filter paper into 1 L beaker with hot tap H_2O (55–70°). Fold filter paper in half and rub together; then wash with hot tap H_2O into beaker. Repeat several times until filter paper appears clean. Discard paper and bring vol. of hot H_2O to 600 ml. Add 30 ml HCl and 1 ml antifoam, **40.003(e)**. Boil on hot plate, **40.002(s)**, 10 min with constant stirring; then remove from heat. Pour contents of beaker onto No. 230 plain weave sieve, **40.002(q)**, and wash with forcible hot H_2O spray (55–70°), **40.002(a)**, until all starchy material has passed thru and only bran remains (color of sample will change from light tan to dark brown). Transfer material from sieve to 2 L trap flask with alcohol, (c), dil. to ca 1 L, and add 50 ml HCl. Heat to 60–70° on hot plate (do *not* boil), remove flask from heat, and add 50 ml mineral oil, **40.003(w)**. Stir magnetically, **40.004(b)**, 3 min. Fill flask with alcohol, (c), stir gently by hand 1 min, let flask stand 10 min, and trap off, rinsing neck of flask with alcohol. Perform second extraction, using 25 ml mineral oil. Stir gently by hand 1 min, let stand 15 min, and trap off.

Table 1. Collaborative results for recovery of insect fragments, rodent hairs, and adult insects (15, 15 and 4 added, respectively/subsample) from wheat germ

Coll.	Insect Fragments	Rodent Hairs	Adult Beetles
1	13	11	2
	18[a]	15	3
	8	13	4
	16[a]	16[a]	4
2	16	15	3
	14	13	4
	11	11	2
	15	14	2
3	11	14	3
	10	12	3
	12	14	4
	9	12	3
4	15	14	4
	12	14	4
	15	15	4
	15	15	4
5	15	13	4
	15	15	4
	15	15	4
	13	15	4
6	14	10	2
	13	13	2
	14	15	1
	14	10	4
7	13	11	4
	14	15	4
	15	12	4
	14	8	4
8	14	14	3
	14	14	4
	13	14	4
	15	14	3
Av. rec., %	90.6	88.8	83.9
Coeff. of var., %	14.04	13.88	25.76

[a] Excess over 15 fragments and/or hairs is probably due to carryover on sieve from previous subsample.

[1] The section numbers within the method are those for the 11th ed. of *Official Methods of Analysis*, 1970: sec. **40.002(q)** (10th ed.) =**36.001(n)**, **40.002(s)** =**36.007(e)**, **40.003(w)** =**36.002(s)**; for **40.002(a)** and **40.003(e)**, see *JAOAC* **52**, 424–425 (1969). Cautionary notes, if present, refer to the new chapter on safety, Chapter 46.

Rinse neck of flask with undild isopropanol or alcohol. Filter trappings thru ruled paper and examine microscopically.

Results and Discussion

Collaborative Study

Eight collaborators each examined 3 subsamples of raw wheat germ and 1 subsample of the finished product. Each subsample was spiked with 15 insect elytral squares (0.5 × 0.5 mm), 15 rodent hairs (1–2 mm), and 4 adult beetles.

Table 1 shows the individual recoveries and average per cent recoveries for light filth spike elements obtained by collaborators, using the proposed method. No adverse comments regarding the method were made by any of the collaborators.

The average time required to analyze 4 subsamples was 6½ hr.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee F and was adopted by the Association. See *JAOAC* **53**, 396 (1970).

Conclusions and Recommendations

The proposed method for both raw and processed wheat germ yielded 90.6, 88.8, and 83.9 average per cent recoveries of insect fragments, rodent hairs, and adult insects, respectively. In addition, the method yields clean extraction papers and requires a minimum of analytical time. The method is recommended for adoption as official first action.

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Acidic, Mineral Oil Extraction of Light Filth from Bread and Donuts

By JOEL J. THRASHER (Division of Microbiology, Food and Drug Administration, Washington, D.C. 20204)

Collaborative results are presented on a new method for the extraction of light filth from bread and donuts. The new method consists of product dispersion by steam bath heating or autoclaving, wet sieving on No. 230 sieve, digestion with HCl, followed by extraction of filth with mineral oil from water. Minimum recovery of spike filth elements was 82%. The new method also proved to be faster than method 36.025 and resulted in cleaner filter papers than 36.026. The proposed method is recommended for adoption as official first action.

There are presently 2 official methods for the extraction of post-milling extraneous materials from breads and donuts. These methods, **36.025** and **36.026**, utilize pancreatin digestion and autoclaving, respectively. One method, **36.025**, states in part, ". . . wash twice alternately with alcohol and $CHCl_3$ in that order, and then rinse thoroly with alcohol . . .". This removes fat from the sieve retainings; however, the Associate Referee found that defatting was not efficient using these reagents. A study was made to deter-

mine a way to remove sufficient fat from the products.

Last year a collaborative study was completed on a method for alimentary pastes. This method, similar to the proposed procedure for bread and donuts, resulted in higher and more reproducible recoveries than methods **36.025** and **36.026**. This year, 10 collaborators analyzed bread and donuts by the proposed method. Products were dispersed either by acid autoclaving or heating in a steam bath. Samples were then wet sieved on a No. 230 plain weave sieve and filth was extracted from hot water with mineral oil. A mixture of 2 acid-stable emulsifiers released enough fat for easy removal by immediate hot water wet sieving.

METHOD[1]

40.040 ***Light Filth***

Add 1 L hot (55–70°) tap H_2O to 2 L beaker. Add 20 ml DM-710, **40.003(t)**, and 5 ml CO-730, **40.003(s)**, and mix well. Add 225 g sample, breaking any crust to <1 sq in. Stir well. Proceed with either autoclave, (**a**), or steam bath, (**b**).

(**a**) *Autoclave.*—Add 30 ml HCl with stirring. Add 1 ml antifoam soln, **40.003(e)**. Autoclave as in **40.002(b)**(*1*) or (**b**)(*2*).

(**b**) *Steam bath.*—Add 90 ml HCl with stirring. Heat in steam bath for 10 min. Add 1 ml antifoam soln. Boil 15 min on stirring hot plate, keeping beaker covered with watch glass.

Wet sieve on No. 230 plain weave sieve, **40.002(q)**, with hot H_2O (55–70°). Sieve until effluent is clear and foam is gone. Transfer sieve retainings to original beaker. (*Caution:* Do not allow sample in beaker or sieve to cool.) Add 30 ml HCl and dil. to 1 L with H_2O. Stir on stirring hot plate, **40.002(r)**, and bring to boil. Boil 6 min, add 50 ml mineral oil, **40.003(w)**, and continue heating until boiling resumes. Transfer beaker to cool stirring plate, **40.002(r)**. Stir magnetically, **40.004(b)**, 3 min.

Promptly transfer beaker contents to percolator, **40.002(h)**(*3*), contg ca 250 ml H_2O. Rinse beaker into percolator and bring vol. to 1700 ml mark with H_2O. After 1 min, stir percolator contents with glass rod. Place rod in beaker and set aside to receive final oil drain. Let stand 2 min. Drain oil to 250 ml mark and discard drainings. Refill percolator with H_2O. Continue drain and refill cycles until lower aq. phase is almost clear. Drain oil to 250 ml mark. Drain oil into original beaker. Wash percolator sides with min. of 50 ml H_2O and alcohol or isopropanol. If sides do not appear clean, follow with H_2O and 5% detergent wash, **40.003(o)**. Filter onto ruled paper and examine microscopically.

[1] The section numbers within the method are those for the 11th ed. of *Official Methods of Analysis*, 1970: sec. **40.002(h)**(*3*) (11th ed.) = **36.001(o)** (10th ed.), **40.002(q)** = **36.001(n)**, **40.002(r)** = **36.007(d)**, **40.003(w)** = **36.002(s)**, **40.004(b)** = **36.007(e)**; for **40.002(b)**(*1*) and (*2*), **40.003(e)**, and **40.003(o)**, see *JAOAC* **53**, 424 (1970). Cautionary notes, if present, refer to the new chapter on safety, Chapter 46.

Results and Discussion

Collaborative Study

Capsules containing insect fragments, rodent hairs, and *Tribolium* adults and larvae were sent to each collaborator with instructions to add the spike capsules to locally purchased bread and donuts. These collaborators used the proposed method to analyze 2 spiked bread and donut samples and 1 blank of each product by each dispersion procedure, for a total of 12 subsamples. Their results are shown in Table 1. Certain data indicated in the table and related footnotes were dropped from final evaluation because the individual values were outliers and not representative of the normal group values.

Table 2 presents data from 4 intralaboratory collaborators, comparing the 2 official methods, **36.025** and **36.026**, with the 2 options of the proposed method.

Some collaborators stated that the proposed method may fragment adult insects; however, this should not be a serious problem, since the majority of food samples processed by this method will contain very few adult insects. The collaborative results indicated that, for optimum results, the dispersed samples should be sieved immediately after removal from the autoclave or steam bath. A delay in sieving, which allows the sample to cool appreciably, or sieving with cold water would probably result in significantly more plant debris on the filter paper(s) and lower filth recovery. Analysis of the 2 products tested, bread and donuts, agreed quite closely in recovery as shown in Table 1. Average recoveries of adult insects, larvae, insect fragments, and rodent hairs from bread were 89, 96, 90, and 86%, respectively. Average recoveries of adult insects, larvae, insect fragments, and rodent hairs from donuts were 97, 96, 86, and 85%, respectively.

Recommendation

The method for extraction of light filth from bread and donuts is recommended for adoption as official first action.

Table 1. Collaborative results for recovery (%) of added filth from samples of bread and donuts by the autoclave (Au) and steam bath (Sb) techniques

	Tribolium Adults (5 Added)				*Tribolium* Larvae (5 Added)				Elytral Squares (20 Added)				Rodent Hairs (20 Added)			
	Bread		Donuts		Bread		Donuts		Bread		Donuts		Bread		Donuts	
Coll.	Au	Sb	Au	Sb	Au	Sb	Au	Sb	Au	Sb	Au	Sb	Au	Sb	Au	Sb
1	5	5	5	5	5	5	5	5	16	17	13	19	17	18	20	20
	5	4	4	5	5	5	5	5	20	17	17	20	14	18	17	20
2	4	5	5	5	5	5	5	4	20	19	17	18	18	17	16	20
	5	5	5	5	5	5	5	5	18	20	17	18	20	15	16	20
3	5	5	5	5	5	5	4	5	19	19	19	19	18	19	18	20
	5	5	5	5	4	5	4	5	19	L[a]	15	19	20	19	19	20
4	4	5	5	5	4	1[b, c]	4	4	13	2[b, c]	12	14	16	5[b]	15	13
	3[b]	1[b]	3[b]	3[b]	4	4	4	4	15	17	15	2[b, c]	14	15	7[b]	4[b, c]
5	5	5	5	5	5	3	5	3[b]	19	19	14	14	19	16	14	18
	5	3[b]	5	4	3[b]	2[b]	2[b]	3[b]	18	17	19	17	20	18	17	19
6	5	5	5	4	5	5	4	5	16	18	18	16	19	14	20	17
	5	5	5	4	5	4	5	3[b]	17	11[b]	13	15	19	16	17	19
7	5	5	5	5	5	5	5	5	19	17	18	16	20	19	16	18
	5	5	5	5	5	5	5	5	16	15	19	20	18	16	18	20
8	4	4	4	5	4	5	4	5	15	20	15	20	7	16	16	15
	5	5	5	5	5	5	5	5	20	17	15	17	18	19	13	15
9	5	5	4	5	5	5	5	5	19	16	17	20	19	17	20	18
	5	5	5	5	5	4	5	5	18	17	20	18	19	19	18	20
10	5	L[a]	5	5	5	L[a]	4	5	15	L[a]	18	6[b]	16	L[a]	18	16
	5	L[a]	5	5	5	L[a]	5	3[b]	16	L[a]	18	15	15	L[a]	13	15
11	5	5	5	5	5	5	5	5	20	20	19	20	20	20	20	19
	5	5	5	5	5	5	5	5	20	19	20	20	20	20	19	19
12	5	5	5	5	5	5	5	5	20	18	20	19	18	17	19	19
	5	5	5	5	5	5	5	5	20	20	17	19	19	20	19	14
13	5	5	5	5	5	5	5	5	19	18	16	18	9	19	19	15
	5	5	5	5	5	5	5	5	17	18	19	16	4	16	17	20
14	5	5	5	3[b]	5	4	5	3[b]	18	12	20	19	18	17	19	3[b]
	5	5	5	3[b]	5	5	5	1[b]	20	19	19	13	20	20	20	2[b]
Av.	4.89	4.08	4.89	4.88	4.85	4.75	4.74	4.86	18.11	17.78	17.11	17.65	16.93	17.60	17.51	16.59
Std dev.	0.319	0.282	0.319	0.439	0.748	0.532	0.447	0.351	0.564	1.86	2.29	2.32	4.08	1.78	2.10	4.65
Coeff. of var., %	6.52	5.73	6.53	9.01	15.4	11.2	9.43	7.22	3.11	10.4	13.4	13.1	24.09	10.11	11.95	28.03

[a] L = lost during analysis.
[b] Eliminated from statistical evaluation as outliers.
[c] Instructions not followed.

Table 2. Collaborative results for recovery of added filth from 8 samples, comparing the proposed method (Prop.) with 36.025 (P) and 36.026 (H)

	Bread				Donuts				Bread				Donuts			
Coll.	P	H	Prop.[a]		P	H	Prop.[a]		P	H	Prop.[a]		P	H	Prop.[a]	
	Tribolium Adults (5 Added)								Elytral Squares (20 Added)							
11	5	4			5	5			19	13			17	16		
	5	4			5	5			17	16			19	18		
12	5	5			5	5			16	12			17	15		
	5	2[b]			5	5			20	6			18	18		
13	5	5			5	5			18	7			16	15		
	5	5			5	5			15	16			15	16		
14	5	2[b]			5	5			15	19			17	19		
	5	5			5	5			16	16			19	16		
Av.	5	4.66	4.89	4.08	5	5	4.89	4.89	17.0	13.1	18.11	17.78	17.3	16.7	17.11	17.65
Std dev.	0	0.577	0.319	0.282	0	0	0.319	0.439	1.85	4.62	0.564	1.86	1.39	1.55	2.29	2.32
Coeff. of var., %	0	12.4	6.52	5.73	0	0	6.53	9.01	10.9	35.2	3.11	10.4	8.03	9.28	13.4	13.1
	Tribolium Larvae (5 Added)								Rodent Hairs (20 Added)							
11	5	5			5	5			16	16			16	18		
	5	5			5	5			17	18			17	20		
12	5	5			4	5			17	15			18	14		
	5	3[b]			5	5			15	7[b]			19	19		
13	5	5			5	5			18	14			17	19		
	5	5			5	5			14	16			16	18		
14	5	5			5	5			18	17			18	19		
	5	5			5	5			19	15			18	17		
Av.	5	5	4.85	4.75	4.90	5	4.74	4.86	16.8	15.85	16.93	17.60	17.4	18.0	17.51	16.59
Std dev.	0	0	0.748	0.532	0.11	0	0.447	0.351	2.28	1.81	4.08	1.78	1.06	1.85	2.10	4.65
Coeff. of var., %	0	0	15.4	11.2	2.29	0	9.43	7.22	13.6	11.4	24.09	10.11	6.10	10.3	11.95	28.03

[a] Values from Table 1.
[b] Eliminated from statistical evaluation.

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The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee F and was adopted by the Association. See *JAOAC* **53**, 396 (1970).

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PESTICIDE FORMULATIONS

Gas Chromatographic Determination of Dioxathion and Chlorfenvinphos in Emulsifiable Formulations and Livestock Dips

By J. E. PATERSON (African Explosives and Chemical Industries, Ltd., Research Department, P.O. North Rand, Transvaal, South Africa)

A gas chromatographic method is described for the quantitative determination of dioxathion and chlorfenvinphos in emulsifiable concentrates and livestock dips. A convenient quantity of the emulsifiable formulation is dissolved in xylene and the aqueous dip is extracted with xylene for gas chromatographic analysis, using a mixed silicone oil stationary phase and a flame ionization detector. The coefficients of variation for the dioxathion and chlorfenvinphos determinations in the emulsifiable concentrate are 2.4 and 1.0%, respectively. Recoveries of the former from a fouled dip ranged from 97 to 103% and recoveries of the latter ranged from 92 to 97%.

The principal biologically active constituent of dioxathion (Delnav®, Hercules AC 528) is 2,3-*p*-dioxanedithiol S,S -bis (O,O-diethyl phosphorodithioate). The technical material contains about 72% of the active ingredient, which is composed of 46% *trans*-isomer and 26% *cis*-isomer. The active constituent of chlorfenvinphos (Supona®, GC 4072) is 2-chloro-1-(2,4-dichlorophenyl) vinyl diethyl phosphate. The technical material is also a mixture of isomers consisting of about 90% β-isomer and 6.5% α-isomer.

Delnav is the trademark of the Hercules Powder Co.

Supona is the trademark of the Shell International Chemical Co.

Emulsifiable concentrate formulations containing both pesticides were prepared for trials in the control of ticks on cattle. A reliable and specific method of analysis was required to assess the different formulations produced and to study the changes in aqueous dips during field trials. A gas chromatographic method for the determination of dioxathion, using a microcoulometric detector, has been recommended (1), and chlorfenvinphos has also been determined by gas chromatography (2, 3). It therefore seemed likely that these 2 compounds could be determined in admixture by a gas chromatographic procedure, particularly as they are very different chemically.

Initially a nonpolar silicone oil (SE-30) was used as a stationary phase for this analysis. This, however, was not entirely satisfactory because dioxathion was not completely separated from the solvent. The mixed silicone oil liquid phase described by Henly, Kruppa, and Supina (4) was then used and this gave an excellent separation of the solvent from 1 of the dioxathion isomers and the 2 chlorfenvinphos isomers (*see* Fig. 1).

Although the methods presented here were designed for an emulsifiable concentrate containing 15% (w/w) of each pesticide and aqueous emulsion dips containing about 0.025% (w/v) of each pesticide, they may be readily adapted for other concentrations.

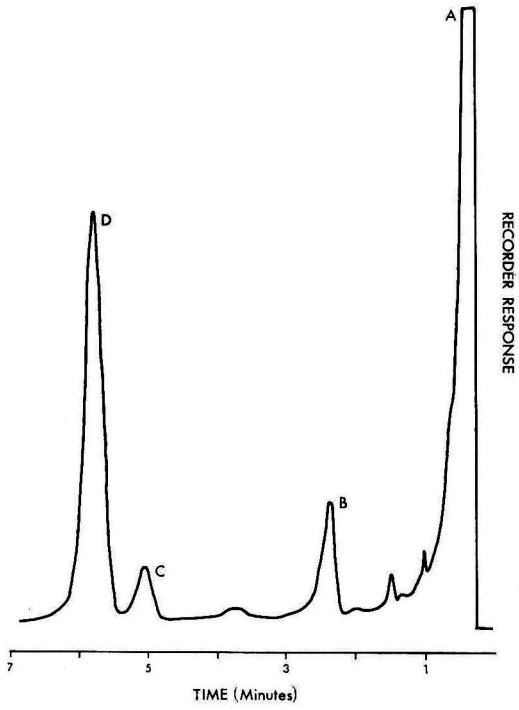

FIG. 1—Chromatogram of pesticide mixture: A, solvent (xylene) and *cis*-isomer of dioxathion; B, *trans*-isomer of dioxathion; C, chlorfenvinphos (α-isomer); D, chlorfenvinphos (β-isomer).

METHODS

Apparatus and Reagents

(a) *Gas chromatograph.*—Perkin-Elmer Model 881 equipped with dual flame ionization detectors and dual coiled glass columns, 6′ × 2 mm id, packed with 11% (QF-1 + OV-17) on 80–100 mesh Gas Chrom Q (Applied Science Laboratories, Inc., State College, Pa.). Operating conditions: nitrogen flow 30 ml/min, column 220°C, detector 230°C, injection port 270°C, recorder 2.5 mv, and chart speed 15″/hr.

(b) *Dioxathion.*—Technical grade (Hercules Powder Co., Inc., Wilmington, Del.). Technical material used in preparation of formulation should be used as standard.

(c) *Chlorfenvinphos.*—Technical grade (Shell International Chemical Co., Ltd., Shell Centre, London S.E.1, England). Technical material used in preparation of formulation should be used as standard.

(d) *Emulsion-splitting solution.*—10% (w/v) lead nitrate in 4% nitric acid solution.

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Analysis of Emulsifiable Concentrates

Accurately weigh amount of sample containing ca 0.30 g of each component into 50 ml volumetric flask. Dilute to volume with xylene. Prepare 3 standard solutions containing dioxathion and chlorfenvinphos in the range 0.10 to 0.50 g/50 ml xylene.

Chromatograph each sample and standard solution 3 times, using 1.5 μl per injection. Plot average peak area for each component against concentration of dioxathion and chlorfenvinphos in standard solutions. Use peak of β-isomer for measurement of chlorfenvinphos. Calculate amount of each component in sample by comparing average peak areas with standard curves.

Analysis of Aqueous Livestock Dips

Transfer 100.0 ml aliquot of well mixed sample to suitable glass-stoppered bottle. Add 10 ml emulsion-splitting solution and 25.0 ml xylene. Shake mixture 1 hr on mechanical shaker and centrifuge 20 min. Transfer upper xylene layer to small flask. Prepare 3 standard solutions containing dioxathion and chlorfenvinphos in the range 0.010–0.035 g/25 ml xylene.

Proceed with gas chromatography as above.

Results and Discussion

The precision of the method for emulsifiable concentrates was determined by replicate analyses of a single sample. The average dioxathion concentration was 14.1%, the standard deviation was 0.34, and the coefficient of variation was 2.4%. The average chlorfenvinphos concentration was 14.8%, with a standard deviation of 0.14 and a coefficient of variation of 1.0%. To check the accuracy of the method an emulsifiable concentrate containing exactly 14.6% dioxathion and 15.2% chlorfenvinphos was prepared in the laboratory. The average of 6 determinations was 14.8% of the former and 15.2% of the latter, which is an acceptable level of accuracy for the purposes mentioned.

It was found that extracts of fouled dips could be injected without any cleanup procedure. This shortened the analysis time considerably. The efficiency of the extraction procedure was checked by fortifying a fouled dip with varying amounts of both pesticides. Table 1 summarizes the average recoveries.

The standard deviation of the method on a fouled dip was 0.002 for dioxathion and 0.001 for chlorfenvinphos. This was determined by a number of replicate analyses on a single sample.

Table 1. Recovery of dioxathion and chlorfenvinphos from fouled dip

	Dioxathion			Chlorfenvinphos		
Sample	Added, mg	Found, mg	Recovery,[a] %	Added, mg	Found, mg	Recovery,[a] %
1	4.4	4.5	103	4.5	4.3	95
2	10.1	9.8	97	10.6	9.8	92
3	13.5	13.5	100	14.2	13.8	97

[a] Each recovery value is the average of 4 determinations.

Acknowledgment

Permission of African Explosives and Chemical Industries, Ltd., to publish this paper is gratefully acknowledged.

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FRUIT AND FRUIT PRODUCTS

Collaborative Study of Betaine in Orange Juice

By GRAYSON R. ROGERS (Food and Drug Administration, 60 Eighth Street, N.E, Atlanta, Ga. 30309)

An ion exchange-colorimetric method for determining betaine in orange juice was studied by 11 collaborators on 4 orange juice samples and 2 synthetic water solutions consisting of sucrose, dextrose, and various amino acids found in orange juice. Average recoveries in the collaborative study were 96.7 and 95.9%. Results show that the precision standard deviation among laboratories is generally acceptable. The distribution of the actual data is greater than normally expected, but random errors appear to be responsible since no significant systematic error can be detected in the data. The method is recommended for adoption as official first action.

The method of Lewis (1) for determining betaine was selected for study and application to the problem of determining betaine in orange juice. Betaine and amino acids are retained on a cation exchange resin while carbohydrates are removed with water. Elution of this column with 2% ammonia removes betaine and other amino acids. The eluate, after concentration to a small volume, is passed through a second ion exchange column consisting of a mixture of a cation and an anion exchange resin. Betaine passes through while other ionic species are retained. The eluate from this column is concentrated and betaine is precipitated with ammonium reineckate. After the precipitate is washed free of excess reagent, it is dissolved in 70% acetone for the colorimetric determination at 525 nm.

METHOD[1]

(Applicable to orange juice)

22.053 ***Reagents***

(a) *Ammonia soln.*—2%. Dil. 140 ml NH_4OH to 2 L with H_2O.

(b) *Ammonium reineckate soln.*—2.5%. Shake 2.5 g in 75 ml H_2O 30 min. Filter thru paper and dil. to 100.0 ml. Adjust pH to 1.0 with HCl and filter thru fine porosity fritted glass crucible. Prep. fresh before betaine pptn. Do *not* use reagent contg ppt.

(c) *Acetone soln.*—70%. Dil. 70 ml to 100 ml with H_2O.

[1] The section numbers within the method are those for the 11th ed. of *Official Methods of Analysis*, 1970: **22.003**(a) (11th ed.) =**20.003**(a) (10th ed.). Cautionary notes, if present, refer to the new chapter on safety, Chapter 46.

(d) *Aqueous ether.*—Add 1 ml H_2O to 140 ml ether.

(e) *Ion exchange resins.*—(*1*) *Amberlite IR-120 C.P. medium porosity (20–50 mesh, wet).*—Prep. 250 g in H form by treating with 2 bed vols 2*N* HCl (ca 500 ml). Soak 2 hr. Drain resin and wash with H_2O until neut. and Cl-free. (*2*) *Amberlite IRA-400 (20–50 mesh, wet).*—Prep. 250 g in OH form by treating with 2 bed vols 2*N* NaOH (ca 500 ml). Drain and wash NaOH-free with H_2O. Mix with IRC-50 C.P. immediately for column II prepn. (*3*) *Amberlite IRC-50 C.P.*—Prep. 125 g in H form by treating with 2 bed vols 2*N* HCl. Drain and wash Cl-free with H_2O.

(f) *Betaine std soln.*—1 mg anhyd. betaine/ml. Weigh 0.2623 g betaine.HCl in 200 ml vol. flask and dil. to vol. with H_2O.

22.054 *Preparation of Columns*

(a) *Column I.*—Use 18 mm id chromatgc tube with medium or coarse porosity fritted glass and with stopcock. Add aq. slurry Amberlite IR-120 C.P. (H) to 12.5 cm bed depth (wet resin). To regenerate resin, pour thru 100–200 ml 1*N* HCl and wash Cl-free with H_2O.

(b) *Column II.*—Intimately mix 2 vols Amberlite IRA-400 (OH) with 1 vol. IRC-50 (H) and transfer to column as above to 7.5 cm bed depth. Resins have different densities and excess H_2O causes undesirable sepn. Bed must be intimate mixt. Resin mixt. cannot be regenerated. Use for 2 detns only.

22.055 *Preparation of Sample*

Prep. juice as in **22.003**(a).

22.056 *Determination*

Add accurately measured amt prepd juice (10–20 ml) contg 5–7 mg betaine to small beaker. Dil. to ca 30 ml with H_2O and adjust to pH 3.0 with 0.1*N* HCl, using pH meter. Transfer to column I. Collect eluate at ca 3 ml/min. When liq. reaches top of resin, wash column with 200 ml H_2O or until carbohydrate-free. Discard eluate and wash soln. Elute betaine by washing column with ≥150 ml 2% NH_4OH, ensuring eluate is alk. Follow with 100 ml H_2O. Reduce eluate to ca 25 ml by boiling. Cool, adjust to pH 7.0 with 0.1*N* HCl, and transfer to column II. (Reduce vol. in erlenmeyer and then transfer to small beaker for pH adjustment.)

Collect eluate at 1 ml/min. When liq. reaches top of resin bed, wash with 50 ml H_2O. Conc. combined eluates and washings to 15–20 ml, cool, and adjust to pH 1.0 with 1*N* HCl. Cool to 0±3° and gradually add, with stirring, 20 ml 2.5% NH_4 reineckate, adjusted to pH 1.0 and cooled to 0±3°. Let stand 3 hr at 0±3°. Filter while cold thru medium porosity 60 ml fritted glass crucible with vac. Transfer ppt with small amts cold filtrate. Wash ppt with three 5 ml portions aq. ether. Dissolve ppt in 10 ml 70% acetone and transfer to 25 ml vol. flask. Dil. to vol. with 70% acetone. Det. *A* at 525 nm on spectrophtr, using 1 cm cell against 70% acetone as ref. (Make readings within 4 hr.) Det. amt betaine from std curve.

22.057 *Preparation of Standard Curve*

Transfer 2.5, 5.0, 7.5, 10.0, 12.5, and 15.0 ml betaine std soln to beakers, using 10 ml buret. Add H_2O to ca 20 ml and proceed as in **22.056**, beginning, ". . . adjust to pH 1.0 with 1*N* HCl."

Plot of mg anhyd. betaine/ml against *A* should be straight line.

Results and Discussion

Preliminary Study

The method was tested for accuracy and precision before the collaborative study was conducted, as recommended by Youden (2). The mean recovery of betaine from 10 synthetic solutions was 98±3%. An intralaboratory study by another analyst showed recoveries of 98.5, 100, and 99.8% on samples similar to those used in the collaborative study.

Collaborative Study

Six samples were sent to each participating laboratory for the collaborative study. A betaine hydrochloride standard was also provided. Samples 1–4 were orange juice and Samples 5 and 6 were synthetic solutions of betaine in a mixture of sucrose (5%), dextrose (5%), choline citrate (0.2%), and 9 amino acids (0.02% each of L-glutamine, DL-serine, DL-alanine, DL-asparagine, L-(+)-glutamic acid, L-(+)-arginine HCl, L-(−)-proline, L-(+)-histidine HCl, and aspartic acid) normally found in orange juice. Betaine levels were selected so that the results could ultimately be grouped in pairs to facilitate the analysis of variance according to Youden's recommendation for collaborative tests (2). Samples 5 and 6 contained 100 and 90 mg anhydrous betaine/100.0 ml, respectively. In addition to the above 6 samples, each collaborator was sent a synthetic solution prepared similarly to Samples 5 and 6 to be used as a practice sample.

Collaborators were instructed to perform duplicate determinations on the practice sample and not to proceed with the other samples until they had achieved a recovery of 95–105% of the added betaine and were satisfied with their precision.

Table 1. Collaborative results for betaine in orange juice (mg/100 ml)

Coll.[a]	Paired Samples A (1, 2)	B (3, 4)	C (5, 6)[b]	Trial Samples, % Recovery
1	41.8, 37.9	76.2, 77.0	98.8, 85.2	96.5, 96.5
2	34.5, 38.0	68.0, 83.0	91.0, 92.0	99.0, 95.0
3	37.5, 36.5	74.0, 75.0	95.0, 89.0	98.3, 101.5
4	47.1, 59.1	91.3, 86.3	101.8, 75.0	101.8, 101.8
5	41.8, 31.8	68.1, 84.5	109.8, 90.0	87.3, 74.5, 76.5, 74.4, 83.1
6	33.0, 36.0	75.0, 72.0	79.0, 68.0	100.0, 99.0
7	36.9, 34.3	74.8, 85.0	94.0, 82.0	99.2, 98.1
8	39.1, 39.1	77.0, 81.3	103.1, 94.8	99.5, 94.8
9	32.5, 40.0	99.0, 86.0	97.0, 101.0	103.0, 104.0
10	36.6, 30.8	75.0, 72.5	97.5, 85.8	97.5, 96.2
Mean	38.2	79.1	91.5	
Std dev.	7.85	9.32	10.8	

[a] The values reported by Collaborator 11 were out of line with the rest of the results and are not included.
[b] Average recovery for Sample 5 = 96.7%; average recovery for Sample 6 = 95.9%.

They were instructed to perform single determinations on the 6 other samples and report results as mg anhydrous betaine/100.0 ml.

Results of the collaborative study are shown in Table 1 and are grouped in related pairs. The precision standard deviation and systematic error deviation are shown in Table 2. The results of Collaborator 4 for pair A could have been eliminated from the data by the Dixon test for outliers and by examination of 2 sample plots. However, they could not be eliminated in the detailed statistical analysis of the pairs. Therefore, the results were included.

Four collaborators had no problems with the method; they had no comments and reported no difficulties. Collaborator 1 felt that the Amberlite IRC-50 C.P. and the Amberlite IRA-400 should be mixed immediately after their preparation to prevent breakdown in the latter resin. Collaborator 3 had the same comment, along with a suggestion concerning reducing the volume of eluates from the columns and subsequent pH adjustment. He suggested using Erlenmeyer flasks to concentrate the column eluates to small volumes and then transfer to a small beaker for pH adjustment. Both suggestions have been incorporated in the method. Collaborator 7 felt the method could be written more explicitly as pertains to ion exchange terms, such as "bed volume" and "bed depth." This suggestion was incorporated in the method. Collaborators 8 and 9 reported some difficulty in filtering and washing the precipitate. This laboratory has found that this can be avoided by filtering immediately after removing the precipitate and solution from the refrigerator or ice bath. Collaborator 5 studied the effect of the length of time the precipitate stood and the time between dissolving the precipitate in acetone. His conclusion was that a length of time over 3 hr for the precipitation had little effect on the result but that allowing the acetone solution to stand 2 days would affect the absorbance reading. The author can confirm this and has added an appropriate statement to the method. Collaborator 5 also reported difficulty in obtaining good results on the practice sample but was able to obtain good recoveries on a standard he prepared himself. The author has no explanation for this. The general comment of the collaborators was that the method was too lengthy. This is inherent in most ion exchange methods.

Table 2. Precision (S_r) and systematic error (S_b) standard deviation at different betaine levels for collaborative method (each pair = 9 degrees of freedom)

Pair	S_r	S_b	F Ratio[a]
A	4.58	6.38	2.94
B	6.54	6.50	1.98
C	6.39	8.76	2.88

[a] $F = S_d^2/S_r^2$.

Summary and Recommendation

The overall precisions (Tables 1 and 2) are affected by laboratory biases, variations in preparation of standards, and random errors associated with most methods. The statistical

test for the presence of systematic errors (Table 2) shows that systematic error deviations are insignificant for all 3 pairs of test samples at the 95 and 99% confidence levels. This is supported by the results of Samples 5 and 6 and the results on the trial sample. It is recommended that the method for betaine in orange juice be adopted as official first action.

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Levels of Brominated Vegetable Oils in Soft Drinks by X-Ray Fluorescence Spectrometry and Gas-Liquid Chromatography

By H. B. S. CONACHER, J. C. MERANGER, and J. LEROUX[1] (Research Laboratories, Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Ontario, Canada)

A rapid screening method using X-ray fluorescence spectrometry has been developed for the detection and semiquantitative estimation of brominated vegetable oils in soft drinks. This method and a quantitative GLC technique have been applied to the determination of the brominated oil content in a wide range of soft drinks. Of 46 drinks examined, 23 contained brominated vegetable oils at levels between 7 and 85 mg/10 fluid oz of drink.

Brominated vegetable oils are used extensively in soft drinks as dispersing agents for the flavoring citrus oils. Although classified as safe by the U.S. Food and Drug Administration (1) and presently permitted by the Canadian Food and Drug Directorate (2), recent concern has arisen as to the possible toxicity of these brominated oils (3).

At the present time there is very little available data on the levels and distribution of brominated vegetable oils in soft drinks and this is mainly due to a lack of methodology. Recently, however, a quantitative gas-liquid chromatographic technique (GLC) has been described for the determination of these oils (4) and used to provide some information on the brominated oil content of a few orange drinks. Application of this GLC technique alone in a survey of a wide range of drinks would prove time-consuming because of uncertainty regarding their brominated oil contents. It was therefore considered essential to develop some rapid, semiquantitative technique for preliminary screening of the drinks prior to GLC analysis.

Of several methods described for the determination of total bromide (5–8), an X-ray spectroscopic technique based on that reported by Mathies and Lund (8) was selected as the most suitable.

This paper presents a detailed survey of soft drinks for their brominated oil content, analyzed by quantitative GLC (4), after a preliminary

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screening procedure based on X-ray fluorescence spectrometry.

METHOD

Reagents and Materials

(a) *Diethyl ether.*—Reagent grade; distilled before use.

(b) *Brominated oils and standard solutions.*—Brominated olive, sesame, and cottonseed oils were obtained from Abbott Laboratories; brominated corn oil was prepared in the laboratory (9). Standard solutions of brominated sesame oil were prepared by diluting various weights of this oil (range 0–400 mg) to 10 ml with diethyl ether.

(c) *Soft drinks.*—All samples were purchased in local stores.

Apparatus

(a) *Gas chromatograph.*—Varian Model 1740, with dual flame ionization detectors and following columns and conditions: dual 3′ × ⅛″ stainless steel columns packed with 3% JXR on 80–90 mesh Anakrom ABS support, injection temperature 250°C, detector temperature 290°C, column temperature programmed from 150 to 275°C at 8°/min, helium carrier gas ambient flow rate 35 ml/min. Peak areas were measured by disk integrator.

(b) *X-Ray spectrometer.*—Norelco vacuum X-ray spectrograph with Philips transistorized electronic circuit panel and scintillation counter, operated under following conditions: (*1*) tungsten target tube: 50 kv and 20 ma; (*2*) lithium fluoride-analyzing crystal (2d spacing 4.0276Å); (*3*) scintillation counter: 960 v dc; (*4*) samples: brominated oil solutions spotted on 1.25″ diameter paper disks; and (*5*) bromide peak intensity values (cps), measured at 29.98° (2θ), using fixed time 20 sec. Background values were obtained with blank disk.

(c) *Graduated pipet.*—0.2 ml Mohr-type, graduated to 0.01 ml.

(d) *Paper disks.*—1.25″ diameter cut from Whatman 3 mm chromatography paper.

(e) *Wooden holder.*—3 × 3 × ½″ plywood with plywood walls ½″ high on 2 adjacent sides.

(f) *Propipette.*—Rubber pipet filler (Canadian Laboratory Supplies Limited, Ottawa).

Procedure

(a) *Standard curve for brominated sesame oil.*—Position paper disk on wooden holder in corner between walls and clamp graduated pipet fitted with Propipette 1.5 cm above center of disk. Pipet, with continuous flow, 0.2 ml aliquot standard brominated oil solution onto center of disk. Dry disk in air 5 min at 20°C and measure cps in X-ray spectrometer. Repeat procedure on another 0.2 ml aliquot and obtain average cps. Plot latter against brominated oil content of 10 ml solution, for different concentrations of the oil, to obtain standard curve.

(b) *Extraction.*—Pour 280 ml soft drink (10 fluid oz) into 500 ml separatory funnel, saturate with NaCl, and extract with 100 ml diethyl ether by shaking 2 min. Repeat extraction 2 times with 75 ml portions of diethyl ether. Transfer combined ether extracts to 500 ml separatory funnel and extract with 30 ml 5N NaOH by shaking 2 min. Discard aqueous alkaline layer and wash ether with 50 ml portions of distilled water until neutral to litmus paper. Dry ether extract over anhydrous Na_2SO_4, filter off latter, and evaporate dried extract in flash evaporator at 50°C. Transfer residue quantitatively with diethyl ether to 10 ml graduated flask and dilute to volume with the same solvent. Retain for analysis by X-ray fluorescence spectrometry and GLC.

(c) *Approximate brominated oil content.*—Pipet 0.2 ml aliquot of 10 ml extract (b) onto paper disk and proceed as in (a) to obtain average cps. Read brominated oil content (as brominated sesame oil) from brominated oil standard curve (a).

(d) *Brominated oil content.*—Evaporate remainder (9.6 ml) of 10 ml extract (b) in rotary evaporator at 50°C and add appropriate amount of methyl pentadecanoate internal standard (ca ⅕ weight of approximate brominated oil content (c)). Convert to methyl esters by reaction with sodium methoxide-methanol and analyze by GLC as described by Conacher *et al.* (4).

Calculate brominated oil content as follows:

$$\text{mg Brominated oil/10 fluid oz} = (AT \times WI \times 1.042)/AI$$

where AT = sum of peak areas in sample; WI = weight of internal standard; and AI = peak area of internal standard.

Experimental

GLC Method

Weight response factors, relative to methyl pentadecanoate, for methyl 9,10-dibromo- and methyl 9,10; 12,13-tetrabromostearates were calculated to be 1.45 and 1.92, respectively. Allowance was made for 10% of the tetrabromo derivatives eluting under the dibromo derivative peak.

These factors are slightly lower than those obtained previously (4) and are probably due to the use of 2 different GLC instruments.

X-Ray Fluorescence Spectrometry

Initial experiments carried out with brominated sesame oil established that a linear relationship between X-ray measurement of bromide and oil concentration was obtained at oil concentrations up

to 400 mg/10 ml.[2] As the highest brominated oil concentration from extraction of any soft drink was expected to be approximately 50 mg/10 ml[3] (4), no attempt was made in this study to establish the linear relationship at higher concentrations.

The accuracy of this method of bromide analysis in microgram quantities is bound mainly to that of sample preparation. The standard deviations from intensity measurements of 10 samples from 1 low brominated oil concentration (10 mg/10 ml) and from 10 samples of 1 higher brominated oil concentration (125 mg/10 ml) were found to be 4.0 and 7.0%, respectively. These errors were considered quite acceptable for this type of screening procedure.

Results and Discussion

The brominated oil contents of the soft drinks, determined by the X-ray fluorescence technique and by quantitative GLC, are presented in Table 1.

With the carbonated beverages, the results indicate that brominated vegetable oils are used mainly in citrus-flavored soft drinks at levels ranging from approximately 10 to 50 mg/10 fluid oz. Brominated oils could not be detected in any of the colas, ginger ales, or root beers examined.

By comparison of the fatty acid compositions of the 4 standard brominated oils (Table 2) with those from carbonated drinks (Table 3) and allowing for the natural variation of seed oils, it can be concluded that the most common brominated oils used are brominated sesame oil (13 drinks), and an oil whose composition does not correspond to any of the standard brominated oils (6 drinks). Using the standard brominated oil compositions (Table 2), it would appear that an oil of this composition would result from a mixture of 2–3 parts of brominated corn oil and 1 part of brominated olive oil. The remaining 2 carbonated beverages contain brominated cottonseed oil (orange H) and brominated olive oil (miscellaneous C), respectively.

The 2 samples of noncarbonated drinks are, however, more unusual. Relative to the carbonated drinks, both contain high amounts of brominated oil (ca 80 mg/10 fluid oz) in addition to similar amounts of an oil with a high proportion of shorter chain fatty acids (C_{12}, C_{14}, C_{16}). It is possible that the total oil content is comprised of an equal mixture of brominated sesame oil and a seed oil of the Palmae family. This family is usually characterized by the presence of high amounts of these shorter chain acids (10).

Table 1. Levels of brominated oils in soft drinks[a]

Type of Drink		Brominated Vegetable Oil (mg/10 fluid oz) Extract 1 X-Ray[b]	Extract 1 GLC	Extract 2 GLC
Carbonated Beverages				
Citrus				
Orange	A	36.8	34.0	33.5
	B	44.0	45.5	46.1
	C	45.6	40.5	43.5
	D	54.6	51.3	52.1
	E	18.2	19.1	18.8
	F	28.8	29.2	29.1
	G	35.4	34.4	34.6
	H	7.8	9.0	9.5
	I	38.4	37.0	41.5
	J	33.0	32.8	33.6
	K	47.0	46.0	49.0
	L	24.8	23.9	23.5
Lime	A	23.4	27.1	28.9
	B	24.8	24.8	23.7
Lemon-lime	A	14.5	15.0	15.6
	B	<1	—	—
	C	<1	—	—
Grapefruit-lime	A	18.4	18.7	18.0
Lemon	A	7.0	7.8	7.6
	B	16.0	17.0	14.8
	C	<1	—	—
	D	<1	—	—
	E	<1	—	—
Miscellaneous	A	7.2	8.0	7.4
	B	29.8	34.5	33.2
	C	24.8	28.5	27.8
Colas	A–I	<1	—	—
Ginger ales	A–G	<1	—	—
Root beers	A, B	<1	—	—
Noncarbonated Beverages				
Citrus				
Orange	A	83.0	79.9[c]	78.3[c]
Pineapple-orange	B	86.0	77.7[c]	80.3[c]

[a] Each sample was extracted in duplicate. In Extract 2, methyl pentadecanoate was added as internal standard prior to extraction.

[b] Values below 10 cps, i.e., 1 mg/10 ml were considered insignificant and are given as <1 mg.

[c] Total oil determined by GLC was actually twice the amount given. Brominated sesame oil is assumed to comprise 1/2 total oil.

[2] Concentrations are reported as mg/10 ml as this volume is the final dilution with both standard oil and soft drink extracts.

[3] 50 mg brominated oil/10 fluid oz drink, extracted into 10 ml ether.

Conclusions

In general, agreement between the brominated oil contents determined by the 2 different methods is very good and, as would be predicted, the correlation is better with those drinks containing brominated sesame oil than with those possessing other brominated oils. Although it should be noted that the presence of any other ether-extractable bromide in the drinks would lead to erroneously high results from the X-ray procedure, the good agreement obtained with the GLC technique precludes this possibility in the drinks examined and indicates the potential of the X-ray technique as a rapid screening technique in survey work.

Table 2. Composition of standard brominated oils

Brominated Oil	Fatty Acid Composition[a] 16:0	18:0[b]	Dibromo Deriv.	Tetrabromo Deriv.
Olive	8.6	5.0	74.6	11.8
Sesame	5.9	5.0	42.7	46.4
Cottonseed	14.5	1.9	19.4	64.2
Corn	6.3	1.6	21.7	70.4

[a] Area per cent.
[b] Includes unsaturated C_{18} esters.

Acknowledgments

The authors are indebted to R. K. Chadha and A. G. Giroux for technical assistance.

Table 3. Nature of brominated oils in soft drinks

Type of Drink		Fatty Acid Composition[a] 16:0	18:0[b]	Dibromo Deriv.	Tetrabromo Deriv.	Brominated Oil
Carbonated Beverages						
Orange	A	6.8	8.3	35.0	49.9	corn and olive
	B	6.2	4.5	38.1	51.2	corn and olive
	C	6.5	5.1	37.4	51.0	corn and olive
	D	8.9	6.8	35.0	49.3	corn and olive
	E	6.0	7.6	39.7	46.7	sesame
	F	6.4	6.9	40.1	46.6	sesame
	G	5.8	6.7	42.1	45.4	sesame
	H	14.2	13.9	17.8	54.1	cottonseed
	I	5.8	6.6	41.5	46.1	sesame
	J	5.7	6.9	42.2	45.2	sesame
	K	6.3	7.3	43.0	43.4	sesame
	L	6.5	7.9	40.2	45.4	sesame
Lime	A	6.3	4.9	38.4	50.4	corn and olive
	B	6.5	4.8	37.0	51.7	corn and olive
Lemon-lime	A	6.7	7.0	43.5	42.8	sesame
Grapefruit-lime	A	5.6	4.9	43.3	46.2	sesame
Lemon	A	6.6	12.3	37.7	43.4	sesame?
	B	6.5	8.0	42.1	43.4	sesame
Miscellaneous	A	7.1	10.4	39.5	43.0	sesame
	B	7.5	8.1	39.3	45.1	sesame
	C	8.6	6.8	75.2	9.4	olive

Type of Drink		Fatty Acid Composition[a] 12:0	14:0	16:0	18:0[b]	Dibromo Deriv.	Tetrabromo Deriv.	Brominated Oil
Noncarbonated Beverages								
Orange	A	25.3	9.8	8.5	11.7	21.9	22.8	sesame and non-brominated oil
Pineapple-orange	A	24.5	10.3	8.8	10.9	22.0	23.5	sesame and non-brominated oil

[a] Area per cent.
[b] Includes unsaturated C_{18} esters.

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Collaborative Study of Two New Methods for the Determination of Phosphorus in Fruits and Fruit Products

By BEN ESTRIN and FREDERICK E. BOLAND (Division of Food Chemistry and Technology, Food and Drug Administration, Washington, D.C. 20204)

A collaborative study was conducted to compare a spectrophotometric molybdovanadate method and the official gravimetric quinoline molybdate fertilizer method 2.025(b), slightly modified, with the official volumetric method 20.032. Six collaborating laboratories analyzed dilute acid solutions of the ash of 5 different kinds of fruit products and an aqueous solution containing only KH_2PO_4 by each of the 3 methods. Satisfactory agreement between laboratories and good agreement between methods were obtained. From the results of this collaborative study it appears that the quinoline molybdate and the molybdovanadate methods are more accurate and precise than the official volumetric method. It is recommended that both the spectrophotometric molybdovanadate method and the gravimetric quinoline molybdate method for the determination of phosphorus in fruits and fruit products be adopted as official first action.

At present, the 2 official AOAC methods for determining the amount of phosphorus in fruits and fruit products are the colorimetric method **20.033–20.035** and the volumetric method **20.031–20.032** (1). Neither of these methods is entirely satisfactory. The colorimetric method is time-consuming and requires the use of hazardous perchloric acid and other concentrated acids. A serious disadvantage of the official volumetric method is that it is not sufficiently sensitive to accurately measure the low levels of phosphorus present in some types of food products.

Recently 2 accurate, rapid, and precise methods were adopted for total phosphorus in fertilizers: the spectrophotometric molybdovanadate method **2.018–2.022** and the gravimetric quinoline molybdate method **2.023–2.025**. In 1968, Estrin and Brammell (2) adapted these slightly modified methods to fruit analysis and compared them with the official volumetric method. Since the 2 proposed methods yielded results which were in good agreement with those obtained by the official volumetric method, it was decided to subject these methods to collaborative study.

Collaborative Study

Sample solutions of 5 different kinds of fruit products, prepared according to **20.003**, were selected for this study. Aliquots of these sample solutions were dried and ashed at 525°C as in **20.017** and the ash was dissolved in dilute acid. One sample, which was included to determine per cent recovery, consisted of a standard solution of P_2O_5 formulated by dissolving a weighed amount of KH_2PO_4 in a known volume of water.

Necessary dilutions were made by the Associate Referee in the preparation of collaborative samples. These steps were taken to eliminate ashing, preparation of the ash solution, and dilutions as variables and thus obtain a more valid comparison of the methods tested. Eighteen samples in 4 oz polyethylene bottles were sent to each of 6 collaborators. Each sample consisted of a test solution so that each collaborator was only required to pipet the prescribed aliquot from the sample and run the determination.

Collaborators were directed to determine phosphorus in each sample by the 3 methods: the spectrophotometric molybdovanadate method, the quinoline molybdate method, and the official volumetric method.

METHODS[1]

Spectrophotometric Molybdovanadate Method

(Do not clean glassware with P-contg detergents.)

22.040 ***Apparatus and Reagents***

(a) *Spectrophotometer.* — Beckman Instruments Model B or DU, or equiv., with matched 1 cm cells.

(b) *Molybdovanadate reagent.*—Dissolve 60 g NH_4 molybdate.$4H_2O$ in 900 ml hot H_2O, cool, and dil. to 1 L. Dissolve 1.5 g NH_4 metavanadate in 690 ml hot H_2O, add 300 ml HNO_3, cool, and dil. to 1 L. Gradually add molybdate soln to vanadate soln with stirring. Store at room temp. in polyethylene or g-s Pyrex bottle. (Reagent is stable indefinitely in polyethylene, but in Pyrex, ppt gradually forms after several months. Discard reagent if ppt forms.)

(c) *Phosphate std solns.*—(*1*) *Stock soln.*—0.5 mg P_2O_5/ml. Dissolve 0.2397 g pure (if assay $<100\%$ KH_2PO_4, 0.2397 g $\times$ 100/% KH_2PO_4 = correct wt) and dried (2 hr at 105°) primary std KH_2PO_4 in H_2O and dil. to 250 ml. (*2*) *Working solns.*—Dil. 0, 5, 10, 15, 20, 25, 30, and 35 ml stock soln to 500 ml to obtain 0.00, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, and 0.35 mg P_2O_5/10 ml, resp.

22.041 ***Preparation of Standard Curve***

Pipet 10 ml of each working soln into 25 ml erlenmeyers and stopper immediately to prevent evapn. As rapidly as possible for entire series, pipet 5.0 ml molybdovanadate reagent into each, stopper, and mix. Let stand $\geq$10 min for color development and read A of each soln within 1 hr.

Fill 4 matched cells with 0.00 mg standard. Set spectrophtr at 400 nm and adjust to 0 A with 1 cell. Read each cell A against this cell. Use cell with lowest A with 0.00 mg std in future measurements. If A of 0.00 mg std in other cells are >0.001 against this std in ref. cell, subtract their A from subsequent readings. Det. A of each std with instrument adjusted to 0 A for 0.00 mg std. After every 3 detns, refill cell contg 0.00 mg std to avoid error due to evapn and temp. changes. Plot A against mg P_2O_5/10 ml (vol. working std soln).

(*Note:* Use Pyrex dropper to fill and empty cells. Do *not* remove cells from holder. Use dropper tube with greater capacity than cell to prevent liq. from entering bulb. Bulb should be just large enough that cell can be filled or emptied in one operation. Rinse cell with succeeding std or sample soln. Use different dropper to fill and empty ref. cell.)

22.042 ***Preparation of Sample***

Proceed as in **22.020**. (Add 1 teaspoon sucrose to samples low in sugar to speed ashing.) Dissolve ash in 10 ml HCl (1 + 3) and evap. to dryness on steam bath. Dissolve residue in 10 ml HCl (1 + 9) on steam bath and transfer to 100 ml vol. flask. Cool, dil. to vol., and mix. Filter thru dry paper if any insol. matter is present. If ash has >3.5 mg P_2O_5, dil. to >100 ml or make secondary dilns so 10 ml aliquot contains <0.35 mg P_2O_5. (*See* Watt, B. K., and Merrill, A. L., *Composition of Foods*, USDA Handbook No. 8, pp. 6–67, Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402, rev. Dec. 1963, for data on P content of fruit products and other foods.) If ash wt is not desired, use smaller sample aliquot to reduce drying and ashing time.

22.043 ***Determination***

Into sep. 25 ml erlenmeyers pipet 10 ml aliquots std solns contg 0.00 and 0.20 mg P_2O_5/10 ml. Develop color as for std curve. Adjust instrument to 0 A for 0.00 mg std and det. A of 0.20 mg std. (A of this std should be within $\pm 1\%$ of A of std curve; if not, prep. new std curve.) Develop color and det. A of sample ash solns concurrently with and in same manner as for std solns. Calc. as follows:

(a) *From std curve.*—mg P_2O_5/100 g sample = 100 $\times$ (mg P_2O_5/10 ml from std curve/g sample in 10 ml ash soln).

(b) *From formula.*—mg P_2O_5/100 g sample = $A \times S \times 100/W$, where A refers to sample soln at 400 nm; S = slope of std curve = $\Sigma r/n$, Σr = sum of ratios of mg P_2O_5/10 ml to A of each std, and n = number of std solns used in calcns; and W = g sample in 10 ml ash soln.

[1] The section numbers within the method are those for the 11th ed. of *Official Methods of Analysis*, 1970: **22.020** (11th ed.) = **20.017** (10th ed.), **2.023**(c) = **2.023**(c), **2.025**(b) = **2.025**(b). Cautionary notes, if present, refer to the new chapter on safety, Chapter 46.

Gravimetric Quinoline Molybdate Method

22.044 ***Reagents***

See **2.023**(c).

22.045 ***Preparation of Sample***

Prep. as in **22.042**, but transfer HCl (1 + 9) soln of residue to 500 ml erlenmeyer. Filter into 500 ml erlenmeyer if any insol. matter is present. If sample ash has >25 mg P_2O_5 (*see* Watt and Merrill), dil. to 100 ml or other definite vol. and pipet aliquot contg <25 mg P_2O_5 into 500 ml erlenmeyer. Dil. soln to ca 100 ml with H_2O.

22.046 ***Determination***

Proceed as in **2.025**(**b**), except boil 3 min. Report results as mg P_2O_5/100 g.

Results and Discussion

The results obtained from 6 collaborators for the determination of phosphorus in fruit products are shown in Table 1.

The mean values obtained by the official volumetric method for all samples except orange juice are slightly higher than by the other 2 methods. For orange juice the mean values from the molybdovanadate method (51.3 mg P_2O_5/100 g) and the official volumetric method (51.2 mg P_2O_5/100 g) are essentially the same. The results by all 3 methods on all the samples are generally in good agreement with each other.

The mean value for per cent recovery of P_2O_5 from a standard solution of known composition for the official volumetric method (102.0%) is slightly higher than for the molybdovanadate method (101.3%) and the quinoline molybdate method (100.4%). On the basis of this 1 sample, it appears that the molybdovandate and quinoline molybdate methods are more accurate than the official volumetric method.

Using standard deviation as a measure of precision, the quinoline molybdate and the molybdovanadate methods are more precise than the official volumetric method. In 5 of the 6 samples the standard deviations for these 2 new methods were lower than for the official volumetric method.

Recommendation

It is recommended that the molybdovanadate and the quinoline molybdate methods for the determination of phosphorus in fruit products be adopted as official first action.

Table 1. Comparison of phosphorus analysis (mg P_2O_5/100 g) by the proposed spectrophotometric molybdovanadate method (MV), the gravimetric quinoline molybdate method (QM), and the official volumetric method (V)

	Grape Jelly			Apple Jelly			Orange Juice			Raspberries			Strawberry Preserves			P_2O_5 Std Soln, % Rec.		
Coll.	MV	QM	V	MV	QM	V	MV	QM	V	MV	QM	V	MV	QM	V	MV	QM	V
1[a]	12.78	13.04	12.74	7.31	7.36	7.46	50.75	50.99	50.55	42.29	42.44	42.38	23.75	23.96	24.11	100.0	100.6	100.9
2	13.06	12.83	13.12	7.42	7.44	7.00	51.25	50.90	51.90	42.71	42.33	42.92	23.61	24.11	24.22	100.0	101.2	101.4
3	12.94	12.56	13.50	7.64	7.00	8.06	52.00	50.20	52.20	43.33	41.67	42.92	24.31	23.44	24.61	101.3	99.0	102.6
4	13.19	12.83	14.56	7.64	7.23	7.78	50.62	50.70	49.30	42.70	42.28	44.08	24.30	23.82	25.56	100.9	100.5	103.0
5	13.06	12.83	13.67	7.42	7.28	7.78	51.75	49.60	51.40	41.67	42.25	42.17	24.16	23.72	24.56	102.7	129.4[b]	101.4
6	13.11	12.88	12.50	7.50	7.39	7.89	51.50	50.90	51.60	42.29	42.42	43.17	24.16	24.00	24.39	102.7	100.8	102.8
Mean	13.02	12.83	13.35	7.49	7.28	7.66	51.31	50.55	51.16	42.50	42.23	42.94	24.05	23.84	24.58	101.3	100.4	102.0
Std dev.	0.15	0.13	0.76	0.13	0.16	0.38	0.56	0.55	1.07	0.54	0.27	0.67	0.31	0.26	0.54	1.22	0.84	0.89

[a] Associate Referee.
[b] Omitted from statistical calculations.

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The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee D and was adopted by the Association. See *JAOAC* 53, 389–390 (1970).

This report of the Associate Referee, B. Estrin, was presented at the 83rd Annual Meeting of the AOAC, Oct. 13–16, 1969, at Washington, D.C.

Note on Paper Chromatographic Detection of Adulteration in Dark Colored Fruit Juices

By J. FITELSON (Filtelson Laboratories, Inc., 254 W. 31 St., New York, N.Y. 10001)

A second collaborative study was carried out on the official first action method for dark colored fruit juices. The method is based mainly on differences in the anthocyanin patterns of adulterated fruit juices when compared with authentic samples. Natural colors used for such adulteration show more complex patterns than those of the dark juices, so that such addition can usually be detected. Anthocyanidin patterns yield much less information than the anthocyanin patterns. Eleven collaborators correctly detected adulteration in a blackberry juice with added invert sugar and elderberry juice, and 10 of them correctly found adulteration in a raspberry juice with added apple juice and grape skin extract. The method is recommended for adoption as official final action.

A paper chromatographic method for the separation of the natural coloring material in dark colored fruit juices, based on the official method for Concord grape juice (1), was adopted as an official first action method last year (2, 3). Unlike Concord grape juice, other dark colored fruit juices show relatively simple anthocyanin patterns, so that this part of the method is usually sufficient to detect addition of foreign natural colors with complex anthocyanin patterns. In some cases, it may be necessary to develop the chromatograms of the anthocyanidins to confirm the findings in the anthocyanin examination.

A second collaborative study was carried out this year with 2 authentic juices and 2 unknowns, preserved with salicylic acid. One sample was made from raspberry juice and an equal volume of apple juice, with intensity of color restored by addition of a grape skin extract. The other test sample was a blackberry juice with 30% added invert sugar solution and elderberry juice added to original color intensity. Both of these foreign colors are alleged to have been used in some commercial samples. Collaborators were instructed to concentrate on the anthocyanin patterns and to use the anthocyanidin part of the methods only if necessary to arrive at some definite conclusion.

All collaborators correctly identified the blackberry juice as adulterated with a foreign color, while 10 of the 11 collaborators also correctly found that the raspberry sample was adulterated. The ultraviolet examination of the anthocyanin chromatogram gave the most useful information. A few collaborators also developed the anthocyanidin chromatograms, but limited information was obtained here. Several of the collaborators had never performed this type of test before, but obtained satisfactory results without difficulty.

Recommendations

In view of the excellent results obtained in this study, it is recommended that the method be adopted as official final action, and that no further work be done on this subject.

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DRUGS

Rapid Colorimetric Assay for Nitroglycerin, Suitable for Content Uniformity Testing

By CLYDE E. WELLS, HARVEY M. MILLER,[1] and YVONNE H. PFABE (National Center for Drug Analysis, Food and Drug Administration, 1114 Market St., St. Louis, Mo. 63101)

The column chromatographic isolation method of Hohmann and Levine has been combined with a modified version of the colorimetric determination of Bell, resulting in a convenient assay for nitroglycerin in tablets. The method agrees closely with the AOAC infrared method and is sensitive enough for analysis of individual tablets (0.15 mg nitroglycerin). The standard deviation is 0.91% at the 1.2 mg level.

Nitroglycerin in pharmaceutical preparations is usually determined by the AOAC infrared method (1) or the USP method of Hohmann and Levine (2, 3).

In the AOAC method nitroglycerin is extracted with carbon disulfide and the absorption at 7.89 μm is measured. While this method has a degree of specificity, it lacks sensitivity and is subject to wide variability of results as shown by the collaborative study (4). In the USP method nitroglycerin is eluted from a water-Celite column with isooctane. The nitroglycerin is then extracted into a phenoldisulfonic acid solution for color development. This method has the advantage of using potassium nitrate as a standard instead of the nitroglycerin adsorbate used in the AOAC method. However, neither the AOAC nor the USP method is sensitive enough for the analysis of individual tablets.

A more sensitive method, used for individual tablet analyses, has been developed by Bell (5). This method is based on the alkaline hydrolysis of nitroglycerin. The nitrite ion formed during hydrolysis is used to diazotize an aromatic amine which is then reacted with N-1-naphthylethylenediamine to form a colored product. The method suffers from the fact that it is empirical and the lack of a separation procedure could lead to interferences.

Woodson and Alber (6) have developed a polarographic method suitable for individual tablet analyses. This method does not require isolation of the nitroglycerin but does require the use of polarographic equipment not readily available.

In our laboratory it was found that the hydrol-

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ysis of nitroglycerin with tetramethylammonium hydroxide in nonaqueous systems is rapid and reproducible, yielding 2 moles of nitrite ion from each mole of nitroglycerin. A colorimetric method based on this hydrolysis has been developed. The nitrite ion formed is used to diazotize *p*-chloroaniline which is then reacted with N-1-naphthylethylenediamine. Interferences are removed by Hohmann and Levine's column chromatographic procedure (2, 3).

The method is rapid and precise. It yields results in close agreement with the AOAC method (1) and is sensitive enough for the analysis of individual tablets containing 150 μg nitroglycerin.

METHOD

Reagents

(a) *Nitroglycerin adsorbate.*—About 10% nitroglycerin adsorbed on lactose. May be standardized by USP method (3).

(b) *Isooctane.*—ACS reagent grade 2,2,4-trimethyl pentane.

(c) *1-Propanol.*—ACS reagent grade.

(d) *Celite 545.* — Acid-washed (Johns-Manville Corp.).

(e) *Tetramethylammonium hydroxide (TMAH) solution.*—Dilute 25 ml 10% TMAH to 1 L with 1-propanol.

(f) *Amine solution.*—Dissolve 50 mg N-1-naphthylethylenediamine dihydrochloride in 10 ml concentrated HCl and add to 90 ml 1-propanol containing 50 mg *p*-chloroaniline. Prepare fresh daily.

(g) *Standard solution.*—Weigh accurately an amount of adsorbate equivalent to ca 6 mg nitroglycerin, transfer to 500 ml volumetric flask containing 200 ml isooctane, and shake 1 min; dilute to volume with isooctane. Prepare a fresh standard weekly.

Extraction

Accurately weigh portion of finely powdered tablet material equivalent to ca 1.2 mg nitroglycerin into 50 ml beaker. Add 2.0 ml water and swirl 1 min; add 3 g Celite and mix well. Transfer in 2 portions to chromatographic column containing bottom layer of 3 g Celite and 2.0 ml water and tamp. Drywash beaker with ca 1 g Celite and place on column. Wipe beaker and tamping rod with pledget of glass wool and transfer to column. Place 100 ml volumetric flask under column and elute with 100 ml isooctane. Dilute to volume with isooctane.

Color Development

Pipet 5.0 ml each of standard solution, sample, and isooctane into separate 25 ml volumetric flasks. Pipet 10.0 ml TMAH solution into each flask and let stand 10 min. Add 5.0 ml amine solution to each flask, let stand 30 min, and dilute to volume with isooctane. Determine absorbance of standard and sample solutions in 1 cm cells against reagent blank at 560 nm.

For individual tablets proceed as above except take 10 ml for color development and dilute standards appropriately.

Results

The following procedure was used to confirm the stoichiometric conversion of nitroglycerin to nitrite in the colorimetric method: Sodium nitrite solution (0.1*M*), which had been standardized against sulfanilamide, was diluted with TMAH solution to obtain a solution containing 3.5 μg sodium nitrite/ml. A 10.0 ml aliquot of this dilute nitrite solution was transferred to a 25 ml volumetric flask containing 5.0 ml isooctane. The color was developed according to the procedure above, beginning with "let stand 10 min." A 5.0 ml aliquot of nitroglycerin standard solution (60 μg) was also carried through the color development procedure. The conversion to nitrite was calculated from the formula:

$$\text{Moles } NO_2/\text{mole nitroglycerin} = (A_N)(MW_N)(\mu g\ S)/[(A_S)(MW_S)(\mu g\ N)]$$

where A_N and A_S = absorbance of nitroglycerin and sodium nitrite solutions, respectively, and MW_N and MW_S = molecular weight of nitroglycerin and sodium nitrite, respectively. Determinations using 3 separate sodium nitrite solutions gave 1.95, 1.97, and 1.98 moles nitrite/mole nitroglycerin.

To test the precision of the colorimetric and AOAC methods, 11 portions of a ground composite sample (0.4 mg nitroglycerin/tablet) were analyzed by the colorimetric method and 5 portions by the AOAC method. For the colorimetric method the average recovery was 0.397 mg/tablet, with a range of 0.395–0.407 mg/tablet and a relative standard deviation of 0.91%. For the AOAC method the average recovery was 0.394 mg/tablet with a range of 0.386–0.405 mg/tablet and a relative standard deviation of 2.0%.

To check the application of the colorimetric method to individual tablet analysis, 9 portions of a composite sample (0.376 mg nitroglycerin/tablet found by the colorimetric method), each equivalent to 1 tablet weight, were analyzed. The average recovery was 0.380 mg/tablet with

Table 1. Recovery of nitroglycerin by the colorimetric method

Added, mg	Recovered, mg	Recovery, %
0.1040	0.1006	96.7
0.1069	0.1047	97.9
0.2496	0.2471	99.0
0.3785	0.3706	97.9
	Av.	97.9
	Std dev.	0.83

a range of 0.377–0.386 mg/tablet and a relative standard deviation of 0.80%.

Four portions of adsorbate, equivalent to 0.1–0.4 mg nitroglycerin, were taken through the procedure to determine the recovery of nitroglycerin at low levels. These results are reported in Table 1.

The colorimetric and AOAC methods were compared on 28 commercial samples from 3 manufacturers. These comparative results are reported in Table 2.

Conclusions

These experiments show that the colorimetric and AOAC methods give results which agree within 1% on the average and that the colorimetric method is more precise. The colorimetric method is also sensitive enough to be used for individual tablet analysis and the color development may be automated (7). The results on the commercial samples show that the colorimetric method is applicable to a wide range of samples. No interferences with the method were found in any of these samples.

Table 2. Comparison of colorimetric and AOAC methods for nitroglycerin in commercial samples

Manuf.	Label Claim, mg/ Tablet	% of Label Claim Found		Colorim. – AOAC
		Colorim.	AOAC	
A	0.6	105.7	108.3	−2.6
	0.6	104.2	106.1	−1.9
	0.6	108.8	111.2	−2.4
	0.6	112.0	111.3	+0.7
	0.6	112.2	109.2	+3.0
	0.4	95.0	99.1	−4.1
	0.4	101.2	103.0	−1.8
	0.4	99.5	98.5	+1.0
	0.4	110.2	107.0	+3.2
	0.4	105.0	107.5	−2.5
	0.4	105.0	104.0	+1.0
	0.4	102.2	103.0	−0.8
	0.4	113.5	111.0	+2.5
	0.4	94.5	96.3	−1.8
	0.4	102.8	104.2	−1.4
	0.4	102.0	105.0	−3.0
	0.3	105.8	107.0	−1.2
	0.3	102.2	103.0	−0.8
	0.3	108.7	111.7	−3.0
	0.3	97.6	100.0	−2.4
	0.3	107.0	106.6	+0.4
	0.15	109.9	111.7	−1.8
	0.15	108.1	108.0	+0.1
B	0.6	105.5	103.0	+2.5
	0.4	71.5	71.7	−0.2
	0.3	97.8	98.2	−0.4
C	0.4	97.7	99.5	−1.8
	0.3	98.9	101.2	−2.3
Av. diff. ($\bar{D}$)				−0.78*
Std dev. (SD) of diff.				1.92

* Significant at 5% level as determined by the paired t-test where $t = \sqrt{N}\left(\frac{\bar{D}}{SD}\right)$.

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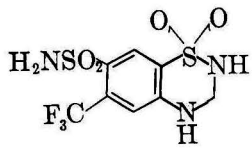
Extraction and Spectrophotometric Determination of Benzthiazide, Hydrochlorothiazide, and Hydroflumethiazide in Pharmaceuticals

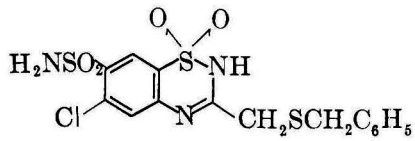
By F. RAYMOND FAZZARI (Division of Drug Chemistry, Food and Drug Administration, Washington, D.C. 20204)

A method has been developed for the determination of benzthiazide, hydrochlorothiazide, and hydroflumethiazide in pharmaceuticals. The procedure is rapid and specific and employs established separation techniques. The active ingredient is eluted from a basic Celite column with an acetic acid-ether solvent and extracted from the organic phase into NaOH for the spectrophotometric determination.

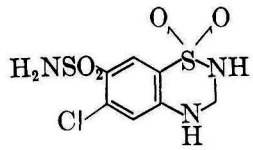
An improved analytical method was needed for the determination of hydroflumethiazide in pharmaceuticals. A specific quantitative method was developed for hydroflumethiazide; this same procedure was applied to other thiazides and found applicable to benzthiazide and hydrochlorothiazide, all closely related compounds.



Hydroflumethiazide



Benzthiazide



Hydrochlorothiazide

The official USP XVII method for hydrochlorothiazide is a polarographic procedure which does not include a quantitative separation. Perez *et al.* (1) assayed the color reaction product of the drug and anthrone in H_2SO_4. Kala (2) made a colorimetric determination by diazotization and then coupling with alkali thymol solution. In addition, he made gravimetric and titrimetric determinations. A bromatometric procedure has been reported by Kertesz (3). Other colorimetric methods (4, 5) involving hydrolysis and/or steam distillation have also been developed and published.

For benzthiazide and hydroflumethiazide, applicable methodology is limited. Bermejo (6) determined hydroflumethiazide by hydrolyzing the compound to 4-amino-6-trifluoromethyl-1,3-disulfamate benzene and then determining the color after diazotizing and coupling with chromotropic acid.

Benzthiazide was determined by Vasiliev (7) by extracting the compound from dosage form with acetone and then titrating with 0.1*N* NaOH, using phenol red.

The present method employs simpler and more direct techniques, is more specific, and is free of the analytical problems associated with the aforementioned methods. The procedure involves the use of both column partition chromatography and liquid-liquid extraction. The active ingredient is eluted from a basic Celite column with an acetic acid-ether solvent, extracted from the organic phase into NaOH, and determined by UV spectrophotometry.

Experimental

Apparatus and Reagents

(a) *Chromatographic columns and tamping rod.*—25 × 200 mm glass chromatographic column and suitable tamping rod.

(b) *Chromatographic siliceous earth.*—Celite 545, acid-washed (Johns-Manville Corp.).

(c) *Solvents.*—Ether, $CHCl_3$, isooctane, 0.2*N* NaOH, and glacial acetic acid.

(d) *Standards.*—For UV comparison, prepare standards in 0.2*N* NaOH saturated with ether as follows: benzthiazide 3.0 mg/200 ml, hydroflumethiazide 2.0 mg/200 ml, hydrochlorothiazide 2.0 mg/200 ml.

Procedure

Sample preparation.—Finely powder 20 tablets to pass No. 60 sieve. Transfer portion equivalent to 75 mg active ingredient for benzthiazide and 50 mg for hydrochlorothiazide and hydroflumethiazide, with aid of 0.2*N* NaOH, to 50 ml volumetric flask. Shake to achieve complete dissolution and dilute to volume.

Preparation of column.—Lower layer: Mix 2 g Celite with 1 ml 0.2*N* NaOH in 150 ml beaker, transfer to column, and tamp to uniform mass. *Upper layer:* Mix 3 g Celite with 2 ml sample preparation, transfer to column, and tamp. Dry wash container in which the sample preparation was mixed with 1 g Celite and 2–3 drops water; transfer wash to column and tamp. Add glass wool plug to column.

Procedure. — (Use water-saturated solvents throughout.) Pass 50 ml $CHCl_3$, followed by 50 ml ether, through column; discard eluate. Use 250 separatory funnel as receiver and elute column with 0.1 ml acetic acid in 100 ml ether. (Wash tip of column with ether.) Add 65 ml isooctane to eluate and extract organic phase with three 50 ml portions of 0.2*N* NaOH; combine NaOH in 200 ml volumetric flask and dilute to volume.

Concomitantly determine absorbance of sample and standard in 1 cm cells with suitable spectrophotometer, using 0.2*N* NaOH as blank.

Wavelength of maximum absorbance and absorptivities of individual compounds are as follows:

Compound	λ *Max., nm*	*Absorptivity*
Benzthiazide	295	29.6
Hydrochlorothiazide	273	49.1
Hydroflumethiazide	273	45.4

Identification.—Acidify portion of sample preparation with 1*N* HCl and extract with 50 ml ether. Evaporate ether to dryness, add 5 ml 95% alcohol, and evaporate again. Compare IR absorption spectrum of potassium bromide matrix of residue with that of reference standard previously recrystallized from 95% alcohol.

Discussion

The accuracy and precision in the recovery of the compounds are shown in Tables 1 and 2. These data were obtained by taking known amounts of each compound through the procedure. The assay values of commercial dosage forms are also reported and tabulated in these same tables. Table 3 represents the data obtained in the analysis of simulated tablet mixtures.

Table 1. Recovery of known amounts of standards

Added, mg	Recovered, mg	Recovered, %
	Benzthiazide	
3.14	3.14	100.0
3.14	3.14	100.0
3.14	3.14	100.0
3.11	3.13	100.5
3.09	3.11	100.4
3.02	3.03	100.2
1.57	1.58	100.5
1.57	1.57	100.0
1.57	1.57	100.0
	Hydroflumethiazide	
1.52	1.53	100.5
1.52	1.52	100.0
1.52	1.52	100.0
3.05	3.07	100.5
3.05	3.07	100.5
3.05	3.07	100.5
4.01	4.01	100.0
4.01	4.02	100.3
4.01	4.00	100.0
	Hydrochlorothiazide	
1.66	1.67	100.6
1.66	1.67	100.6
1.66	1.66	100.0
3.32	3.33	100.3
3.32	3.33	100.3
3.32	3.32	100.0
4.00	3.99	99.5
4.00	4.00	100.0
4.00	4.00	100.0

The method is rapid, reasonably specific, and employs well established techniques of quantitative separation and determination. The method will not, however, distinguish between active ingredient and the 4-amino-6-chloro-*m*-benzenedisulfonamide decomposition product which may be present with hydrochlorothiazide and hydroflumethiazide.

Prior to the analysis for the active ingredient, samples and standards were analyzed for the disulfonamide by the Rehm and Smith procedure (5) which has an absolute sensitivity of approximately 15 μg. An amount of sample equivalent to 50 mg active ingredient was extracted into ether from acid solution (as described in the procedure). The ether was then evaporated and the residue was analyzed. Standards were analyzed directly.

There was no decomposition product associated with benzthiazide. Hydrochlorothiazide and hydroflumethiazide gave negligible responses less

Table 2. Recovery from commercial dosage forms

Sample Weight, g	mg Found per Tablet	Per Cent of Declared
Benzthiazide (50.0 mg Declared)		
0.2041	50.1	100.2
0.2070	49.2	98.4
0.2037	50.1	100.2
0.3003	49.2	98.4
0.2993	50.2	100.4
0.3005	49.1	98.1
Hydroflumethiazide (50.0 mg Declared)		
0.2371	50.2	100.4
0.2780	49.9	99.8
0.3061	50.0	100.0
0.3011	50.5	101.0
0.2922	49.5	99.5
0.3250	50.5	101.0
Hydrochlorothiazide (50.0 mg Declared)		
0.4472	51.8	103.6
0.4599	52.0	104.0
0.4483	51.7	103.4
0.2285	51.5	103.1
0.2256	51.9	103.8
0.2275	51.7	103.4

Table 3. Assay of simulated tablet mixtures (10 determinations/sample)

Compound	Av. % Rec.	Std Dev.	Range
Benzthiazide	100.1	1.23	98.8–101.9
Hydrochlorothiazide	100.2	1.08	98.7–101.2
Hydroflumethiazide	100.1	0.97	98.7–101.8

than 0.04%, the same response given by the pure standards.

This same procedure was applied to the active ingredient that had been stored 5 hr in 0.2*N* NaOH at room temperature. The results were the same; less than 0.04% disulfonamide was detected. In addition there was no change in the intensity or position of the UV band for the active ingredient. Hence, under the conditions of the method, decomposition to the disulfonamide is not a problem.

The literature (5, 8) does report on the instability of hydrochlorothiazide in alkaline solutions. The studies, however, were carried out in 1.0*N* NaOH at elevated temperatures, 60 and 100°C, respectively. Mollica, Rehm, and Smith (8) determined the forward rate of reaction (k_1) to be 2.24×10^{-2} hr in 1.0*N* NaOH at 60°C. This results in a half-life of approximately 31 hr. In view of the fact that this is a first order reaction, extrapolation back to room temperature would result in a half-life of approximately 16 days. In 0.2*N* NaOH at room temperature the half-life would, if anything, be longer and decomposition in the first several hours would be negligible. Hence, the findings by this author that there is no discernible decomposition of the active ingredient to the disulfonamide in 0.2*N* NaOH at room temperature over several hours are consistent with what has been previously reported.

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Partition Chromatography and Determination of Noscapine in Cough Preparations

By STANLEY C. ELLISTON and MAUREEN L. COLES (Quality Control Laboratory, A. Wander Limited, King's Langley, Hertfordshire, WD4 8LJ, England)

The proposed method for combinations of noscapine with neutral and other basic drugs uses 2 columns: sulfamic acid and NaOH. Noscapine is eluted from the sulfamic acid column with chloroform, while other basic drugs are retained. Acidic substances and acetaminophen are trapped on the NaOH column. Recoveries of noscapine ranged from 98.4 to 100.3% on an authentic linctus and suspension. The method has been tested on 6 proprietaries and the low deviations between parallel determinations indicate a satisfactory degree of precision.

Noscapine (synonym, narcotine), a non-narcotic morphine alkaloid, made a brief appearance in an official compendium in the British Pharmaceutical Codex (BPC) of 1934, but was dropped from subsequent editions and did not appear again in an official pharmacopeia for many years. However, the drug appears to have attracted greater attention recently and was included in the British Pharmacopoeia, 1963, and the USP, 17th revision. It finds application in at least one official preparation (Noscapine Linctus BPC) and in several proprietaries.

The current BPC method for noscapine in Noscapine Linctus involves ether extraction and evaporation of ether, followed by UV determination of an HCl solution of the dry residue. Although such a method is applicable to preparations containing a single drug, it is not suitable in the presence of other UV-absorbing species which can be extracted from alkaline solutions by organic solvents.

This paper describes a method for the determination of noscapine, based on the technique of partition chromatography, and provides a striking demonstration of the power of the method to separate a single drug from interfering substances, even in complex mixtures. Most of the methods published on this technique (1–12) were developed on the basis of empirical observations. However, 2 recent papers (13, 14) have now expounded the theoretical and mathematical basis of the technique. The initial studies for this work were already under way when these latter papers were published. Nevertheless, it was felt, considering the general paucity of available information regarding this compound, and as existing literature values appear to be very uncertain (15), that an attempt should be made to determine the physico-chemical constants of noscapine and to develop a distribution diagram (13, 14). However, this was thwarted by some unusual properties of noscapine. The solubility in water is too low to permit a potentiometric determination of pK. From absorbance measurements we estimate the solubility to be about 1 in 100,000.

In attempting to follow the procedure for the spectroscopic determination of pK_a described by Albert and Sergeant (16), we found that there is a difference between the spectrum in acid and alkali (0.1*N* NaOH: maximum, 276 nm, minimum, 259 nm, rising to cut off at 244 nm; 0.1*N* HCl: maximum, 314 nm, minimum, 268 nm, cut off at 249 nm), but on adding acid solution of noscapine to buffer, the change to intermediate form was very slow, indicating that some phenomenon other than a simple charge transfer was taking place. It is tentatively postulated that what was observed may be due to the slow hydrolysis of the lactone ring, but the further investigation of this is beyond the scope of this present paper.

The method described below was therefore developed on the empirical basis that has so often proved fruitful during the last 10 years.

Two of the papers referred to above mention the use of sulfamic acid as a stationary phase. We have examined its behavior toward other drugs in which we are interested and we have found, using chloroform as eluant, that as well as pyrilamine (10), chlorpheniramine (10), and codeine (2), it also retains pheniramine and phenylpropanolamine, whereas noscapine and acetaminophen are eluted.

In developing the method the experimental work was carried out on preparation D (Table 1) which is the most complex proprietary encoun-

Table 1. Preparations analyzed for noscapine content

Preparation	Active Ingredients Claimed
A: BPC Linctus	Noscapine, 3 mg/ml
B: Sirup	Noscapine, 25 mg/7 ml
C: Sirup	Noscapine, 25 mg/10 ml; Pseudoephedrine, 49.2 mg/10 ml; Carbinoxamine maleate, 6 mg/10 ml
D: Suspension (hydrated Mg-Al silicate suspending agent)	Noscapine, 20 mg/4 ml; Pyrilamine maleate, 6.25 mg/4 ml; Pheniramine maleate, 6.25 mg/4 ml; Phenylpropanolamine hydrochloride, 12.5 mg/4 ml; Acetaminophen, 160 mg/4 ml; Terpin hydrate, 90 mg/4 ml
E: Tablet	Noscapine, 25 mg/tab
F: Sustained action tablet	Noscapine, 30 mg/tab; Theophylline, 200 mg/tab
G: Bi-tab, sustained action core	Noscapine, 20 mg/tab; Pyrilamine maleate, 6.25 mg/tab; Pheniramine maleate, 6.25 mg/tab; Phenylpropanolamine hydrochloride, 12.5 mg/tab; Acetaminophen, 160 mg/tab; Terpin hydrate, 90 mg/tab

tered containing noscapine. It was felt that if the problems involved in analyzing this preparation were overcome, no difficulty should be experienced with the other preparations.

The first approach was to follow, for example, Levine (2) and attempt to release basic drugs from an NaOH column followed by trapping of unwanted drugs on an acidic column. However, it was found that when mixtures containing significant quantities of acetaminophen were made alkaline before incorporation into a basic column, then brown decomposition products were eluted which interfered with spectrophotometric measurements. However, when acetaminophen in chloroform solution is passed through an NaOH column, it is trapped and no measurable breakdown occurs.

When noscapine and acetaminophen pass through a sulfamic acid column, they are accompanied by acidic excipients such as saccharin and sorbic acid; these and the acetaminophen can be removed by passage through an NaOH column. Phenylephrine and glyceryl guaiacolate are also associated with noscapine in a few formulations. Phenylephrine is trapped by an NaOH column (4, 8) and glyceryl guaiacolate has no absorbance at 314 nm; therefore, these drugs do not interfere. It was found that when the eluate has been passed through the NaOH column, the maximum absorbance had shifted from 314 to 310 nm. The eluate was therefore acidified with methanol and HCl to ensure that all noscapine present was in the fully protonated state. There is also some reduction in the absorbance of noscapine after passing through the column system; the reason for this is not known. This phenomenon is both constant and reproducible, but it does imply that a standard must be taken through the whole procedure in an identical manner to the sample, and instructions to this effect are included in the experimental section below.

The following procedure based on these observations was chosen.

Experimental

Apparatus and Reagents

(a) *Chromatographic columns.*—*See* sec. **32.013** (a) and (b) (11); we have found it convenient to use a column 11 cm long for column I and 7 cm long for column II.

(b) *Standard solution.*—Accurately weigh ca 0.1 g noscapine and transfer to 100 ml flask. Dissolve and dilute to volume with 10% sulfamic acid.

(c) *Water-washed chloroform.*—Shake ACS reagent grade chloroform with an equal quantity of water for 2 min. Let layers separate and filter chloroform layer slowly through plug of cotton wool. Reject first 10 ml of filtrate. Use chloroform from same bottle throughout, or mix chloroform from 2 bottles before commencing. Use within 3 days.

Preparation of Columns

Column I—liquid preparations: Pack wad of fine glass wool into base of column. Transfer accurately weighed representative portion of sample containing ca 5 mg noscapine to 100 ml beaker. Add sulfamic acid solution with concentration adjusted so that total volume of preparation is ≥5 ml and final concentration of sulfamic acid is 10%. Mix by swirling gently, in the case of suspensions, using a flexible spatula to break up any lumps. Do *not* heat.

Add 6.5 g Celite 545 per 5 ml solution and mix with flexible spatula until mixture is fluffy. Pack into column in about 5 equal quantities, tamping after each addition to compress mixture to uniform mass. Cover surface with pad of fine glass wool. Retain beaker, spatula, tamper, and any apparatus used to assist in packing column (e.g., powder funnel).

Column I—tablets: Pack wad of fine glass wool into base of column. Transfer accurately weighed representative portion of finely ground sample containing ca 5 mg noscapine to 100 ml beaker. Add 5 ml 10% sulfamic acid solution. It is essential to dissolve noscapine at this stage. Mix well with flexible spatula, ensuring that all fine powder is thoroughly wetted, and let stand ca 30 min, stirring occasionally.

Add 6.5 g Celite and continue as described above for liquid preparations.

Column II: Pack wad of fine glass wool into base of column. Add 3 g Celite to 2.5 ml 1*N* NaOH. Mix and pack into column as described above.

Standard columns—Column I: Pipet 5 ml standard solution into 100 ml beaker. Add 6.5 g Celite and continue as for sample column I. *Column II:* same as for sample column II.

Procedure

(Use water-washed chloroform throughout.)

Treat both sample and standard columns in the following identical manner. Mount column I directly over column II. Place under column II 100 ml volumetric flask containing 5 drops concentrated HCl and 10 ml methanol. Pass 80 ml chloroform through both columns. Use first portions to rinse beaker, spatula, tamping rod, etc., used in preparing column I and transfer wash to columns. When eluting, pour chloroform into column I to 2–3 cm above bed and let drain before adding next portion. Take care that level does not build up in column II. Discard column I and continue eluting column II until flask contains nearly 100 ml. Dilute eluate to volume with chloroform and mix.

Prepare reference solution by diluting 5 drops concentrated HCl and 10 ml methanol to 100 ml with chloroform.

Determine absorbance of sample and standard eluates against reference solution at wavelength of maximum absorbance, ca 314 nm.

Note: Weighing is recommended for liquid samples because of difficulty of accurately pipetting viscous sirups. Results can easily be converted to mg/ml by multiplying mg/g by density of sirup.

Table 2. Recovery of noscapine from laboratory preparations

Preparation	Noscapine		
	Added, mg/ml	Found, mg/ml	Recovery, %
A	3.00	3.01	100.3
		2.98	99.3
D	5.05	4.97	98.4
		5.00	99.0

Results and Discussion

The method has been applied to 7 preparations, 6 proprietary and 1 official (Table 1). Samples of preparations A and D containing known amounts of noscapine were prepared in the laboratory, and the recovery values obtained are given in Table 2. The results on the other preparations are given in Table 3.

With suspension D and tablets G, evaporation

Table 3. Analysis of various commercial preparations for noscapine

Preparation	Noscapine		
	Claimed, mg/unit	Found, mg/unit	Found, % of Label Claim
A	3/ml	2.93/ml	97.7
		2.91/ml	97.0
B	25/7 ml	26.6/7 ml	106.4
		26.5/7 ml	106.0
C	25/10 ml	23.9/10 ml	95.6
		23.8/10 ml	95.2
D sample 1	5/ml	4.76/ml	95.2
		4.74/ml	94.8
D sample 2	5/ml	4.75/ml	95.0
		4.84/ml	96.8
E	25/tab	25.9/tab	103.6
		25.8/tab	103.2
F	30/tab	28.5/tab	95.0
		28.5/tab	95.0
		28.5/tab	95.0
		28.6/tab	95.3
G sample 1	20/tab	20.5/tab	102.5
		20.6/tab	103.0
G sample 2	20/tab	21.4/tab	107.0
		21.5/tab	107.5

of the final eluate to dryness and application of the phosphotungstic-phosphomolybdic acid reagent **32.017** (**a**) (11) showed that terpin hydrate is present, but as this has no UV absorbance it does not interfere. With one exception the absorption spectra of the final eluates indicate the presence of no absorbing species other than noscapine. The exception was preparation B. By placing solutions of noscapine in the reference cell and adjusting the concentration until no evidence of the noscapine spectrum could be seen, it was concluded from the shape of the difference curve that the other absorbance observed was due to an unknown excipient with a single maximum at 270 nm and no absorbance at 314 nm. It was therefore concluded that it would not interfere in the determination.

The method has been described in a form which makes it as versatile as possible and which will succeed even with a complex pharmaceutical containing an excipient which makes "classical" manipulations involving separatory funnels impossible because of emulsion-forming properties.

Acknowledgments

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Cautionary Note Concerning Color Stability in Reineckate Salt Analyses

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The absorbance of solutions of reineckate salts in acetone or in aqueous acetone varies with time. Procedures should be modified so that absorbance measurements are made as soon as possible after dissolving the salt and so that the time lapse between solution and measurement is uniform for all samples and standards. The effect of time and temperature of precipitation on the absorbance of the reineckate salts of betaine, choline chloride, and homatropine methylbromide is shown.

Many quaternary ammonium compounds are determined by photometric measurement of the reineckate salt dissolved in acetone or aqueous acetone. All these methods are based on the work of Beattie (1) who determined choline as the reineckate salt by visual colorimetry. Bandelin and his coworker (2, 3) applied the method to the photometric determination of homatropine methylbromide and of betaine and choline mixtures. The NF procedure (4) for the assay of Homatropine Methylbromide Tablets and the USP procedure (5) for the assay of Carbachol Ophthalmic Solution utilize the photometric measurement of the reineckate salt in acetone.

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During a recent determination of betaine by the reineckate method, it was noted that the absorbance of the aqueous acetone solutions decreased markedly with time at room temperature. Variation of color intensity with time was noted by Beattie (1) who suggested a series of permanent visual standards based on methyl red. None of the photometric methods listed emphasizes the importance of time on the absorbance, but Fritz (6) suggested that measurements should be made at a specific time after solution of the precipitated reineckate salt.

This paper reports the variation of absorbance with time in acetone and aqueous acetone for the reineckate salts of betaine, choline chloride, and homatropine methylbromide and points out the necessity of close control of the time lapse between solution of the precipitated salt and photometric measurement in reineckate salt procedures.

METHOD

Reagents and Apparatus

(a) *Ammonium reineckate reagent.*—Prepare saturated solution by agitating 2.5 g ammonium reineckate (J. T. Baker Chemical Co.) with 100 ml water for 10 min at room temperature. Filter solution through fine porosity sintered glass filter just before use. Prepare fresh for each set of determinations.

(b) *Betaine standard solution.*—1.00 mg/ml. Dissolve 131.1 mg betaine hydrochloride (K & K Laboratories, Inc.) and dilute to 100.0 ml with water.

(c) *Choline chloride standard solution.*—1.00 mg/ml. Use USP Reference Standard.

(d) *Homatropine methylbromide standard solution.*—1.00 mg/ml.

(e) *Trisodium phosphate.*—25% (w/v).

(f) *Dilute reineckate-trisodium phosphate solution.*—Dilute 2.0 ml ammonium reineckate reagent plus 200 ml trisodium phosphate (25%) to 1 L with water.

(g) *Acetone.*—USP. Also 70% (v/v) and 75% (v/v) in water.

(h) *Aqueous ether.*—Add 1 ml water to 140 ml USP ethyl ether.

(i) *Spectrophotometer.*—Cary Model 15.

Procedures

Betaine.—Pipet a volume of betaine standard solution containing amount of betaine desired into 100 ml beaker and dilute to ca 30 ml. Add 1.0 ml 3*N* HCl and refrigerate until temperature is approximately 8°C. Refrigerate reineckate reagent during same period. Add slowly, with agitation, 5.0 ml chilled reineckate reagent and refrigerate 1 hr. Filter through medium porosity sintered glass filter, using bell jar rather than suction flask. Complete transfer of last portions of precipitate, using small quantities of filtrate to insure that none of the betaine reineckate dissolves during transfer step. Wash with three 5 ml portions of aqueous ether. Discard washings and filtrate. Replace receiver with 25 ml volumetric flask and add 5 ml acetone to filter. Allow 2 or 3 min for salt to dissolve before applying suction. Wash filter with 3 further 5 ml portions of acetone to insure complete removal of reineckate salt. Rinse stem of filter with acetone into flask and then dilute solution to volume with acetone. Determine absorbance at 525 nm immediately, using suitable spectrophotometer. (Aqueous acetone solutions (70 or 75%) were utilized in place of acetone in some of the procedures employed in this investigation.)

Choline chloride.—Use same procedure as for betaine except omit 3*N* HCl and make solution basic with 1.0 ml 25% trisodium phosphate before refrigeration. Also wash precipitate with 2.0 ml portions of dilute reineckate-phosphate solution in place of ether.

Homatropine methylbromide.—Use same procedure as for betaine except use 1.0 ml 20% H_2SO_4 (v/v) instead of HCl. Also wash precipitate with 2.0 ml portions of ice water in place of ether.

Effect of Time and Solvent on Absorbance

The absorbance of acetone solutions and aqueous acetone solutions of the reineckate salts produced from betaine, choline chloride, and homatropine methylbromide standards was measured periodically for several days. Also betaine standards were refrigerated for various times between precipitation and filtration to check the effect of refrigeration time after the precipitate was formed.

Effect of Temperature of Precipitation on Absorbance

Standard solutions of homatropine methylbromide were carried through the standard procedure with the exception that the precipitation was carried out at room temperature rather than after refrigeration. The absorbance was measured periodically for several days.

Results and Discussion

The effect of time upon the absorbance of acetone solutions of the reineckate salts of choline chloride, betaine, and homatropine methylbromide is shown in Fig. 1. The curves represent the absorbance of the reineckate salts produced from 10.0 mg choline chloride, 10.0 mg homatropine methylbromide, and 5.0 mg betaine. In each case the final volume is 25.0 ml. Similar variations of

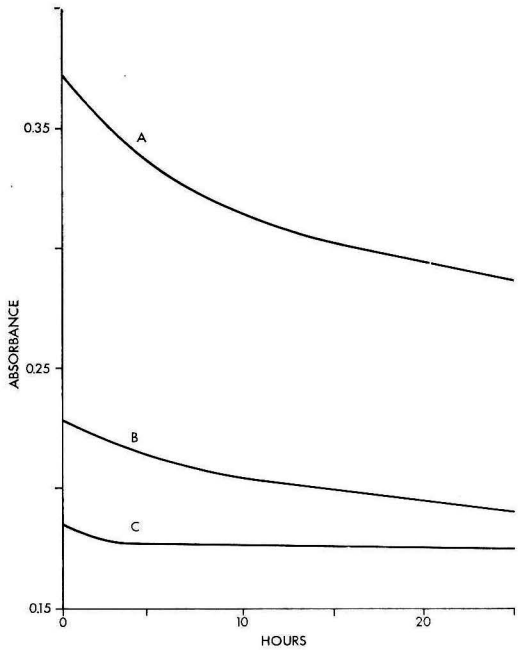

FIG. 1—Variation of absorbance with time for reineckate salts in acetone: A, 10.0 mg choline chloride; B, 5.0 mg betaine; C, 10.0 mg homatropine methylbromide. All solutions were diluted to 25.0 ml before measurement.

absorbance with time were found for other amounts ranging from 5.0 to 20.0 mg of each compound.

The effect of solvent on the variation of absorbance with time is shown in Table 1, which lists the absorbance and percentage of original absorbance at various times for the reineckate salts produced from 5.0 mg betaine dissolved in acetone and in 70% acetone in water. The original absorbance of the salt in acetone is higher than that of the salt in the 70% acetone solution. The absorbance decreases at approximately the same rate for the first few hours, but after 24 hr or more, the absorbance of the salt in acetone is significantly less than that of the 70% acetone solution. The reineckate of homatropine methylbromide showed the same type of decrease in absorbance with time and solvent as the betaine reineckate.

The absorbance of the reineckate salt of choline chloride decreased similarly when dissolved in acetone, but increased for the first few hours when dissolved in 75% acetone in water. The increase in absorbance was accompanied by the formation

Table 1. Variation of absorbance of betaine reineckate with time in acetone and in 70% acetone in water

	Acetone		70% Acetone	
Time, hr	Abs.	% Original Abs.	Abs.	% Original Abs.
0	0.226	—	0.217	—
1	0.224	99.1	0.212	97.7
3	0.218	96.5	0.210	96.7
4	0.215	95.1	0.208	95.9
24	0.191	84.5	0.194	89.4
96	0.167	73.9	0.181	83.4
120	0.161	71.2	0.170	78.3

of a haziness in the solution and the subsequent separation of a precipitate. The precipitate was removed by filtration after 24 hr, at which time the absorbance of the clear solution was less than the absorbance of the solution of the salt in acetone, which had aged for the same time interval.

The necessity for refrigeration before precipitation and during the aging of the precipitate is shown in Table 2, which lists the variation of absorbance with time for acetone and 70% acetone solutions of reineckate salts of homatropine methylbromide which were formed in refrigerated solutions and in solutions at room temperature. The absorbance of the solutions of salts produced at room temperature is approximately 65% of the absorbance of solutions of salts produced in refrigerated solutions. Extended periods of refrigeration before or after precipitation of the

Table 2. Variation of absorbance with time for the reineckate salt of homatropine methylbromide precipitated with and without refrigeration

	Acetone		70% Acetone	
Time, hr	Abs.	% Original Abs.	Abs.	% Original Abs.
Precipitated at 8°C				
0	0.184	—	0.185	—
1	0.181	98.4	0.184	99.5
2	0.178	96.7	0.181	97.8
24	0.175	95.1	0.167	90.3
48	0.157	85.3	0.147	79.5
72	0.152	82.6	0.144	77.8
Precipitated at 25°C				
0	0.119	—	0.119	—
1	0.116	97.5	0.116	97.5
2	0.114	95.8	0.114	95.8
24	0.106	89.1	0.105	88.2
48	0.098	82.3	0.086	72.3
72	0.096	80.7	0.083	69.7

reineckate salt has no effect on the absorbance. Also, precipitation at various temperatures below 10°C has no effect on the original absorbance.

The wavelength of maximum absorbance of betaine reineckate in the 70% acetone solution increases with time as the decomposition progresses from an original absorbance maximum of 522 nm to a value of 532 nm after 120 hr. The wavelength of maximum absorbance for the other reineckates did not vary with time or extent of decomposition.

Based on the results of this investigation, it is recommended that a standard be determined simultaneously with any sample assayed by the reineckate method, that absorbance readings be obtained at a specified time after the solution of the reineckate salt in acetone or acetone-water solutions, and that the time lapse between solution and absorbance measurement should be as short as feasible.

Whenever a series of standards must be determined at the same time as the sample, such as in the assay of Carbachol Ophthalmic Ointment (5), the experiment must be designed so that the time lapse between dissolving the salt and measurement of absorbance is the same for all standards and samples.

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Collaborative Study of the Analysis of Acetaminophen in a Sirup and in Combination with Other Drugs in a Tablet

By JOHN R. HOHMANN (Division of Drug Chemistry, Food and Drug Administration, Washington, D.C. 20204)

Five samples were included in this collaborative study: 2 sirups containing acetaminophen only at 2 levels, 1 commercial tablet containing acetaminophen combined with 4 other drug materials, and 2 synthetic mixtures similar to this tablet, containing acetaminophen at 2 levels. The precision of the analyses of all samples and the accuracy for the 2 sirups and 1 of the tablet mixtures were satisfactory. The recoveries obtained for the second tablet sample were low. In view of the precision for this sample and the accuracy for the others, it is concluded that this sample had been improperly prepared. It is recommended that the method be adopted as official first action.

Acetaminophen is formulated alone and in combinations with other drugs in both liquid and solid dosage forms. The NF XI and XII (1) have had methods for the determination of acetaminophen in the single-component tablet. At the present time there is no official method for the determination of acetaminophen alone in liquids nor in combinations in either the liquid or solid dosage forms.

A procedure for the analysis of acetaminophen in combination with other drugs was published by Levine and Hohmann (2). In this method, acetaminophen is separated from acidic, basic, and neutral substances by a buffered partition chromatographic system. In the report on Acidic and Neutral Nitrogenous Drugs (3), the General Referee recommended that this method be subjected to a collaborative study.

This procedure, slightly modified, was used satisfactorily to determine acetaminophen in a sirup, elixir, and solution. The same basic column and solvent system was satisfactory for determination of acetaminophen in a tablet containing amphetamine, butabarbital, aspirin, and salicylamide. The procedure for the sirup and for the tablet combination was then subjected to a collaborative study.

Two samples of sirup simulating a commercial product were prepared; one contained 240 mg acetaminophen/10 ml sirup and the other contained 216 mg. A commercial tablet containing *d*-amphetamine sulfate (5 mg), butabarbital (32 mg), aspirin (162 mg), salicylamide (162 mg), and acetaminophen (162 mg), and 2 comparable synthetic tablet mixtures, one containing 27.13% acetaminophen and the other 28.38%, were used in the study.

The collaborators were given 1 of the sirups and tablet mixtures and a sample of the powdered commercial tablet. The acetaminophen supplied as a standard was the same as that used in preparing the synthetic samples.

METHOD[1]

36.179 *Reagents*

(a) *Bicarbonate-carbonate buffer.*—pH 10.1. Weigh 1.0 g $NaHCO_3$ and 4.5 g Na_2CO_3 into 100 ml vol. flask and dil. to vol. with H_2O.

(b) *Acidic methanol.*—1.0 ml 0.1*N* HCl/100 ml MeOH. Prep. enough to ensure same MeOH is used thruout for std and sample.

(c) *Diatomaceous earth.*—Celite 545, acid-washed.

(d) *Acetaminophen std soln.*—0.008 mg acetaminophen/ml. Accurately weigh 40 mg acetaminophen std into 100 ml vol. flask. Dil. to vol. with acidic MeOH and mix well. Transfer 2.0 ml to 100 ml vol. flask and dil. to vol. with acidic MeOH.

36.180 *Preparation of Chromatographic Column*

Pack fine glass wool plug in base of chromatgc tube (25 × 250 mm) with aid of tamping rod ca 18″ long and having disk with diam. ca 1 mm less than tube. To 3.0 g Celite 545 add 2.0 ml buffer soln and mix until fluffy mixt. is obtained. Transfer mixt. to column and tamp gently to compress material to uniform mass. Transfer 2.0 ml sample soln to 100 ml beaker, add 1 drop HCl, and mix. Add 3.0 g Celite 545, mix thoroly, and transfer to column. Scrub beaker with 1 g Celite 545 and 2 drops H_2O. Transfer to column, tamp, and top column with fine glass wool pad.

36.181 *Preparation of Sample*

(a) *Sirup.*—Transfer 15.0 ml 0.1*N* NaOH to 25 ml vol. flask. Dil. to vol. with acetaminophen sirup, avoiding wetting flask neck above graduation mark while adding sirup, and mix. Transfer 10.0 ml of this diln to 100 ml vol. flask, dil. to vol. with H_2O, and mix.

(b) *Tablets.*—Weigh and finely pulverize ≥20 tablets. Accurately weigh portion of powder contg ca 240 mg acetaminophen and transfer to 250 ml vol. flask. Add 2 ml 1.0*N* NaOH and ca 100 ml H_2O. Shake, dil. to vol. with H_2O, and mix.

36.182 *Determination*

(*Caution: See* **46.011, 46.039,** and **46.054.**)

(Use H_2O-washed solvs thruout.)

Pass 100 ml $CHCl_3$ thru column and discard eluate. Elute acetaminophen with 150 ml ether, collecting eluate in 400 ml beaker. Evap. soln to dryness on steam bath under air stream. Dissolve residue in acidic MeOH, transfer quant. to 50 ml vol. flask, and dil. to vol. with same solv. Transfer 10.0 ml of this soln to 50 ml vol. flask, dil. to vol. with acidic MeOH, and mix. Scan spectrum of sample and std solns from 350 to 240 nm in 1 cm cells, using acidic MeOH as blank.

mg Acetaminophen in portion of sirup or tablet taken = $31.25 \times C \times (A_u/A_s)$, where C = mg/ml std soln, and A_u and A_s = absorbances of sample and std, resp., at max. of ca 249 nm.

Results and Discussion

The results reported by the collaborators are presented in Tables 1–3. Only 1 value (1 result of Collaborator 5 for the sirup) was discarded on the basis of Chauvenet's criterion (4).

Table 1. Collaborative results (%) for acetaminophen recovered from sirups

Coll.	240 mg/10 ml	Coll.	216 mg/10 ml
1	100.0	8	102.3
	97.8		101.5
2	98.3	9	100.3
	98.3		100.1
3	99.5	10	96.9
	99.1		94.9
4	97.4	11	96.3
	94.8		92.1
5	100.0	12	106.9
	99.2		100.3
	99.2		
	114.6[a]		
6	98.5	13	101.3
	98.5		101.3
7	99.2	14	102.2
	99.2		100.4
Av.	99.6	Av.	99.8
Std dev.	1.29	Std dev.	3.67

[a] Excluded on the basis of Chauvenet's criterion.

[1] The section numbers within the method are those for the 11th ed. of *Official Methods of Analysis*, 1970. Cautionary notes, if present, refer to the new chapter on safety, Chapter 46.

Table 2. Collaborative results (%) for acetaminophen recovered from synthetic tablet mixtures

Coll.	28.38%	Coll.	27.13%
8	102.8	2	97.1
	103.1		95.4
9	100.0	11	90.9
	99.1		92.9
10	94.9	12	98.3
	96.1		98.0
3	101.5	4	94.1
	102.0		95.5
5	99.2	13	94.8
	99.6		99.5
	98.3		
	100.4		
14	101.3	6	91.6
	101.7		93.3
1	96.3	7	95.5
	97.1		96.2
15	101.4		
	103.9		
16	98.9		
	97.3		
Av.	99.8	Av.	95.2
Std dev.	2.47	Std dev.	2.52

Collaborator 1 found that the pH of the buffer was 10.3, not 10.1, and adjusted the pH, using sodium bicarbonate. Levine and Hohmann (2) noted that the accuracy of the 10.1 value read on the pH meter is not certain because of the high sodium content. Use of the prescribed amounts of sodium bicarbonate and sodium carbonate should be relied upon rather than adjustment to pH 10.1 with a pH meter.

Two collaborators noticed a tendency of the eluate to bump during the evaporation step. One found that stirring while evaporating eliminated the problem.

Another collaborator had difficulty with small particles clogging the pipet when an aliquot of the sample preparation for tablets was taken and suggested filtering the suspended material.

Collaborator 11 suggested transferring the sirup to the volumetric flask with a pipet and washing the pipet. The purpose of the proposed procedure was to avoid the possible errors introduced when the viscous sirups were pipetted. Another suggested weighing the sirup, but this would require the determination of the specific gravity of the sirup.

Collaborator 5, who obtained poor duplicate results on his initial analysis of the sirup, was concerned that evaporation on the steam bath might decompose the acetaminophen. His second set of results was obtained by evaporating only in a current of air without the aid of a steam bath.

Another collaborator felt that more exact measurement of the acid added to the sample solution was indicated. However, the sample solution is very weakly basic and a considerable excess of acid is being added in the 1 drop.

Collaborator 6 found that up to 2 ml water remained in the beaker after evaporation of the eluate. This collaborator did not attempt to remove this residual moisture but diluted the residue to volume as it was. This collaborator also noted that the absorbance at 218 nm was slightly higher in the sample than in the standard. This was typical of spectra submitted by all the collaborators.

The recoveries obtained for the solutions and 1 of the synthetic tablet mixes were satisfactory. The average recovery for the first synthetic tablet, containing 27.13% acetaminophen, was low. Use of the *t*-test (5) indicated that this low average result was not due to random error. It was at first thought possible that large particle crystalline acetaminophen may have been used in preparing this sample and the resultant lower rate of solubility may have accounted for the low results. However, investigation did not show this to be the case. There is no reason to believe that

Table 3. Collaborative results (%) for acetaminophen recovered from a commercial tablet, labeled as containing 162 mg acetaminophen/tablet

Coll.	% of Declared	Coll.	% of Declared
1	98.5	8	100.4
	96.5		98.6
2	97.5	9	99.3
	97.9		98.9
3	98.5	10	96.1
	100.9		96.7
4	95.4	11	98.9
	98.1		95.3
5	98.3	12	100.2
	98.8		102.2
	99.2		
	96.3		
6	96.6	13	98.6
	93.2		98.6
7	99.4	14	99.4
	99.4		100.7
		Av.	98.3
		Std dev.	1.9

this sample differed from the other synthetic tablet samples in such a way as to affect the performance of the assay. It is assumed that the sample was incorrectly prepared.

Recommendations

Since the recoveries from the sirups and the 1 tablet mixture are satisfactory and since the poor recovery from the 1 tablet sample was probably the fault of the sample and not the method, it is recommended that the method described be adopted as official first action for the determination of acetaminophen in sirups and in tablet combinations containing amphetamine, aspirin, salicylamide, and butabarbital. It is further recommended that the possibility of using the proposed method with minor modifications for the determination of other components commonly found in combination with acetaminophen be investigated and, if satisfactory, be subjected to collaborative study.

Acknowledgments

The Associate Referee wishes to express his sincere appreciation to the following collaborators for their cooperation in this study: Paul Turi, Sandoz Pharmaceuticals, Division of Sandoz, Inc., and the following, all of the Food and Drug Administration: Rosemary Dempsey, Philadelphia; Joseph J. Diliberto, Buffalo; Eldon L. Farmer, II, Los Angeles; Paul Goebel, Jr., Kansas City; Mary C. Jakes, Cincinnati; Phyllis S. Jones, Baltimore; Gertrude Lowell, New York; Robert K. Munns, Denver; Carl Ponder, Atlanta; Jacob S. Sage and Oscar R. Sammul, Division of Drug Chemistry, Washington, D.C.; James R. Shearer, Chicago; Andrew Rodrigues, Boston; Richard D. Thompson, Minneapolis; and Patricia A. Williams, Dallas.

I would also like to express my appreciation to Joseph Levine, Director of the Division of Drug Chemistry, Washington, D.C., for his helpful suggestions on conducting this collaborative study.

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The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee B and were adopted by the Association. See *JAOAC* **53**, 381 (1970).

This report of the Associate Referee was presented at the 83rd Annual Meeting of the AOAC, Oct. 13–16, 1969, at Washington, D.C.

Collaborative Study of the Analysis of Pentaerythritol Tetranitrate and Meprobamate in Tablets

By JAMES L. HAMILTON, JR. (Food and Drug Administration, 900 Madison Ave., Baltimore, Md. 21201)

Meprobamate and PETN are separated by selective elution from a mixed Celite-sodium hydroxide-phosphoric acid column, using chloroform and benzene, respectively, as eluting solvents. PETN and meprobamate are detected and quantitatively measured by IR spectrophotometry. A collaborative study gave an average recovery of 99.3% for PETN and 99.95% for meprobamate. It is recommended that the method be adopted as official first action.

The positive identification of pentaerythritol tetranitrate (PETN) in combination with meprobamate has been unsuccessful in the past, due to the inability to separate the 2 compounds (1).

The procedure presented is a modification of a method reported by Levine (2). He used 85% phosphoric acid to retain the meprobamate, while eluting PETN with benzene. However, this author had no success in removing the meproba-

mate from the column, even with twice the volume of eluting solvent suggested by Levine.

The column chromatographic-IR method presented below quantitatively measures the carbonyl groups at about 5.82 μm (1720 cm^{-1}) for meprobamate and the nitrate groups at about 6.02 μm (1665^{-1}) for PETN. For positive identification of each ingredient, KBr disks of the assay solutions are prepared and the IR spectra of each are scanned from 2 to 15 μm (5000 to 660 cm^{-1}) and compared to their respective standards.

A collaborative study was conducted with 14 collaborators. Each collaborator assayed a standard mixture (containing meprobamate, PETN, and lactose) and 3 commercial samples for each ingredient. The commercial samples consisted of 180 tablets which were ground to a powder and passed through a 60 mesh sieve. Each collaborator received 1 vial of each standard, 1 vial of the standard mixture, and 1 vial of each of the commercial samples. The collaborators performed single quantitative determinations on each ingredient and positive qualitative identifications by the KBr disk technique.

METHOD[1]

(*Caution:* PETN may explode when heated strongly, even when dissolved.)

36.407 *Apparatus*

(a) *Recording infrared spectrophotometer.*—With two 1.0 mm liq. cells with NaCl windows, preferably matched or of known *A* difference, KBr disk holder, and equipment suitable for prepg KBr disk.

(b) *Chromatographic tube.*—25 × 200–250 mm with 5 × 40 mm stem.

(c) *Tamping rod.*—*See* **36.015**(b).

36.408 *Reagents*

(*Caution: See* **46.011**, **46.040**, and **46.056**.)

(a) *Dilute phosphoric acid.*—3 + 1. Dil. 3 vols 85% H_3PO_4 with 1 vol. H_2O.

(b) *Water-washed benzene.*—Shake equal vols benzene and H_2O 1 min in separator. Discard lower phase. Use within 2 days of prepn.

(c) *Water-washed chloroform.*—Shake equal vols. $CHCl_3$ and H_2O 1 min in separator. Discard upper layer. Use within 2 days of prepn.

(d) *Anhydrous chloroform.*—Filter H_2O-washed $CHCl_3$ thru anhyd. Na_2SO_4.

(e) *Pentaerythritol tetranitrate (PETN) std soln.*—10 mg/50 ml. Ext PETN from com. PETN (usually 10% on lactose or other inert diluent) with $CHCl_3$ to give ca 20 mg pure PETN. Filter and evap. to dryness under air current with little or no heat. Dry in vac. desiccator 2 hr. Accurately weigh ca 10 mg, using microbalance, dissolve in anhyd. $CHCl_3$, and dil. to 50 ml with this solv. Destroy excess PETN by dissolving in acetone and burning in large vessel behind safety barrier, using effective fume removal device.

(f) *Meprobamate std soln.*—80 mg/100 ml. Dissolve 80 mg NF XII Meprobamate Ref. Std in anhyd. $CHCl_3$ and dil. to 100 ml with this solv.

(g) *Diatomaceous earth.*—Celite 545, acid-washed, or equiv.

36.409 *Determination*

(Use H_2O-washed solvs unless designated anhyd. *Caution: See* **46.011**, **46.039**, **46.040**, **46.045**, and **46.056**.)

Loosely pack small amt fine glass wool in base of chromatgc tube to support Celite. Weigh 3 g Celite into 100 ml beaker, add 2.0 ml 1*N* NaOH, mix with metal spatula until fluffy, and pack uniformly in tube. Weigh 5 g Celite into 250 ml beaker, add 7.0 ml dil. H_3PO_4, mix until fluffy, and pack uniformly on column. (Do *not* pack too tightly as column will elute too slowly.) Accurately weigh portion powd sample contg ca 10 mg PETN into 150 ml beaker. Add 4 ml benzene and heat gently with swirling ca 1 min. Cool, add 4 g Celite, mix until fluffy, transfer quant. to column, and pack uniformly. Dry-wash beaker with 1 g Celite and 0.5 ml benzene, transfer to column, and pack uniformly. Wipe sample beaker and all app. used in column prepn with glass wool, and pack on column.

Pass 75 ml benzene thru column and collect eluate in 150 ml beaker until elution ceases. Rinse column tip with small portions benzene into beaker and set aside. This fraction contains PETN.

Place 250 ml beaker under column. Add 4.0 ml H_2O to column and let it be absorbed. Pass 150 ml $CHCl_3$ thru column and collect eluate in 250 ml beaker. Rinse column tip with $CHCl_3$ into beaker. This fraction contains meprobamate.

Evap. each fraction on steam bath under gentle air current to ca 10 ml and take to dryness with little or no heat from steam bath. Place beakers in vac. oven 30 min at 30° and ≤15″ Hg. Remove from oven. Add ca 10 ml anhyd. $CHCl_3$ to PETN beaker and heat gently to dissolve residue. Quant. transfer with anhyd. $CHCl_3$ to 50 ml vol. flask and dil. to vol. with anhyd. $CHCl_3$.

Dissolve meprobamate residue with ca 20–25 ml anhyd. $CHCl_3$. If theoretical wt of meprobamate in sample wt taken is 100 mg, quant. transfer to 100

[1] The section numbers within the method are those for the 11th ed. of *Official Methods of Analysis*, 1970: **36.015**(b) (11th ed.) = **32.013**(b) (10th ed.). Cautionary notes, if present, refer to the new chapter on Safety, Chapter 46.

ml vol. flask with anhyd. $CHCl_3$ and dil. to vol. with this solv. If theoretical wt of meprobamate is ca 200 mg, use 250 ml vol. flask and proceed as above.

Scan sample and std solns in 1.0 mm cells from 5.0 to 6.5 μm (2000–1540 cm^{-1}) on IR spectrophtr, using anhyd. $CHCl_3$ as ref.

Calc. PETN by subtracting *A* at 5.5 μm (1818 cm^{-1}) from *A* at ca 6.02 μm (1660 cm^{-1}) and compare with std *A*. (*Note:* PETN sample solns may contain very small peak at ca 5.8 μm (1722 cm^{-1}). This is contaminant of meprobamate and does not interfere with PETN detn. Also, a peak may appear at ca 6.25 μm (1600 cm^{-1}). This is H_2O peak. Disregard this peak in calcg PETN net *A*.)

Calc. meprobamate by subtracting *A* at 5.5 μm (1818 cm^{-1}) from *A* at ca 5.82 μm (1718 cm^{-1}) and compare with std *A*.

36.410 ***Identification***

(a) *PETN.*—Prep. both std and sample KBr disks from respective assay solns. Evap. 4–5 ml of each soln in small mortar, add 200 mg KBr, mix thoroly, and press. Scan spectrum from 2 to 15 μm (5000–667 cm^{-1}). Compare sample and std curves. (*Note:* Sample IR curve may deviate from std curve. This deviation is caused by meprobamate contaminant. However, all major peaks in std and sample should be evident.)

(b) *Meprobamate.*—Prepare KBr disks as above from 1 ml sample and std assay solns. Scan and compare as in (a).

Results and Recommendation

The results of the collaborative study are presented in Table 1. Comments received from the collaborators and the observations of the Associate Referee are given below.

Collaborator B used the chloroform solution for the qualitative tests for meprobamate with the knowledge and consent of the Associate Referee.

Collaborator E suggested that it would be better to say "at a minimum of 15 inches of mercury" instead of "at 15 inches of mercury."

Collaborator G commented that a more efficient drying of the assay solutions with basic aluminum oxide would be desirable when expensive equipment, such as matched KBr or variable pathlength IR cells, are involved. She also reported that there was excess water in her assay solutions and this presented a problem in the preparations of the KBr disk for the PETN identity. No other collaborator mentioned this problem. Perhaps her sodium sulfate was not

Table 1. Results of collaborative study of PETN and meprobamate in tablets

	Standard Mixture; mg Recovered		Sample B; mg Found/Tab		Sample C; mg Found/Tab		Sample D; mg Found/Tab	
Coll.	PETN	Meprobamate	PETN	Meprobamate	PETN	Meprobamate	PETN	Meprobamate
A	10.3	202.6	10.4	209.1	9.55	199.3	19.7	182.8
B	10.6	200.2	10.5	197.4	10.5	193.0	20.5	211.6
C	10.3	204.2	10.0	200.8	10.3	200.2	21.5	201.1
D	9.69	198.5	9.89	201.0	10.1	200.2	19.9	196.8
E	10.1	186.5[a]	10.3	183.1[a]	10.5	186.5[a]	20.9	185.8[a]
F	10.1	199.8	10.3	195.8	10.1	196.5	20.4	195.3
G[b]	10.0	192.1	10.2	190.3	10.2	192.7	20.8	190.9
G[b]	10.0	199.2	10.0	194.0	10.2	194.5	20.5	200.4
H	9.24	200.8	9.66	194.2	9.47	190.1	19.3	193.3
I	9.66	199.1	9.47	196.2	9.43	194.6	18.7	196.1
J	10.1	192.2	10.0	196.1	9.20	195.7	19.4	193.3
K	9.84	195.3	10.5	200.0	10.1	198.7	20.2	193.3
L	9.51	196.0	9.81	193.4	10.1	191.8	20.2	198.1
M	9.18[a]	197.3	9.28[a]	193.4	9.43[a]	191.3	18.3[a]	189.9
N	9.63	198.3	9.85	211.5	10.1	196.5	19.4	193.9
Mean rec.	9.93	198.3	10.1	198.1	9.99	195.4	20.1	195.5
Std dev.	0.36	3.48	0.32	6.01	0.41	3.36	0.75	6.53
Mean rec., %	99.3	99.95	101	99.05	99.9	97.70	100	97.75
% Coeff. of var.	3.62	1.75	3.17	3.03	4.10	1.72	3.73	3.34
Added	10.0	198.4	—	—	—	—	—	—
Declared	—	—	10	200	10	200	20	200

[a] Rejected as outliers on basis of Youden's rank sum test.
[b] Two different instruments used to quantitatively measure ingredients.

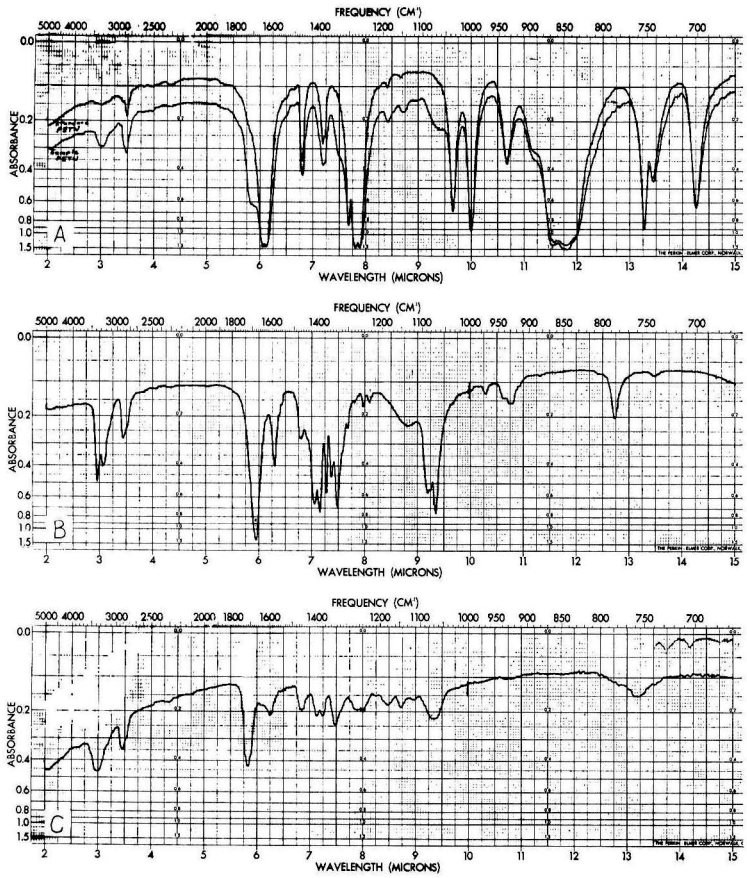

FIG. 1—Infrared spectra of A, sample and standard PETN; B, standard meprobamate; and C, contaminant isolated from meprobamate used to prepare standard mixture.

anhydrous or not enough anhydrous sodium sulfate was used to dry the volumes of chloroform needed for the assay solutions. She and other collaborators suggested that item (**a**) under *Apparatus* be changed to "equipment suitable for preparing KBr disks," since many people are now using minipresses. Also, she and others suggested that wave numbers should be given along with wavelengths.

Collaborator I indicated that a statement should be included as to the effect of using water-washed and anhydrous chloroform within 1 or 2 days after preparation due to the formation of phosgene. He stated that he had used acid-washed Celite 545, although it was not specified in the method. The Associate Referee could detect no difference in either the acid-washed or nonacid-washed Celite during the development of the method. However, as pointed out by Collaborator I, "use of the nonacid-washed support may lead to some contamination in using this column, since Celite can vary from batch to batch." To avoid this problem, the method now specifies acid-washed Celite. He also asked if nitrogen could be used in place of air in the evaporations. The Associate Referee sees no reason why it could not be used, but he has not tried it.

Collaborators J and K mentioned that the PETN standard and the PETN sample (Fig. 1A) identifications were dissimilar. Collaborator K stated that the sample PETN disk showed an impurity which might possibly be meprobamate, since the impurity absorbed strongly at the same wavelengths (5.8, 8.5, and 9.4 μm) as the standard meprobamate (Fig. 1B). This impurity is definitely not meprobamate, but it is a contaminant

of meprobamate (Fig. 1C). This contaminant was isolated by the Associate Referee from meprobamate available, using the above procedure plus Levine's column. If the impurity were meprobamate, it would definitely be trapped on Levine's column. Meprobamate, when "cleaned up," does not give this interference in the PETN disks when analyzed by the above method. The meprobamate used to make the standard mixture was not "cleaned up" but was of known purity. However, the meprobamate used as a standard in the collaborative study was cleaned up by the above method and assayed as 100%.

Collaborator K suggested passing the column eluates through an anhydrous sodium sulfate column, thus avoiding the evaporation step. This would be fine if benzene did not absorb so strongly in the region used for quantitation of the ingredients. He also suggested using 200 ml chloroform to elute the meprobamate, since he noticed the sample had collected on the tip of the column after using 150 ml of chloroform. He stated that he had repeated the assay using his suggested volume and got consistently higher results. The Associate Referee has also noticed the above, but if the tip of the column is rinsed as stated in the method, recoveries of 99+% are obtained with 150 ml chloroform. Contact with Collaborator K confirmed the suspicion that he had not rinsed the tip of the column after the 150 ml. The results reported for Collaborator K in Table 1 are the results he reported using 200 ml chloroform.

At least 2 collaborators experienced difficulty in extracting the standard PETN from the inert ingredient. This problem is overcome if the commercial PETN is extracted from an aqueous solution.

Collaborator N suggested using a longer column. The author has used both 200 and 250 mm length columns with equal success. All other collaborators had no suggestions on the method as presented. Most collaborators, 13 of the 14, stated that the method was straight forward and easy to follow.

The changes suggested by the collaborators have been incorporated in the method reported.

The Associate Referee recommends that both the qualitative and quantitative determinations of the above method be adopted as official first action.

Acknowledgments

The Associate Referee wishes to thank the following collaborators (all from the Food and Drug Administration): James M. Moore, Baltimore (now with Bureau of Narcotics and Dangerous Drugs, Washington, D.C.); Sal Marchese, Boston; Pamela F. Wojtowicz, Buffalo; Danute G. Orentas, Chicago; Sherry O. Smith, Cincinnati; Marshall Jeffus, Dallas; Wilson L. Brannon, Washington, D.C.; Richard E. Olson, Kansas City; Richard Thompson, Minneapolis; R. Wade Noxon, St. Louis; Seymour Fishman, New York; Robert W. McCullough, Philadelphia; Rod Chu, San Francisco; and Ruth E. Anderson, Seattle.

We would also like to thank Janet A. Springer, Bureau of Science, Washington, D.C., for the statistical analysis of the results, and Joseph Levine, Division of Drug Chemistry, Washington, for the initial chromatographic idea.

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The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee B and was adopted by the Association. See *JAOAC* **53**, 381 (1970).

This report of the Associate Referee was presented at the 83rd Annual Meeting of the AOAC, Oct. 13–16, 1969, at Washington, D.C.

Comparative Study of Micro Infrared Techniques

By WILSON L. BRANNON (Division of Drug Chemistry, Food and Drug Administration, Washington, D.C. 20204)

A comparative study was made of several techniques used in the isolation and identification of microgram quantities of drugs by infrared spectroscopy. The isolation techniques include preparative GLC and TLC. In the case of TLC, silica gel interference was nearly eliminated by using a sintered glass filter and vacuum. The identifications were enhanced by concentrating the sample in the infrared beam center. The sample was centered either with the aid of a cardboard silhouette or punched out or drilled indentations in the disk.

In view of widespread interest in identifying small quantities of drugs by infrared spectroscopy, a comparative study of 10 existing procedures for sample preparation was undertaken. The purpose of this investigation was to obtain meaningful spectra with the least amount of sample and using the simplest equipment and technique possible.

Techniques involving micro quantities can mean using from 1 to 50 μg compound. The methods presented here illustrate the identification of quantities ranging from 0.5 to 15 μg. To insure that spectra would be comparative, 15 μg samples were prepared by each of the procedures. Generally acetanilide was used as the test compound because a chloroform solution of this drug, when evaporated, would yield crystals rather than an amorphous glass.

Experimental

Apparatus and Reagents

(a) *Cardboard window.*—0.1 mm thick × 13 mm diameter, with 2 × 10 mm hole (No. 195713, Beckman Instruments, Fullerton, Calif.).

(b) *Gas chromatograph.*—Hewlett-Packard F&M Model 402, with flame ionization detector, 1:1 effluent stream splitter (Nos. 2–5259, 2–5258, and 1–4583–1), and Model 402 oven.

(c) *Infrared spectrophotometer.*—Perkin-Elmer Model 621 with 6× beam condensers (No. 211–0047).

(d) *Metal disk.*—0.5 mm thick × 13 mm diameter, with 0.5 mm hole (Perkin-Elmer No. 186–1042) and with 1.5 mm hole (Perkin-Elmer No. 186–1043).

(e) *Micro bell jar.*—72 mm id × 94 mm inside height (No. 2161, Arthur H. Thomas, Philadelphia, Pa.).

(f) *Micro filter funnel.*—Pyrex, 135 mm high, 90 mm stem, with 3M sintered glass filter (Kimble Glass, No. 28450, Fisher Scientific Co.).

(g) *Potassium bromide.*—Infrared quality (Harshaw Chemical Co., Cleveland, Ohio).

(h) *Silver chloride sheet.*—1 × 1″ (Harshaw Chemical Co.).

(i) *Silica gel.*—GF-254 (Merck, Darmstadt, Germany; available from Brinkmann Instruments, Inc., Westbury, N.Y.).

(j) *Steel cylinders.*—13 mm diameter × 20 mm long (*see* reference 1 for additional specifications).

(k) *Wick Stick.*—Potassium bromide (Harshaw Chemical Co.).

Procedures

In the first method, a cardboard window is used to support the sample mixture (2). Fifteen μg sample dissolved in chloroform is placed in a mortar, using a Hamilton syringe. The sample is thoroughly mixed with 3–4 mg KBr and carefully transferred to the center of the hole in the cardboard window. The mortar is rinsed with about 20 mg KBr, and this mixture is packed around the preceding sample. The disk is then pressed in the usual manner. A spectrum obtained with the cardboard window method is shown in Fig. 1A.

The metal disks provided for making micro disks in conjunction with beam condensers are familiar to IR spectroscopists. In the second method, a disk with a 1.5 mm hole is used to support the matrix and normal optics are used for IR spectroscopy. A 6 mg KBr sample mixture is transferred to the 1.5 mm hole and packed, and the disk is pressed at 20,000 psi between 2 steel cylinders (1). If the disk is not clear, an excess of KBr can be pressed on the other side to obtain a clear disk. The spectrum is then obtained, using normal optics, an attenuator, and 2× slit widths, as seen in Fig. 1B.

Two procedures were also studied, using the 6× beam condensers. In one method (1), 6 mg KBr is mixed with the sample and pressed in a 0.5 mm hole disk, using steel cylinders as previously described. The results are illustrated in Fig. 1C. This procedure evolved in this laboratory from a somewhat more involved technique (1) which was originally designed to handle 0.5–1

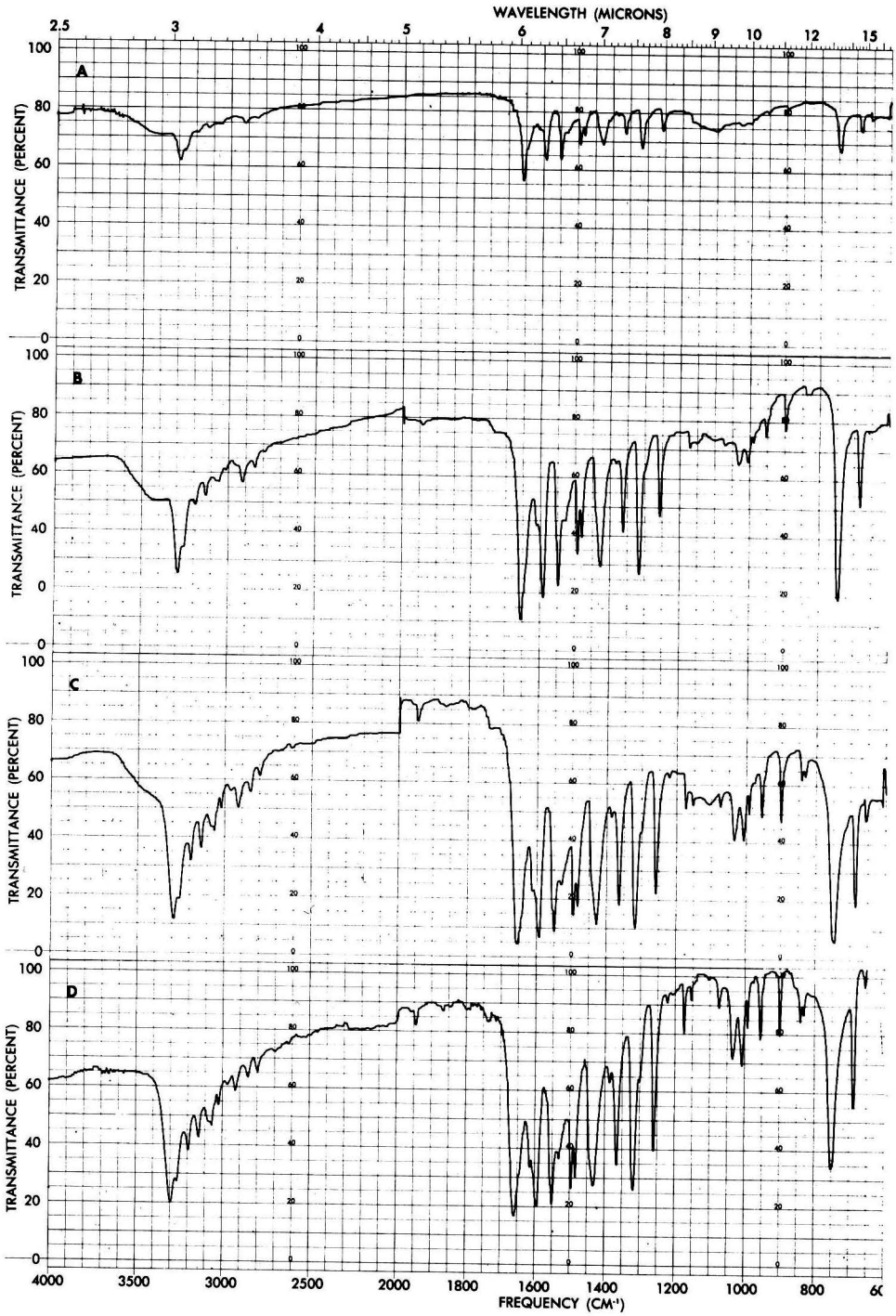

FIG. 1—IR spectra of 15 μg acetanilide prepared by different techniques: A, 2 × 10 mm hole cardboard window; B, 1.5 mm hole disk; C, 0.5 mm hole disk, using 6× beam condenser; and D, cone-punched silver chloride disk, using 6× beam condenser.

μg samples. Although the latter has seldom been used recently, the original methodology is of interest. A blank disk is prepared to receive the sample by placing 10 mg KBr, taken directly from a 105°C oven, over the 0.5 mm hole of the disk. The disk is then pressed in the usual way. With a 20 gauge needle a conical hole is bored and 1 μl solution, containing about 1 μg sample, is applied in small increments into the hole. The sample is dried by a heat lamp. Ten mg KBr is placed over the hole and the disk is pressed. The scan is made, using a beam condenser and a reference blank disk.

The other procedure involving the beam condenser was introduced by Chen and Gould (3). A silver chloride sheet is used to prepare the supporting matrix. After an indentation is made in the silver chloride with a punch, the indented sheet is placed on a heat source which is kept slightly above the boiling point of the solvent. The sample is placed in the center of the punched hole with a hypodermic syringe. Then, with a cone-shaped punch, a disk is hammered out and the resulting cone is placed between 13 mm diameter disks with holes slightly smaller than the prepared disk. Silver chloride reduces the interference from water as seen in Fig. 1D.

We studied extractive procedures coupled to micro infrared identification. Rice (4) proposed making a tear drop outline around a thin layer chromatogram spot (step 1, Fig. 2) and scraping off all the silica gel around it, thus leaving the silica gel in the form of a tear drop (step 2, Fig. 2). Potassium bromide is placed on the plate next to the tear drop, connecting to and extending the point (step 3, Fig. 2). By dropping solvent on the tear drop, the sample is eluted onto the KBr. In our experience, however, the sample did not move off the silica gel, even after several solvent applications; this could be seen by observing the tear drop in an ultraviolet viewer. By returning the tear drop plate to the elution chamber (step 4, Fig. 2) and by adjusting the solvent level to touch the base of the tear drop we could elute the sample to the point (step 5, Fig. 2).

To reduce interference from silica gel and to prevent the solvent creeping up the sides of the tear, more silica gel was removed, leaving the shape of an arrowhead (step 6, Fig. 2). Then, the sample was eluted onto the KBr. The KBr was dried over phosphorus pentoxide, removed, and placed in a 1.5 mm hole disk. After the disk

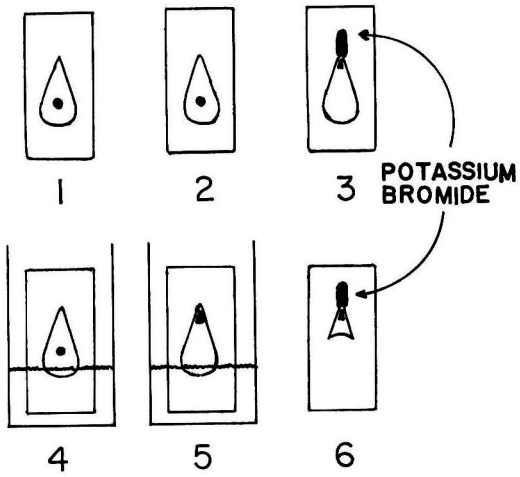

FIG. 2—Comparison of steps in the Rice method (steps 1–3) and the modified method (steps 4–6).

was pressed, the spectrum seen in Fig. 3A was obtained.

In addition to poor elution onto the KBr, the sample also spreads out on the plate. de Klein (5) suggested using a semicircular mound of KBr around the tear drop to allow the sample to elute from the spot onto the plate; from here it is sucked up into the KBr powder. After several attempts no productive results were obtained. There were strong absorption interferences at 1100 cm^{-1} in all cases.

To remove the silica gel, a micro filtration apparatus is used which consists of a bell jar and a small sintered glass microfunnel. Fifteen μg sample is spotted on a silica gel plate and the plate is developed. The plate is dried over phosphorous pentoxide and the sample spot is scraped from the plate, placed in the sintered glass filter, eluted with redistilled methanol, and filtered directly into a mortar. A disk is prepared, using 5 mg KBr and a 1.5 mm hole disk as previously described. A spectrum obtained in this procedure is shown in Fig. 3B. A fluorescent dye, eosin Y, was used to observe the sample transfer.

With the extractive procedures, the sintered glass filter removes the problem of silica gel impurity. Also, this has the advantage of less surface area in contact with the sample, thus reducing loss and contamination. Though de Klein recommended not using alcohols, redistilled methanol created no problems. However, silica gel extractions repeatedly show an impurity, referred to by

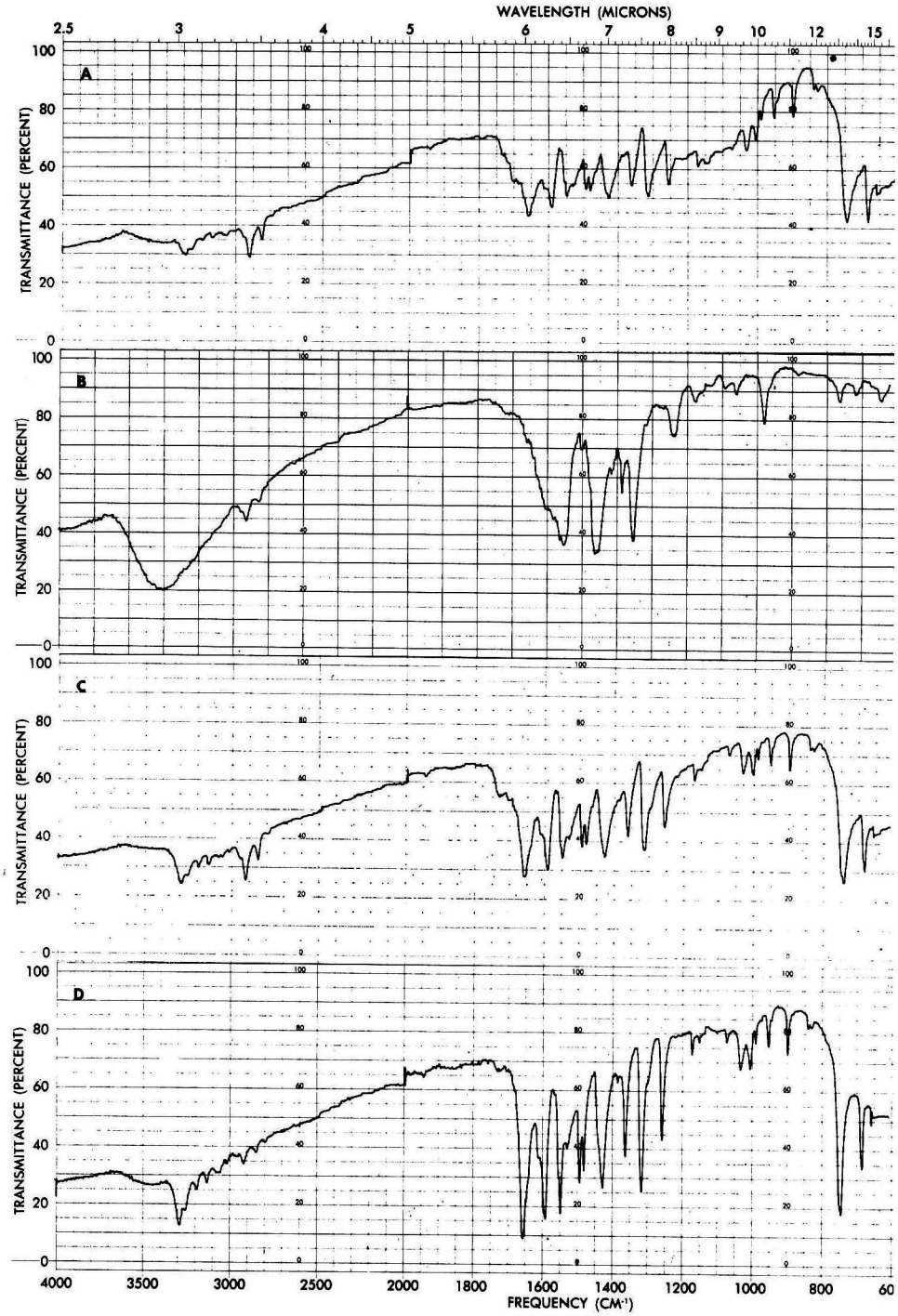

FIG. 3—IR spectra of samples prepared by different techniques: A, 15 μg acetanilide extracted from TLC tear drop; B, 15 μg eosin extracted from TLC, using sintered glass filter and 1.5 mm disk; C, 15 μg acetanilide eluted on Wick Stick, using 1.5 mm disk; and D, 30 μg acetanilide injected and collected from GLC.

Snavely and Grasselli (6); this material is aluminum nitrate, found to be a component of distilled water in our laboratory.

In a novel approach (7), 15 μg sample is eluted up a Wick Stick. The point is broken off, placed into a 1.5 mm hole disk, and pressed, resulting in a relatively clear disk. The spectrum obtained, using normal optics, is shown in Fig. 3C. The Wick Stick is particularly adaptable to the 1.5 mm hole disk technique.

Another means of extracting the sample is gas-liquid chromatography. It looks most promising because it has less background material. The sample, 30 μg acetanilide for example, can be injected into a high efficiency gas chromatograph with a 1:1 effluent stream splitter. The sample is collected on a melting point capillary tube, washed out with chloroform into a mortar, and analyzed by IR. The resulting curve using a 1.5 mm hole disk and normal optics is in Fig. 3D.

Conclusions

We have compared 10 micro techniques for the analysis of pharmaceuticals by infrared spectroscopy. In some of the methods described, suggestions were made and tried for possible improvements. In general, disk preparations involving the centering of the sample were more difficult than those in which the sample was first incorporated into potassium bromide and then pressed. Any of the extraction procedures described could be used, but the actual choice would depend on the analyst's needs. If silica gel interference is a problem, then only the sintered glass filter technique could be used. Gas-liquid chromatography appears to be most promising for the elimination of extraction impurities.

For identification purposes, full scale deflection curves of 15 μg samples were obtained without using specialized equipment.

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Collaborative Study of the Determination of Morphine in Opium

By EDWARD SMITH (Division of Drug Chemistry, Food and Drug Administration, Washington, D.C. 20204)

The partition chromatographic procedure which was successfully applied in last year's collaborative study of the determination of morphine in paregoric was applied to the determination of morphine in opium. Two methods of preparation of the opium solution before partition were studied: homogenization in a high-speed blender and use of DMSO as a solvent. Reproducible results were obtained with the use of DMSO solutions. The standard deviations obtained for samples containing 7.82, 13.88, 12.45, 14.87, and 15.74% anhydrous morphine were 0.171, 0.184, 0.207, 0.220, and 0.307, respectively. From the results obtained, the method using DMSO in the preparation of the sample solution together with the partition chromatographic method is recommended for adoption as official first action.

A partition chromatographic procedure for the determination of morphine in opium and in paregoric was reported at the International Drug Symposium on Alkaloids and Related Bases at the 81st Annual Meeting of the AOAC (1). A review of the principles and pertinent literature

was included in that presentation. A collaborative study of the assay of paregoric using this 3-column system, with slight modifications, was carried out in 1968. The results were good and the method was adopted as official first action (2).

A collaborative study applied to the assay of opium was initiated this year. The same modified chromatographic system which was applied in the paregoric study was used. The sample preparation was that described in the original article (1): homogenation in a measured volume of water, using a high-speed blender. The samples used in the study were 5 lots of powdered opium obtained from the Division of Narcotic Drugs of the United Nations. These samples were prepared from the natural opium originating in different geographical areas and had a wide range of composition (3). Each collaborator received either 3 or 5 of the samples and was requested to analyze duplicate aliquots of each sample solution. In some cases, collaborators in the Associate Referee's laboratory analyzed solutions prepared by other collaborators.

In this initial collaborative study, in which the blender was used for the preparation of the sample solutions, varying results obtained with some of the samples were shown to have resulted directly from the method of sample preparation. When individual sample solutions were analyzed by different analysts reproducible results were obtained. Several collaborators noted that particles of unblended opium adhered to the parts of the blender after the blending operation and were thus not available for the subsequent analytical treatment. In these instances low results were obtained in the subsequent partition steps. The behavior of the individual lots of opium differed. Sample A, which is the most gummy of the samples, gave the widest variation.

Levine and Doyle (4) reported that dimethyl sulfoxide (DMSO) is an excellent solvent for a wide variety of drug materials and that, under the proper conditions, mixtures of DMSO with water can be used as an immobile phase in partition chromatography. Using this solvent we found that virtually complete dissolution[1] of the opium can be achieved, thereby assuring availability in the ensuing partition steps.

The effect of DMSO on the partition chromatography was investigated. Sample solutions previously prepared with the blender were analyzed both as is and with DMSO added to the solution before mixing with Celite. The same values were obtained, showing that DMSO causes no interference in the partition system. Portions of Sample A, which is the most resinous and the most difficult to dissolve using the blender, were dissolved in DMSO. Water was added to the solutions to provide the final concentrations of DMSO varying from 10 to 100%, and the solutions were analyzed. Results (Table 1) indicate that the concentration of DMSO present had no effect on the recovery of the morphine. In addition to this, quantitative recoveries of known amounts of morphine in DMSO solutions of varying concentrations were obtained. Therefore, the directions for the preparation of the sample solution allow a latitude in the final DMSO concentration, but suggest the use of a volume of DMSO which will provide 20% DMSO in the final solution.

Table 1. Effect of concentration of dimethylsulfoxide on per cent anhydrous morphine found in Sample A

% Morphine Found With Final Concn of DMSO in Soln as:			
10%	20%	30%	100%
12.52	12.64	12.63	12.60
12.61	12.83	12.67	12.62
—	—	12.58	—

The effect of prolonged heating of the DMSO extract was investigated. It was found that it could be heated in a steam bath or in a water bath for several hours without any deleterious effect. Therefore the length of time for heating the sample to effect dissolution of the opium was left unrestricted, with a period of 15 min suggested.

The samples used in the collaborative study were powdered and therefore could readily be transferred to a volumetric flask prior to the addition of the DMSO. Since gum opium cannot be handled this way, dissolution of the sample in a beaker before quantitative transfer to the volumetric flask facilitates the handling of these samples.

A second collaborative study was initiated, utilizing the revised method of sample preparation described below and the same column partition method previously described (2). The method

[1] A small amount of insoluble material such as fine leaf fragments, sand-like particles, and gelatinous particles may remain undissolved.

as reported incorporates the method for determination of morphine in opium studied this year with the method for morphine in paregoric adopted as official first action (2).

METHOD[2]

(*Caution: See* **46.011, 46.039, 46.040,** and **46.052.**)

36.031 ***Apparatus***

(a) *Chromatographic tubes.*—*See* **36.015.**

(b) *Diatomaceous earth.*—Celite 545, acid-washed.

36.032 ***Reagents***

(a) *Triethylamine.*—Purified as in **36.016(a).**

(b) *Morphine std soln.*—0.08 mg anhyd. morphine/ml. Accurately weigh morphine base or salt equiv. to 4 mg anhyd. morphine into 50 ml vol. flask. Add 10 ml MeOH, 1 ml HCl, and 1 ml Et_3N and dil. to vol. with $CHCl_3$. Alternatively, prep. stock soln by dissolving accurately weighed std equiv. to ca 40 mg anhyd. morphine in 0.5 ml Et_3N in 100 ml vol. flask, and dil. to vol. with MeOH. Pipet 10 ml this stock soln into 50 ml vol. flask, add 1 ml Et_3N and 1 ml HCl, and dil. to vol. with H_2O-satd $CHCl_3$.

(c) *Citrate buffer.*—0.1*M*, pH 4.4. Mix equal vols. 0.1*M* Na citrate (2.94 g $Na_3C_6H_5O_7.H_2O$/100 ml) with 0.1*M* citric acid (2.10 g $H_3C_6H_5O_7.H_2O$/100 ml).

36.033 ***Preparation of Sample***

(a) *Opium (Official First Action).*—Accurately weigh ca 2 g opium into 100 ml vol. flask. Add 20 ml dimethyl sulfoxide (DMSO) and heat in beaker of boiling H_2O or in steam bath ca 15 min. Swirl gently to dissolve, keeping opium particles in contact with DMSO and not letting particles remain on flask walls. Inspect soln carefully. If undissolved material remains, continue heating. Small amt insol. material, such as fine leaf fragments, sand-like particles, and gelatinous particles, may remain undissolved; add more DMSO, if necessary. Cool, add H_2O to ca 90 ml, and mix. Let soln reach room temp., dil. to vol. with H_2O, and mix. (If foaming occurs on mixing, use 1 drop ether or alcohol to dispel foam.)

If sample is in pieces too large to fit in neck of vol. flask, accurately weigh into 100 ml beaker, add 20 ml DMSO, and heat in boiling H_2O or steam bath. Use stirring rod to disperse sample while heating. Decant into 100 ml vol. flask. If undissolved opium remains in beaker, heat with addnl 3 ml portions DMSO as needed until soln is complete as possible. (DMSO concn in final soln can vary over wide range without adverse effect.) Dil. to vol. with H_2O as above.

Filter prepd soln thru paper, rejecting first 20 ml filtrate. Use 2 ml aliquot for prepn of Column I.

(b) *Paregoric (Official Final Action).*—Evap. 10.0 ml paregoric, contg ca 4 mg morphine, to ca 2 ml on steam bath under stream of air. If evapn continues beyond 2 ml, dil. to 2 ml with H_2O. Cool soln to room temp. and then use for prepn of Column I.

36.034 ***Preparation of Columns***

(a) *Column I.*—*(1) Lower layer.*—Mix 3 g Celite and 2 ml citrate buffer; transfer to tube and tamp as in **36.017.** *(2) Upper layer.*—Add 0.5 ml citrate buffer to 2.0 ml aliquot of sample ext, **36.033(a)** or **(b).** Add 3 g Celite, mix, and transfer to tube. Dry-wash beaker with 1 g Celite and add to column; tamp and add glass wool pad.

(b) *Column II.*—Mix 3 g Celite and 2 ml 1.0*M* K_2HPO_4 (17.42 g/100 ml); transfer to tube, tamp, and add glass wool pad.

(c) *Column III.*—Mix 3 g Celite and 2 ml 0.5*M* NaOH; transfer to tube, tamp, and add glass wool pad.

36.035 ***Determination***

(Use H_2O-satd solvs thruout. Rinse each column tip with $CHCl_3$ before discarding columns or changing receivers.)

Pass 100 ml ether, followed by 100 ml $CHCl_3$, thru Column I. Discard eluates. Mount Columns II and III in series below Column I. Pass thru columns 5 ml 20% (v/v) Et_3N in $CHCl_3$, followed by four 10 ml portions 1% Et_3N in $CHCl_3$. Let each portion pass thru completely before next addn. Continue elution without delay. Discard Column I, and pass three 5 ml portions 1% Et_3N in $CHCl_3$ thru remaining columns. Discard Column II. Wash Column III successively with 10 ml 1% Et_3N in $CHCl_3$, 50 ml $CHCl_3$, 2 ml 10% HOAc in $CHCl_3$, and 50 ml 1% HOAc in $CHCl_3$. Discard all eluates.

Place as receiver under Column III 50 ml vol. flask contg 10 ml MeOH and 1 ml HCl. (Remove metal leashes from vol. flasks to prevent contamination during transfer to cuvets.) Elute column with 5 ml 20% Et_3N in $CHCl_3$, followed by 33 ml 1% Et_3N in $CHCl_3$. Dil. eluate to vol. with $CHCl_3$.

Scan spectrum of eluate and morphine std from 360 to 255 nm, using $CHCl_3$ as ref. (Film of Et_3NHCl may adhere to walls of cuvets. Rinse cuvets carefully with H_2O and alcohol; then wipe clear before scanning.) Correct A at max. of ca 285 nm by extrapolating base line from 340 to 310 nm to this wavelength. Calc. wt anhyd. morphine in aliquot taken from formula: $W = (W_s \times A_u/A_s) \times f$, where W_s = wt morphine in std soln, A_u and A_s = cor-

[2] The section numbers within the method are those for the 11th ed. of *Official Methods of Analysis*, 1970: sec. **36.015** (11th ed.) = **32.013** (10th ed.), **36.016(a)** = **32.014, 36.017** = **32.015.** Cautionary notes, if present, refer to the new chapter on safety, Chapter 46.

rected A of sample and std, resp., and f = factor to convert wt std to its equiv. in anhyd. morphine (if hydrated morphine or morphine salt is used as std).

Results and Discussion

Due to the limited quantity of sample available, the sample size used by the collaborators was 0.5 or 1.0 g instead of the 2.0 g specified in the method; the volume of solution was decreased to 25 or 50 ml, respectively, instead of the 100 ml specified with the 2 g sample. While this will increase the sampling error, the shortage of material made this decrease necessary.

Several analysts suggested that the method should include a precaution to keep the opium particles in contact with the DMSO and not to allow the particles to get on the walls of the flask, viz., "Don't swirl until after heating 15 minutes, then swirl gently making sure that all of the sample is dissolved." Most of the collaborators heated the DMSO solution for more than the 15 min minimum suggested.

Due to the wording of the original instructions there was some question raised by several collaborators as to when (or if) complete dissolution of the sample in DMSO takes place. Many of the collaborators observed that, although the procedure stated "heat until dissolution is complete," a small amount of insoluble material was still present after the dissolution step. When the DMSO solution was decanted and the residue was heated with fresh DMSO, this material remained insoluble. It consists of small particles of leaf and other foreign matter, rather than opium itself.

The amount of DMSO used by the collaborators varied from 20 to 50% and, in general, the differences did not affect the results. With 1 laboratory the results with the 50% DMSO agreed well with those of the other collaborators, while the results with 20 and 30% DMSO did not. All the other collaborators used DMSO equivalent to 20–35% DMSO.

In the original collaborative study, in which the blender was used to disperse the sample, sample A was reported as being the most difficult to disperse. In contrast, Samples B and E were reported as being the most difficult to dissolve when DMSO was used to prepare the opium sample solutions.

Upon the addition of water to the DMSO solution some resinous material separates and tends to float to the top in the foam. A drop of ether or ethanol dispels the foam and makes the adjustment of volume easier.

One analyst initially reported results which were from 74 to 87% of those obtained by the other collaborators. This analyst had not used water-saturated solvents as specified in the procedure. After repetition of the analyses, using the properly prepared solvents, her results were in agreement with those of the other collaborators.

Another analyst reported varying results and attributed this to the fact that he "supersaturated" the chloroform with water, allowing droplets of water to be transferred to the column and thereby changing the conditions of the partitioning. When he used water-saturated chloroform which was clarified he obtained reproducible results.

One collaborator reported that he could not obtain reproducible results when he performed his chromatography in a hood, but did obtain reproducible results when the chromatographic step was run on a regular bench top. He attributes the variation to evaporation of the solvents during the elution in the hood with resultant cooling of the columns.

One collaborator suggested that, in view of the lability of morphine in alkaline solution, it be eluted from the alkali column without delay. This has been incorporated in the proposed method.

Two collaborators suggested the use of a reagent blank instead of chloroform as the blank. If purified triethylamine is used as specified, a reagent blank reads essentially the same as chloroform vs. chloroform, therefore making the preparation of a reagent blank unnecessary. The amount of triethylamine in a blank would not necessarily be the same as that in the eluate and, therefore if it were not properly purified, it could over- or undercompensate for the absorbance of the samples.

Several collaborators suggested that the method should clearly specify that the outside of the cuvets should be rinsed before scanning. Simply wiping the cuvets does not always remove the film of triethylamine HCl which can be present and this residue will cause higher baseline absorbance in the resulting ultraviolet scan.

Some collaborators made the background absorbance correction by drawing a line through the intersection of the baseline at 310 and 340

nm, instead of following the instructions which require the extrapolation of the baseline itself. In these instances, in order to make these collaborators' measurements on the same basis as those of the other collaborators, the Associate Referee corrected the ultraviolet curves for the baseline extrapolation. In most instances this did not result in a significant difference, because the standard and the sample curves were both adjusted in the same manner or the deviation from the actual baseline was slight. If there was a significant rise in the absorbance at 310 nm in the ultraviolet curve, the use of the point of intercept method could lead to erroneous low readings by overcrowding the baseline.

One collaborator measured the absorbance of his solutions on both a recording spectrophotometer (results included in Table 2) and on a single beam manual spectrophotometer (Beckman DU). He constructed the baseline by taking readings at 5 nm intervals from 340 to 310 nm. The values obtained in this manner were in good agreement with those obtained with the recording spectrophotometer.

There is a precaution written in the method requiring the removal of the metal leashes from the volumetric flasks to prevent the contamination of the solutions with iron. In spite of this, the presence of the ferrous ion was detected in the spectrum of 1 sample from 1 collaborator and in all samples and standards from another collaborator. These samples were not included in the tabulation of results.

In general the collaborators found that the partition chromatographic step was rapid and reproducible. The results obtained are shown in Table 2. As pointed out previously (1), since opium is a complex natural product, the preparation of a sample suitable for standard recovery experiments is precluded. The samples tested in this collaborative study were obtained from the Division of Narcotic Drugs of the United Nations,

Table 2. Collaborative results of determination of morphine in opium; results reported as per cent anhydrous morphine

Coll.[a]	Sample A	Sample B	Sample C	Sample D	Sample E
1	12.45	15.67	14.78	—	—
	12.25	15.59	14.85	—	—
2	12.06	14.38[b]	—	—	7.64
	12.12	14.31[b]	—	—	7.68
3	—	15.53	14.98	13.76	—
	—	15.79	14.85	13.64	—
4	12.51	—	—	13.86	7.97
	12.48	—	—	13.88	7.93
5	—	—	14.82	13.79	7.79
	—	—	14.87	13.97	7.84
6	12.43	15.86	—	13.84	—
	12.54	15.82	—	13.88	—
7	—	16.19	15.02	—	7.82
	—	—	15.11	—	8.00
8	—	15.76	—	13.90	7.69
	—	15.66	—	13.96	7.74
9	12.60	—	14.99	—	7.95
	12.56	—	14.87	—	7.93
10	12.63	15.88	14.92	13.81	7.69
	12.80	16.11	14.81	13.91	7.58
11	12.40	15.08	14.77	13.43	7.59
	12.54	15.04	14.62	13.52	7.52
12	12.22	15.44	14.41	14.00	7.56
	12.35	15.56	14.34	14.02	7.39
13	12.56	15.63	14.79	14.00	7.70
	12.62	15.65	14.77	13.96	7.72
14	12.51	16.02	14.78	13.76	7.84
	12.62	16.09	14.76	13.85	7.87
15	12.64	15.85	15.02	14.16	7.85
	12.83	15.67	15.00	13.97	7.89
16	12.55	15.90	14.91	14.20	8.01
	12.55	15.73	14.91	14.01	7.77
17	12.39	16.24	15.11	13.22[b]	7.98
	12.22	16.18	15.21	13.16[b]	7.98
18	12.24	15.48	14.96	14.26	8.09
	12.77	15.82	14.77	14.26	8.10
19[c]	12.56	15.79	14.74	13.89	7.99
	12.52	15.96	14.98	13.56	8.10
20	12.09	15.30	15.42	13.79	7.74
	11.87	15.19	15.28	13.67	7.80
21	11.13[b]	13.61[b]	14.78	13.76	7.06[b]
	11.24[b]	13.35[b]	14.30	13.84	7.17[b]
Av.	12.45	15.73	14.87	13.88	7.82
Std dev.	0.2224	0.3070	0.2330	0.1941	0.1766
Coeff. of var., %	1.79	1.95	1.57	1.40	2.26

[a] Collaborators 1–16 used a 0.5 g sample; Collaborators 17–20 used a 1.0 g sample; and Collaborator 21 used a 2.0 g sample.

[b] These values were excluded in the calculation of the average and the standard deviation on the basis of Dixon's test for extreme values (6). Their average was considered to be an outlier at the 5% significance level.

[c] This collaborator reported individual results of duplicate samples instead of duplicate aliquots of individual samples.

Table 3. Comparison of the results of collaborative studies of the determination of morphine[a] in the UN opium samples; results reported as per cent anhydrous morphine

	Present Study[a]				WHO Study[b]			
Sample	Av.	Std Dev.	No. Coll.	Coeff. of Var.	Av.	Std Dev.	No. Coll.	Coeff. of Var.
A	12.45	0.207	16	1.66	12.55	0.988	8	7.87
B	15.74	0.307	16	1.95	16.01	0.277	8	1.73
C	14.87	0.220	14	1.48	15.06	0.236	8	1.56
D	13.88	0.184	16	1.33	13.90	0.332	8	2.39
E	7.82	0.171	17	2.18	7.63	0.179	8	2.34

[a] The data reported are based on the average of the duplicates reported by the collaborators, omitting those values which were excluded in Table 2 on the basis of Dixon's test for extreme values (6).
[b] The data below are based on average values reported by the collaborators (3).

which prepared them for collaborative study by the WHO for the International Pharmacopeia. The WHO study of these same lots of opium utilized the Schultz and Schneckenburger (5) gravimetric procedure and involved the use of standardized alumina for the separation of the morphine. As a point of reference, the results of the partial report of this study (3) which is now available are compared with the results of this collaborative study in Table 3.

Recommendations

It is recommended that the proposed partition chromatographic procedure, using DMSO for sample preparation, for the determination of morphine in opium be adopted as official first action and that further study be made on the preparation of the sample solution.

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The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee B and were adopted by the Association. See *JAOAC* **53**, 381 (1970).

This report of the Associate Referee was presented at the 83rd Annual Meeting of the AOAC, Oct. 13–16, 1969, at Washington, D.C.

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Note on Gas Chromatographic Determination of Antihistamines Employing a Dual Column Direct Injection System

By THOMAS J. REISS (Food and Drug Administration, 850 Third Ave., Brooklyn, N.Y. 11232)

A GLC method is presented for the rapid direct analysis of antihistamines in pharmaceuticals in which the common interfering components are separated by gas chromatography. In previous papers the interferences are first separated by extraction before injection on the column. A dual column system makes it possible to separate each of the antihistamine peaks from each of the 7 most common accompanying component peaks. A simple 1-step extraction procedure is given for the separation of flavoring materials in sirups. Since only 1 or 2 antihistamines are present in any given sample, peak overlapping does not usually present a problem. Deviations from linearity on each column tested were minimal.

Several papers have been published reporting the use of gas chromatography for the analysis of the antihistamines (1–3). In these procedures, interfering components are separated by extraction. This is time-consuming, is often incomplete, and can result in losses of the antihistamines being determined. Examples of all-purpose columns for pharmaceuticals which have been used for the gas chromatographic separation of antihistamines are 3% Hi Eff 8BP (4) and 3% OV-17 (5). The method described in this paper permits the rapid direct analysis of antihistamines, except sirups, without preliminary separation.

The dual flame ionization detection system of the F & M Model 5750 gas chromatograph permits the simultaneous use of 2 columns with individual detectors. The 2 columns are inserted in the oven and brought to the same temperature. A direct injection of a water solution of the antihistamines into the proper column permits the complete separation of each of the antihistamine peaks from all interfering component peaks by gas chromatography.

METHOD

Reagents

(a) *Standard solution.*—Prepare solution containing 0.3 mg antihistamine reference standard/ml and 0.3 mg internal standard/ml in water.

(b) *Internal standard.*—3 mg/ml. Use antihistamine with closest retention time to component of interest but still completely resolved from it.

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Apparatus

(a) *Gas chromatograph.*—F & M Model 5750 with dual hydrogen flame detectors and 1 mv recorder. Conditions: range setting 10; attenuation 8; column oven 210°C, injection port 240°C, detector 230°C; helium carrier gas 30 psig, 3.0 rotometer reading (50 ml/min); hydrogen 12 psig, 3.4 rotometer reading (40 ml/min); air 32 psig, 4.0 rotometer reading (420 ml/min).

(b) *Columns.*—(*1*) 4′ × ¼″ od glass, containing 2% SE-30 + 2% Carbowax 20M on 80–100 mesh Diatoport S (1). (*2*) 4′ × ¼″ od glass, containing 10% silicone oil DC-200 on 60–80 mesh Diatoport S (2).

Procedure

Finely powder sample to pass 60 mesh sieve. Accurately weigh portion equivalent to ca 30 mg antihistamine. Transfer to 100 ml volumetric flask, add 10 ml internal standard and ca 50 ml water, and

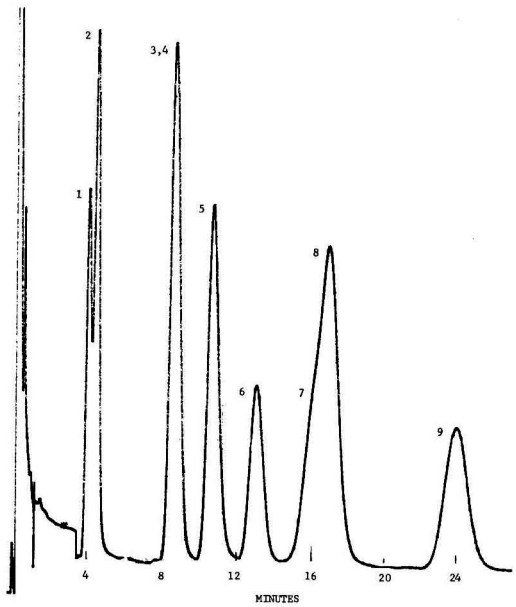

FIG. 1—Gas chromatogram of antihistamines on 2% SE-30 + 2% Carbowax 20M column: 1, pheniramine maleate; 2, diphenhydramine HCl; 3, 4, methapyrilene HCl and chlorpheniramine maleate; 5, diphenylpyraline HCl; 6, brompheniramine maleate; 7, phenindamine tartrate; 8, chlorcyclizine HCl; 9, pyrilamine maleate.

Table 1. Retention times (RT) on 2% SE-30 + 2% Carbowax 20M column

Compound	RT, cm	Relative RT
Aspirin	0.48	0.09
Phenylpropanolamine HCl	not detected	
Pheniramine maleate	2.59	0.47
Diphenhydramine HCl	2.84	0.52
Methapyrilene HCl	5.49	1.00
Chlorpheniramine maleate	5.49	1.00
Dextromethorphan HBr	6.77	1.23
Diphenylpyraline HCl	6.78	1.23
Salicylamide	7.69	1.40
Phenacetin	7.90	1.44
Brompheniramine maleate	8.27	1.51
Glyceryl guaiacolate	9.41	1.71
Caffeine	10.29	1.87
Phenindamine tartrate	10.30	1.88
Chlorcylizine HCl	10.69	1.95
Pyrilamine maleate	15.08	2.75

place on shaking machine 30 min. Dilute to volume with water, mix, and filter. Inject 4 μl sample solution into gas chromatograph under conditions described.

Results and Discussion

The retention times of the 9 most common antihistamines (Fig. 1) plus the 7 common accompanying components (Table 1), relative to chlorpheniramine maleate, were determined on the 2% SE-30 + 2% Carbowax 20M column suggested by Celeste (1). With this column, dextromethorphan HBr, salicylamide, phenacetin, glyceryl guaiacolate, or caffeine present in the formulation will interfere with the antihistamine peaks. With the 10% DC-200 column, 4 of the interfering substances elute before the first antihistamine peak, and dextromethorphan HBr is eluted with a shift in position relative to the antihistamines.

Table 2. Retention times (RT) on 10% DC-200 column

Compound	RT, cm	Relative RT
Aspirin	0.72	0.09
	1.03	0.13
Phenylpropanolamine HCl	0.94	0.12
Salicylamide	1.20	0.15
Glyceryl guaiacolate	2.37	0.30
Phenacetin	2.45	0.31
Caffeine	3.93	0.50
Pheniramine maleate	4.20	0.53
Diphenhydramine HCl	4.95	0.63
Methapyrilene HCl	7.35	0.93
Chlorpheniramine maleate	7.87	1.00
Diphenylpyraline HCl	10.85	1.38
Brompheniramine maleate	10.85	1.38
Dextromethorphan HBr	12.15	1.54
Phenindamine tartrate	12.59	1.60
	13.72	1.74
Chlorcyclizine HCl	16.19	2.06
Pyrilamine maleate	16.19	2.06

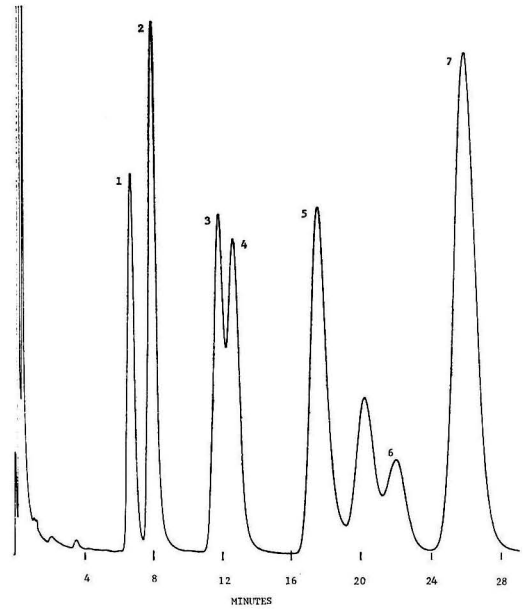

FIG. 2—Gas chromatogram of antihistamines on 10% DC-200 column: 1, pheniramine maleate; 2, diphenhydramine HCl; 3, methapyrilene HCl; 4, chlorpheniramine maleate; 5, diphenylpyraline HCl and brompheniramine maleate; 6, phenindamine tartrate; 7, chlorcyclizine HCl and pyrilamine maleate.

The retention times of the antihistamines and other components (Table 2) were determined on the 10% DC-200 column, again relative to chlorpheniramine maleate. The chromatogram (Fig. 2) shows the separation of the 9 antihistamines on this column. The use of the 2 columns together permits separations not possible with either column alone.

Retention time is not the only criterion of an adequate separation. There can also be interference due to relative concentration. Four of the interfering compounds elute before the first antihistamine, pheniramine maleate, on the DC-200 column. However, the greater concentration of these compounds relative to the antihistamines resulted in interference with the pheniramine maleate and diphenhydramine HCl peaks. Therefore, when the sample contains either of these 2 antihistamines with any of the 4 mentioned interferences, the 2% SE-30 + 2% Carbowax 20M column would be the column of choice.

This method can be applied to a variety of tablet and sirup preparations. Interferences from flavoring materials in the sirups, if present, are removed by a

simple 1-step extraction procedure. The sample is dissolved in water, the internal standard and 5 ml 1*N* NaOH are added, and the solution is extracted with 25 ml ether. The standard is treated similarly. Since only 1 or 2 antihistamines are usually present, resolution is not the most important consideration and the overlapping of antihistamine peaks on the column does not present a problem.

In a number of runs, employing several solvents, there were small deviations from linearity on the 2% SE-30 + 2% Carbowax 20M column; the 10% DC-200 column showed good linearity. For one chloroform solution of the sample, the analysis was not reproducible on the 10% DC-200 column.

Acknowledgment

The author wishes to thank Edward Rennard for suggesting the DC-200 column.

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MYCOTOXINS

Determination of Aflatoxin in Cottonseed by Ferric Hydroxide Gel Cleanup

By JAMES VELASCO (Market Quality Research Division, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md. 20705)

The procedure for determination of aflatoxin in cottonseed has been simplified by removal of interfering gossypol pigments with ferric hydroxide gel. The need for purification of the aflatoxin fractions through silica gel columns is eliminated, thereby reducing the time and cost of analysis. Greater amounts of aflatoxin B_1 are recovered by this method than by the official AOAC method.

A single standard method of analysis for determination of aflatoxin in seed products in general has not yet been adopted because of the great variety of interfering pigments and fluorescent compounds present in seeds.

In cottonseed products, the AOAC method (1, 2) for estimation of aflatoxin utilizes lead acetate to remove gossypol pigments and silica gel columns to remove interfering fluorescent compounds (3). Though the final extracts obtained by this procedure are very clean, a great deal of time, material, and manipulation is required for its completion.

Simplification of aflatoxin methodology for cottonseed and cottonseed meals has been under investigation by this laboratory. A simplified procedure for cottonseed meals has previously been reported (4); however, it is not adaptable to cottonseed.

Investigation of cottonseed extracts revealed that the major interference in detection of aflatoxin by thin layer chromatography (TLC) was due to the pigment gossypol. It was thought that a more effective removal of this pigment from extract solutions could possibly eliminate the need for further cleanup through silica gel columns.

Use of ferrous salts to inactivate gossypol in cottonseed meals was first reported by Withers and Brewster (5). The formation of an iron-gossypol complex (6, 7) suggested the possibility of eliminating gossypol from solution by either complexing or adsorbing it on the surface of an iron hydroxide gel. This reaction was investigated and the procedure described here was subsequently developed.[1]

[1] Since this paper was submitted a method describing the removal of gossypol with ferric chloride has been published (Yatsu, L. J., Jacks, T. J., and Hensarling, T., *J. Amer. Oil Chem. Soc.* **47**, 73–74 (1970)).

Experimental

Two approaches to the binding of gossypol with iron hydroxide gel were tried. In the first, an extract containing gossypol was added directly to a solution containing iron hydroxide gel and the adsorption of gossypol on the gel was determined. In the second, ferrous ions were added to a gossypol extract to form the soluble iron-gossypol complex and then hydroxyl ions were added to form the insoluble iron hydroxide gel. Both approaches effectively removed gossypol from solution. The second method, however, was more efficient and simpler. A test of the amount of gossypol removed from an acetone-methanol-water (2 + 2 + 1) extract of cottonseed (Table 1) showed that more than 90% of the gossypol was adsorbed by the gel. The amount of gel needed to remove gossypol from solution was determined and the concentration and volumes of ferric chloride and sodium hydroxide were adjusted to facilitate the calculation of the aflatoxin content.

Table 1. Amount of free gossypol adsorbed by ferric hydroxide gel from a cottonseed extract of acetone-methanol-water (2 + 2 + 1)

Filtrate From:	Aniline, ml	Absorbance at 440 nm	Equiv. Gossypol,[a] mg	Gossypol Adsorbed, %
Extract[b] and gel[c]	—	0.000	—	—
Extract	2	0.363	1.25	—
Extract and gel	2	0.033	0.08	93.6
Extract $FeCl_3$ + NaOH	2	0.035	0.12	90.4

[a] Gossypol determined by AOCS method Ba 7-55.
[b] 100 ml (equivalent to 23.4 g cottonseed).
[c] Gel formed by the addition of 10 ml 10% $FeCl_3$ + 13.1 ml 2.9% NaOH.

In addition to iron, the hydroxides of aluminum and copper were investigated. Results were best with ferric iron. Ferric hydroxide forms a very dense and flocculent gel in the acetone-methanol-water (2 + 2 + 1) solvent.

Addition of ferric chloride solution to an extract of cottonseed turns the yellow extract black due to formation of soluble ferric-gossypol complex and, with the volumes indicated in the method, changes the pH from about 7 to 2.5. Ferric hydroxide is formed upon addition of sodium hydroxide; however, the hydroxide does not gel readily until the pH of the extract is increased above 4. Filtration of the extract-gel through folded filter paper results in an almost colorless filtrate. Occasionally, the first few milliliters of filtrate might contain pigment and are therefore poured back into the filter paper. However, with proper stirring, this condition is minimized.

A determination was made to measure the effect on aflatoxin results by forming gels at pH values higher than 5. The results are shown in Table 2. As the pH of the extract-gel solution is increased from 5 to 9 (aliquots A–C), by the addition of sodium hydroxide, the amount of aflatoxin detected decreases. This decrease could be attributed either to a change in pH, since aflatoxin fluoresces stronger in an acid medium (8), or to adsorption of aflatoxin by the gel. To determine how much of this decrease was due to pH, a similar set of aliquots (D–F) was increased in pH, filtered, and then re-acidified to a pH of 4 with hydrochloric acid. The results of this test indicate that major aflatoxin losses are due to loss of fluorescence at higher pH levels. However, significant losses of aflatoxin B_1 are also noted when the extracts are re-acidified. These losses confirm the observation (8) that aflatoxin solutions made alkaline and then brought back to an acid pH lose as much as ⅔ of their fluorescence.

Control of pH is therefore important in the formation of the ferric gel since neither a high pH nor re-acidification of extracts is desirable. This control, however, is not difficult once the volumes and concentration of ferric chloride and sodium hydroxide are adjusted.

A satisfactory gel is formed at a pH range of 4.5 to 5.0. Analysis of extracts at this pH range showed no significant differences in aflatoxin results (data not shown). pH 4.7 is therefore arbitrarily selected as the pH at which the gel is formed. Formation of the gel can be made without the use of a pH meter by addition of a predetermined volume of sodium hydroxide (see *Method*). We have encountered a variation of ±0.2 pH units in the extract-gel solutions routinely analyzed; this small variation does not affect the analysis.

Tests were also made to determine whether heating of the extract-gel solution improved removal of gossypol. Extract-gel solutions were heated as high as 50°C without effectively decreasing the color of the filtrate; in fact, heated filtrates contained more color than unheated.

Table 2. Effect of pH on adsorption of aflatoxin by ferric hydroxide gel

Aliquot[a]	10% $FeCl_3$, ml	2.9% NaOH, ml	pH	Filtered & 2.5% HCl, ml	pH	Densitometer Count		
						B_1	B_2	Total
A	10	13.1	5.0	—	5.0	121	107	228
B	10	14.9	7.0	—	7.0	94	93	187
C	10	15.9	9.0	—	9.0	54	55	109
D	10	13.1	5.0	0.1	4.0	90	111	201
E	10	14.9	7.0	0.2	4.0	92	118	210
F	10	16.0	9.0	0.45	4.0	105	96	201

[a] 100 ml each.

In order to extract the maximum amount of aflatoxin from the gel-purified extract, the combined concentration of acetone and methanol must be reduced to about 20%. Tests to determine whether this reduction should be made by evaporation or simply by dilution with water indicate that the methods are equally effective. The simpler dilution step is therefore used.

METHOD

Apparatus and Reagents

(a) *Long wave ultraviolet lamp.*—3660Å.

(b) *Filter paper.*—24 cm, folded, S&S No. 588, or equivalent.

(c) *Burets.*—100 ml capacity, with Teflon stopcocks.

(d) *Reagents.*—ACS grade acetone, methanol, ferric chloride, and sodium hydroxide; and technical grade chloroform.

(e) *Extraction solvents.*—Acetone-methanol-water (2 + 2 + 1); aqueous ferric chloride (15%); and aqueous sodium hydroxide (4%).

(f) *Aflatoxin standards.*—About 1 μg B_1 and 0.3 μg B_2/ml chloroform-acetonitrile (98 + 2).

(g) *pH 5 buffer solution.*—20 ml 0.2*M* acetic acid and 30 ml 0.2*M* sodium acetate.

Procedure

Preparation of Sample

Weigh 150–200 g (±1 g) gin-run cottonseed and grind either in Bauer mill or Waring Blendor. In Waring Blendor, grind seed in 20 g increments (with >20 g, seed will not fall into blade zone). Separate meats or kernels from lint and hulls with coarse sieve (U.S. No. 6) and record weight of lint and hulls. Obtain weight of meats by difference. With Bauer mill, grind meats to pass U.S. No. 16 sieve; meats ground in the Waring Blendor are of adequate fineness.

Extraction

Weigh 30 g (±0.1 g) ground meats into 500 ml Erlenmeyer flask. Add 250 ml acetone-methanol-water (2+ 2 + 1) solution and shake 30 min on mechanical shaker. Filter solution through 24 cm folded filter paper. Measure 150 ml filtrate in 250 ml graduated cylinder and transfer to 250 ml beaker (polyethylene beakers may be used to facilitate cleaning). Add 10 ml 15% ferric chloride solution from buret and stir solution well. Add 4% NaOH from buret and increase pH to 4.6–4.8, using pH meter for measurement. (For routine analysis, this amount can be predetermined and added without need for pH meter; see *Standardization* below.) Stir well and let stand for ≥5 min. Filter solution through 24 cm filter paper. If first few milliliters filtrate show signs of pigment, pour back into filter paper. Transfer 100 ml filtrate into 500 ml separatory funnel, using 100 ml graduated cylinder. Dilute solution in separatory funnel with 250 ml distilled water. Extract diluted solution twice with 50 ml portions of chloroform and collect chloroform layer in 250 ml beaker. (Use of stainless steel beakers will reduce time required for evaporation.) Evaporate chloroform on water or steam bath and transfer extract residue into 10 ml vial with ca 6 ml chloroform. Evaporate chloroform in vial, let cool to room temperature, and use for TLC. (TLC development of extract residues should be made same day; if not, store extracts in freezer without evaporating chloroform.)

Standardization of pH Meter

Standardize pH meter with pH 5 buffer solution. Place electrodes in beaker containing 150 ml extract solution and 10 ml 15% ferric chloride solution. Add 4% NaOH solution from buret (15–17 ml) until slight buffering action is observed at pH 4.6–4.8. Take point at which this buffering action occurs as end point. Briskly stir solution by hand or magnetic stirrer while making final pH measurements. Make several determinations, preferably with different sample extracts, and calculate average volume. This volume can be used for formation of gels without need for pH measurement. Readjust NaOH volume with pH meter when new batches of ferric chloride or NaOH are used. Since ferric gel tends to

adhere to glass surface of pH electrodes, occasionally immerse electrodes in 0.1*N* HCl to ensure proper function of meter.

TLC Development

Pipet 1 ml chloroform-acetonitrile (98 + 2)[2] into vials containing sample extract. Spot 5 μl sample on silica gel thin layer (0.025–0.500 mm) plate with appropriate amounts of B_1 and B_2 standards. Place plate in unlined tank and develop with diethyl ether-methanol-water (96 + 3 + 1) solvent for 45 min.

Estimation of Aflatoxin

Place plate in appropriate chamber and expose to long wave UV light. Estimate aflatoxin contents of the sample spots either visually or with densitometer (1). Use weight of seed rather than meats for final calculation of aflatoxin.

Equiv. wt meats = 30 × (150/250) × [100/(160 + vol. NaOH added)].

Equiv. wt cottonseed = equiv. wt meats/% meats in cottonseed. Substitute equivalent weight cottonseed for *W* in formula to calculate B_1 and B_2 by AOAC method (1).

Results and Discussion

This method of cleanup was compared with the lead acetate-silica gel cleanup procedure of the AOAC method (1, 2). The cottonseed samples used for this test had been found to be heavily contaminated with aflatoxin and had been stored for 8 months.

Each sample was thoroughly mixed in a modified Henry mixer and a 200 g weight was taken for analysis. The sample was dehulled and ground in a Bauer mill. The ground sample was thoroughly blended and split into quarters: 30 g sample, from opposite quarters, was taken for analysis for each of the methods. The results of this comparison are shown in Table 3. Samples 1–20 were chosen at random, while Samples 21–25 were selected because of their low aflatoxin content.

In 23 out of 25 samples, aflatoxin B_1 values were higher with ferric hydroxide gel than with lead acetate-silica gel. Many of these differences were large. For 6 samples, yields of aflatoxin B_1 from the ferric hydroxide gel procedure were double the yields from the lead acetate-silica gel procedure.

Table 3. Aflatoxins B_1 and B_2 determined in contaminated cottonseed after cleanup by lead acetate-silica gel or ferric hydroxide gel

Sample	B_1, μg/kg		B_2, μg/kg	
	Lead Acetate-Silica Gel	Ferric Hydroxide Gel	Lead Acetate-Silica Gel	Ferric Hydroxide Gel
1	874	1162	119	170
2	508	635	568	164
3	2166	2375	522	412
4	478	957	181	322
5	990	1232	238	278
6	323	610	297	193
7	5467	5555	1159	928
8	1861	2281	1690	1484
9	329	869	193	254
10	462	993	185	246
11	1148	1845	692	725
12	477	1212	166	198
13	4432	4340	806	806
14	1125	1216	353	311
15	614	716	167	106
16	1242	1175	165	129
17	569	839	112	139
18	831	999	255	242
19	1544	1802	362	408
20	1596	2408	490	536
21	13	31	7	10
22	15	22	3	9
23	34	49	12	17
24	38	57	6	13
25	16	27	2	5

The preponderance of high B_1 results by the ferric hydroxide gel procedure was at first attributed to the use of 40% methanol in the extraction solvent. Feuell (9) found that methanol extracts aflatoxin more completely from peanut meal than acetone.

A comparison was, therefore, made of the extraction capacity of acetone-methanol-water (2 + 2 + 1) with that of acetone-water (85 + 15) used in the lead acetate-silica gel procedure. Contaminated samples of cottonseed were extracted with both solvent systems and the extracts were purified by the ferric hydroxide gel procedure.

The results of this experiment (data not shown) revealed that there is no significant difference in the extraction capacity of the 2 solvent systems. Apparently, the addition of water to both systems reduces the extraction difference between methanol and acetone. The use of 40% methanol, however, reduces the amount of dark pigments extracted from cottonseed meats.

[2] Since this study was completed, a paper has appeared on the use of benzene-acetonitrile (98 + 2) for the preparation of aflatoxin standards (Pons, W. A., Jr., *et al.*, *JAOAC* **53**, 293–299 (1970)). This solvent system was also specified for use in the AOAC official methods for aflatoxins ("Changes in Methods," *JAOAC* **53**, 437 (1970)). Although the present investigations were performed with chloroform as a solvent, the mixed solvent should be as suitable.

Table 4. Recovery of aflatoxin from silica gel columns (1), using ferric-purified extracts of contaminated cottonseed

Composite Ext	B_1 Added, μg	B_1 Recd, μg	B_1 Recd, %	B_2 Added, μg	B_2 Recd, μg	B_2 Recd, %
1	25.7	11.4	44.4	6.7	5.6	83.6
2	16.4	8.6	52.4	2.5	1.7	68.0
3	24.2	13.9	57.4	4.1	3.4	82.9

The next step in our investigation of why there were such wide differences between these 2 procedures was to determine the extent to which aflatoxin results are affected by purification of extracts through silica gel columns. Pons *et al.* (10) reported that recoveries of B_1 added to cottonseed meats, by the lead acetate-silica gel procedure, ranged from 87 to 91%, using densitometric measurements. In a later collaborative study (2) and using essentially the same procedure, he reported that B_1 recoveries among 7 laboratories ranged from 71.4 to 130.0% with an average recovery of 93.8%.

Standard solutions of crystalline B_1 were used in both of the above recovery experiments; 100% recoveries were obtained at the 0.2–0.6 μg level. Since there is evidence to suggest that aflatoxin might be bound to other compounds in natural extracts (9), especially when water is used, this factor could influence the recovery of natural aflatoxin from silica gel columns.

The development of this new cleanup procedure enabled us to obtain some measure of the effect of purifying natural extracts containing known amounts of aflatoxin, through silica gel columns. Cottonseed meats from the samples shown in Table 3 were made up into 3 composite samples. The composite samples were extracted and purified by the ferric hydroxide gel procedure. Aliquots of the ferric-purified extracts were then added to silica gel columns and the amounts of B_1 and B_2 recovered are shown in Table 4. The highest recovery of aflatoxin obtained from these columns was 84% for aflatoxin B_2 and 57% for aflatoxin B_1.

Figure 1 is a photograph of the recovery experiment. Aliquot 1B, which was purified through a silica gel column, contains a prominent spot (R_f 0.56) above the B_1 spot (R_f 0.41). This spot, which is absent in the original ferric-purified aliquot 1A, could be a product of degradation in the silica gel column. The intensity of the spot indicates that it was derived from either the B_1 or the B_2 fraction. Since the greatest chromato-

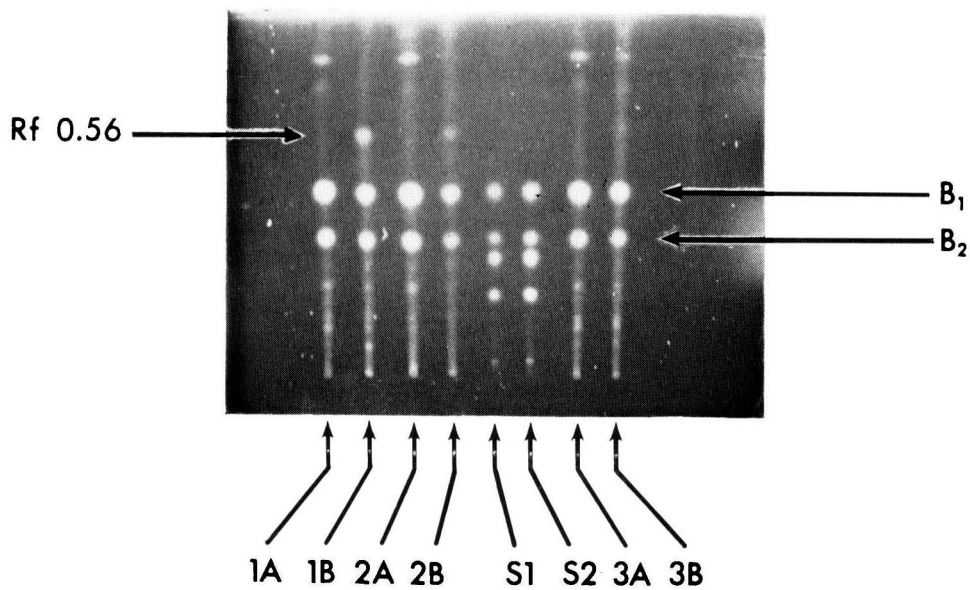

FIG. 1—Thin layer chromatogram of aflatoxin recovery from cottonseed. 1–3, Composite samples of contaminated cottonseed purified by ferric hydroxide gel procedure; A, aliquots purified with ferric hydroxide; B, aliquots further purified and recovered from silica gel columns; and S1 and S2, alfatoxin B_1 and B_2 standards, respectively.
TLC conditions: aliquots equivalent to 15 mg cottonseed meats spotted on silica gel plates, developed 45 min in unlined tank, with diethyl ether-methanol-water (96 + 3 + 1) as solvent.

graphic loss was from fraction B_1, this spot appears to be a product of B_1 degradation. Similar spots, although not as intense, appear in aliquots 2B and 3B, which were also passed through silica gel columns.

Low recoveries of B_1 from silica gel columns are apparently reflected in the results by the AOAC lead acetate-silica gel procedure for most of the samples shown in Table 3.

In view of these findings, previous studies on stored contaminated cottonseed will be reevaluated, using the ferric hydroxide gel procedure. Preliminary data had indicated that the content of aflatoxin B_1 decreased substantially during storage of cottonseed.

Concurrently, stored cottonseed meals contaminated with aflatoxin also are being analyzed by the ferric hydroxide gel procedure.

Acknowledgments

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Ochratoxins A and B Confirmation by Microbiological Assay Using *Bacillus cereus mycoides*[1]

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TLC identification of presumptive ochratoxins A and B can be supplemented with overnight results obtained by a single and rapid bioassay of these toxins, using *Bacillus cereus mycoides* LSU as the test organism. Furthermore, this bioassay can provide an accurate quantitative estimation of ochratoxins. The calculated coefficient of variation was 5.9%. The optimal medium, conditions, and ochratoxin concentrations for this assay are suggested. This test is sensitive to as little as 1.5 and 3.0 μg ochratoxins A and B, respectively.

Much emphasis has been focused on finding a rapid biological test and more sensitive substitute for ducklings as the test organism in mycotoxin bioassays. The microbiological assay technique was recently adapted to supplement the results of thin layer chromatographic (TLC) detection and confirmation of the presence of mycotoxins in food materials or fermentation extracts. Burmeister and Hesseltine (1) found that *Bacillus megaterium* NRRL B-1368 was the most sensitive to aflatoxins of 329 different microorganisms tested. Clements (2, 3) pursued further the possible use of *B. megaterium* NRRL B-1368 as the test organism in the microbiological assay of aflatoxins on a qualitative basis, and the medium, analytical conditions, and aflatoxin concentrations for this test were published. Clements (4)

[1] Adapted from the senior author's doctoral thesis, Louisiana State University, 1969.

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used this organism in ochratoxin A assay and reported a sensitivity of 4 μg for a crude extract of ochratoxin A. Shotwell *et al.* (5) recently followed the microbiological method of Clements (3) as one of the confirmatory tests of ochratoxin A crude extracts.

However, the use of *Bacillus cereus mycoides* as the test organism for the bioassay of ochratoxins A and B has never been reported. Therefore, this study was undertaken to investigate the feasibility of *B. cereus mycoides* LSU in the microbiological assay of ochratoxins from both a qualitative and quantitative basis.

METHOD

Materials

(a) *Agar medium.*—Dissolve 30.5 g antibiotic agar medium A, prepared as in **33.112**(a) (6), in 1 L distilled water and sterilize 15 min at 15 psi (121°C).

(b) *Bacillus cereus mycoides LSU.*—(Supplied by the Feed and Fertilizer Laboratory, Louisiana State University, Baton Rouge, La. 70803). Inoculate slant culture tubes and incubate overnight at 30°C.

(c) *Ochratoxin A and B standards.*—Dilute ochratoxins A and B separately in hexane-chloroform (1 + 1) to obtain concentrations of 0.168 and 0.365 μg/μl, respectively. Pure crystalline ochratoxin A and qualitative ochratoxins A, B, and C were kindly furnished by Dr. I. F. H. Purchase, Division of Toxicology, National Research Institute, Pretoria, South Africa. Ochratoxin B was purified by column chromatography on Florisil and further purified first by acid-aqueous hexane partition and then by pH-adjusted aqueous hexane partition (7).

Apparatus

(a) *Petri dishes.*—Sterile disposable plastic, 100 × 15 mm.

(b) *Filter paper disks.*—¼″ diameter, custom-made from Whatman filter paper No. 2 and heated at 90°C overnight. (Commercially available filter paper disks can be obtained from Carl Schleicher and Co., Keene, N.H., ¼″ disks No. 740-E.)

(c) *Vernier caliper.*—Scale 0 to 120 mm, vernier 0.1 mm.

Inoculum Preparation and Plating

(a) *Inoculum.*—Prepare inoculum of *B. cereus mycoides* LSU as in **33.119**(b)(*2*) (6), except use 2 L Erlenmeyer flasks instead of Roux bottles. (Working inoculum concentration is spore suspension which, when diluted 1:20 with sterile distilled water, gives 67% transmittance at 655 nm, using 1 cm cuvet and Bausch & Lomb Spectronic 20.)

(b) *Seeding.*—Use ca 1% inoculum to seed agar as follows: Inoculate 1 ml working inoculum into 100 ml melted agar (ca 50°C); thoroughly mix agar and spores, and plate immediately.

(c) *Plating.*—Pipet 4 ml seeded agar into each plastic Petri dish and immediately distribute agar uniformly over entire surface of plate, by first using slight horizontal shaking motion and then rotating motion. To prevent seeded agar from cooling too fast, repeatedly warm outside of bottle containing agar by passing it through Bunsen burner flame. To avoid lumping, use same technique to warm pipet before transferring seeded agar. Let plates harden on level surface ca 10 min. Store plates in inverted position in refrigerator to prevent moisture from condensing and damaging surface of seeded agar.

Assay Procedure

(a) *Qualitative and quantitative assay.*—(Since a similar procedure of impregnating the filter paper disks as recommended by Clements (3) is followed, both the qualitative and quantitative assays can be combined into one assay.) Place filter paper disks on wire mesh and slowly apply solvent (control) or solutions (sample or standard) dropwise, using microliter syringe. Dry disks 15 min and place 6 disks (impregnated side down) on seeded agar surface of each dish. Use 2 concentration levels (20 and 40 μl) for sample assay of each of the following: solvent (control), sample, and reference standard on the 4 plates to be assayed. For quantitative assay, obtain standard curve each time new stock standard solution or inoculum is prepared. To obtain standard curve, assay 4 standard concentrations (10, 20, 30, and 40 μl) and 2 controls (20 and 40 μl solvent) on each of 4 assay plates.

(b) *Incubation and zone measurements.*—Incubate plates overnight (18 hr) at 35°C. Measure diameter of inhibition zone with vernier caliper and subtract diameter of filter paper disk from this value to obtain corrected inhibition zone.

(c) *Standard curve.*—Plot corrected inhibition zone (mm) against ochratoxin concentration (μg) on linear graph paper. Use standard curve to determine concentration of sample.

Discussion

Preliminary trials indicated that the growth inhibition response of *B. cereus mycoides* LSU to ochratoxins A and B was greater than that of *B. megaterium* NRRL B-1368, *Sarcina lutea* ATCC No. 941, and *Micrococcus flavus* ATCC No. 10240, using the same medium and conditions. Whereas no inhibition response to the growth of *B. cereus mycoides* by the solvent was

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Table 1. Relation between the ochratoxin A concentration and the corrected inhibition zones (coefficient of variation = 5.9%)

Plate	Corrected Inhibition Zone[a] (mm) for μg Ochratoxin A:			
	1.68	3.36	5.04	6.72
1	2.7	3.5	4.4	6.1
2	1.9	3.4	4.2	4.9
3	2.7	3.6	4.3	5.7
4	2.6	3.5	4.3	5.5

[a] Corrected inhibition zone is equal to the diameter of the zone of inhibition minus the diameter of the paper disk.

observed, the sensitivity of this bacterium to ochratoxins A and B was found to be above 1.5 and 3.0 μg, respectively.

Clear and distinct inhibition zones were noticed after 10 hr of incubation at 35°C. However, all inhibition zone measurements were taken after 18 hr of incubation at this temperature. Table 1 shows the corrected zone diameters in 4 plates produced by 4 different equally spaced concentrations of ochratoxin A, and Fig. 1 shows a photograph of 2 of these plates. The statistical analysis of the data indicated that the relationship between the corrected inhibition zone diameter and the ochratoxin concentration was only linear ($P < 0.01$). The linear equation found was as follows:

$$y = 1.50 + 0.595x$$

where x = μg standard ochratoxin A, and y = corrected growth inhibition zone in mm. A highly significant correlation ($P < 0.01$), correlation coefficient of 0.97, was found between the 2 variables.

The analysis of variance of the data also revealed that the same ochratoxin concentration produced different inhibition zones, which were significant ($P < 0.05$) among plates. However, the experimental error, as measured by the coefficient of variation, was only 5.9%. In actual assay conditions, as suggested in this experiment, experimental error smaller than 5.9% can be expected by assaying the sample and standard in the same plates. This would eliminate errors due to differences between plates.

This microbiological assay technique was also used qualitatively to supplement the TLC identity of ochratoxins from crude extracts of foods or fermentation. Table 2 shows the inhibition zones produced by different concentrations of

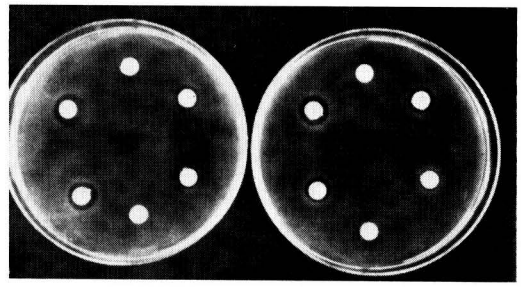

FIG. 1—Microbiological assay of 4 standard ochratoxin A concentrations, using *B. cereus mycoides* as the test organism. In each Petri dish, starting from the bottom counterclockwise, the filter paper disks are as follows: blank (control), ochratoxin A (1.68 μg), ochratoxin A (3.36 μg), blank control, ochratoxin A (6.72 μg), and ochratoxin A (5.04 μg).

purified extracts of *Aspergillus ochraceous* Wilh cultures in comparison to different concentrations of standard ochratoxins A and B. Extract 231 chloroform fraction (mostly ochratoxin A), the water fraction (ochratoxin B), and pigments (hexane fraction) from a pH-adjusted aqueous acetone-hexane mixture produced growth inhibition of *B. cereus mycoides*. However, Extract Culture 1B, a presumptive ochratoxin B, did not produce any detectable growth inhibition of *B. cereus mycoides*. These results were further substantiated by UV and IR spectroscopy and by a chemical derivative preparation method (7). No

Table 2. Microbiological assay of several purified mold extracts

Extract	Corrected Inhibition Zone[a] (mm) for: Extracts, μl		Std Ochratoxin A, μg	
	20	40	3.36	6.72
229 chloroform	4.3	1.1	3.0	5.5
229 water	3.2	6.1	2.5	5.3
230 water	3.5	6.1	2.5	4.6
231 hexane[b]	2.8	5.3[c]	3.7	5.2
231 chloroform	4.3	6.8	3.2	4.5
231 water	3.8	6.3	2.5	4.5
Culture 1B	0	0	3.3	5.0
Ochratoxin B (0.639 μg/ml)	6.8	12.7	3.3	4.9

[a] Corrected inhibition zone is equal to the zone of inhibition minus the paper disk diameter. Each corrected inhibition zone here is the average of 4 measurements.

[b] Hexane fraction contained mostly yellow pigments and perhaps some ochratoxin C.

[c] Average of only 2 measurements; the other 2 were too irregular to be measured.

further confirmatory tests were performed on the other mold extracts due to excessive pigments.

Since it appears that some of the pigments produced by toxigenic strains of *A. ochraceous* Wilh are also toxic to *B. cereus mycoides*, the quantitative determination of ochratoxin A or B as such by this method should involve further purification of the partially purified extracts before assaying them.

Acknowledgments

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Rapid Method for the Isolation of Pure Aflatoxins

By M. STEYN (Division of Toxicology, South African Medical Research Council, Private Bag 380, Pretoria, Republic of South Africa)

A chromatographic method is described for the preparation of pure aflatoxins from crude aflatoxin-containing extracts. The stationary phase consists of aluminum oxide (basic, according to Brockman) containing 6.25% oxalic acid dihydrate. The main advantage of this method is that pure samples of individual aflatoxins may be obtained in less than 2 hr. An additional advantage is economy of materials.

The most important toxic metabolites of certain strains of the fungi *Aspergillus flavus* and *A. parasiticus* are undoubtedly the aflatoxins. Separation of the aflatoxins from crude extracts of media on which appropriate mold strains have been grown is a time-consuming operation which seldom results in the isolation of toxins of more than 90% purity when the normal chromatographic method using silica, alumina, or formamide-impregnated cellulose is employed (1).

Rodricks (1) described a method to isolate pure aflatoxins from crude aflatoxin-containing extracts by chromatography on acid alumina. Although excellent results are obtained by the use of this method, it is quite a lengthy procedure and also involves the use of considerable volumes of solvents.

Experiments in this laboratory have shown that aluminum oxide standardized by Brockman's procedure is excellently suited for the chromatographic isolation of individual aflatoxins when it contains about 6% oxalic acid. The use of this material as the stationary phase enabled the most active aflatoxins (B_1, G_1, and M) to be obtained as white crystalline solids of high purity (95–99%) in less than 2 hr. One recrystallization from benzene resulted in products of virtually 100% purity, based on TLC examination and molar absorptivity measurements.

Aflatoxins B_2 and G_2 are produced in minor quantities by the strains of aflatoxin-producing fungi at our disposal. The result is that only small quantities of these aflatoxins may be obtained from these fungi in a pure state, since a considerable amount of contamination with either B_1

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and/or G_1 occurs. However, aflatoxins B_1 and G_1 are readily hydrogenated almost quantitatively, which is a more practical way of preparing aflatoxins B_2 and G_2 (1).

Aflatoxin M_1 can be separated from aflatoxin M_2 only by paper chromatography as described by Holzapfel and coworkers (2).

Experimental

Preparation of Aflatoxin-Containing Extracts

The method described by Shotwell *et al.* (3) was used to produce aflatoxins and to prepare an extract therefrom. Aliquots of 20 ml containing about 0.5 g solids (40% of which were aflatoxins) were used for each experimental separation.

Preparation of Mixed Adsorbents

Oxalic acid dihydrate was mixed with aluminum oxide (activity grade II–III, basic (Merck, Darmstadt, W. Germany), according to Brockman) in proportions from 0 to 20% (w/w) by the procedure detailed below. The required quantity of aluminum oxide was weighed into a suitable beaker and the oxalic acid was weighed, transferred to a 1 L flask, and dissolved in about 400 ml acetone. The aluminum oxide was added to this solution and the resultant slurry was shaken well and transferred to a stainless steel pan with a large surface area from which the acetone was allowed to evaporate completely at room temperature. When the mixed adsorbent was completely free from acetone it was ready for use. This adsorbent remains in satisfactory condition for at least 3 months; however, this adsorbent should be stored in a desiccator over silica gel or equivalent dehydrating agent.

Column Chromatography

Both mixed-solvent and single-phase elution methods of aflatoxin separation were investigated.

(a) *Mixed-solvent elution.*—A column ca 60 cm and 3–5 cm diameter was provided with a cotton wool plug and was packed with 50–70 g mixed adsorbent suspended in benzene. The excess benzene was drained. A few grams of mixed adsorbent was added to the 20 ml extract to be chromatographed and the solvent was completely removed under reduced pressure in a rotary evaporator. This adsorbent was suspended in benzene and transferred to the column. The excess benzene was drained from the bottom of the column so that the levels of the liquid and solid surfaces were approximately even.

The column was eluted with 100–200 ml of each of the following solvents in succession: benzene, benzene-chloroform (1 + 1), chloroform, chloroform-methanol (98 + 2), chloroform-methanol (95 + 5), and chloroform-methanol (90 + 10), at a constant head. All the eluates were collected in 5–10 ml fractions while a flow rate of 2–8 ml/min was maintained. The aflatoxins contained in each fraction were identified by TLC on silica, as described by Pons, Robertson, and Goldblatt (4).

Fractions containing similar aflatoxins were combined, evaporated to near dryness, and either precipitated by adding diethyl ether or crystallized from benzene (overnight).

(b) *Single-phase elution.*—Procedures were identical to those employed in (a), except that a single solvent (either benzene or chloroform) was used to eluate the aflatoxins. Elution of the more polar aflatoxins was facilitated by a final elution with chloroform-methanol (95 + 5).

Estimation of Recoveries

The method of Pons, Robertson, and Goldblatt (4) was used to determine the quantites of the individual aflatoxins recovered from an aliquot of the extract after application of the procedures detailed above. The same method was also used to analyze an aliquot of the original extract. Thus it was possible to calculate the per cent recovery of each aflatoxin. A Photovolt Model 530 densitometer was used for all determinations.

Results and Discussion

The recovery of the 3 main aflatoxins was improved by increasing the oxalic acid content of the adsorbent to 20% or higher (Table 1). It was determined, however, that the separation of the individual aflatoxins from one another was less effective as the oxalic acid content of the adsorbent increased. Reference to Table 1 shows that use of the adsorbent containing 6.25% oxalic acid results in a high recovery rate (more than 95% for B_1 and G_1 and 90% for M) and yields products with a purity of 96%, which can be increased to almost 100% by recrystallization. Therefore, this concentration of oxalic acid in the adsorbent

Table 1. Influence of the oxalic acid content of the stationary phase on recovery of aflatoxins

Oxalic Acid, %	Aflatoxins Recovered, %		
	B_1	G_1	M
0	65	0	0
3	75	65	15
5	90	90	40
6	95	95	80
6.25	>95	>95	90
7.5	>95	>95	>90
10	>95	>95	>90
20	>95	>95	>90

is recommended. If only aflatoxin B_1 is required, no addition of oxalic acid is necessary, but recovery of the toxin is only 65–70%.

The 2 methods of elution which were employed proved equally effective and either may be used, depending on convenience. However, the mixed-solvent elution was slightly more rapid than single-phase elution.

The main advantage of the method described is that pure samples of individual aflatoxins may be obtained from extracts in less than 2 hr. A further advantage of the method is economy of materials, as relatively small volumes of solvents are required and the mixed adsorbent can effectively separate the aflatoxins with loads of extracts of up to 5% on a dry weight basis.

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PRESERVATIVES AND ARTIFICIAL SWEETENERS

Determination of α-Hydroxy Acids in Foods by TLC and Manometric Measurement

By M. TROP and I. M. LEVINGER (Department of Life Sciences, Bar-Ilan University, Ramat-Gan, Israel)

An analytical method for determination of α-hydroxy and α-keto acids is developed based on separation of the acids from other components by acidic alcoholic extraction and by adsorption on and elution from an anion exchange resin, followed by thin layer chromatography. Acids are identified by ceric ammonium nitrate reagent sprayed on the chromatogram. Quantitative determination is performed by manometric measurement of gas released in reaction with that reagent.

α-Hydroxy acids such as citric, malic, and tartaric or their salts are used in some food products. They are added to control pH (active acidity) and taste, as well as to contribute other desirable properties to the finished food products. In general, they are added to fruit juices, soft drink, jams, jellies, candies, confections, etc. (1).

The classical methods (2, 3) for the determination of these acids are based on separation by means of selective extraction, adsorption, and sedimentation, which are not specific for each of the hydroxy acids. Moreover, the reagents used in these methods are not specific. Even the general reagents and detection systems used in thin layer chromatography (4) are not specific for α-hydroxy mono- and dicarboxylic acids. Recently, Trop *et al.* (5, 6) developed a new reagent (ceric ammonium nitrate) which is specific for α-hydroxy, α-carbonyl acids, and mercaptans. This new method allows the identification and determination of each of these substances on a thin layer chromatogram. The acids are identified by color change on the chromatogram. The quantitative determination is performed by manometric measurement of the gas released in reaction of the acids with ceric ammonium nitrate reagent.

In this paper, an analytical method for α-hydroxy and α-keto acids in food is described, based on separation of the acids from other components of food by acidic alcoholic extraction,

followed by adsorption on and elution from an anion exchange resin. After separation, the method of Trop *et al.* (6) may be applied.

METHOD

Apparatus and Materials

(a) *Anion exchange column.*—Prepare 2 × 27 cm column of Dowex-1-X8, chloride form, 50–100 mesh 8% cross-linked particles (E. Merck, Darmstadt, W. Germany), equilibrated with 0.05*N* NaOH solution.

(b) *Thin layer equipment.*—Add 12.5 g each of silica gel G and kieselguhr (both from E. Merck) to porcelain mortar and mix with pestle. Add 35 ml distilled water and stir slowly 1 min. Dilute further by adding 15 ml distilled water and stir moderately 1 min more. Place suspension into spreader and coat adsorbent (0.25 mm thickness) on 20 × 20 cm glass plates. Activate coated plates 1 hr at 95–100°C and store in desiccator until use.

(c) *Developing solvents.*—(*1*) Benzene-95% ethanol-concentrated ammonium hydroxide (10 + 20 + 5); (*2*) butyl acetate-absolute methanol-concentrated ammonium hydroxide (15 + 20 + 5); and (*3*) butyl acetate-acetic acid-water (30 + 20 + 10).

(d) *Chromogenic sprays.*—(*1*) *Ceric ammonium nitrate.*—10%, in absolute ethanol or methanol. (*2*) *Indole.*—0.25%, in absolute methanol or ethanol.

(e) *Carbon dioxide-forming reagents.*—Prepare fresh nitric acid-water solution (1 + 4, v/v) and dissolve in it enough ceric ammonium nitrate to make 10% (w/v) solution. Store this solution in glass-stoppered bottle.

(f) *Warburg vessel.*—13.8–15.4 ml total volume (B. Braun, Melsungen, W. Germany).

(g) *Standard solutions.*—Prepare separate 0.5, 0.2, 0.1, and 0.05*N* water solutions of reference grade malic acid, citric acid, tartaric acid, and another α-hydroxy acid if necessary. Solutions represent 2.50, 1.00, 0.50, and 0.25 μmoles α-hydroxy acid, respectively.

Procedure

Add 20 ml water to 5–10 g ground solid or gel sample or to 5–10 ml original liquid in 100 ml beaker and stir. Heat over boiling water bath 15 min, while stirring. Let solution reach room temperature and add concentrated HCl, dropwise and with stirring, until pH is less than 3. (The appearance of a precipitate does not interfere.) Add 4 volumes (ca 100 ml) 95% ethanol, with vigorous stirring, stir 10 min, and let stand 30 min. Filter suspension through Whatman No. 1 paper. Wash precipitate 3 times in 50 ml beaker with 10 ml 80% ethanol. Combine filtrates and evaporate in vacuum evaporator at 60°C. Dissolve residue in 15 ml 0.05*N* NaOH and pass solution through anion exchange column. Rinse beaker with 15 ml 0.05*N* NaOH and add to column. Wash column with 200 ml 0.05*N* NaOH, adjusting flow to 25 ml/min for the entire process. Wash column again with 300 ml distilled water, pass through 175 ml 0.1*N* HCl, and follow with 250 ml 0.3*N* HCl. Immediately after adding the 0.3*N* HCl to column, collect first 200 ml eluate, discarding rest.

Dry eluate in vacuum evaporator at 40°C. Dissolve residue in 15 ml distilled water and evaporate again as mentioned above, using small flask. Dissolve residue in 1 ml distilled water. Spot 5, 10, and 20 μl concentrate several times on TLC plates; make parallel spots of 5 μl standard solutions of corresponding acids. Develop duplicate plates in chromatographic tanks with the 3 developing solvents.

Air-dry plates and spray 1 plate of each pair with ceric ammonium nitrate solution. After 10 min, spray with indole solution. (The above acids appear as bright spots on dark brown background.) Scrape adsorbent corresponding to spots detected from second plate of each pair into centrifuge tube. Add 1 ml nitric acid reagent (e), mix 5 min, and centrifuge 5 min at 5000 rpm. Pipet 0.5 ml supernatant into center compartment of Warburg vessel and place 0.5 ml ceric ammonium nitrate reagent in side arm of that vessel. Arrange thermobarometer and add 0.5 ml ceric ammonium nitrate reagent. Equilibrate vessel 10 min at 30°C and mix the 2 solutions in each Warburg vessel. Measure gas volume produced after 20–30 min.

Prepare standard curve for gas volumes evolved from standard solutions.

Results and Discussion

Citric, malic, and tartaric acids give bright spots within the limits of 0.1–1.0 μmole. Table 1 presents the R_f values as well as the linear ranges of carbon dioxide evolved from the acids by the gasometric measurement. The accuracy of the gasometric measurements is ±6%. Different fruit samples were also analyzed for their α-hydroxy acid content; *see* Table 2.

Table 1. R_f values and linear ranges of carbon dioxide evolved from citric, malic, and tartaric acids

Acid	R_f Value[a]			Linear Range, μmoles
	A	B	C	
Citric	0.42	0.15	0.51	0.1–1.2
Malic	0.83	0.22	0.58	0.2–2.0
Tartaric	0.40	0.17	0.44	0.15–2.0

[a] The acids are detected on plates of silica gel G-kieselguhr G (1 + 1) with the following solvents: A: benzene-ethanol-ammonium hydroxide (10 + 20 + 5); B: butyl acetate-methanol-ammonium hydroxide (15 + 20 + 5); C: butyl acetate-acetic acid-water (30 + 20 + 10).

Table 2. Analysis of tartaric, malic, and citric acids in some fruits[a] (μmole acid/g fruit)

Fruit	Citric	Malic	Tartaric
Muscate grapes	0.30	7.6	103
Dabuki grapes	0.05	12	41
Queen of vineyard grapes	0.82	1.0	67
Sultania grapes	1.05	23	63
Marmande tomatoes (green)	38	16	—
Marmande tomatoes (ripe)	11	5.5	0.15

[a] The fruits were all picked September 10–30, in Israel.

The entire method described must be used to prove that other α-hydroxy or α-keto acids (possibly in food) do not appear at the same spots. Acids other than α-hydroxy or α-keto do not interfere with qualitative or quantitative analysis, as they do not react with ceric ammonium nitrate. Although this reagent also reacts with mercaptans, tryptophan, and tyrosin, these are not present in food in such a form and concentration which would interfere in the results. Acid-reducing substances such as sugars and certain aldehydes would not interfere, as they are not adsorbed on the anion exchange column. Proteins and polysaccharides are precipitated by alcohol in the initial stages of the extraction.

The method described above is suitable for routine analyses if many samples are to be analyzed simultaneously.

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OILS, FATS, AND WAXES

Comparison of Separation Procedures for Identification of Oils by Gas Chromatography

By CHARLES W. THORPE (Division of Food Chemistry and Technology, Food and Drug Administration, Washington, D.C. 20204)

Four methods were evaluated for detecting adulteration of butter fat with vegetable fats. The procedures involve GLC determination of free or combined β-sitosterol after isolation of the sterols by either preparative GLC or digitonin precipitation. Each of the methods was sensitive enough to detect about 1 mg added β-sitosterol/100 g butter fat. Also studied was the free and combined β-sitosterol content of a number of crude and refined vegetable oils. The TLC-GLC method for determining cholesterol in fats and oils, which was collaboratively studied in 1968, is being recommended for official first action status. The GLC portion of this procedure replaces the obsolete one in methods 26.065–26.070 for detecting vegetable fats in butter fat.

The adulteration of butter fat with other fats has long been a matter of concern to regulatory officials. Before the advent of gas chromatography a number of methods for detecting butter fat adulteration were proposed, but these methods generally did not detect adulteration below 10% (1, 2).

One general procedure for detecting butter fat adulteration involves analysis of the sterol content of a fat. Cholesterol is the major sterol of animal fats, while vegetable oils contain a group of sterols commonly called phytosterols, the major ones being campesterol, stigmasterol, and β-sitosterol. Boemer and Winter (3, 4) and Windaus (5) showed that the melting point of mixtures of cholesterol and phytosterol acetates could be used to detect small amounts of vegetable oils in animal fats.

Vanden Heuvel and coworkers separated cholesterol from the phytosterols by gas-liquid chromatography (GLC) (6–8). Following this development, a number of methods have been reported, whereby determination of the β-sitosterol content of a fat by GLC has served as an index of contamination by vegetable oils.

Eisner *et al.* (9) separated the sterols, after saponification, by Florisil chromatography of the isolated unsaponifiable matter. Cannon (10) employed digitonin precipitation, after saponification, to isolate the sterols, followed by GLC of the sterol acetates. This method was adopted as official first action by the AOAC (11). Parodi (12) isolated the sterols from unsaponifiable matter by preparative thin layer chromatography (TLC), followed by GLC of the trimethylsilyl ethers. Thorpe *et al.* (13) reported a method for detecting animal fats in vegetable oils, based on GLC of the free sterols isolated by preparative TLC of isolated unsaponifiable matter. This method was subjected to an AOAC collaborative study (14) and its application to detection of vegetable oils in butter fat is under study. Katz and Keeney (15) detected vegetable oil adulteration of butter fat by GLC of the free sterols isolated by passing the sample over a digitonin-impregnated Celite 545 column and eluting the sterols with dimethylsulfoxide. LaCroix (16, 17) evaluated and modified the digitonin column method and conducted a collaborative study of the modified procedure. He concluded that the procedure could detect 5% adulteration of butter fat with vegetable oil, based on detection of a minimum of 4 mg β-sitosterol/100 g butter fat. He commented that the collaborators reported difficulty with quantitation of samples containing less than 4 mg β-sitosterol/100 mg butter fat.

Because neither the modified digitonin column method (17) nor the TLC-GLC procedure (13) has been compared to the official first action AOAC method, **26.065–26.070**, for determining β-sitosterol in butter, replicate test samples were prepared for a comparative study of sterol cleanup procedures for determining recoveries of added β-sitosterol.

Experimental

β-Sitosterol was added to butter fat at 5 levels ranging from 0 to 5.95 mg/100 g. These butter fats were analyzed in duplicate by the following 4 methods, using the same GLC procedure for analysis of all samples.

A. AOAC Method

The official first action method, **26.065–26.070**, was modified as follows: A 2.5 g sample was analyzed, reagent quantities were reduced accordingly, and the digitonin precipitation step was shortened to a 2 hr refrigeration period. Our experience indicated that these changes made the procedure faster and easier without any loss of accuracy. The sterol acetates were not recrystallized before GLC.

B. TLC-GLC Method

The method of Thorpe *et al.* (13) was modified to permit determination of β-sitosterol.

C. Digitonin Column Method

The method for free sterols of Katz and Keeney (15), as modified by LaCroix (17), was used.

D. Digitonin Precipitation Method

The digitonin precipitation procedure for free sterols has not been previously published but we have used it extensively in determining the free sterol content of fats and oils. The method is outlined below:

Five ml 2% digitonin dissolved in ethyl alcohol is added to 2.5 g butter fat in 15 ml glass-stoppered centrifuge tube containing three 4 mm glass beads. The sample is heated 15 min in beaker of water on steam bath (ca 80°C) and then cooled to room temperature. Five ml hexane is added and sample is shaken vigorously 1 min and then centrifuged 3 min at 2000 rpm. The liquid layer is carefully decanted and discarded. The precipitate is then washed with two 10 ml portions of hexane by shaking, centrifuging, and discarding hexane layer each time. One ml pyridine and 2 ml acetic anhydride are added to precipitate and sample is heated 20 min in beaker of water on steam bath (ca 80°C) and then cooled to room temperature.

Five ml water and 5 ml hexane are added to sample and, after shaking, mixture is centrifuged 3 min at 2000 rpm. Solution is transferred to 60 ml separatory funnel and centrifuge tube is rinsed with two 5 ml portions of water and one 5 ml portion of

Table 1. Recoveries by 4 methods of β-sitosterol added to butter fat

β-Sitosterol Added	β-Sitosterol Recovered (mg/100 g Butter Fat)			
	(A) Modified AOAC	(B) TLC-GLC	(C) Digitonin Column	(D) Digitonin Pptn
0	0.03, 0.09	0.04, 0.12	0.41, 0.49	0.46, 0.53
1.19	1.18, 1.13	1.18, 1.20	1.35, 1.49	1.51, 1.61
2.38	2.28, 2.43	2.27, 2.40	2.34, 2.56	2.61, 2.57
3.57	3.39, 3.71	3.41, 3.53	3.46, 3.50	3.75, 3.87
5.95	5.64, 5.92	5.63, 5.93	5.45, 5.84	5.78, 6.20

hexane; each rinse is added to separatory funnel. Aqueous layer is removed from separatory funnel and discarded and hexane layer is washed with 1 additional 10 ml portion of water which is also discarded. Hexane layer is transferred to 3 dram vial through funnel containing small pledget of glass wool. Separatory funnel is washed with 3 ml hexane which is added to vial, and hexane is evaporated to dryness on steam bath under stream of nitrogen.

All test samples prepared by the 4 methods listed were analyzed by the GLC procedure described by Thorpe *et al.* (13). The same GLC column was used for all GLC analyses, which were run consecutively. GLC conditions were as follows: Barber-Colman Model 5000 gas chromatograph with 6′ × 3 mm id glass column containing 3% JXR on 100–120 mesh Gas Chrom Q. Column, 258°C; injector, 265°C; detector, 265°C; nitrogen, 25 psi, 40 ml/min. Retention times (min) were: cholesterol, 17.6; cholesterol acetate, 22.9; campesterol, 22.5; campesterol acetate, 29.2; β-sitosterol, 27.8; β-sitosterol acetate, 36.1. The number of theoretical plates for cholesterol was 2800. The separation factor for cholesterol-campesterol was 3.0. Sensitivity was adjusted so that 0.1 μg β-sitosterol gave a deflection of about 5% FSD.

Discussion

Sample size for methods *A*, *B*, and *D* was 2.5 g, and for method *C*, 900 mg. Cholestane (0.1 mg) was added as an internal standard to each sample before GLC analysis. Final volumes for methods *A*, *B*, and *D* were 200 μl, and for method *C*, 100 μl. Results are presented in Table 1. All values are expressed in mg β-sitosterol/100 g butter fat. Similar values of β-sitosterol (0.03–0.12 mg/100 g) were found in the unadulterated butter fat, using methods *A* and *B* which determine total β-sitosterol, and higher values (0.41–0.53) were found by methods *C* and *D*. The butter fat sterols isolated by methods *C* and *D* were examined on XE-60, QF-1, OV-22, and Hi-Eff 8BP (cyclohexanedimethanol succinate) columns, and the retention times of components were compared to β-sitosterol. The results indicated that very little (<0.1 mg/100 g) or no β-sitosterol was present in the butter fat. The blank results from methods *C* and *D* were apparently due largely to impurities which were carried through to the GLC step.

At levels above 1 mg β-sitosterol/100 g, the results obtained were roughly comparable for all 4 methods. The results suggest that as little as 1–2 mg β-sitosterol/100 g can be detected by any of the methods.

Methods *C* and *D*, which do not have a saponification step and, therefore, determine only free sterols, were faster by at least a factor of 2 than methods *A* and *B*. On an average, an experienced analyst required 3–4 hr to prepare 6 samples for the GLC determination step by methods *C* or *D*, while about 8 hr were required to prepare 6 samples by methods *A* or *B*. Method *D* is somewhat faster and simpler than method *C*, which requires 12 separate column elutions to eliminate unwanted lipids and isolate the free sterols.

Rapid methods *C* and *D* use the free β-sitosterol content of an oil as an index of adulteration. The total sterol content and particularly the free sterol content of an oil are known to be reduced during processing, mainly in the alkali refining, deodorizing, and winterization steps. This indicates that the free β-sitosterol content may not always be a suitable index where adulteration with a refined or partially refined vegetable oil is suspected.

Since very little information is available on the levels of free and bound (esterified) sterols in crude and refined vegetable oils, we have analyzed 4 authentic vegetable oils for β-sitosterol content. Each oil was analyzed as the crude oil as well as at 3 stages of refining. The levels of β-sitosterol found are presented in Table 2. Analysis for total β-sitosterol was made by the TLC-GLC method (13) and for free sterols by the digitonin precipitation procedure outlined earlier. GLC conditions were the same as pre-

Table 2. Free and total β-sitosterol in vegetable oils

Oil	Processing[a]	β-Sitosterol (mg/100 g) Total	Free	Bound (By diff.)
Corn	A	526	235	291
	B	498	199	299
	C	477	178	299
	D	407	137	270
Soybean	A	130	78	52
	B	106	63	43
	D	94	62	32
	D	91	51	40
Cottonseed	A	376	226	150
	B	341	198	143
	C[b]	288	149	139
	D[b]	277	134	143
Peanut	A	146	106	40
	B	121	83	38
	C	103	69	34
	C	96	59	37

[a] A = crude, B = alkali refined, C = alkali refined and bleached, D = alkali refined, bleached, and deodorized.
[b] Also winterized.

viously described. Cholestane (0.4 mg) was added to each sample as an internal standard and the final volume of each sample before GLC was 1.0 ml.

The results in Table 2 tend to confirm earlier findings that refined vegetable oils have a lower β-sitosterol content than crude oils and indicate that free sterols are more readily removed in processing than combined sterols. Butter fat containing 2% of either the fully refined soybean or peanut oil would be expected to give 1–1.2 mg β-sitosterol/100 g of sample when analyzed by methods determining only free sterol content, or 1.8–1.9 mg/100 g β-sitosterol when analyzed by methods determining total sterol content. The 2% adulteration level would be a practical limit for quantitative determination by either of the free sterol determination methods, while the methods for total sterols could be expected to determine adulteration at the 1% level for these oils.

The vegetable oils analyzed (Table 2) were selected at random and may not be typical. Accordingly, we are extending our survey of β-sitosterol content in vegetable oils so that we may establish expected levels of free and combined sterols as a function of the type of oil and stage of processing. With this data we can determine the utility of methods which use only free β-sitosterol content as an index of adulteration.

Based on review and statistical analysis of the collaborative study (14) of the TLC-GLC method for determining cholesterol in vegetable fats and oils, together with a review of collaborators comments, it has been found that this method is reliable and accurate. This method is recommended for official first action status.

It is also recommended that the obsolete GLC method in **26.065–26.069** be replaced with the GLC portion of the TLC-GLC method (13), modified for determination of β-sitosterol. The GLC procedure in the latter method provides for columns and operating conditions which result in greater resolution of sterol components, permits quantitation of sterols, using an internal standard, and replaces the argon ionization detector with the preferred and generally used flame ionization detector.

Recommendations

It is recommended—

(1) That the TLC-GLC method (13) for determining cholesterol in vegetable fats and oils, which was collaboratively studied (14) in 1968, be adopted as official first action.

(2) That the GLC portion of the TLC-GLC method (13), modified for determination of β-sitosterol (see *below*), replace the GLC method in **26.065–26.069.**

(3) That further study be made on the levels of free sterols in vegetable fats and oils as a function of the stage of processing and on methods for determining free sterol content.

METHOD[1]

28.071 ***Preparation of Sample***

Obtain fat from butter by **16.167.** Weigh 5–10 g filtered fat into 150 ml beaker and proceed as in **28.068,** beginning "add 4 g KOH . . ." (Sterol acetate need not be recrystd unless mp is also desired.)

28.072 ***Reagents***

(a) *GLC column packing.—(1) Stationary phase.—* JXR or OV–1 dimethylpolysiloxane or OV–17 methylphenylpolysiloxane. *(2) Support.—*100–120 mesh Gas Chrom Q. Com. prepd packing of 3%

[1] The section numbers within the method are those for the 11th ed. of *Official Methods of Analysis,* 1970: **28.068** (11th ed.) = **26.068** (10th ed.), **16.167** = **15.167.** Cautionary notes, if present, refer to the new chapter on safety, Chapter 46.

The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee C and were adopted by the Association. See *JAOAC* **53,** 387 (1970).

stationary phase available from Applied Science Laboratories, Inc. or Supelco, Inc., Bellefonte, PA 16823.

(b) *Ethyl acetate.*—Distd in glass (Burdick and Jackson Laboratories, Inc., or equiv.).

(c) *Cholestane std soln.*—0.4 μg/μl. Weigh 40.0 mg cholestane std (Applied Science Laboratories, Inc.) into 100 ml vol. flask and dil. to vol. with EtOAc.

(d) *Cholestane internal std soln.*—0.2 μg/μl. Dil. 10.0 ml std soln (c) to 20.0 ml with EtOAc.

(e) *β-Sitosterol acetate std soln.*—2.0 μg/μl. Weigh 22.2 mg β-sitosterol acetate std (Nutritional Biochemicals Corp., ca 90% pure) into 10 ml vol. flask and dil. to vol. with EtOAc. Com. β-sitosterol acetate is mixt. of campesterol acetate (earlier eluting minor component) and β-sitosterol acetate. Det. purity of std by chromatographing 2–3 μl std soln. Det. area of campesterol acetate and β-sitosterol acetate peaks by drawing lines tangent to sides of peak and intersecting baseline. Det. areas of resulting triangles by multiplying ht by ½ base. Concn β-sitosterol acetate = $(C_s \times P_s)/(P_s + P_c)$, where C_s = mg sterol acetate std/ml, P_s = area β-sitosterol acetate peak, and P_c = area campesterol peak.

(f) *Cholestane-β-sitosterol acetate std mixture.*—0.2 μg cholestane and 1.0 μg β-sitosterol acetate/μl. Mix equal vols (c) and (e).

28.073 ***Apparatus***

(a) *Gas chromatograph.*—Barber-Colman Co. Model 5000, or equiv., with H flame ionization detector and 1 mv strip chart recorder. Temps: column, 220–260°; flash heater and detector, 240–270°; flow rates: N (ultra high purity grade), 20–25 psi to elute β-sitosterol acetate in 16–20 min; H, ca 40–45 ml/min; air, 300–340 ml/min.

Adjust electrometer sensitivity so that 2.5 μg β-sitosterol acetate gives ca 50% deflection (10^{-9}–10^{-10} amp full scale deflection with 1 mv recorder). Repeat injections until constant peak hts are obtained on successive injections of identical vols of std mixt.

(b) *Preparation of column.*—(*Caution: See* **46.039** and **46.040.**) Pack glass column, 6′ × 4 mm id, with com. 1–3% stationary phase on 100–120 Gas Chrom Q or dissolve 0.4–1.2 g polysiloxane in 200 ml toluene or CH_2Cl_2-toluene (1 + 1). Heat to dissolve (polysiloxane dissolves slowly in solv. mixt). Add soln to 40 g Gas Chrom Q and let stand 10 min with occasional gentle stirring. Dry in rotary evaporator held in 50° bath or heat on steam bath with occasional gentle stirring and remove residual solv. in vac. oven at 50°.

Carefully wash inside of column and small amt glass wool with 5% soln dimethyldichlorosilane in toluene, rinse with MeOH until rinsings are neut. to indicator paper, and air-dry. Plug column exit with small plug of silanized glass wool and thru-hole septum, and plug injection side arm with ½ hole septum. Add coated packing material thru injection port, using funnel and plastic tubing and tapping column very gently during addn. Add ¼ packing material at time, remove funnel, and apply ca 5–10 psi N to injection port while tapping gently to settle packing. Pack to 2.5 cm below injection side arm and plug with silanized glass wool.

(c) *Conditioning of column.*—Heat ≥8 hr at 260° with ca 5–10 psi N flowing thru column. Shut off pressure, raise temp. to 290°, and continue heating ≥8 hr. Reduce temp. to 260°, adjust N to 5–10 psi, and heat addnl 8–12 hr.

(d) *Performance.*—Chromatograph ca 2 μl β-sitosterol acetate std soln to det. retention times and resolution of column. Min. of 1600 theoretical plates is required for β-sitosterol acetate peak. Theoretical plates = $(C/B)^2 \times 16$, where C = cm β-sitosterol acetate peak from injection point and B = cm triangulated base width of β-sitosterol acetate peak.

28.074 ***Determination***

Pipet 1.0 ml cholestane internal std soln into 3 dram vial contg sterol acetates, rotate vial to wash down sides, and swirl to dissolve. Inject 2–3 μl soln and 2–3 μl std mixt., (f), at least in duplicate. Identify β-sitosterol acetate peak in sample soln from retention time in std mixt. If ht of sample peak is >60% full scale, add addnl 1.0 ml internal std soln to sample soln, and rechromatograph sample and std mixt. solns. Measure peak hts of cholestane and β-sitosterol acetate peaks in mm.

mg β-Sitosterol acetate/100 g sample = $(H_i/H_x) \times (C_x/C_i) \times (S_x/S_i) \times (Q_i/Q) \times 100$, where H_i and H_x = ht (mm) cholestane and β-sitosterol acetate peaks, resp., in std mixt.; S_x and S_i = ht (mm) β-sitosterol acetate and cholestane peaks, resp., in sample; C_x and C_i = μg β-sitosterol acetate and cholestane/μl, resp., in std mixt.; Q_i = μg cholestane std/μl in sample; and Q = mg sample/μl.

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Methodology for Chlorinated Aromatics in Fats, Oils, and Fatty Acids

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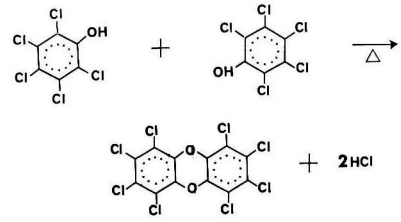
The official first action electron capture GLC (EC-GLC) method for chick edema factors (polychlorodibenzo-*p*-dioxins) has been reviewed. This general procedure, which was collaboratively studied in 1967, has undergone several minor modifications which result in better recoveries of polychlorodibenzo-*p*-dioxins and increased specificity in interpretation of the gas chromatographic results. The EC-GLC method can be used as a screening test or, where a typical pattern of GLC peaks is obtained, as a preliminary test, but confirmatory tests are needed to demonstrate structure and toxicity of polychlorodibenzo-*p*-dioxins. Preliminary work with combined GLC-mass spectrometry indicated that this technique might provide a suitable test, if adequate sample cleanup can be accomplished. A chicken embryo assay has been developed to the point where toxicity can be observed 3–5 days after injection of eggs. A preliminary procedure has been developed for isolation and gas chromatography of chlorophenols in fats and fatty acids. Polychlorophenols have been found to be precursors of chlorodibenzo-*p*-dioxins. A nonspecific microbiological test for chlorophenols, employing *Bacillus megaterium*, was evaluated. Chlorophenols were found to produce uniformly graded growth inhibition of the test organism in the range 1–100 μg.

The widespread use of toxic organochlorine compounds in agriculture and industry requires development of sensitive methods for their detection in a wide variety of commodities. In addition, it is equally important that methods be developed to detect toxic breakdown or conversion products of organochlorine compounds. One of the most urgent needs in the fat and oil industry is for a rapid and specific method for polychlorodibenzo-*p*-dioxins (chick edema factors) in fats, oils, and fatty acids. The official first action microcoulometric and electron capture methods for chick edema factors (CEF) are essentially screening procedures (1–3). Both methods, at present, require a rather time-consuming 3-week chick bioassay (4) for confirmation.

The purpose of this report is to review the current status of chemical and biological methods for chlorophenols and chlorinated dibenzo-*p*-dioxins in fats, oils, and fatty acids.

CEF consist of a mixture of chlorinated dibenzo-*p*-dioxins; these occur occasionally as trace contaminants in fats. Recently, a communication (5) from this laboratory reported the results of a preliminary study which demonstrated the possibility that CEF could arise from residues of pentachlorophenol and 2,3,4,6-tetrachlorophenol in fats and fatty acids. Chlorophenols and

their salts, when heated, undergo condensation reactions and form chlorinated derivatives of dibenzo-*p*-dioxin. The following equation illustrates this condensation reaction:



Technical grade pentachlorophenol contains about 10% 2,3,4,6-tetrachlorophenol which also undergoes thermal condensation reactions and forms hexachloro derivatives of dibenzo-*p*-dioxins. The condensation of 2,3,4,6-tetrachlorophenol with pentachlorophenol forms 2 heptachloro derivatives of dibenzo-*p*-dioxin.

An electron capture GLC method has been developed for pentachlorophenol and 2,3,4,6-tetrachlorophenol in fats, oils, and fatty acids (6). However, recoveries of the 2 volatile polychlorophenols were low and varied over a wide range; nevertheless, the method appears to be satisfactory for qualitative measurements at the 0.5 ppm level. Several samples of oleic acid known to contain CEF were analyzed and were found to be contaminated with residues of pentachlorophenol. The method requires further study.

The official electron capture and microcoulometric methods for CEF were developed before their chemical structures were known. The methods are screening procedures and are based on the observation that toxic fats contain a number of chlorinated components (now known to be polychlorodibenzo-*p*-dioxins) which have greater retention times than chlorinated pesticides. The electron capture method has received wide acceptance. It is approximately 2000 times more sensitive and requires less sample than the microcoulometric method. In addition, electron capture gas chromatographic equipment is simpler and is in general use in many laboratories.

Recently, it has come to our attention that a number of laboratories that routinely use the electron capture method for control work are not aware of improvements (3) that have been made in the original procedure (2). Recent changes which have not been published include a minor modification of the sulfuric acid cleanup step and a slight modification of the procedure for packing the GLC column. The modified method, which includes these changes, replaces all existing GLC methods for the chemical assay of CEF.

METHOD[1]

(*Caution: See* **46.011, 46.015, 46.030, 46.039,** and **46.040.**)

28.109 *Principles*

Fat, oil, fatty acid, or lipid is treated with H_2SO_4 and extd with pet ether. Ext is purified on Al_2O_3 column, further treated with H_2SO_4, and examined by electron capture GLC. Peaks with retention times relative to aldrin (R_a) between 8 and 45 indicate presence of chick edema factors (hexa-, hepta-, and octachlorodibenzo-*p*-dioxins).

28.110 *Reagents and Apparatus*

(Rinse all glassware with appropriate solvs before use. Do not store solvs in polyethylene containers.)

(a) *Petroleum ether.*—Distd in glass, bp 30–60° (Burdick and Jackson Laboratories, Inc., or equiv.)

(b) *Ethyl ether for alumina chromatography.*—Ether (≤2% alcohol) (Burdick and Jackson Laboratories, Inc., or equiv.), or absolute ether (≤0.01% alcohol).

(c) *Carbon tetrachloride.*—Distd in glass (available from Burdick and Jackson Laboratories, Inc.).

(d) *Isooctane.*—Distd in glass (Burdick and Jackson Laboratories, Inc., or equiv.)

(e) *Aldrin std soln.*—0.05 μg/ml isooctane.

(f) *Chick edema factor low positive reference sample.*—1.5% ref. toxic fat (available from Division of Pesticides, Food and Drug Administration, Washington, DC 20204) in USP cottonseed or other vegetable oil. (*Caution:* Do not contact toxic fat.)

(g) *Activated alumina.*—Fisher Scientific Co. No. A-540; do *not* substitute. Activate 100 g portions by heating 4 hr at 260°. Transfer without cooling to dry container and close tightly. Check activity of Al_2O_3 by analysis of low pos. ref. sample, (f), examining Al_2O_3 fractions 2 and 3. With sufficiently activated Al_2O_3, chick edema factor elutes predominantly or entirely in fraction 3 as indicated by gas chromatograms. (Chromatograms should show series of peaks with R_a between ca 8 and 45.)

(h) *Alumina chromatographic column.*—To dry tube, 17 mm od × 250 mm long, fitted at bottom with coarse porosity fritted glass disk and Teflon stopcock (tube without disk but with glass wool

[1] The section numbers within the method are those for the 11th ed. of *Official Methods of Analysis*, 1970. Cautionary notes, if present, refer to the new chapter on safety, Chapter 46.

plug at bottom may also be used), add redistd pet ether, dried before use with anhyd. Na_2SO_4, until tube is ⅔ full. Transfer 15 g Al_2O_3 to tube in small portions, tapping to settle. After last portion has settled and air bubbles stop rising to surface of solv., add 5 g anhyd. Na_2SO_4. Drain excess pet ether until it is just above surface of Na_2SO_4.

(i) *Gas chromatographic column.*—Glass, 7–9′ × ¼″, packed with 2.5% SE-52 silicone gum rubber on 60–80 mesh Gas Chrom Q (Applied Science Laboratories). Coat support with substrate as follows: Dissolve 2.5 g silicone gum rubber in 300 ml CH_2Cl_2-toluene (1 + 1) with heat. Add 97.5 g Gas Chrom Q and let stand 10 min with occasional gentle stirring. Dry in rotary evaporator held in 50° bath. Apply vac. to chromatgc column and add small amts of coated support while tapping column at packing level after each addn. Fill to within 1″ on exit side and 3″ on entrance side and fill remaining space with silanized glass wool. Condition column at operating pressure 2–5 days at 250°.

(j) *Gas chromatograph.*—With tritium source concentric-type electron capture detector. Operate instrument in accordance with instructions of manufacturer. Obtain stable baseline before use. Choose operating voltage that will cause between 0.6 and full scale deflection for 0.1 ng aldrin (2 μl std aldrin soln) at sensitivity setting of 1×10^{-9} amp full scale. Keep column temp. at 200±1° and adjust N flow so that aldrin elutes in 1–1.5 min (0.25–0.33″/min chart speed). Inject 2 μl aldrin std soln before each ref. or test sample.

28.111 ***Determination***

(*Caution: See* **46.011**, **46.039**, and **46.073**.)

(a) *Analysis of reference toxic fat.*—Dissolve 2.5 g ref. 1.5% toxic fat in 10 ml CCl_4 in 500 ml g-s erlenmeyer and proceed as in (b), (c), and (d). Dissolve residue in 250 μl isooctane and inject 5 μl (equiv. to 50 mg original sample) into gas chromatograph. Resulting chromatogram should exhibit series of peaks with R_a ca 8–45. Peaks at 8–13 are due to hexachlorodibenzo-*p*-dioxin isomers; 2 peaks at 17–22 to the 2 hepta-isomers; and peak at 35–45 to octa-isomer.

(b) *Preliminary sulfuric acid cleanup.*—Dissolve 2.5 g sample in 10 ml CCl_4 in 500 ml g-s erlenmeyer. Add 10 ml H_2SO_4, stopper, and shake 30 sec. Add 125 ml pet ether, stopper, and shake vigorously ca 1 min. Let sep. and decant upper layer into 500 ml erlenmeyer, avoiding transfer of lower layer. Repeat extn with addnl 125 ml portion pet ether. Evap. combined pet ether exts to 5 ml.

(c) *Alumina chromatography.*—Before use, dry all solvs by shaking with anhyd. Na_2SO_4. Transfer evapd pet ether ext to Al_2O_3 column, (h), using total of 10 ml pet ether. Let drain to just above level of Na_2SO_4. Keeping liq. level above Na_2SO_4 at all times, elute with 100 ml pet ether (fraction 1), 50 ml 5% Et ether in pet ether (fraction 2), and 100 ml 25% Et ether in pet ether (fraction 3). (Flow rates of 8–9 ml/min are satisfactory.) Discard fractions 1 and 2 and collect fraction 3 in 125 ml erlenmeyer. Add several boiling chips and evap. to ca 2 ml on steam bath. Transfer residue to 10 ml g-s graduated cylinder and further evap. to 3 ml.

(d) *Additional sulfuric acid cleanup.*—Add 2 ml H_2SO_4 to pet ether soln, stopper, and shake vigorously 30 sec. Let sep. and decant upper layer into 10 ml beaker, avoiding transfer of any H_2SO_4. Add 2 ml pet ether to cylinder, swirl vigorously, let sep., and decant upper layer into beaker. Add 0.5 g solid $NaHCO_3$ to beaker and stir ca 0.5 min. Let stand 5 min and decant pet ether layer into 2 or 4 dram vial. Wash $NaHCO_3$ with 2 ml pet ether and decant washings into vial. Evap. solv. just to dryness at room temp. in vial under N.

(e) *Gas chromatography.*—Dissolve residue in 250 μl isooctane, stopper vial, and rotate to wet sides with solv. Inject 1 μl soln (equiv. to 10 mg sample) into gas chromatograph, (j). Peaks with R_a of 8–45 indicate presence of chick edema factor. Compare R_a values with those from ref. toxic fat, (f). If peaks indicative of chick edema factor are not observed, inject 5 μl soln (equiv. to 50 mg sample). (Types of samples found by experience to be free of components characteristic of toxic fats may be examined by initial injection of 5 μl.)

Perform reagent blank detn with each set of samples. Smooth baseline should be obtained in region R_a 8–45.

Discussion

Analysis of the low positive reference sample serves as an overall check on instrument performance and sample cleanup. The chromatogram from the low positive reference fat should show a distinct peak pattern as illustrated in Fig. 1. The lower chromatogram (B) represents an injection equivalent to 50 mg of the original low positive reference fat. Chromatogram A represents a mixture of synthetic polychlorodibenzo-*p*-dioxins prepared by pyrolysis of 2,3,4,6-tetrachlorophenol and pentachlorophenol. As stated previously, peaks 1 through 4 are due to 4 positional isomers of hexachlorodibenzo-*p*-dioxins. The isomer associated with the small shoulder (peak 3) is probably caused by the presence of a tetrachlorophenol other than the 2,3,4,6-isomer in the starting material. Peaks 5 and 6 are due to 2 positional isomers of heptachlorodibenzo-*p*-dioxin. Peak 7 is due to octachlorodibenzo-*p*-dioxin.

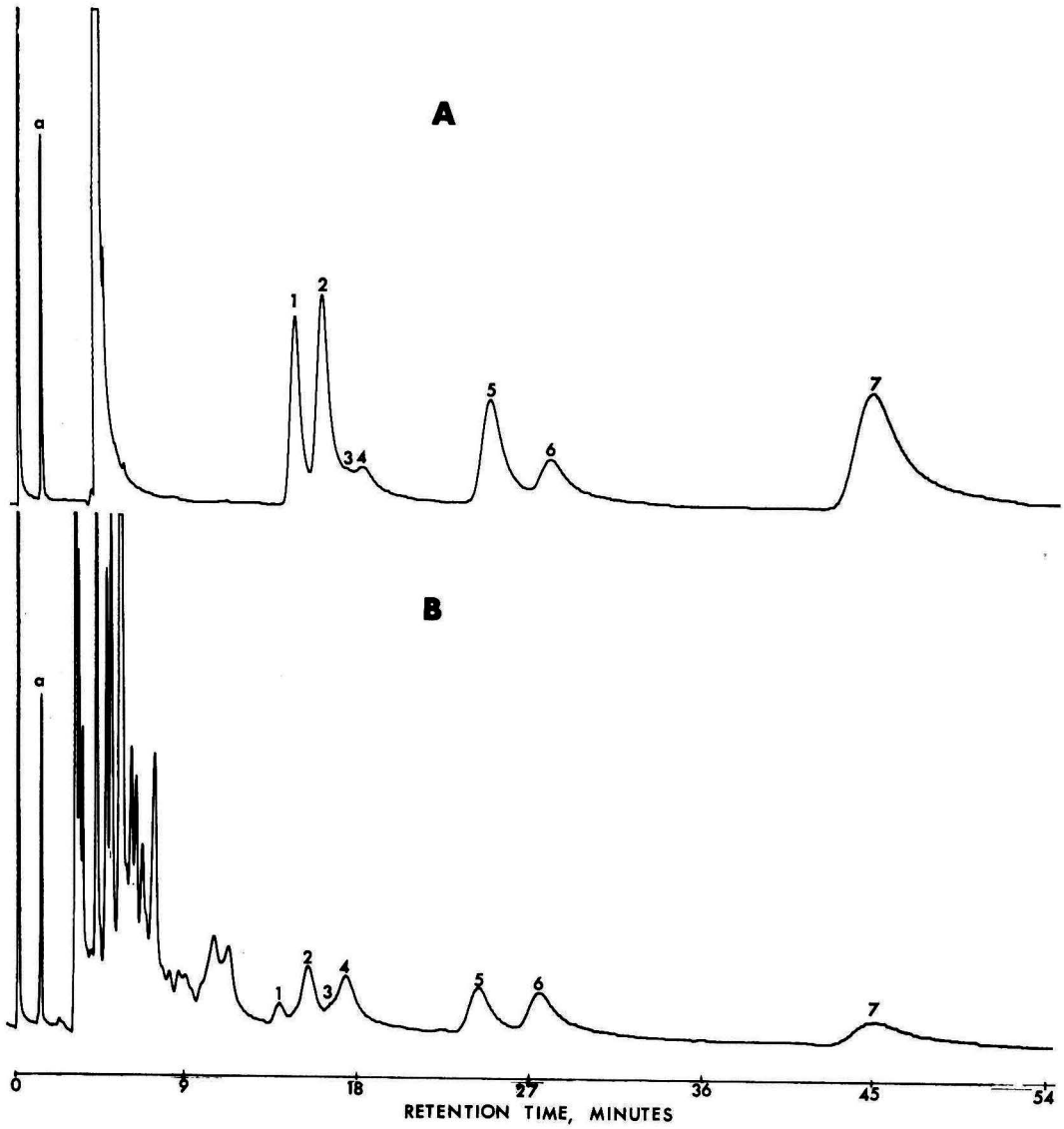


FIG. 1—Chromatograms of typical chick edema factors EC-GLC peak patterns: A, 5 ng of 1:1 mixture of 2,3,4,6-tetrachlorophenol pyrolysate and pentachlorophenol pyrolysate; B, 1.5% low positive reference, about 50 mg starting material.

Aldrin is used to calibrate the instrument sensitivity for chick edema factor analyses. The similarity of detector response vs. applied voltage for aldrin and an extract from the 1.5% reference toxic fat (low positive reference sample) is illustrated in Fig. 2.

Chemical and Biological Confirmatory Methods

The need for rapid chemical and biological confirmatory tests has led to investigation of mass spectrometry as well as 2 biological toxicity assays. Preliminary work has suggested that rapid EC-GLC screening for chick edema factors can be carried out initially; if the presence of chick edema factors is indicated, then larger portions of sample would be fractionated and cleaned up for chemical and biological confirmation.

Mass Spectrometry

Results of a preliminary investigation of combined GLC-mass spectrometry (GLC-MS) indi-

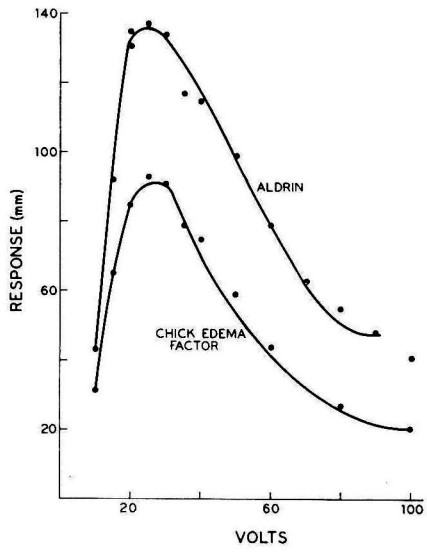


FIG. 2—Response for changes in voltage at a sensitivity of 1 × 10^{-9} AFS.

cated that this technique might be suitable if adequate sample cleanup can be accomplished. A 7′ coiled glass column packed with 2.5% SE–52 on 60–80 mesh Gas Chrom Q was used with an Atlas CH–4 mass spectrometer and single-stage Llewellyn (silicone membrane) separator to examine standards and an extract from a 2.5 g sample (positive for chick edema factor by EC-GLC). The GLC oven temperature was 220°C with a helium flow at 75 ml/min. Injection temperature was 235°C and silicone membrane temperature was about 150°C.

GLC retention times as well as molecular weight and number of chlorine atoms in the molecule were determined for a standard mixture and an extract from the 2.5 g test sample. One-fifth of the sample extract from the test sample was injected; it was estimated that 2 μl test sample contained 0.4 μg hexa-, hepta-, and octachlorodibenzo-*p*-dioxins in addition to other unidentified constituents. A summary of results is in Table 1. Comparison of fragmentation pattern and relative abundance of the ions from standards and sample might afford additional specificity; impurities in the test sample prevented such evaluation at this time.

Chicken Embryo Assay

Extracts from a reference toxic fat and several test samples were subjected to the chicken embryo assay (7); 111 g samples of fat were fractionated according to **26.093–26.094** and alumina fraction 3, and cleaned up with sulfuric acid (2). Small portions of each extract were retained for EC-GLC analysis and the remainder, in chloroform solution, was subjected to the chicken embryo assay (10–15 eggs per sample were tested by injection of portions of the sample extract in the air cell) at 3 levels equivalent to ca 40, 30, and 10 g starting sample. Assay results are shown in Table 2. These results indicate that the chicken embryo test can provide a sensitive indication of toxicity as well as a measure of specificity due to observations of localized and generalized edema. In many instances evidence of toxicity can be observed (by periodic candling) in 3–5 days.

Bacillus megaterium Toxicity Test

The use of a nonspecific biological test employing *Bacillus megaterium* was evaluated (8). This test involves observation of inhibition of a seeded Petri dish holding a *B. megaterium* spore suspension in agar medium. Filter paper disks of sample extracts are placed on the surface of the agar plates and inhibition zones are observed after 18 hr incubation at 37°C.

Five samples (111 g each) were fractionated according to the general procedure **26.093–26.094**. These test samples consisted of 2 highly toxic fats,

Table 1. Mass spectrometric analysis of isolated components

Sample	GLC Peak[a]	Identity	Molecular Weight Found	No. Chlorine Atoms Indicated
Standard mixture	1	Hexachlorodibenzo-*p*-dioxin	388	6
	2	Hexachlorodibenzo-*p*-dioxin	388	6
	3	Heptachlorodibenzo-*p*-dioxin	422	7
Test sample	1	Hexachlorodibenzo-*p*-dioxin	388	6
	3	Heptachlorodibenzo-*p*-dioxin	422	7

[a] Peak numbers refer to Fig. 1.

Table 2. Chicken embryo assay of extracts

Sample	EC-GLC Analysis for Chick Edema Factor	Estimated Level of Chlorodibenzo-*p*-dioxins in Fat, ppm[a]	% Mortality, Chicken Embryo Assay	Assay Observations
Reference toxic fat	positive	2.4	100	edema observed
Test fat No. 1	positive	0.1	73	edema observed
Test fat No. 2	positive	0.7	100	edema observed
Test fat No. 3	positive	0.6	93	edema observed
Reagent blank	negative	—	30	—
Chloroform solvent	—	—	20	—
Control eggs	—	—	7	—

[a] Hexa-, hepta-, and octachlorodibenzo-*p*-dioxins.

Table 3. Results from *B. megaterium* test

Sample[a]	Observation
Nontoxic USP cottonseed oil Fr. 4 (2.5 g extract)	Barely visible around disk
TEF-F797, Fr. 2 (2.5 g extract)	Barely visible around disk
100 μg synthetic (CDPD)[a] reference std	Barely visible around disk
1 μg 2,3,4,6-TCP	Barely visible around disk
10 μg 2,3,4,6-TCP	16 mm inhibition zone
100 μg 2,3,4,6-TCP	36 mm inhibition zone

[a] CDPD = chlorodibenzo-*p*-dioxins; TCP = tetrachlorophenol.

1 low toxic reference material (1.5% TEF in USP cottonseed oil), 1 nontoxic oil, and a reagent blank. In addition to the 3 alumina fractions obtained with petroleum ether, 5% ethyl ether in petroleum ether, and 25% ethyl ether in petroleum ether, a fourth fraction eluted with 400 ml 100% ethyl ether was obtained. The 4 alumina fractions were cleaned up twice with sulfuric acid (2). The residues of fractions 2, 3, and 4 were spotted on filter disks (7.5 mm diameter) at 2 levels equivalent to 2.5 and 54 g starting sample. EC-GLC analysis of alumina extracts indicated that chick edema factors (polychlorodibenzo-*p*-dioxins) are predominantly concentrated in alumina fraction 3. In addition, solvent blanks, a synthetic reference standard consisting of hexa-, hepta-, and octachlorodibenzo-*p*-dioxin, and a sample of technical grade 2,3,4,6-tetrachlorophenol were spotted at the following concentrations: 0.01, 0.1, 0.5, 1.0, 10.0, and 100 μg.

After incubation, inhibition was observed in 6 cases as shown in Table 3. The sensitivity of the *B. megaterium* test for CEF appears to be limited to 100 μg and even then only a very small zone of inhibition was noticed. It appears, however, that a rapid confirmation test for chlorophenols can be developed for amounts of about 1 μg or more. These are the low levels at which aflatoxin B_1 shows inhibition of *B. megaterium*.

Recommendation

It is recommended that the modified electron capture GLC method for chemical assay of CEF reported here be adopted as official first action to replace all existing GLC methods.

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The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee C and was adopted by the Association. See *JAOAC* **53**, 387 (1970).

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DRUGS IN FEEDS

Collaborative Study of Methods for Determination of Ronnel in Feeds and Mineral Mixtures

By ALBERT J. GEHRT (Moorman Mfg. Co., 1000 N. 30th St., Quincy, Ill. 62301)

A GLC method (electron capture detector) for ronnel in cattle feeds at the 0.040% level and a UV method for ronnel in mineral mixtures (1–40% ronnel) were studied by 9 collaborators. Ronnel is extracted from feed by shaking with acetone, followed by direct GLC measurement. The method is simple and rapid and the electron capture detector is much more sensitive and gives cleaner ronnel measurements than the flame ionization detector used last year. Agreement between laboratories was good; coefficients of variation were 7.7, 7.1, 11.1% with ronnel recoveries of 95–100%. Analyses of feed samples after storage (several months) showed decreases in ronnel, found to be due to partial ronnel binding. Ronnel is extracted from minerals with methanol, followed by UV measurement. The coefficient of variation was 5.4%. The Associate Referee recommends that the mineral method be adopted as official final action and that the feed method be continued as official first action for 1 more year. An investigation of the binding effect is continuing.

Analytical methods for ronnel have been studied collaboratively since 1961. A method for ronnel in mineral feed mixtures at concentrations of 1–40% was adopted as official final action in 1962, *JAOAC* **46**, 459–462 (1963). This method involved methanol extraction of ronnel from the sample followed by UV measurement of the methanol extract. The method was improved in 1965 by addition of an ion exchange resin step for removing any 2,4,5-trichlorophenol formed by ronnel decomposition during storage. The improved method was adopted as official first action in 1965, *JAOAC* **49**, 318–325 (1966). This method is not intended for use in the presence of organic feed ingredients nor at the lower levels of ronnel normally present in complete feeds.

Methods for determining ronnel in organic feed mixtures have been extensively studied, as described in the 1968 Associate Referee's report, *JAOAC* **52**, 435–438 (1969). These studies involved cleanup by steam distillation of the 2,4,5-trichlorophenol moiety of ronnel followed by measurement of its UV absorbance, cleanup on a Florisil column followed by UV measurement, and gas-liquid chromatography (GLC). The UV methods were discarded because the steam distillation method was laborious and gave somewhat low ronnel recoveries in complete feeds and the Florisil cleanup did not separate the ronnel from other UV-absorbing compounds well enough to permit accurate measurement.

In 1966 a GLC method involving Florisil cleanup, followed by measurement of ronnel by GLC (electron capture detector), was studied by collaborators with satisfactory results. However, because it was still felt that the Florisil cleanup for the UV method could be made to work, recommendation of official action for the GLC method was deferred. Subsequent to the 1966

Ronnel (O,O-dimethyl-O-(2,4,5 trichlorophenyl) phosphorothioate) is an organic phosphate used in control of cattle grubs and external parasites such as flies, lice, and ticks. It is the trademark of the Dow Chemical Company abroad.

study it was found that a GLC method that measures ronnel without cleanup, using the flame ionization detector, had been developed in the laboratories of the Dow Chemical Co. This method was studied by collaborators in 1968 and was adopted as official first action at the 1968 meeting.

During the past year the GLC method was studied further in the laboratory of the Associate Referee. It was found that an unknown ingredient in the hay used in the cattle feed samples interfered with the ronnel separation when the flame ionization detector was used. However, the electron capture detector showed no such interference. Furthermore, the electron capture detector is much more sensitive than the flame ionization detector. For these reasons the electron capture detector was used in this year's study.

1969 Collaborative Study

As previously described, both the UV method for ronnel in mineral feed mixtures and the GLC method for ronnel in organic feeds are currently in official first action status. Therefore, this year's study included both methods. Nine collaborators representing 3 Food and Drug Administration laboratories, 3 State feed control laboratories, 2 industrial laboratories, and 1 commercial testing laboratory participated in the 1969 study. Three samples of complete cattle feed containing 0.040% ronnel and 1 sample of a mineral mixture containing 5% ronnel and 1% 2,4,5-trichlorophenol were sent to the collaborators. The cattle feeds contained a wide variety of ingredients including ground grains, ground hay, vegetable proteins, urea, molasses, vitamins, and minerals. The mineral mixture contained both major and trace minerals. Collaborators were requested to analyze the cattle feed samples by the GLC method described and the mineral mixture by both the UV and GLC methods. Single determinations were to be made on 2 different days if possible.

Gas Chromatographic Method[1]

(For feed samples contg <1% ronnel)

38.126 ***Principle***

Ronnel is extd from feed with acetone and dild with hexane for electron capture GLC detection.

38.127 ***Apparatus***

Gas chromatograph.—With electron capture detector. Operating parameters: Column 4–6′ glass, 3–4 mm id packed with 5% (w/w) SF-96 silicone fluid on 60–80 mesh Chromosorb W; temps: column 195°, inlet 220°; carrier gas Ar-CH_4 (95 + 5), 60 ml/min; and attenuation to give ca 50% scale deflection with 1 ng ronnel. (Retention time for ronnel should be 5–10 min. Adjust column temp. if necessary.) Significant variations in replicate injections indicate instability of instrument or faulty injection and these should be rechecked.

38.128 ***Reagents***

(a) *Hexane.*—Spectral grade.

(b) *Ronnel std solns.*—(*1*) *Stock soln.*—1 mg/ml. Dissolve 0.1000 g ronnel std sample (available from Dow Chemical Co., Sample Coordinator, Agricultural Products Dept.) in hexane and dil. to 100 ml. (*2*) *Intermediate soln.*—20 μg/ml. Dil. 2.0 ml stock soln to 100 ml with hexane. (*3*) *Working soln.*—1 ng/5 μl. Dil. 1.0 ml intermediate soln to 100 ml with hexane.

38.129 ***Determination***

Weigh 10.0 g sample into 8 oz screw-cap extn bottle, or equiv. Add 200.0 ml acetone and shake 4 hr. Remove from shaker and centrf. portion 10 min. Pipet 1.0 ml into 100 ml vol. flask and dil. to vol. with hexane.

Inject 5 μl portions in following sequence: ronnel working std soln, dild sample ext, duplicate of dild sample ext, and ronnel working std soln. Record chromatogram of each.

Measure ronnel peak hts and calc. % ronnel as follows, using av. peak hts (PH) from std replicates and ext replicates for each detn:

$$\% \text{ Ronnel} = (PH_{sample}/PH_{std}) \times 0.04$$

Sample wt and aliquot specified are for feeds contg 0.04% ronnel. With feeds contg other levels of ronnel use 10.0 g sample but select aliquot or aliquots to give expected ronnel concn of 1 ng/5 μl final diln. Calc. % ronnel = $(PH_{sample}/PH_{std}) \times$ g ronnel in std injected × F × 100, where F = 1/[(g sample/ml in final diln) × (μl sample injected × 10^{-3})].

Ultraviolet Method[1]

(For mineral feed mixts contg 1–40% ronnel)

38.130 ***Principle***

Ronnel is extd from feed with MeOH, cleaned up with ion exchange resin, and detd by UV measurement.

[1] The section numbers with the method are those for the 11th ed. of *Official Methods of Analysis*, 1970. Cautionary notes, if present, refer to the new chapter on safety, Chapter 46.

This report of the Associate Referee was presented at the 83rd Annual Meeting of the AOAC, Oct. 13–16, 1969, at Washington, D.C.

38.131 ***Reagents***

(a) *Methanol.*—Absolute. Coefficient C as detd in calibration with MeOH should be 0.0322–0.0330. If outside this range, adjust to this value by adding 5–15 drops HCl/L MeOH. Det. C on same day samples are analyzed.

(b) *Ronnel std solns.*—*See* **38.128**(b).

(c) *Ion exchange resin.*—Dowex 2-X8, 50–100 mesh. Obtainable in chloride form. Convert to acetate form as follows: Plug 10 × 300 mm chromatgc tube, equipped with Teflon stopcock, with glass wool. Fill with aq. slurry of resin to ht of ca 120 mm. Wash resin with ca 500 ml 10% aq. NaOAc soln until effluent gives only faint test for Cl with $AgNO_3$ after acidification with HNO_3. (Use ca 4 lb/sq in. N pressure to speed washing.) Keep resin covered with liq. during prepn, storage, and analysis. Extrude or wash resin into 100 ml beaker; wash 3 times with H_2O by decantation, then 3 times with MeOH. Pour MeOH slurry back into tube and wash with 50 ml HOAc-MeOH (1 + 4), followed by 50 ml MeOH. Keep resin column (now ready for use) covered with MeOH.

Resin can be used repeatedly unless samples contain considerable salt. Check activity periodically by passing 10 ml of soln of 50 mg trichlorophenol in 100 ml MeOH thru column, washing with three 10 ml portions MeOH, and dilg eluate to 100 ml with MeOH. If soln has noticeable peaks at 292 and 298 nm, discard resin. To prep. larger amts resin, use larger column. Store resin in closed container under MeOH. It is stable at least 1 month.

38.132 ***Calibration***

Weigh 0.1000 g ronnel std into 100 ml vol. flask, dissolve in MeOH, dil. to vol., and mix. Dil. 10.0 ml to 100 ml with MeOH. Det A against MeOH in 1 cm Si cell at 302, 282, and 262 nm.

Calc. coefficient C (g ronnel/100 ml final soln/A unit) = 0.01/net A, where net A is $A_{282} - [(A_{302} + A_{262})/2]$. Coefficient C should be ca 0.0326.

38.133 ***Determination***

Weigh 10.0 g sample into 8 oz extn bottle, or equiv., add 100.0 ml MeOH, stopper, and shake vigorously 15 min on mech. shaker. Decant thru folded 18.5 cm Whatman No. 2V paper, or equiv., into 500 ml vol. flask. Repeat extn twice, shaking 3 min each time with 100 ml MeOH. Wash residue from bottle onto paper and wash thoroly with three 50 ml portions MeOH. Dil. to vol. with MeOH and mix. Pipet 20 ml aliquot onto ion exchange column, (c), and collect eluate in 250 ml vol. flask. Wash column with three 10 ml portions MeOH (do not let MeOH level drop below resin surface). Dil. to 250 ml with MeOH and mix. (If sample is reasonably fresh or if presence of 2,4,5-trichlorophenol is not suspected, ion exchange resin cleanup step may be omitted.) Det. net A against MeOH as in **38.132**.

$$\% \text{ Ronnel} = (\text{net } A \times C \times 100)/\text{g sample in 100 ml final soln}$$

Sample wt and aliquot specified are for mineral mixts contg 5% ronnel. With mineral mixts contg other levels of ronnel use 10.0 g sample but select aliquot to give expected ronnel concn of 0.01 g ronnel/100 ml of final diln. Calc. % ronnel from above formula.

38.134 ***Gas Chromatography***

If GLC measurement of ronnel is desired, dil. 1.0 ml undild MeOH sample ext to 100 ml with hexane. (Dil. aliquot of MeOH ext with hexane same day to avoid hydrolysis of ronnel in MeOH.) Dil. 1.0 ml of this dild ext to 50 ml with hexane. Measure response from 5 µl injections of ronnel working std soln and final diln of ext as in **38.129**. Calc. % ronnel = $(PH_{sample}/PH_{std}) \times 5$.

Sample wt and aliquots specified are for mineral mixts contg 5% ronnel. With mineral mixts contg other levels of ronnel use 10.0 g sample but select aliquot to give expected ronnel concn of 1 ng/5 µl final diln. Calc. % ronnel by general formula shown in **38.129**.

Results and Discussion

Results from each participating laboratory together with standard deviations, coefficients of variation, and recoveries are given in Table 1. Changes in the chromatographic equipment and/or operating conditions as reported by the individual collaborators are also shown.

With few exceptions the agreement between replicates in a single laboratory and between laboratories as demonstrated by the standard deviations was very satisfactory. The clean separations of the ronnel plus the much higher sensitivity with the electron capture detector are definitely advantageous. The collaborators appeared satisfied with the method. Collaborators 4 and 8 suggested use of 250 or 300 ml glass-stoppered Erlenmeyer flasks for extraction instead of the 8 oz screw-cap extraction bottles.

Most of the collaborators analyzed the samples within 1 month after they were prepared. Additional analyses were made in the laboratory of the Associate Referee on the feed mixtures after 2 and 3 months' storage (in sealed cans) at room temperature. These results are shown in Table 2 and indicate a slow but steady decrease in apparent ronnel content. This tendency for feed samples to show lower ronnel recovery after storage

Table 1. Collaborative results for per cent ronnel found in mixtures

Coll.	Instruments, Parameters	Sample 1 (0.040%)		Sample 2 (0.040%)		Sample 3 (0.040%)		Sample 4 (5.0%) GLC		Sample 4 (5.0%) UV	
		Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
1	F&M Model 810	0.0383 (96)[a]	0.0365 (91)	0.0365 (91)	0.0367 (92)	0.0404 (101)	0.0379 (95)	4.91 (98)	4.80 (96)	4.86 (97)	4.74 (95)
2	Barber-Colman 6000, N_2 45 ml/min	0.0389 (97)	0.0404 (101)	0.0395 (99)	0.0423 (106)	0.0403 (101)	0.0416 (104)	4.89 (98)	4.76 (95)	4.93 (99)	4.95 (99)
3	Jarrell Ash 28–730	0.037 (93)		0.041 (103)		0.037 (93)		4.93 (99)		5.12 (102)	
4	Barber-Colman 10, N_2 carrier gas, 165°C column	0.032 (80)	0.035 (88)	0.039 (98)	0.039 (98)	0.033 (83)	0.033 (83)	5.06 (101)	3.95 (79)	5.52 (110)	4.65 (93)
5	Aerograph Hy-Fi	0.036 (90)	0.038 (95)	0.040 (100)		0.029 (73)	0.033 (83)	4.43 (89)		4.70 (94)	4.78 (96)
6	MicroTek MT 220	0.0417 (104)	0.0433 (108)	0.0434 (109)	0.0405 (101)	0.0383 (96)	0.0369 (92)	4.73 (95)	4.50 (90)	4.33 (87)	4.25 (86)
7	Barber-Colman 5000	0.0422 (106)	0.0434 (109)	0.0484 (121)	0.0414 (104)	0.0478 (120)	0.0422 (106)	4.63 (93)	4.63 (93)	4.66 (93)	4.75 (95)
8	Barber-Colman 5000, N_2 carrier gas	0.037 (93)	0.038 (95)	0.037 (93)	0.037 (93)	0.039 (98)	0.037 (93)	4.31 (86)	4.75 (95)	4.52 (90)	4.97 (99)
9	Barber-Colman 5000, N_2 carrier gas	0.0395 (99)	0.0410 (103)	0.0354 (89)	0.0376 (94)	0.0414 (104)	0.0404 (101)	4.66 (93)			
	Av., %	0.0381	0.0395	0.0400	0.0392	0.0385	0.0378	4.73	4.57	4.83	4.73
	Std dev.[b]	0.00298		0.00281		0.00423		0.172		0.262	
	Coeff. of var., %	7.7		7.1		11.1		3.7		5.4	
	Av. rec., %	96.5		99.5		95.3		93.4		96.2	

[a] Values in parentheses indicate per cent recovery.
[b] Calculated from average of each collaborator's results.

has been noted in previous studies and must be due to either decomposition or binding of the ronnel. More rigorous extraction methods with various solvents have been investigated in the Dow laboratories and by the Associate Referee but these have not yielded higher results. A test to determine whether part of the ronnel was bound was made in the laboratory of the Associate Referee. Sample 2 was extracted with acetone as specified in the collaborative method. The feed residue was then hydrolyzed by boiling with NaOH and the hydrolysate was acidified and extracted with hexane. The hexane extract was analyzed for 2,4,5-trichlorophenol by GLC (glass column packed with 3.8% SE-30 on Chromosorb W). A sufficient quantity of the trichlorophenol was found to substantially account for the missing ronnel in the sample, indicating that some of the ronnel or its trichlorophenol moiety is bound.

Experiments are presently in process in the Dow laboratories to determine the specific nature of the apparent binding of the ronnel. Also, in the laboratory of the Associate Referee fly-killing tests are in process to determine whether the "bound" ronnel remaining in the feed has any parasite-killing power.

Conclusions and Recommendations

The method for ronnel in mineral feed mixtures has been used successfully since 1961 and has consistently yielded accurate results with both fresh and stored samples.

As demonstrated by the collaborators' results shown in this report, the method for ronnel in organic feed mixtures gives excellent ronnel recoveries with reasonably fresh samples. Therefore, it should be completely satisfactory in quality control. Furthermore, because of possible

Table 2. Per cent ronnel found after storage

Time Stored	Sample 1	Sample 2	Sample 3
Initial analysis	0.0383 (96)[a]	0.0365 (91)	0.0404 (101)
	0.0365 (91)	0.0367 (92)	0.0379 (95)
2 months	0.0335 (84)	0.0313 (78)	0.0367 (92)
3 months	0.0313 (78)	0.0278 (70)	0.0326 (82)

[a] Values in parentheses indicate per cent recovery of original ronnel.

ronnel instability, vendors of ronnel concentrates recommend that the organic feed mixtures not be stored longer than 14 days.

Since the chromatographic column and the detector were changed in this year's study, the method should not be advanced to official final action at this time. Continuation as first action will permit confirmation of the method and resolution of the "binding" problem.

It is recommended—

(1) That the method for ronnel in mineral feed mixtures described in this report be adopted as official final action.

(2) That the method for ronnel in organic feed mixtures adopted as official first action in 1968 be modified as described in this report and held in first action status for another year.

The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee B and were adopted by the Association. See *JAOAC* **53**, 383 (1970).

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Collaborative Study of the Colorimetric Determination of Sulfadimethoxine in Feeds

By M. OSADCA and E. DE RITTER (Food and Agricultural Products Development Department Hoffmann-La Roche, Inc., Nutley, N.J. 07110)

A method is described for determination of sulfadimethoxine in medicated poultry feeds. After extraction and purification of the extract, sulfadimethoxine is determined colorimetrically after diazotization and coupling with N-(1-naphthyl)ethylenediamine. The method was studied collaboratively by 11 laboratories on 4 feed samples containing graded drug levels. The method yields good recovery and precision and is recommended for adoption as official first action.

Sulfadimethoxine (N^1-(2,6-dimethoxy-4-pyrimidinyl)sulfanilamide) is effective in poultry feeds as an anticoccidial as well as an antibacterial agent. A combination of 5 parts sulfadimethoxine plus 3 parts ormetoprim(2,4-diamino-5(4′,5′-dimethoxy-2′-methylbenzyl)pyrimidine) (Rofenaid®) is particularly effective in chickens.

A quantitative method for determining sulfadimethoxine in feeds is described below. Following a brief enzyme digestion, the drug is extracted with acetone, the extract is purified by solvent transfer, and the drug is determined colorimetrically, using the Bratton-Marshall reaction. The method yields excellent recoveries of drug from medicated feeds. For example, 46 feeds mixed at the Roche experimental farm to contain 0.0125% sulfadimethoxine yielded an average of 101.8% of the claimed level with a standard deviation of ±5.1%. Another 46 feed mixes made to contain 0.00625% sulfadimethoxine yielded an average of 98.5% of claim ±12.5% (standard deviation). In both sets of samples these results on the medicated feeds were corrected for the blanks obtained on assay of parallel samples of unmedicated feeds.

The Bratton-Marshall reaction is subject to interference by other compounds containing amine groups, such as procaine penicillin and arsanilic acid. The extraction and subsequent purification procedure has been designed to practically remove significant interference from these compounds as well as from natural constituents of poultry feeds. Blank assays of unmedicated feeds in most cases have fallen in the range of 0–0.0004% when calculated as sulfadimethoxine.

In order to evaluate the analytical method by a collaborative study, 4 feed samples were prepared with graded levels of Rofenaid equivalent to 0.003, 0.006, 0.012, and 0.018% sulfadimethoxine in a commercial broiler starter ration. Eleven collaborators participated in the study. Samples of each of the medicated feeds and the corresponding unmedicated feed and a quantity of sulfadimethoxine standard were given to each collaborator, together with a copy of the method, instructions, and a data sheet. A single assay of each feed on each of 2 separate days was requested. Dilutions in the procedure were prescribed for the 2 middle levels of sulfadimethoxine, and collaborators were instructed to use 1 set of dilutions for the 2 samples at the lower potencies and the other set of dilutions for the 2 higher potencies. The specific levels of drug addition were not indicated.

METHOD[1]

(For sulfadimethoxine levels of 0.0125 and 0.00625%, equiv. to levels of Rofenaid® of 0.02 and 0.01%, resp.)

38.135 *Reagents and Apparatus*

(a) *Ficin soln.*—0.2%. Disperse 500 mg ficin (Calbiochem, fig latex) in H_2O (preheated to 40°) and dil. to 250 ml. Use 10 ml of this warm soln in detn. (*Caution:* Ficin is very potent proteolytic enzyme which attacks living tissues. Avoid contact with skin and eyes and breathing dust.)

(b) *Petroleum ether.*—Bp 35–60°, purified on silica gel column.

(c) *Trichloroacetic acid soln.*—3%. Dissolve 30 g CCl_3COOH in H_2O and dil. to 1 L. (*Caution: See* **46.082.**)

(d) *Sodium nitrite soln.*—0.1%. Dissolve 100 mg $NaNO_2$ in H_2O and dil. to 100 ml. Prep. daily.

(e) *Ammonium sulfamate soln.*—0.5%. Dissolve 500 mg $NH_4SO_3NH_2$ in H_2O and dil. to 100 ml.

(f) *N-(1-Naphthyl)ethylenediamine dihydrochloride soln (Bratton-Marshall reagent).*—0.1%. Dissolve 100 mg N-(1-naphthyl)ethylenediamine.2HCl in H_2O and dil. to 100 ml. Store in amber bottle. Prep. weekly.

(g) *Sulfadimethoxine std soln.*—Accurately weigh 125 mg sulfadimethoxine NF Ref. Std and transfer quant. into 100 ml vol. flask. Add ca 70 ml acetone and shake until completely dissolved. Dil. to vol. with acetone and mix. Pipet 20 ml soln into 200 ml vol. flask, dil. to vol. with acetone, and mix. Pipet 10 ml (or 5 ml if working at 0.00625% level) of last diln into 200 ml vol. flask contg 10 ml 0.2% ficin. Add ca 120 ml acetone and 2 ml 40% NaOH, and mix. Dil. to vol. with acetone and mix. Pipet 25 ml final diln into 50 ml g-s centrf. tube, evap. almost to dryness under N stream in 50° H_2O bath, and proceed as in **38.136**, beginning "Pipet 15 ml pet ether into centrf. tube . . ." Resulting clear filtrate is std soln.

(h) *Reagent blank.*—Into 200 ml vol. flask pipet 10 ml 0.2% ficin and 2 ml 40% NaOH. Dil. to vol. with acetone and mix. Pipet 25 ml of this soln into 50 ml g-s centrf. tube, evap. almost to dryness under N stream in 50° H_2O bath, and proceed as in **38.136**, beginning "Pipet 15 ml pet ether into centrf. tube . . ." Resulting clear filtrate is blank soln.

(i) *Spectrophotometer.*—With 5 cm cells, or Evelyn photoelec. colorimeter with 540 nm filter, or equiv.

38.136 *Preparation of Sample*

(*Caution: See* **46.004, 46.018, 46.039,** and **46.046.**)

Pipet 10 ml 0.2% ficin into high-speed blender. Accurately weigh 10 g sample into blender, spreading carefully on surface of liq. Let sample soak 10 min.

Add ca 120 ml acetone. Blend 2 min, adjusting speed with variable transformer, so that acetone does not wet screw cap. (*Caution:* To release pressure, stop blending after 3–4 sec and unscrew cap momentarily.) Blend 2 min and remove screw cap. Push down into acetone all solid particles adhering to container wall, using rubber policeman. Replace screw cap and continue blending 1 min. Remove screw cap, pipet 2 ml 40% NaOH into container, and continue blending 2 min. Push down into acetone all solid particles adhering to wall of container, using rubber policeman.

Transfer blender contents quant. into 250 ml g-s graduate, using small portions acetone to total vol. of 200 ml. Stopper, mix well, and let liq. and solids sep. Wrap tip of 50 ml pipet with glass wool and transfer ca 40 ml ext into 50 ml g-s centrf. tube. Centrf. 5 min at 2000 rpm. Pipet 25 ml clear acetone

[1] The section numbers within the method are those for the 11th ed. of *Official Methods of Analysis*, 1970. Cautionary notes, if present, refer to the new chapter on safety, Chapter 46.

ext into another centrf. tube and evap. almost to dryness (only few drops of oily, sirupy liq. left) under N stream in 50° H_2O bath. Pipet 15 ml pet ether into centrf. tube and dissolve or disperse residue in it.

Pipet 25 ml 0.2*N* NaOH into centrf. tube, stopper, and shake on mech. shaker 5 min. Centrf. tube at 2000 rpm 10 min. Transfer by pipet lower NaOH layer (ca 24 ml) into another centrf. tube and centrf. at 2000 rpm 10 min. Pipet 20 ml clear soln into 100 ml vol. flask. Dil. to vol. with 3% CCl_3COOH. Mix and let stand 10 min. Filter entire soln thru Whatman No. 42 paper, discarding first 10 ml filtrate. If turbid, filter thru second paper. Clear filtrate is sample soln.

38.137 *Determination*

(a) *Reading on spectrophotometer with 5 cm cells.*— Pipet following vols (ml) of indicated solns into 6 sep. labeled 25 ml vol. flasks:

Soln	*Sample 1*	*Sample 2*	*Sample Blank*	*Std 1*	*Std 2*	*Reagent Blank*
Sample	15	15	15	—	—	—
Std	—	—	—	15	15	—
Blank	—	—	—	—	—	15

Pipet 1 ml 0.1% $NaNO_2$ soln into each, mix, and let stand 3 min. Pipet 1 ml 0.5% NH_4 sulfamate soln into each, mix, and let stand 2 min. Pipet 1 ml 0.1% Bratton-Marshall reagent into all except sample blank, and 1 ml H_2O into sample blank. Mix and let stand 10 min in dark. Dil. each flask to vol. with H_2O and mix. Measure *A* of each soln at 540 nm in 5 cm cells against reagent blank in ref. cell.

(b) *Reading on Evelyn photoelectric colorimeter.*— Proceed as in (a) except do not dil. after standing, but read at existing vol.; 50 ml g-s centrf. tubes may be used in place of vol. flasks. Transfer solns from flasks or tubes into matched colorimeter tubes. Set instrument with 540 filter to 100% *T* (0 *A*) with tube contg reagent blank. Det. *A* (= 2 − log *T*) of each of other tubes contg samples, sample blank, and stds.

(c) *Calculation.*—Higher levels of sulfadimethoxine:

$$[(A_x - A_b) \times S]/(1000 \times A_s \times W) = \%$$ sulfadimethoxine

Lower levels of sulfadimethoxine:

$[(A_x - A_b) \times S]/2000 \times A_s \times W) = \%$ sulfadimethoxine, where A_x, A_b, and A_s refer to sample, reagent blank, and std, resp.; W = g original sample; and S = mg std weighed.

Table 1. Collaborative results for per cent sulfadimethoxine in feeds

Coll.	Sample Number 262-73	262-74	262-75	262-76
1	0.0031	0.0059	0.0117	0.0182
	0.0028	0.0056	0.0116	0.0167
2	0.0034	0.0065	0.0115	0.0181
	0.0025	0.0058	0.0112	0.0169
	0.0023	0.0048	0.0113	0.0175
3	0.0034	0.0059	0.0123	0.0157
	0.0028	0.0059	0.0118	0.0173
4	0.0036	0.0070	0.0135	0.0215
	0.0026	0.0053	0.0102	0.0166
5	0.0060[a]	0.0068	0.0118	0.0182
	0.0054[a]	0.0068	0.0118	0.0182
6	0.0040	0.0062	0.0117	0.0173
7	0.0035	0.0040	0.0101	0.0150
	0.0036	0.0053	0.0103	0.0185
8	0.0056[a]	0.0056	0.0134	0.0125[a]
	0.0054[a]	0.0058	0.0132	0.0126[a]
9	0.0033	0.0063	0.0110	0.0160
	0.0032	0.0063	0.0110	0.0160
10	0.0041	0.0067	0.0135	0.0206
	0.0034	0.0069	0.0130	0.0202
	0.0033	0.0064	0.0130	0.0207
	0.0033	0.0062	0.0139	0.0217
11	0.0029	0.0052	0.0105	0.0163
	0.0024	0.0047	0.0094	0.0161
	0.0022	0.0045	0.0099	0.0150
	0.0020[a]	0.0044	0.0078[a]	0.0153
Av.	0.0031	0.0058	0.0116	0.0176
Std dev.	0.00049	0.00083	0.00127	0.0020
Coeff. of var., %	17.2	14.3	11.1	11.5

[a] Omitted from average (outside ±2 std dev.).

Results and Recommendation

The results obtained by the 11 collaborators on the 4 samples of feed are listed in Table 1 together with the average values, standard deviations, and coefficients of variation. Most collaborators experienced no difficulty with the method. One collaborator reported difficulty in wetting the 10 g sample with 10 ml ficin solution, and one felt that the evaporation of 25 ml acetone was rather time-consuming. Two collaborators obtained high results on the lowest potency sample, and as indicated, these were outside the 2 standard deviation limits and were omitted from the average. Only 4 other values in Table 1 fell outside such limits. The ratios of potencies of the 4 samples were designed to be 0.5:1.0:2.0:3.0. The average values found in the study are in the ratios 0.53:1.0:1.98:3.04, which represent excellent agreement with expected values.

Since the results obtained were generally satisfactory and the majority of collaborators reported no difficulty with the method, the Associate Referee recommends that the method be adopted as official first action.

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The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee B and was adopted by the Association. See *JAOAC* **53**, 383 (1970).

This report of the Associate Referee, E. De Ritter, was presented at the 83rd Annual Meeting of the AOAC, Oct. 13–16, 1969, at Washington, D.C.

Improved Colorimetric Method for the Determination of Nitarsone in Feeds

By G. M. GEORGE and J. L. MORRISON (Department of Biochemistry, Salsbury Laboratories, Charles City, Iowa 50616)

An improved procedure for the determination of nitarsone in poultry feeds has been developed. The drug is extracted from feed with 50% dimethylsulfoxide; interferences are removed by column chromatography of the extract on alumina. The drug is eluted from the column with 4% NaOH and assayed colorimetrically with the Bratton-Marshall reaction after reduction of the nitro group with aqueous 4% titanous chloride. The average recovery of nitarsone from laboratory-medicated feed was 97.0% of theoretical with an average coefficient of variation of 3.4%. The method was applicable to both fresh feeds and feeds stored for periods up to 6 months. This method was subjected to a collaborative study for the determination of nitarsone in medicated feeds. The average recovery from 6 collaborators was 103.5% with an average coefficient of variation of 12.7%. Most of the collaborators reported satisfactory results and therefore this method is recommended for adoption as official first action.

Nitarsone (4-nitrophenylarsonic acid) is the active ingredient in Histostat®-50 which is used for the prevention of blackhead in turkeys and chickens (1). Although nitarsone in feed can be determined by the total arsenic procedure (2), that method is tedious and time-consuming. Cavett in 1956 (3) published a colorimetric method for the determination of nitarsone in feeds which has been satisfactory for mash feeds since its introduction. However, as the use of pelleted feeds increased, it became increasingly apparent that this method was providing low recoveries (75–90%) from pelleted and crumbilized feeds. The proposed procedure overcomes this problem of low recovery in pelleted and crumbilized feeds and also eliminates the lengthy sodium hydrosulfite reduction step.

This report of the Associate Referee, G. M. George, was presented at the 83rd Annual Meeting of the AOAC, Oct. 13–16, 1969, at Washington, D.C.

METHOD

Principle

Nitarsone is extd from feed with 50% dimethylsulfoxide (DMSO) and sepd from interferences by Al_2O_3 chromatgy. The nitro group is reduced with aq. 4% $TiCl_3$ and resulting amine is assay colorimetrically at 530 nm, using Bratton-Marshall reaction. Arsanilic acid and carbarsone interfere.

Reagents

(a) *Nitarsone std solns.*—(*1*) *Stock soln.*—1 mg/ml. Weigh 100 mg nitarsone std (available from Salsbury Laboratories) into 100 ml vol. flask and dil. to vol. with 4% NaOH. (*2*) *Working soln.*—50 μg/ml. Dil. 10 ml stock soln to 200 ml with 4% NaOH.

(b) *Activated alumina.*—Alcoa grade F-20, 80–200 mesh (available from Fisher Scientific Co. as Alumina, adsorption, Fisher No. A-540). To det. suitability of Al_2O_3, perform entire detn on 100 μg nitarsone. Recovery should be >95%.

(c) *Dimethylsulfoxide (DMSO) soln.*—50%. Dil. with equal vol. H_2O. (*Caution:* DMSO can be harmful. Avoid skin contact by wearing heavy rubber gloves. Use effective fume removal device.)

(d) *Titanous chloride soln.*—4% aq. Prep. fresh daily from 20% soln open ≤3 months and kept refrigerated, or from solid $TiCl_3$. If >1 min required for color disappearance in detn, use fresh source of $TiCl_3$. (*Caution:* $TiCl_3$ is corrosive. Wear disposable plastic or rubber gloves. Avoid contact with eyes.)

(e) *Sodium nitrite soln.*—0.1% aq. Prep. weekly.

(f) *Ammonium sulfamate soln.*—0.5% aq. Prep. weekly.

(g) *Coupling reagent.*—*See* **33.013(b)**.

Preparation of Standard Curve

Pipet 0, 2, 5, 10, 15, 20, and 25 ml working soln into sep. 100 ml vol. flasks and dil. to vol. with 4% NaOH. Pipet 10 ml from each flask into sep. 50 ml vol. flasks, add 15 ml 4% NaOH, and dil. to vol. with H_2O. Pipet 4 ml from each flask into sep. test tubes and develop color as in *Determination*, beginning ". . . add 2 drops 4% $TiCl_3$ . . ." Std concns correspond to 0, 0.004, 0.010, 0.020, 0.030, 0.040, and 0.050% nitarsone in feeds. Plot *A* against % drug in feed.

Preparation of Sample

Accurately weigh 5 g finely ground feed into 100 ml vol. flask. Add 75 ml 50% DMSO, place sample on wrist-action mech. shaker, and shake at room temp. 30 min. Dil. to vol. with 50% DMSO and mix. Transfer 30–40 ml to 50 ml centrf. tube and centrf. 10 min at 2000 rpm.

Determination

Add Al_2O_3 to 20 × 400 mm chromatgc tube with fritted glass disk to depth of 7 cm. Tap tube wall to settle Al_2O_3; then add 1 cm layer of sand. Prewash column with 50 ml 50% DMSO before use.

Pipet 10 ml supernatant from prepn of sample onto prewashed column. For feeds contg >0.04% nitarsone, use smaller aliquot. Let sample enter column and then wash into column with several 5 ml portions H_2O. Wash column with 75 ml H_2O and discard eluate.

Elute nitarsone with 65 ml 4% NaOH, discarding first 15 ml. Collect remaining eluate in 100 ml vol. flask, letting column run dry. Nitarsone is eluted with ca 25–30 ml eluant. Dil. eluate to vol. with H_2O and mix.

Pipet 4 ml dild eluate into 2 test tubes, add 2 drops 4% $TiCl_3$ to each, and shake or mix on Vortex mixer until black color disappears. Add 2 ml HCl to each and mix thoroly. Add 0.5 ml 0.1% $NaNO_2$, (e), and mix. After 5 min, add 0.5 ml 0.5% NH_4 sulfamate and mix. After 2 min, add 0.5 ml 0.1% coupling reagent, **33.013(b)**, to one tube and 0.5 ml H_2O to second tube for blank. Let color develop 15 min; then read *A* of sample and blank at 530 nm on spectrophtr. Correct sample *A* for blank *A* and det. % nitarsone in sample from std curve.

Results and Discussion

Nitarsone is completely extracted from feeds by shaking with 50% dimethylsulfoxide solution at room temperature for 30 min. Longer shaking periods at room temperature or heating and shaking for varying periods of time did not improve the assay results. Interfering materials are removed from the sample by chromatography on an alumina column. Fisher Scientific adsorption alumina, 80–200 mesh, gave the best results for flow rates and sample cleanup.

Several feed samples were assayed by both the method reported by Cavett (3) and the proposed procedure to compare the accuracy and precision. It was noted that both methods gave essentially the same results; however, the precision in the proposed method was better (Table 1). Values were compared for both methods on pelleted feeds. Since these feed samples came from outside sources, the actual amount of drug in the feed was unknown. The values obtained, however, indicate that the higher values observed for the proposed procedure agree with the expected levels, whereas Cavett's method invariably gave low values (Table 2).

In addition to giving low values on pelleted

Table 1. Comparison of proposed method with Cavett's method (3) for determining per cent nitarsone (mash feed containing 0.025% nitarsone)

	Week 1		Week 2		Week 3		Week 4	
	Prop.	Cavett	Prop.	Cavett	Prop.	Cavett	Prop.	Cavett
	0.028	0.030	0.023	0.024	0.027	0.025	0.026	0.021
	0.028	0.031	0.025	0.024	0.026	0.030	0.026	0.021
	0.028	0.028	0.025	0.024	0.027	0.026	0.026	0.022
Av.	0.028	0.030	0.024	0.024	0.027	0.027	0.026	0.021

Table 2. Recovery of nitarsone from commercial pelleted feeds by 2 methods

		Found, %	
Sample	Expected, %	Cavett (3)	Prop.
5273	0.025	0.015	0.025
5425	0.053	0.043	0.047
5426	0.047	0.032	0.047
5427	0.0187	0.013	0.018
5428	0.047	0.035	0.047
5429	0.044	0.028	0.033
0385	0.0187	0.015	0.017
0386	0.0187	0.014	0.017
0396	0.0187	0.021	0.020
0397	0.025	0.017	0.019

feeds, Cavett's method is also unsuitable for feeds containing less than 0.01% nitarsone. The proposed method responds very well for feeds containing as little as 0.0025% nitarsone (Table 3).

Recovery of nitarsone from a laboratory-medicated feed was essentially theoretical. The observed recoveries for 12 assays were 0.023, 0.026, 0.024, 0.024, 0.024, 0.025, 0.024, 0.024, 0.023, 0.025, 0.024, and 0.024%. The average recovery was 97.0%, with an average coefficient of variation of 3.4% and standard deviation of 0.00083.

Control feeds generally showed an average colorimetric response of 0.0006% nitarsone (Table 4). The colorimetric response with graduated amounts of nitarsone was linear over the range 0.0025–0.05% (Table 5).

The following feed additives and antibiotics were assayed by the nitarsone procedure for possible interferences: aklomide, 2-amino-5-nitrothiazole, amprolium, arsanilic acid, butynorate, carbarsone, chlortetracycline, clopidol, cyzine, dimetridazole, dinsed, erythromycin stearate, furazolidone, nicarbazin, nifursol, nihydrazone, nithiazide, nitrofurazone, nitromide, nitrovin, oxytetracycline, procaine penicillin G, reserpine, roxarsone, streptomycin, sulfanitran, tylosin, zinc bacitracin, and zoalene. Only arsanilic acid and carbarsone interfered.

A stability study was run for nitarsone in a commercial ration of mash, pellets, and crumbilized feeds. The feeds were packaged in regular 50 lb feed bags, stored in the attic of a farm building in an attempt to simulate actual storage conditions, and sampled at monthly intervals for analysis. Feeds were assayed in triplicate by both the procedure reported by Cavett and the proposed procedure. The results obtained by the proposed procedure were in close agreement with the expected value (Table 6). After 6 months' storage, the level of nitarsone in the feed (mash, pellets, and crumbles) was unchanged. The feeds

Table 3. Suitability of proposed method for low levels of nitarsone, compared to Cavett (3) method

	% Found	
% in Feed	Cavett	Prop.
0	0.0026	0.0000
0.0025	0.0051	0.0024
0.0050	0.0075	0.0045
0.0100	0.0125	0.0112
0.0200	0.0220	0.0220
0.0400	0.0420	0.0450

Table 4. Colorimetric response for nitarsone in control feed

Sample	Absorbance	Nitarsone, %
4512-R	0.000	0.0000
4134-R	0.003	0.0006
4134-R	0.006	0.0012
4350-R	0.004	0.0008
4203-R	0.004	0.0008
4287-R	0.005	0.0010
4455-R	0.000	0.0000
4382-R	0.003	0.0006
4472-R	0.003	0.0006
1240	0.000	0.0000
Av.		0.00056

Table 5. Colorimetric response for graduated amounts of nitarsone

Added, ppm	Recovered,[a] ppm	Recovered, %
0	5	—
40	38	95.0
100	110	110.0
200	205	102.5
300	315	105.0
400	410	102.5
500	495	99.0
600	550	91.7
800	670	83.7

[a] Average of 4 determinations.

were also subjected to thin layer chromatography (4) to determine the presence of any degradation products that may have formed during storage. No degradation products were detected. This procedure is capable of detecting as little as 1% degradation of nitarsone to arsanilic acid or 4-nitrophenylarsinoxide, 2 potential degradation products.

Collaborative Results and Discussion

This method was submitted for a collaborative study in which 6 collaborators participated. Collaborators were provided with 6 feed samples medicated with nitarsone in a regular turkey ration, a copy of the assay procedure, and a data sheet. One feed sample was of known drug concentration to enable the collaborator to familiarize himself with the assay method; the remaining 5 samples were unknown at 2 different drug concentrations in both mash and pelleted feed. Collaborators were instructed to assay the unknown samples according to the feed assay procedure as written and report a single assay result for each of the 5 feed samples on the data sheet provided.

Table 6. Stability of nitarsone (%) in mash, pelleted, and crumbilized feed[a] by 2 methods

Storage,	Mash		Pellets		Crumbles	
Months	Cavett	Prop.	Cavett	Prop.	Cavett	Prop.
0	0.030	0.029	0.027	0.028	0.025	0.023
1	0.024	0.024	0.022	0.023	0.022	0.023
2	0.027	0.027	0.025	0.025	0.026	0.023
3	0.021	0.026	0.025	0.024	0.024	0.021
4	0.024	0.023	0.022	0.023	0.022	0.023
5	0.029	NA[b]	0.023	NA	0.023	NA
6	0.027	0.025	0.022	0.025	0.023	0.024

[a] Mean values of triplicate assays.
[b] Not assayed.

The known feed sample was medicated to contain 0.01875% nitarsone. The unknown samples were medicated to contain 0.01875% nitarsone in Samples 1 (mash) and 3 (pellets), and 0.025% nitarsone in Samples 2 (mash) and 4 and 5 (pellets). Sample 5 was a blind duplicate of Sample 4 and was included to determine the ability of the collaborator to duplicate his results.

The collaborative results are summarized in Table 7. The table lists the mean assay, standard deviation, coefficient of variation, and per cent recovery for each feed sample.

For feed containing 0.01875% nitarsone the average recovery was 101.9% with an average coefficient of variation of 10.5%. For feeds containing 0.025% nitarsone the average recovery was 104.5% with an average coefficient of variation of 13.9%. The average recovery for all samples was 103.5% with an average coefficient of variation of 12.7%. The average within-laboratory variation (S_r), determined as described by Youden (5), was 0.0019, indicating that the collaborators had no difficulty duplicating their results.

Some collaborators reported that they had problems getting satisfactory results on the feed samples. Through correspondence with them it was learned that the 4% titanium trichloride was prepared from an old opened bottle of 20% $TiCl_3$. The 4% solution of titanium trichloride must be freshly prepared on the day of use from a bottle of 20% $TiCl_3$ that has been open not longer than 3 months. It is also advisable to keep the 20% solution refrigerated after it has been opened. If it takes longer than 1 min for the dark color to disappear during the reduction step, a new bottle of 20% $TiCl_3$ should be used. Using an old 20% solution of titanium trichloride produces variable results.

The collaborators that were having problems were asked to re-assay the samples, using a fresh source of titanium trichloride. The results that were reported the second time were very good. One collaborator did not have time to re-run the assay; therefore, his results were not included in the report.

Recommendation

Since the collaborators that participated in this study reported satisfactory results, the Associate Referee recommends that this method be adopted as official first action and that further study be carried out.

Table 7. Nitarsone collaborative results (%)

Coll.	Mash (0.0187%) Sample 1	Mash (0.025%) Sample 2	Pellets (0.0187%) Sample 3	Pellets (0.025%) Sample 4	Pellets (0.025%) Sample 5[a]
1	0.0210	0.0320	0.0240	0.0320	0.0310
2	0.0184	0.0264	0.0172	0.0282	0.0227
3	0.0205	0.0225	0.0198	0.0255	0.0268
4	0.0185	0.0310	0.0185	0.0210	0.0225
5	0.0167	0.0237	0.0190	0.0260	0.0270
6	0.0169	0.0240	0.0180	0.0240	0.0240
Mean	0.0187	0.0266	0.0194	0.0261	0.0256
Std dev.	0.00178	0.00401	0.00241	0.00374	0.00326
Coeff. of var., %	9.5	15.0	12.3	14.2	12.5
Rec., %	100.0	106.4	103.9	104.5	102.7

[a] Blind duplicate of Sample 4.

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The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee B and was adopted by the Association. See *JAOAC* **53**, 383 (1970).

Collaborative Study of the Determination of Dimetridazole in Feeds by UV Spectroscopy

By J. L. MORRISON (Department of Biochemistry, Salsbury Laboratories, Charles City, Iowa. 50616)

A collaborative study of a previously reported method for the analysis of dimetridazole in feeds has been conducted. The drug is extracted from the feed with methanol, cleaned up by column chromatography on alumina, and measured spectrophotometrically at its ultraviolet wavelength maximum. Nine collaborators participated in this study. The average coefficient of variation was 5.7% for feeds containing 0.015–0.016% dimetridazole and 8.9% for feeds containing 0.05–0.06% dimetridazole. The recovery for these groups was 99 and 97%, respectively. The method is recommended for adoption as official first action.

Dimetridazole (1,2-dimethyl-5-nitroimidazole) is the active ingredient in Emtrymix, a product effective in the prevention and control of blackhead in turkeys (1–3). A polarographic method (4) and an ultraviolet spectrophotometric method (5) for the determination of this drug have been previously described. This report presents the results of a collaborative study conducted on the ultraviolet spectrophotometric method for dimetridazole.

Collaborative Study

Nine collaborators participated in this study. A reference sample of dimetridazole, 2 unknown samples of feed at each of 2 drug levels, 2 practice feed samples containing known levels of dimetridazole, and a control unmedicated feed were supplied to each collaborator. A copy of the method and instructions were also supplied.

METHOD[1]

38.052 *Principle*

Dimetridazole is extd from feeds with MeOH, sepd from interfering substances by two Al_2O_3 chromatgc steps, and detd spectrophtric at its UV wavelength max. Nihydrazone, furazolidone, zoalene, 2-chloro-4-nitrobenzamide, tylosin, and large amts procaine (from procaine penicillin) interfere.

[1] The section numbers within the method are those for the 11th ed. of *Official Methods of Analysis*, 1970. Cautionary notes, if present, refer to the new chapter on safety, Chapter 46.

38.053 *Apparatus and Reagents*

(a) *Ultraviolet spectrophotometer.*—Beckman Instruments DK-2 ratio recording, Beckman DU, or equiv.

(b) *Chromatographic tubes.*—13 × 150 mm and 15 × 250 mm, constricted at bottom to hold glass wool plug and 6 mm od delivery tube.

(c) *Aluminum oxide.*—Suitable for chromatgy (Merck and Co. No. 71707, or equiv.). To det. suitability of Al_2O_3, perform detn of feed that does not contain dimetridazole or other imidazole drugs. If feed appears to contain >0.004% dimetridazole, use another batch of Al_2O_3.

(d) *1,2-Dimethyl-5-nitroimidazole (dimetridazole) std solns.*—(*1*) *Stock soln.*—0.1 mg/ml. Weigh 100 mg dimetridazole std (available from Salsbury Laboratories) into 100 ml vol. flask. Dissolve in H_2O by shaking frequently ca 20 min. Dil. to vol. with H_2O and mix. Pipet 20 ml into 200 ml vol. flask, dil. to vol. with H_2O, and mix. (*2*) *Working std solns.*—Pipet 5, 10, 20, 30, and 40 ml stock soln into sep. 100 ml vol. flasks. Add 5.0 ml 3*N* HCl to each, immediately dil. to vol. with H_2O, and mix. Pipet 5 ml each soln and 5 ml 0.10*N* NaOH into sep. 50 ml erlenmeyers. Stopper and mix. These solns contain 2.5, 5, 10, 15, and 20 μg dimetridazole/ml.

38.054 *Preparation of Standard Curve*

Proceed as in **38.057**, using working std solns and blank prepd by mixing 5 ml 0.15*N* HCl with 5 ml 0.10*N* NaOH.

Read *A* against blank as for recording or manual spectrophtrs. Construct std curve by plotting *A* against μg dimetridazole/ml.

38.055 *Preparation of Sample*

Weigh portion finely ground feed contg 0.5–2.0 mg dimetridazole (usually 5 g) into 100 ml vol. flask. Add 70–75 ml MeOH and place in constant temp. bath 30 min at 60°. Make certain that H_2O level covers flask to ca ⅛″ below MeOH level. Swirl flask 2 or 3 times during first 5 min to heat evenly. Cool to room temp., dil. to vol. with MeOH, and mix. Let stand 5–10 min to let coarse feed particles settle.

38.056 *Chromatography*

Place small glass wool plug in bottom of 15 × 250 mm chromatgc tube and add 8 cm layer Al_2O_3; pack column tightly to prevent streaking. (If streaks

enter effluent, positive bias is introduced.) Decant methanolic ext onto column so that settled feed particles are not disturbed. Collect ca 30 ml eluate in 50 ml vol. flask. Stopper until ready for use.

(*Note:* Dimetridazole sublimes at temps >70°; manner of solv. removal is critical.) For feed contg 0.015% dimetridazole, pipet 15 ml effluent (4 ml if feed contains 0.06%; 3 ml if feed contains 0.10%) into 125 ml suction or r-b flask and evap. under reduced pressure from H_2O aspirator. If 15 ml is taken, use hot plate (low heat) or H_2O-bath to reduce to 3–4 ml. Shake to prevent bumping. When vol. approaches 3–4 ml remove flask from heat and remove last 3–4 ml only with heat from palm of hand. Continue shaking to prevent bumping. Do *not* attempt to attain complete dryness because part of the 2–3 drops of oily residue is dimetridazole.

Wash down walls of flask, beginning at base of neck, with 5.0 ml 0.10*N* NaOH. Swirl to wash walls. Let stand 5 min and add 5.0 ml 0.15*N* HCl. Swirl to mix and again wash flask walls. Stopper until ready for chromatgy.

38.057 ***Determination***

Prep. second Al_2O_3 column by inserting small glass wool plug into bottom of 13 × 150 mm chromatgc tube, add 4 cm layer Al_2O_3, and tap gently to pack column lightly. Pour entire 10 ml soln onto column and let pass thru by gravity. Collect effluent in 50 ml erlenmeyer. Force out liq. adhering to column by applying air pressure with rubber bulb. Swirl flask to mix. Stopper until ready to read. Pass blank soln of 5 ml 0.15*N* HCl and 5 ml 0.10*N* NaOH thru sep. 4 cm Al_2O_3 column as above.

(a) *Using recording spectrophotometer.*—Fill matched pair silica cuvets with reagent blank and with sample soln (always use same cuvet for blank) and scan from 330 to 310 nm. Read *A* at peak and obtain concn of soln in μg/ml from std curve. Calc. % dimetridazole = [(μg/ml from std curve) × diln factor × 100]/(g sample × 10^6). Diln factor = 1.335×10^{-3} for feeds contg 0.015%; 5×10^{-3}, 0.06%; and 6.66×10^{-3}, 0.1%.

(b) *Using manual spectrophotometer.*—Locate peak *A* of sample soln (ca 318 nm), using matched pair silica cells, and set wavelength at peak. Read *A* of sample and blank solns and correct sample for blank. Obtain concn of soln in μg/ml from std curve, and calc. % in feed as above.

Results and Discussion

Previous studies (5) indicated that the average per cent recovery from 2 feeds containing 0.015 and 0.060% dimetridazole were 102.3±2.42% and 98.9±2.56%, respectively. The following drugs were checked for possible interferences with the method: nicarbazin, furazolidone, nitrofurazone, nihydrazone, nithiazide, reserpine, *p*-ureidobenzenearsonic acid, 2,4-diamino-5-(*p*-chlorophenyl)-6-ethylpyrimidine, sulfaquinoxaline, 2-acetylamino-5-nitrothiazole, acetyl-(*p*-nitrophenyl)-sulfanilamide, 3,5-dinitrobenzamide, zoalene, 2-chloro-4-nitrobenzamide, 3-nitro-4-hydroxyphenylarsonic acid, 4-nitrophenylarsonic acid, and arsanilic acid. Of these, nihydrazone, furazolidone, zoalene, and 2-chloro-4-nitrobenzamide interfered. The remaining drugs did not interfere.

The antibiotics tested were aureomycin, procaine penicillin, zinc bacitracin, terramycin,

Table 1. Collaborative results of dimetridazole in feeds: per cent dimetridazole found

Coll.		Sample: A	B	C	D
1		0.0157	0.0140	0.0488	0.0613
		0.0150	0.0144	0.0575	0.0663
		0.0155	0.0145	0.0556	0.0650
	Av.	0.0154	0.0143	0.0540	0.0642
2		0.0155	0.0147	0.0600	0.0560
		0.0163	0.0155	0.0620	0.0635
		0.0153	0.0148	0.0589	0.0515
		0.0139	0.0139	0.0580	
		0.0143	0.0140	0.0581	
	Av.	0.0150	0.0145	0.0594	0.0570
3		0.0161	0.0137	0.054	0.049
		0.0153	0.0127	0.051	0.048
	Av.	0.0157	0.0134	0.0525	0.0485
4		0.017	0.015	0.053	0.055
		0.018	0.016	0.056	0.057
	Av.	0.0175	0.0155	0.0545	0.056
5		0.0152	0.0145	0.0504	0.0590
		0.0156	0.0147	0.0492	0.0595
	Av.	0.0154	0.0146	0.0498	0.0593
6		0.016	0.015	0.062	No
		0.016	0.015	0.063	data
		0.016	0.015	0.063	
		0.016	0.015	0.063	
	Av.	0.016	0.015	0.0628	
7		0.0146	0.0154	0.0480	0.0550
		0.0146	0.0154	0.0450	0.0520
		0.0186	0.0138	0.0500	0.0519
		0.0180	0.0138	0.0533	0.0586
	Av.	0.0164	0.0146	0.0490	0.0543
8		0.0166	0.0166	0.0525	0.0625
9[a]		0.0290	0.0280	0.0950	0.104
		0.0290	0.0280	0.0950	0.110
		0.0280	0.0290	0.0880	0.107
		0.0290	0.0290	0.0880	0.100
	Av.	0.0287	0.0285	0.0915	0.105
Mean, $\bar{X}$		0.0160	0.0148	0.0543	0.0574
Std dev.		0.000809	0.000937	0.0046	0.00527
Coeff. of var., %		5.1	6.3	8.6	9.2

[a] Results not included in statistical evaluation.

streptomycin, and tylosin. Tylosin interfered and procaine, from procaine penicillin, could interfere if a large quantity is in the feed. The results of the collaborative study are given in Table 1. The table also lists the mean assay, standard deviation, and coefficient of variation. One collaborator reported that he could not get satisfactory results because of failure of the alumina to remove all interferences. He commented that blank feeds gave assays equivalent to 0.013–0.025% dimetridazole. Consequently his data have not been included in this study. No other collaborator in this study reported difficulties of this type.

For feeds containing 0.015–0.016% dimetridazole the average recovery was 99% and the coefficient of variation was 5.7%. For feeds containing 0.05–0.06% dimetridazole the recovery was 97% and the coefficient of variation was 8.9%.

Since satisfactory results for the determination of dimetridazole in feeds were obtained by this method, the Associate Referee recommends that this method be adopted as official first action and that further study be conducted.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee B and was adopted by the Association. See *JAOAC* **53**, 383 (1970).

This report of the Associate Referee was presented at the 83rd Annual Meeting of the AOAC, Oct. 13–16, 1969, at Washington, D.C.

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

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

E. E. Martin, Ralston Purina Co., St. Louis, Mo.

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BOOK REVIEWS

Pesticides. S. K. Majumder (Ed.). Academy of Pest Control Sciences, Mysore, India, 1968. ix + 381 pp. Price $8.00 (including postage).

"Residual toxicity of persistent insecticides to man and his animals, migration of the residues from the site of application to remote objects and related toxicological aspects of these chemicals have created great concern to all those who are engaged in the long chain of pesticide industry and science. Pesticide research seems to be taking a new outlook towards its future development."

Beginning with this rather prophetic introduction, the volume of papers contributed to a symposium on pesticides in India in 1964 and published in 1968 brings together several aspects of pesticide research. Were it not for the fact that one must read this work 5 years after it was presented, this might have been a significant contribution to the world's thinking and knowledge of pesticides. In retrospect, the title "Pesticides" reflects a misnomer in that, with the exception of 3 fungicide and 2 rodenticide reports, the volume deals specifically with insecticides.

The volume is divided into 4 sections containing 55 presentations reflecting almost every topic involving insecticide toxicology. The majority of topics are covered superficially and, with the delay in publication, the value of the review articles and certain specific topics such as residue methodology and formulations is minimal.

The broad spectrum coverage of a field such as pesticides through the use of symposia requires that publication of the content be as fast as possible. The loss of time makes this type of publication almost useless. The current extensive use of gas-liquid chromatographs utilizing detection systems specific for halogens, phosphorus, or nitrogen precludes the use of colorimetric methods of analysis for compounds containing these elements such as are presented in Chapters 4.4, 4.5, and 4.6. The advances in analytical methodology in pesticides over the past half decade also negate some of the data presented concerning residues of insecticides on food crops as analyzed by other techniques.

The publication has merit in that it presents certain data not generally available outside of India—although almost all that was presented and cited as original, nonpublished work has since been released and is no longer unique. The titles to several of the articles are somewhat misleading. For example, after reading the title of the penultimate article "Hazards of Use of Pesticides in the Field of Agriculture and Public Health," one reads the article only to find that it consists primarily of DDT and DDE residue data from human fat. That DDT and/or its metabolites in fat constitute a hazard cannot be disputed, but are there no other hazards to be considered? The final article entitled "Answer to Health Hazards by Pesticides" has as one of its 2 lines "There have appeared appreciable data and even well written books on health hazards due to pesticides." Unfortunately, this is not one of them!

RONALD L. BARON
Division of Pesticide Chemistry and Toxicology
Food and Drug Administration

Phosphoric Acid, Phosphates, and Phosphatic Fertilizers. William H. Waggaman. Hafner Publishing Co., Inc., New York, N.Y. 10003. Reprint (1969) of 2nd Ed. (1952). vii + 683 pp. (tables, illustrations). Price $31.50.

This book, under a new publisher, is a facsimile of the second edition copyrighted by the Reinhold Publishing Corporation in 1952. The volume has been a much used standard reference work on phosphates since the first edition appeared in 1927. More recent books have updated the research, development, and economic information on the subject, but this book will most likely remain a favorite reference on the basic principles and early history of phosphate technology.

JOHN O. HARDESTY
Consultant
Fertilizer Technology

A Manual on Methods for Retrieving and Correlating Technical Data. Prepared by the ASTM Special Committee on Numerical Reference Data by Freeman H. Dyke, Jr., ASTM Special Technical Publication No. 468, American Society for Testing and Materials, 1916 Race St., Philadelphia, Pa. 19103, 1969. 70 pp. Price $3.00.

This is an excellent introduction to "information retrieval" with a listing of advantages and disadvantages of numerous types of retrieval equipment and microfilm equipment. It also contains definitions of commonly used terms and a bibliography for further information.

WILLIAM HORWITZ
Office of Foods and Nutritional Sciences
Food and Drug Administration

Applied Spectroscopy Reviews. Vol. 2. Marcel Dekker, Inc., New York, N.Y., 1969. viii + 376 pp. Price $17.50.

The reviews in this volume are well written and fill a definite need for an up-to-date discussion of timely spectroscopic topics. In this volume the discussions of near infrared spectrophotometry, spectroscopic studies of the hydrogen bond, and analysis of ABX spectra in NMR spectroscopy are exceptionally well written.

The inclusion of clear and comprehensive tables and references is to be commended. The thermodynamic data for hydrogen bonding which has been gathered from a large number of references will be especially useful. This is also true of the thermodynamic data given in the near infrared discussion as well as the values for absorption bands, especially overtones, given in the hydrogen bonding article.

The clear examples of the rather complicated calculations involved in the analysis of NMR spectra together with the tables of frequency values will be especially valuable to the workers in this field. This applies also to the example of structural analysis given in the review of molecular vibrations of high polymers.

C. T. KENNER

Department of Chemistry
Southern Methodist University

NEW PUBLICATIONS

Specifications for Identity and Purity of Food Additives and Their Toxicological Evaluations: Some Antibiotics. FAO No. 45 and WHO Technical Report No. 430. Food and Agriculture Organization, Rome, 1969. (Available from UNIPUB, Inc., 650 1st Ave., P.O. Box 433, New York, N.Y. 10016.) 49 pp. Price $1.00.

The 12th Report of the Joint FAO/WHO Expert Committee on Food Additives covers the recommendations on the use of antibiotics as direct (intentional) food additives, use of antibiotics as feed additives, use of antibiotics in veterinary medicine, and medical and veterinary coordination.

Official Publication of Association of American Pesticide Control Officials, Inc., 1969-70. 102 pp. Price $4.00. Available from Robert H. Guntert, State Board of Agriculture, State Office Building, Topeka, Kan. 66612.

This report gives the proceedings of the 23rd annual meeting of this Association as well as information on purposes; by-laws; policies; uniform State insecticide, fungicide, and rodenticide act; model pesticide use and applications act; and official sampling procedures.

Reagents for Organic Synthesis. Vol. 2. Mary Fieser and Louis F. Fieser. Wiley—Interscience, New York, 1969. 538 pp. Price $17.50.

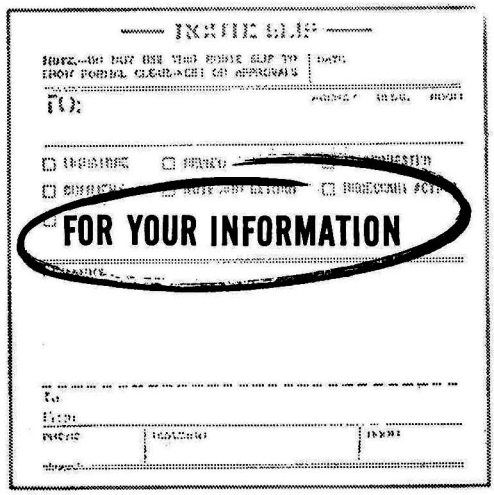
This volume contains 1320 additional references to 390 reagents discussed in the first volume (Aug. 1966) and 550 references to 226 reagents reviewed by the authors for the first time.

Modern Methods of Chemical Analysis. R. L. Pecsok and L. D. Shields. John Wiley and Sons, Inc., 605 Third Ave., New York, N.Y. 10016, 1968. xvi + 480 pp. Price $9.95.

This introductory text is a significant departure from familiar textbooks on quantitative analysis. It presents a blend of topics, all of analytical flavor but geared to every branch of chemistry.

The State of Food and Agriculture, 1969. Food and Agriculture Organization, Rome, 1969. (Available from UNIPUB, Inc., 650 1st Ave., P.O. Box 433, New York, N.Y. 10016.) 196 pp. Price $6.00.

This annual report covers a world review of agricultural production, international trade, developmental assistance, and production forecasts by regions for the year 1968.



Long Range Planning Committee

A meeting was held February 12, 1970, at the Food and Drug Administration, Washington, D.C. Members present were: D. Banes, Chairman, H. A. Davis, L. G. Ensminger, W. Horwitz, K. L. Milstead, S. B. Randle, L. S. Stuart, and M. S. Schechter. P. Chichilo was also present; I. Hoffman was absent.

Mr. Ensminger summarized the results of the survey on how to improve the Annual Meeting. The consensus was that the meeting should continue as in the recent past.

Presently the Subcommittees cannot recommend a method for adoption unless the General Referee approves its adoption. The Committee on the Constitution is preparing a revision which will permit a Subcommittee to overrule the General Referee on this point. It should be noted in the AOAC Handbook that when the General Referee does not concur with the recommendation of the Associate Referee, the Associate Referee is allowed to defend his position before the Subcommittee.

Dr. Randle will prepare a statement on how slides should be prepared for the Annual Meeting; this will be distributed to all Associate Referees. Unless the basic requirements are met, slides will not be shown. Tables of data should be distributed rather than shown on slides.

Some long range ideas for the Annual Meeting include: that one speaker from each symposium speak at a general session; that a program be organized on feeds and pesticides which is a cooperative effort among Rutgers, AFDOUS, and AOAC; that a training session on pesticide analysis be held at the Annual Meeting; that the meeting include a program on chick edema factor in pesticides and soils.

IDF, ISO, AOAC Cooperation

For several years the AOAC has been working with the International Dairy Federation (IDF) and the International Organization for Standardization (ISO) to develop internationally accepted methods analysis for dairy products. This joint effort has resulted in the development of methods for sampling dairy products; methods for determining the fat content in milk, cheese, evaporated milk, sweetened condensed milk, and dried milk; and methods for determining the acid degree value and refractive index of fat from butter and salt in butter. The AOAC has revised and re-published existing AOAC methods so that they comply with the internationally accepted methods (*JAOAC* **49**, 219 (1966); **50**, 200–204 (1967); **52**, 393–394 (1969)) and, on the basis of adequate supporting data, has adopted and published the methods which did not have AOAC status (*JAOAC* **52**, 394–395 (1969)). The IDF has recently completed a revision and re-publication of existing IDF Standards and publication of new Standards to bring them into conformity with the internationally accepted methods. The ISO is in the process of making similar revisions in its Standards. The acceptance and publication of these uniform methods of analysis by the 3 international organizations and by the *FAO/WHO Code of Principles Concerning Milk and Milk Products and Associated Standards* demonstrates that interested international organizations can develop liaison arrangements, work toward the satisfactory resolution of problems, and develop uniform methods of analysis which can be adopted by all interested international organizations.

Research Instrumentation Course

A 3 week summer course in research instrumentation will be conducted at Polytechnic Institute of Brooklyn for educators, engineers, and scientists from all technical fields who need a working knowledge of electronic instrumentation as applied to problems in research.

The course will be held from July 25 to August 15, 1970, on the downtown Brooklyn campus at 333 Jay St. It will be supported in part by the National Science Foundation (NSF) under its College Teacher Programs. Attending free of charge will be 26 college teachers from all over the United States, who will receive a stipend from NSF for 3 weeks plus travel allowances. In addition, applicants from business and industry will be accepted on a tuition-paying basis of $550, covering all laboratory fees, textbooks, and special notes.

The course is open to industrial and academic scientists and engineers from all disciplines. Professor Kenneth R. Jolls of Polytechnic's Chemical

Engineering Department said that "Medical research workers will find the course valuable and are also invited to apply. There are no specific prerequisites beyond a basic understanding of college physics."

The text for the course will be *Electronics for Scientists* by Malmstadt, Enke, and Toren. Professor Jolls, who will teach the course, said this "unique volume presents both reference materials and detailed experiments for laboratory work. It will remain a valuable addition to the student's reference library long after the course has been completed."

Applicants should secure a place in the course at the earliest possible date. The final date for consideration of applications for NSF support was April 20. Industrial participants must file their applications by June 15. Inquiries may be directed to: Professor Kenneth Jolls, Office of Special Programs, Polytechnic Institute of Brooklyn, 333 Jay St., Brooklyn, N.Y. 11201 (Telephone: 212–643–4442 or 643–2266).

Adoption of Methods

The Association adopted 57 new methods as official first action at the 83rd Annual Meeting in October 1969. The sources of the new methods are given below:

FDA	
Washington	22
Districts	13
Industry	15
State	3
USDA	3
Dept. of Interior	1

New methods were adopted on the following topics: Fertilizers 1, Feeds 1, Pesticide Formulations 2, Drug Residues 1, Drugs 8, Drugs in Feeds 5, Cosmetics 3, Decomposition 1, Mycotoxins 4, Food Additives 1, Coffee and Tea 1, Cacao Products 3, Alcoholic Beverages 4, Dairy Products 2, Fish 2, Flavors and Nonalcoholic Beverages 2, Fruits and Fruit Products 3, Oils, Fats, and Waxes 1, Processed Vegetable Products 1, Extraneous Materials 6, Pesticide Residues 3, and Vitamins 2.

Technicon International Congress

The Technicon International Congress 1970, Advances in Automated Analysis, will be held November 2–4, 1970, at the Hilton Hotel, New York City. In the past, the Congress has enabled about 1,000 scientists to present the latest findings in automated analysis. This year the Congress will hold symposia on the following themes: automatic amino acid analysis in research and other applications; the hematology profile; the immunologic profile; pattern recognition in diagnostic medicine; from research tools to routine applications; new directions in clinical chemistry; pollution control: now or never; meeting new requirements for the food industry; drug regulations; new techniques in inorganic analysis; and histology: state of the art.

Abstracts of papers must be received by August 5, 1970. Write to Nicholas B. Scova, International Congress Director, Technicon Corporation, Tarrytown, N.Y. 10591.

International Award for Research in Pesticide Chemistry

The Pesticide Chemistry Division of the American Chemical Society announced that the first recipient of its annual International Research Award in Pesticide Chemistry will be Professor John E. Casida of the University of California, Berkeley. This award to Professor Casida is in recognition for many outstanding contributions to pesticide chemistry. In particular the award is for his important additions to the chemistry and toxicology and degradation and metabolism of a variety of insecticide chemicals, synergists, and respiratory uncouplers, and for his outstanding ability to inspire many students who continue to conduct fundamental studies in this field of research.

This American Chemical Society Division Award is sponsored by Burdick and Jackson Laboratories, Muskegon, Michigan. It consists of a $500 cash award and a scroll, and the recipient is invited to deliver an award address at a national meeting of the American Chemical Society.

The Pesticide Chemistry Division has arranged a symposium on metabolism, in honor of Professor Casida, to be given at the Joint National Meeting of ACS and Chemical Institute of Canada (Toronto, May 24–29, 1970). Following the symposium Dr. B. Wayne Arthur, President of CIBA Agrochemical Co., will introduce Professor Casida, who will speak on "Mixed-Function Oxidase Inhibitors Useful as Insecticide Chemical Synergists." Details of this program appeared in the March 2 issue of *Chemical & Engineering News*.

Nomination forms for submission of candidates for the International Research Award in Pesticide Chemistry for this year are available from: Dr. James P. Minyard, Mississippi State Chemical Laboratory, State College, Miss., and from: Dr. M. B. Green, Imperial Chemical Industries Ltd., Mond Division, Development Dept., The Heath, Runcon, England.

Chemical Contaminants in Foods

A Symposium, "Chemical Contaminants in Foods—Hazard or Not?," will be sponsored by the Food and Drug Directorate, Department of National Health and Welfare, Canada. The meeting will be

held at the Skyline Hotel, 101 Lyon St., Ottawa, Ontario, June 18 and 19, 1970.

Various aspects of chemical contaminants in foods will be explored by internationally known experts including L. Goldberg (Albany); H. Egan (United Kingdom); B. Oser (New York); J. Howard (Washington); P. N. Magee (United Kingdom); F. Sunderman (Hartford); W. B. Deichmann (Miami); and A. B. Morrison (Ottawa).

For further information contact: H. B. Taylor, Symposium Secretary, Food and Drug Directorate, Ottawa, Ontario, Canada.

Second World Food Congress

The Second World Food Congress will be held June 16–30, 1970, at the Netherlands Conference Centre, The Hague. The purpose of the Congress is to draw attention to food and population problems. The Congress will indicate priorities for action and suggest ways of increasing the resources available. The work will be handled by Commissions, based on (*1*) the problems countries must deal with in their development and (*2*) the major types of resources available and how new resources can be found. A preliminary list of invited speakers includes B. R. Sen, former Director-General of FAO; R. Prebisch, Latin American Institute for Economic and Social Planning in Chile; G. Harrar, Rockefeller Foundation; Professor Tinbergen, Rotterdam School of Economics; R. Jackson, Commissioner, UN Capacity Study, Australia; R. K. H. Gardiner, Executive Secretary of E. C. A., Ghana; P. Hoffman, UNDP Administrator; Lord Snow, British writer; A. Sauvy, French Demograph; M. Numata, Japanese Professor of Botanics; E. Marei, Minister of Agriculture, Cairo; S. R. Sen, Planning Commission of India; V. A. Oyenuga, University of Ibadan; D. Umali, University of the Philippines; Professor Van Lier, Netherlands University; A. Philip, President of OECD's Development Centre.

FAO Fact Sheets, a condensed version of the Indicative World Plan, and background papers for the 8 Commissions may be obtained from: Director-General of FAO, Food and Agriculture Organization, 00100 Rome, Italy.

Pesticide Residue Methods

The Department of National Health and Welfare, Canada, has published a comprehensive manual of pesticide residue methods, *Analytical Methods for Pesticide Residues in Foods*. The text was compiled and edited by H. A. McLeod, P. J. Wales, R. A. Graham (Ottawa), M. Osadchuk (Toronto), and N. Bluman (Vancouver), all of the Food and Drug Directorate. In the *Foreward*, A. B. Morrison, Director of Research Laboratories, states "The analytical methods described in this manual are based on a premise which has formed the foundation for the pesticide analysis program of the Directorate for several years: that ever increasing number and use of pesticides necessitates a systematic approach to residue analysis involving broad screening procedures, rather than a multitude of specific methods for specific residues on specific crops."

The manual has a loose-leaf format, and the 15 sections include information on a general analytical technique, such as preparation of sample; extraction, cleanup, and determinative procedures; interpretation of screening results; confirmatory reactions; and information on apparatus. Each section contains a number of alternative procedures, so the analyst may select the one most appropriate to his problem.

The manual is available from The Queen's Printer, Ottawa, Ontario, Canada; price $5.00.

AOAC Sponsors International Meeting

A 4 day international symposium on Identification and Measurement of Environmental Pollutants will be held in Ottawa on June 14–17, 1971. The prime sponsor of the symposium is the AOAC, co-sponsors are the International Union of Pure and Applied Chemistry, the Chemical Institute of Canada, the Agricultural Institute of Canada, and the National Research Council of Canada.

Specialists on pollution from around the world are being invited to present papers on air, water and land pollution, waste disposal, pesticide pollution, noise contamination, repurification of water, biological indicators, etc. The proceedings of the symposium will be published following the meetings.

For additional information, contact: M. K. Ward, Executive Secretary, International Symposium on Identification and Measurement of Environmental Pollutants, c/o National Research Council of Canada, Ottawa, Ontario, Canada.

INSTRUCTIONS TO AUTHORS

Scope of Articles

The Journal of the AOAC will publish articles that present, within the fields of interest of the Association, (*a*) new methods; (*b*) further studies of previously published methods; (*c*) background work leading to development of methods; (*d*) compilations of authentic data; (*e*) cautionary notes and comments on techniques, apparatus, and reagents; (*f*) reviews of methodology in special fields.

Preparation of Manuscript

Authors are required to submit three copies (one of which must be the original—ribbon—copy) of the complete manuscript, including all tables and all illustrations. The manuscript is to be typewritten on one side only of white bond paper, $8 \times 10\frac{1}{2}$ or $8\frac{1}{2} \times 11$ inches, with minimum page margins of 1 inch, and must be **double-spaced** throughout (including title, authors' names and addresses, footnotes, tables, references, and captions for illustrations, as well as the text itself). Tables and captions for illustrations are to be typed on separate sheets, *not* interspersed through the manuscript. Drawings and photographs should be mounted apart from the text or submitted as separate items, *not* interspersed through the text. Proper placement of tables and illustrations is taken care of by the editorial staff at the time the article is in proof.

Style and Format

The text should be written in clear, concise, grammatical English. Unusual abbreviations should be employed as little as possible and must always be defined the first time they appear. Titles of articles should be fully descriptive. The address of the institution (including zip code) from which the paper is submitted should be given and should be in a form to which inquiries, proofs, and requests for reprints can be sent. Information supplementing the title and authors' names and addresses should be given in footnote form.

Methods, Results and/or Discussion, Acknowledgments, and Recommendations (applicable to reports of General and Associate Referees) should be placed in separate sections under appropriate headings typed in capitals and lower case letters, centered on the page, *not* underscored.

Tables: All tables must be cited in the text consecutively. Tables are numbered by arabic numbers, and every table must have a descriptive title, sufficient so that the table can stand by itself without reference to the text. This title should be typed in lower case letters, *not* capitals, with the exception of the word "Table" and the first word of the descriptive portion of the title, of which the first letter is capitalized. Every vertical column in the table should have a heading; abbreviations may be used freely in the headings to save space, but should be self-evident or must be explained in footnotes. Footnotes to both the headings and the body of the table are indicated by lower case letters in alphabetical order; these letters should be underscored and raised above the line of type. Vertical and horizontal rules should be used sparingly; however, horizontal rules are used to bound the table at top and bottom and to divide the heads from the columns. Authors should refer to recent issues of the Journal for acceptable format of tables; good tables should not exceed the normal page width of the Journal, and authors should attempt to revise or rearrange data to fit this pattern.

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The abbreviation for the journal title should be repeated for each reference; the use of *ibid.* has been discontinued. *This Journal* will be referred to as *JAOAC*.

The compendium of methods of the Association should be listed as follows: *Official Methods of Analysis*, 11th Ed., AOAC, Washington, D.C., 1970, with appropriate section numbers; the edition and year are, of course, subject to change.

Methods: Methods should be written in imperative style, i.e., "Add 10 ml ... Heat to boiling ... Read in spectrophotometer." Special reagents and apparatus should be separated from the details of the procedure and placed in sections with appropriate headings; however, common reagents and apparatus (e.g., concentrated HCl, chloroform, ordinary glassware, ovens, etc.), or those which require no special preparation or assembly, need not be listed separately. The steps of the procedure should not be numbered, but should be grouped together to form a logical sequence of two, three, or four operations. Any very long, detailed operation can be given in a separate section with an appropriate heading (e.g., Preparation of Sample; Extraction and Cleanup; Preparation of Standard Curve). Any necessary calculations should be included. Care should be taken that the number of significant figures truly reflects the accuracy of the method.

Abstracts: Each manuscript should be accompanied by a concise abstract (not more than 200 words). The abstract should provide specific information rather than generalized statements.

Symbols and Abbreviations

kg	kilogram(s)
g	gram(s)
mg	milligram(s)
μg	microgram(s)
ng	nanogram(s)
L	liter(s)
ml	milliliter(s)
μl	microliter(s)
m	meter(s)
cm	centimeter(s)
mm	millimeter(s)
μm	micrometer(s)
nm	nanometer(s) (millimicron)
′	foot (feet)
″	inch(es)
lb	pound(s)
oz	ounce(s)
ppm	parts per million
ppb	parts per billion
rpm	revolutions per minute
psi	pounds per square inch
sp gr	specific gravity
bp	boiling point
mp	melting point
id	inside diameter
od	outside diameter
hr	hour(s)
min	minute(s)
sec	second(s)
%	per cent
$\overline{\$}$	standard taper
N	normal
M	molar
m*M*	millimolar

(Note: Spectrophotometric nomenclature should follow the rules contained in *Official Methods of Analysis*, "Definitions of Terms and Explanatory Notes.")

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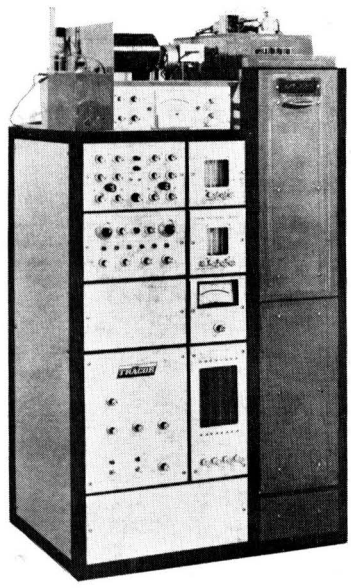

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