ANTIBIOTICS AND DRUGS IN FEEDS

Collaborative Test of a Microbiological Assay for Low Chlortetracycline Concentrations In Final Feeds

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Commercial poultry, cattle, and swine feeds, containing low levels of chlortetracycline (CTC) activity (5, 10, and 20 ppm CTC), were assayed collaboratively in eight laboratories by compensating CTC standard curves prepared with diluent from sodium hypochlorite-inactivated animal feed extracts. The coefficients of variation for poultry, cattle, and swine feeds, individually, were 20, 13, and 10%, respectively. The combined data from those laboratories on both poultry and cattle feeds yielded a coefficient of variation of 12%, while the combined data from those laboratories assaying poultry and swine feeds gave a coefficient of variation of 15%. The inactivation of CTC in animal feed extracts by sodium hypochlorite plus heat appears to be operative in all eight laboratories.

This collaborative study was conducted to evaluate the previously reported procedure (1), in which sodium hypochlorite was used to inactivate antibiotic feed extracts (2), in the preparation of compensating chlortetracycline (CTC) standard curves for feed assays.

In accordance with recommendations for interlaboratory tests (3), feed samples at different concentrations were submitted to eight laboratories.

From commercial feeds of the same composition employed in earlier tests (1), duplicate batches of poultry, cattle, and swine feeds were formulated to contain added chlortetracycline (CTC) activity at 0, 5, 10, and 20 ppm CTC. The 20 ppm CTC level was diluted serially with blank feed to obtain the lower concentrations; all mixtures were blended 15 minutes with a Patterson-Kelley double-cone "V" blender.

Since the method to be tested was designed to conform (wherever possible) with present accepted practices, the reagents and test conditions described in the official procedure for CTC in feeds (4) were used. (The official method, as outlined, was forwarded to each collaborator.) However, where alternatives in the official method are indicated, the selections have been made primarily for convenience and ease in processing a number of samples.

Some of the main differences between the low-level CTC feed assay method under test and the official procedure (4) are as follows:

1. *Section 33.131(b):* The CTC standard curve is extended to include concentrations at 0, 0.005, 0.01, 0.02, 0.04, 0.08, 0.16, and 0.32 μg CTC/ml.

The plain phosphate buffer pH is checked and adjusted, if necessary, with KOH or H_3PO_4 to be not less than pH 4.5 or greater than pH 4.7.

All standard CTC concentrations are prepared in a compensating diluent. This diluent contains the CTC feed extract inactivated by sodium hypochlorite treatment; the extract is then diluted with buffer in the same manner as the CTC feed sample under test.

2. *Section 33.132(b):* The *B. cereus* var. *mycoides* spore suspension is tested so that as little as 0.005 μg CTC.HCl/ml, in plain

buffer, will just show a zone on the preliminary trial plates.

3. *Section 33.133:* A 50 g sample is extracted with a total of 200 ml acid-acetone; the pH of the feed extractant mixture is tested and adjusted, if necessary, to pH 1.0–2.0 (preferably pH 1.5); a 10 ml portion of this extract is mixed with 40 ml buffer before adjusting to pH 4.5 with alkali; and the adjusted solution is quantitatively transferred to make 250 ml final solution. All low-level CTC feed samples used in this study are tested at the same final dilution so that a single compensating CTC standard curve may be used for that particular type of feed.

4. *Section 33.134:* Additional concentrations may indicate an arc in plotting the respective CTC standard curves, and the line of best fit could be drawn by inspection.

Each collaborator received a supply of crystalline CTC hydrochloride to prepare the standard solutions and two pre-weighed jars (50 g feed/jar) for each batch and each CTC concentration under test in that laboratory. Although an extra set of samples was included (in case of shipping accidents), only a single feed sample from each batch at a given concentration was to be tested. All samples were kept refrigerated until tested.

METHOD

Preparation of Feed Samples

Use operating conditions in official method **33.131–33.134** (4). Make certain that all apparatus in contact with the sample is clean and detergent-free. A high speed blender is preferred for extraction; clean glass jar-impeller units and autoclave in pail or tray of water for ca 10 min. at 121°C. Before assaying, rinse drained, cooled blender jar with ca 50 ml acid-acetone and drain. Similarly, autoclave cleaned stainless steel cylinders under distilled water, drain, and then heat-treat and cool to room temperature.

Preparation of Acid-Acetone Feed Extracts

Empty contents of 50 g feed-sample jar into 1 qt blender jar and rinse sample jar with 50 ml acid-acetone extractant; add rinse to blender jar. Place additional 100 ml acid-acetone in blender jar, cover, and blend at high speed for 3 min. (A single-speed Waring Blendor was used in our laboratory.)

Test pH of blended mixture (should be 1.0–2.0); if necessary add concentrated HCl, note volume, and re-blend.

Quantitatively transfer blender jar contents to 250 ml centrifuge bottle with an additional 50 ml acid-acetone, or a lesser amount to compensate for HCl volume used in any pH adjustment; shake centrifuge bottle thoroughly. Centrifuge and collect supernatant.

Mix 10 ml feed extract with 40 ml pH 4.5 phosphate buffer and adjust to pH 4.5–4.7 with 1*N* NaOH. Quantitatively transfer to 250 ml volumetric flask and make to volume with buffer. (Assay solution = 1/100 dilution of feed.)

For CTC standard curve diluent, inactivate[1] portion of extract obtained from most potent feed under test, i.e., *Level 4* (20 ppm CTC), with sodium hypochlorite solution as follows:

Add 80 ml pH 4.5 phosphate buffer to 20 ml acid-acetone feed extract in 600 ml glass beaker. Adjust pH to 4.5–4.7 with 1*N* NaOH. Add 1.0 ml fresh 5.25% sodium hypochlorite solution and *heat in the open beaker* for 30 min. in a boiling water bath; stir and mix thoroughly every 10 min. while heating. Cool beaker contents to room temperature under running tap water and quantitatively transfer to 100 ml volumetric flask; rinse beaker with reagent grade acetone to make 100 ml volume. Dilute 100 ml treated solution with pH 4.5 buffer to make 500 ml CTC standard curve diluent.

Prepare ten-fold serial dilutions of stock CTC standard solution in feed extract diluent to obtain 1.0 μg CTC/ml. From this solution, prepare compensating CTC standard curve dilutions in feed-extract diluent at 0.32, 0.16, 0.08, 0.04, 0.02, 0.01, and 0.005 μg CTC/ml. Use 0.04 μg/ml concentration as the reference solution. Proceed with the standard curve and assay, as in **33.134** (4).

Results and Discussion

The assay results from all collaborating laboratories were received within 8 weeks of the date of shipment (Table 1). Unless otherwise indicated, the results for a given feed were obtained on the same assay day

[1] Since poultry feed batches A and B are mixed from the same basic feed formulation, pool extracts from both *Level 4* feeds in equal parts to inactivate and prepare standard curve diluent; follow the same practice for the other feed under test.

Table 1. Collaborative chlortetracycline (CTC) microbiological assay results on animal feeds

	0		5 ppm		10 ppm		20 ppm	
	Batch		Batch		Batch		Batch	
Lab.	A	B	A	B	A	B	A	B
Poultry Feed								
1	0	0	4.8	5.8	9.0	9.5	21.0	21.0
2	0	0	4.2	4.0	7.1	7.6	14.7	15.4
3	0.14[a]	0.15[a]	5.0	4.3	13.8[a]	11.5	21.7	23.0
4	0	0	4.3	3.4	7.8	6.6	16.0	19.0
5	0.3	0.9	9.4	5.5	11.4	14.6	20.6	21.8
6	0	0	5.1	4.9	10.3	10.5	20.5	18.5
7	0.54	0	4.6	4.2	7.4	8.2	15.3	15.5
8	0	0	4.6	4.3	12.0	10.0	20.0	22.5
CTC ppm Mean			5.25	4.55	9.85	9.8	18.75	19.6
% of Formulated Potency			105	91	98.5	98	93.8	98.0
Cattle Feed								
1	0	0	5.5	6.7	10.5	12.5	22.0	23.0
2	0	0	4.3	4.4	8.7	9.9	19.5	20.4
3	0.10	0.11	3.8	3.8	8.8	9.2	22.0	18.0
4	0	0	2.9	2.4	7.6	6.1	13.5	17.5
8	0	0	4.3	4.9	9.1	11.5	21.5	19.5
CTC ppm Mean			4.16	4.44	8.94	9.84	19.7	19.7
% of Formulated Potency			83.2	88.8	89.4	98.4	98.5	98.5
Swine Feed								
5	0.6	0.5	6.5	5.5	13.8	12.4	23.0	19.5
6	0	0	5.4	6.0	10.0	10.0	19.5	17.5
7	0	0	3.0	3.6	7.1	7.2	14.0	13.0
8	0	0	4.8	4.9	10.3	10.6	18.0	21.4
CTC ppm Mean			4.9	5.0	10.3	10.05	18.6	17.85
% of Formulated Potency			98	100	103	101	93	89

[a] Different assay day and standard curve.

in the respective laboratories. No collaborator indicated difficulty with the sodium hypochlorite-inactivation procedure. However, the blender extraction plus pH adjustment and re-blending process was found cumbersome in 2 of the 8 laboratories.

All microbiological assay values received from the collaborators for the poultry, cattle, and swine feeds tested are presented in Table 1. The mean assay values, when compared to the expected or formulated potency, indicate CTC recoveries of 91–105% for poultry feed, 83–98% for cattle feed, and 89–103% for the swine feed. Although the average recovery appeared to be within reasonable limits for the low CTC concentrations under test, results were variable among laboratories. Since the poultry feed series was tested in all 8 laboratories, the values reported for this feed were ranked and scored (3) (Table 2). For a group of 8 laboratories and 6 materials, the approximate 5% two-tail limits in ranking scores are 12 and 42, with 27 as the average score;

Table 2. Ranked microbioassay results for chlortetracycline (CTC) on poultry feed (3)[a]

	5 ppm		10 ppm		20 ppm		
Lab.	A	B	A	B	A	B	Lab. Score
1	4	1	5	5	2	4	21
2	8	7	8	7	8	8	46
3	3	4.5	1	2	1	1	12.5
4	7	8	6	8	6	5	40
5	1	2	3	1	3	3	13
6	2	3	4	3	4	6	22
7	5.5	6	7	6	7	7	38.5
8	5.5	4.5	2	4	5	2	23

[a] Approximate 5% two-tail limits for 8 collaborators and 6 materials (3); score limits: lower, 12; upper, 42; average, 27.

Table 3. Analysis of variance for chlortetracycline (CTC) microbioassay of animal feed (5, 10, 20 ppm CTC)[a]

Source of Variance	Poultry Feeds			Cattle Feeds			Swine Feeds		
	Degrees of Freedom	Variance	F Values	Degrees of Freedom	Variance	F Values	Degrees of Freedom	Variance	F Values
Batch (B)	1	5.40	<1	1	7.00	3.30	1	0.63	<1
Dilution (D)	2	1.22	<1	2	15.88	7.49	2	9.75	15.48
B × D (Error)	2	14.96		2	2.12		2	0.63	
Std dev.		3.87			1.46			0.79	
Coeff. of var.		0.20			0.08			0.04	
Laboratory (L)	7	92.45	6.12	4	79.20	14.56	3	107.56	27.58
B × L	(7)	(2.78)		(4)	(4.41)		(3)	(6.91)	
D × L	(14)	(4.78)		(8)	(5.13)		(6)	(4.66)	
B × D × L	(14)	(31.58)		(8)	(3.11)		(6)	(1.63)	
Error	35	15.10		20	5.44		15	3.90	
Std dev.		3.89			2.33			1.97	
Coeff. of var.		0.20			0.13			0.10	

[a] Variances in parentheses are incorporated into error.

the score for one laboratory was greater than the upper limit.

Statistical analysis and interpretation of the data (Tables 3–6) indicate insignificant effect(s) due to different feed batches, CTC level, or type of feed; the main source of variance was among the laboratories. Since the varying levels in each feed were obtained by serial dilution of the 20 ppm CTC concentration (instead of independent mixing), it was decided to regard each assay as subject to a dilution factor correction (i.e., 1 ×, 2 ×, and 4 × for the 20, 10, and 5 ppm levels, respectively) to bring all assay values into a homogeneous distribution. The coefficients of variation for poultry, cattle, and swine feeds were 20, 13, and 10%, respectively; in combining the data from laboratories testing poultry and cattle feeds, the coefficient of variation was 12%, and the combined data from those laboratories testing poultry and swine feeds yielded a coefficient of variation of 15%.

Although significant variance was observed among laboratories, the values from 3 of the 8 laboratories (Laboratories 1, 6, and 8) agreed closely with the CTC potencies as formulated and with each other.

The CTC-inactivation procedure, using sodium hypochlorite and heat, appears to be operative in all of the cooperating laboratories for the preparation of compensating CTC standard curves. However, the variation in CTC recoveries may reflect differ-

Table 4A. Poultry feeds: relation of laboratory to chlortetracycline (CTC) level and effects of laboratory, batch, and concentration on CTC assay

Lab.	CTC Concentration in Poultry Feed						Effect of Laboratory		
	CTC, ppm	20 ppm, % Recovery	CTC, ppm	10 ppm, % Recovery	CTC, ppm	5 ppm, % Recovery	Lab.	Average CTC, ppm	Deviation, % from Expected
1	21.0	105.0	18.5	92.5	21.2	106.0	1	20.3	+ 1.5
2	15.0	75.0	14.7	73.5	16.4	82.0	2	15.4	−23.0
3	22.4	112.0	25.3	126.7	18.6	93.0	3	22.1	+10.5
4	17.5	87.5	14.4	72.0	15.4	77.0	4	15.8	−21.0
5	21.2	106.0	26.0	130.0	29.8	149.0	5	25.7	+28.5
6	19.5	97.5	20.8	104.8	20.0	100.0	6	20.1	+ 0.5
7	15.4	77.0	15.6	78.0	17.6	88.0	7	16.2	−19.0
8	21.2	106.0	22.0	110.0	17.8	89.0	8	20.4	+ 2.0
Least significant differences at P =			0.05	0.01			Least significant differences at P =	0.05	0.01
Between labs and dilutions			19	22			Between labs	8.8	10.8
Deviation from expected, %			75	90			Deviation from expected, %	38.5	48.0

Table 4B. Poultry feeds: relation of laboratory to chlortetracycline (CTC) level and effects of laboratory batch and concentration on CTC assay[a]

Effect of Dilution			Effect of Batch		
Dilution Factor	CTC, ppm	Deviation, %		CTC, ppm	Deviation, %
1 × 20 ppm CTC	19.2	−2.0	A	19.8	−1.0
2 × 10 ppm CTC	19.7	−1.5	B	19.1	−4.5
4 × 5 ppm CTC	19.6	−4.0			

[a] Differences and deviations are insignificant.

Table 5. Effects of feed, batch, dilution, and laboratory on chlortetracycline (CTC) assays of combined feed samples

Poultry and Cattle Feed Data Combined			Poultry and Swine Feed Data Combined		
	CTC, ppm	Deviation from Expected, %		CTC, ppm	Deviation from Expected, %
Effect of Feed Type					
Poultry	18.7	− 6.5	Poultry	20.6	+ 3.0
Cattle	18.5	− 7.5	Swine	19.5	− 2.5
No significant difference			No significant difference		
Effect of Batch					
A	18.5	− 7.5	A	20.4	+ 2.0
B	18.8	− 6.0	B	19.7	− 1.5
No significant difference			No significant difference		
Effect of Dilution					
1 × 20 ppm CTC	19.6	− 2.0	1 × 20 ppm CTC	18.8	− 6.0
2 × 10 ppm CTC	18.9	− 5.5	2 × 10 ppm CTC	20.7	+ 3.5
4 × 5 ppm CTC	17.5	−12.5	4 × 5 ppm CTC	20.6	+ 3.0
Least significant difference at P =		0.05 0.01	No significant difference		
Between dilutions		1.8 2.7			
Deviation from expected, %		9.0 13.5			
Effect of Laboratory					
1	21.8	+ 9.0	5	24.7	+23.5
2	17.0	−15.0	6	20.3	+ 1.5
3	19.9	− 0.5	7	14.9	−25.5
4	14.5	−27.5	8	20.2	+ 1.0
8	20.1	+ 0.5			
Least significant difference at P =		0.05 0.01	Least significant difference at P =		0.05 0.01
Between laboratories		2.6 3.2	Between laboratories		3.8 4.7
Deviation from expected, %		12.0 14.5	Deviation from expected, %		15.0 18.5

ences in extraction efficiency for the low CTC concentrations present in these animal feeds.

Recommendations

It is recommended that additional collaborative studies be conducted at these low CTC concentrations in animal feeds.

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The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee B, and was accepted by the Association. See *This Journal*, **49**, 165–167 (1966).

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Table 6. Summarized analyses of variance of microbioassays of chlortetracycline in feed (5, 10, and 20 ppm CTC)[a]

Source of Variance	Poultry and Cattle Feed Data Combined			Poultry and Swine Feed Data Combined		
	Degrees of Freedom	Variance	F Values	Degrees of Freedom	Variance	F Values
Feed (F)	1	0.64	<1	1	14.52	8.59
Batch (B)	1	1.23	<1	1	5.88	3.48
Error (F × B)	1	6.94		1	1.69	
Std Dev.		2.63			1.30	
Coeff. of Var.		0.14			0.07	
Dilution (D)	2	21.13	6.05	2	18.59	2.52
(F × D)	(2)	(1.10)		(2)	(0.49)	
(B × D)	(2)	(0.79)		(2)	(7.41)	
(F × B × D)	(2)	(8.58)		(2)	(14.23)	
Error	6	3.49		6	7.38	
Std Dev.		1.87			2.72	
Coeff. of Var.		0.10			0.14	
Laboratory (L)	4	99.38	20.53	3	193.01	23.74
F × L	4	33.98	7.02	(3)	(5.23)	
(B × L)	(4)	(6.96)		(3)	(7.62)	
(F × B × L)	(4)	(<0.01)		(3)	(1.26)	
(D × L)	(8)	(9.05)		(6)	(11.42)	
(F × D × L)	(8)	(4.37)		(6)	(4.66)	
(B × D × L)	(8)	(4.27)		(6)	(10.04)	
(F × B × D × L)	(8)	(3.05)		(6)	(11.53)	
Error	40	4.84		33	(8.13)	
Std Dev.		2.2			2.85	
Coeff. of Var.		0.12			0.15	

[a] Variances in parentheses are incorporated into error.

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Determination of Diethylstilbestrol in Feeds

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Experimental extractions of diethylstilbestrol from animal feed, containing alfalfa meal and other rich sources of chlorophyll, indicated that a reaction may occur between the drug and a component of the vegetable material. Recovery data, obtained by a new 1.5 hour acid-acetone-chloroform extraction, indicate that an acid-base reaction occurs with the magnesium ion in chlorophyll. Phosphoric acid frees the diethylstilbestrol and permits complete recovery with organic solvents. The drug is separated from the vegetable extracts by elution with ethyl ether from a barium hydroxide column. The final determination is made by a modification of the AOAC irradiation method. Recoveries are comparable to those of the longer AOAC method.

The official, first action AOAC method (1) for diethylstilbestrol (DES) in feeds requires a minimum 16-hour extraction and a multiple funnel cleanup procedure. In addition to these disadvantages, emulsions may form, impurities may still be present, and high blanks may be obtained when DES is irradiated in buffered alcohol. Lower blanks are obtained with acetic acid, but DES is unstable in this medium. The method proposed here overcomes these difficulties.

Cheng and Burroughs (2) proposed a 3–5 hour extraction in a Goldfisch apparatus. DES was separated on a sodium hydroxide-Celite chromatographic column, followed by a colorimetric determination with antimony pentachloride or an ultraviolet irradiation method. These workers concluded that clover and alfalfa hays might interfere with this method.

In developing the present AOAC method (1), Munsey (3) concluded that low recoveries are a result of combination of DES with the feed or from its destruction. In a later collaborative study, using the 16-hour extraction, he concluded that the alfalfa did not interfere in the determination of DES (4).

The irradiation procedure developed by Goodyear, *et al.* (5) and modified by Banes (6, 7) and by Summa and Graham (8) uses Vycor 7900 tubes spaced in a radius around a 15-watt germicidal lamp. Banes (6) showed that the yellow irradiation product of DES is destroyed in acid or strong alkaline solution but is stable to further irradiation in slightly alkaline buffered alcohol solution.

Summa and Graham (8) showed that dienestrol, a dihydroxy synthetic hormone similar to DES, is easily extracted with chloroform from tablet materials on a Celite column. This led the author to believe that if DES were not chemically bound to some feed material, it could be readily extracted. Subsequent work confirmed this fact.

The method presented here utilizes a 1.5-hour acid-acetone-chloroform extraction. The extracted DES is obtained in a purer form by combining column chromatography with separatory funnel cleanup, thus considerably reducing the blank value and the difficulties encountered in radiation.

METHOD

Apparatus and Reagents

(a) *Chromatographic columns.*—2.5 × 30–40 cm.

(b) *Vycor No. 7900 silica test tubes.*—1.8 × 15.0 cm.

(c) *Spectrophotometer.* — Beckman Model DU, or equivalent, with matched 1 cm cells.

(d) *Irradiation lamp without reflector.*—15 watt General Electric Germicidal lamp (15T8). Mount a fluorescent-type lamp holder vertically to the inside wall of a 5-gallon solvent can which has one end replaced with ¾ inch plywood board. Drill six 2 cm diameter holes in the plywood, with the center of each hole being on an arc 13 cm from the surface of the lamp. Cover the exposed portion of the lamp with aluminum foil.

(e) *Diethylstilbestrol standard solution.*—10 μg/ml in ethanol.

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Determination

Weigh accurately 10 g "40-mesh" sample. Transfer to 250 ml 24/40 ⅀ erlenmeyer, add 10 ml 12*N* H_3PO_4, and mix until feed is uniformly wet. Add 30 ml acetone and small magnetic stirring bar. Connect water-cooled condenser to flask; stir and reflux 1 hr, using a hotplate-magnetic stirrer combination. Add 25 ml $CHCl_3$; stir and reflux additional 30 min.

Filter cooled solvent through a 75 ml medium porosity sintered funnel into 100 ml volumetric flask under bell jar. Transfer feed sample, with 10 ml portions acetone-$CHCl_3$ (1 + 1), releasing suction between additions, until diluted to volume. Mix and pipet aliquots containing about 22 μg DES into two 250 ml beakers labeled A and B. To B add 2 ml (20 μg) diethylstilbestrol standard solution. Evaporate each to dryness at room temperature with gentle air current.

Treat each sample as follows: Add 2 ml saturated $Ba(OH)_2$ solution and 3 ml petroleum ether, and mix. Add 4 g acid-washed Celite 545 and mix until uniform. Tamp mixture firmly onto a column of 1 ml water in 2 g Celite. Scrub beaker with few drops water and about 1 g Celite, and transfer to columns. Then scrub beakers with glass wool plug and tamp plug firmly onto columns. Wash beaker with 50 ml petroleum ether. Let wash pass through column and discard.

Rinse beaker with 100 ml water-saturated ethyl ether and transfer to columns. Continue eluting DES with ethyl ether until about 300 ml is collected in 500 ml separator. Save beakers.

Extract DES from ether with three 10 ml portions of 1*N* NaOH saturated with K_2SO_4 and ether. Shake each extract vigorously about 1 min. Combine NaOH extracts in 125 ml separator. Finally wash ether with 5 ml water. Add water to NaOH extracts, mix, and discard ether. Extract NaOH layer with three 10 ml portions of water-washed $CHCl_3$ and pass each $CHCl_3$ washing through a second separator containing 10 ml NaOH. Discard $CHCl_3$ (and small emulsion interface which may form between NaOH and $CHCl_3$). Add the 10 ml NaOH to combined NaOH extracts in 125 ml separator.

Add 2 ml concentrated H_3PO_4 to this separator and extract DES from acidified solution with four 15 ml portions of $CHCl_3$. Pass each extract through second separator containing 10 ml 1*M* K_2HPO_4. Shake, let separate, and pass each $CHCl_3$ extract through 10 g anhydrous Na_2SO_4 in funnels (60°) plugged with glass wool. Collect $CHCl_3$ in original 250 ml beaker. Evaporate $CHCl_3$ with aid of water bath (40–45°C) and gentle air current. Evaporate last 2 or 3 ml at room temperature.

Pipet 10 ml 0.1*M* K_2HPO_4-EtOH (1 + 1) solution into beaker, mix, and filter into 25 ml glass-stoppered erlenmeyers, using S&S 589 (9 cm) Blue Ribbon paper, or equivalent, and keep funnel covered while filtering.

Transfer about half of each filtrate to Vycor tubes, and irradiate exactly 30 min. Determine absorbance of the irradiated and nonirradiated portions at 415 mμ.

Calculate % diethylstilbestrol in sample according to formula:

$$\% \text{ DES} = 20A/[(10{,}000)(B - A)(\text{wt feed in aliquot})].$$

A = absorbance of irradiated A − nonirradiated portion.

B = absorbance of irradiated B − nonirradiated portion.

The constant 20 represents the added 20 μg DES in B.

Confirmation of Identity of DES

Evaporate the nonirradiated portion to about 1 ml, mix with 2 g Celite, and pack into column. Elute DES with $CHCl_3$; collect 50 ml. Evaporate to 2 ml, transfer to graduated centrifuge tube, and continue evaporation to 0.2 ml. Spot 0.1 ml on a double width microscope slide coated with silica gel G, along with a DES standard. Develop the chromatogram with acetone-$CHCl_3$ (3 + 17). Air-dry and irradiate 5 min. with shortwave UV lamp. Two spots will appear with R_f values of about 0.4 and 0.6, the same as standard.

Discussion

This method was developed to eliminate the 16-hour extraction time required by the official method for DES in feeds. Since a solvent or mixture having a high propensity for DES was important, various solvents were investigated by spotting TLC microscope slides with DES in various solvents and comparing the R_f values. Alcohols were not considered because they extract excessive amounts of gummy materials. Acetone had an R_f value of one but was a poor fat solvent. Chloroform had a lower R_f value and was an ideal fat solvent. Preliminary DES extraction tests on a Celite column with an acetone-chloroform mixture (1 + 1) gave very promising results; however, this

Table 1. Diethylstilbestrol determination by the AOAC method, the proposed method as written, and the proposed method without acid extraction

Sample	Type of Premix[a]	% Declared	Diethylstilbestrol, % Found: AOAC Method	Proposed Method	Proposed Method Without H_3PO_4
I Concentrate	A	0.0011	96.4	96.3	74.2
II Concentrate	S	0.0022	91.0	89.7	75.5
			95.5		
II Premix	S	0.22	—	93.2	80.0
III Concentrate	A	0.0011	100.0	99.4	45.5
			100.0		
III Premix	A	0.44	—	97.3	67.0
IV Concentrate	U	0.0011	92.7	93.6	—

[a] A = alfalfa meal; S = vegetable oil and solvent-extracted soybean feed; U = unknown.

solvent mixture would not completely extract DES from feed concentrates and premixes containing alfalfa or other high chlorophyll components. The use of phosphoric acid made DES more readily extractable, thus indicating that the DES was chemically bound with a component in the feed. Additional work confirmed this and showed that it was especially pronounced in feeds prepared with alfalfa meal premixes. The addition of acid was based on the theory that the interfering element was the magnesium in chlorophyll. Difficulties encountered in extracting DES from a magnesium silicate column with chloroform confirmed this theory.

The barium hydroxide-Celite column was used in this method after it was discovered that ethyl ether would elute the DES and leave most of the emulsion-forming vegetable extractives behind. Barium hydroxide was superior to other alkaline substances for this purpose. Chloroform, incidentally, did not extract DES from this column.

In the irradiation step, buffered alcohol was selected because DES was more stable in this medium. When kept in the dark, DES residues may be safely stored overnight in this solvent prior to irradiation. The irradiation lamp and Vycor tubes were suggested by Summa and Graham (8) and their use gave straight-line curves in the range of 10–50 μg.

Four samples of commercial feed concentrates and two premixes were analyzed for DES by the proposed method as written. A second determination for DES was made on each at the same time, using this method without the addition of phosphoric acid to the feed. The second determination was done to demonstrate the necessity of adding the acid. A third determination of DES in each of the concentrates was made by the AOAC method. Results obtained by the three analytical methods are shown in Table 1. Table 2 gives the blank absorbance values obtained with the AOAC and the proposed methods.

The results of the proposed method are comparable with those obtained by the AOAC method. The analysis can be performed within 8 hours because of the decrease in extraction time from 16 hours to 1.5 hours. Column chromatography simplifies the isolation of DES residues through separator cleanups, and this results in lower blank absorbances.

References

(1) *Official Methods of Analysis,* 10th Ed., Association of Official Agricultural Chem-

Table 2. Blank readings from the AOAC method and proposed method

Sample	Blank Absorbance: AOAC Method	Proposed Method
I Concentrate	0.075	0.037
II Concentrate	0.079	0.028
II Premix	—	0.007
III Concentrate	0.151	0.058
III Premix	—	0.005
IV Concentrate	0.115	0.049

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(2) Cheng, E. W., and Burroughs, W., *This Journal*, **38**, 146–150 (1955).
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Analytical Methodology of Oxytetracycline in Feed

By D. C. BILLMAN, JR. (Chas. Pfizer & Co., Inc., Terre Haute, Ind. 47800)

Preliminary evaluations have been conducted of the AOAC method for the assay of oxytetracycline in feed at the level of 10 g/ton. With careful techniques, nonmineral feeds containing 10 g/ton oxytetracycline can be assayed. Mineral feeds containing 10 g/ton oxytetracycline gave erratic results; further studies are planned with the use of compensating standard curves.

Analytical methods are needed to assay oxytetracycline in feeds at the 10–100 g per ton range. AOAC studies in recent years have been conducted on premixes containing substantially high levels of the antibiotic, and mineral interference makes it important to have satisfactory and collaboratively verified methods for low levels of this antibiotic in feeds.

We investigated the sodium hypochlorite heat method[1] to prepare a diluent for the standard. We used 10 g of oxytetracycline per ton, since this would cover the range in which assay difficulties are the greatest. This paper presents a preliminary report of our studies.

Experimental Studies

Two test samples were prepared by blending an oxytetracycline (OTC) premix into mineral and nonmineral feeds to a theoretical level of 10 g OTC activity/ton. The feed ingredients are listed in Table 1. The blends were tested by a modified AOAC cylinder-plate procedure with *B. cereus* var. *mycoides* (ATCC No. 9634); a single 9 ml inoculated agar layer was poured into each sterile flat-bottomed Petri dish (Plastomatic No. 94), equipped with a Brewer Petri metal top with absorbent disc (BBL No. 05-264-A and No. 05-264-C).

A three-plate assay was employed, each plate containing two cylinders filled with plain-buffer OTC reference solution (0.20 μg/ml), and three cylinders with appropriate assay solution.

Single determinations were performed on six different days. Prior to filling the cylinders, the assay solution was divided in

Table 1. Ingredients of mineral and nonmineral feed

Ingredient	%
Mineral Feed	
Calcium carbonate	53.487
Dicalcium phosphate	32.500
Dolomitic limestone	10.000
Ferric oxide	2.000
Zinc oxide	0.030
Cobalt carbonate	0.023
Potassium iodide	0.022
Manganous oxide	0.019
Copper oxide	0.016
Vitamin D_2 supplement	0.028
Mineral oil	1.800
Natural and artificial flavors	0.075
	100.000
Nonmineral Feed	
Yellow corn	63.0
Soybean meal (50%)	24.0
Alfalfa meal (17%)	2.5
Calcium carbonate	6.2
Dicalcium phosphate	3.2
Salt, iodized	0.6
Vitamins and trace minerals	0.5
	100.0

[1] See *This Journal*, **48**, 271–277 (1965).

half: one aliquot was mixed and immediately placed in the cylinders; the second aliquot was filtered through Reeve Angel No. 812 filter paper to remove any turbidity present before placing it in the cylinders.

Compensating standard curves for blank and sodium hypochlorite - inactivated samples[1] were prepared as follows:

Use extractant, buffer, and operating conditions as in method **33.152–33.154** (10th Ed.).

Mix feed thoroughly (grind in mill if necessary just before extraction and assay) and weigh 10 g portion into a 250 ml centrifuge bottle. Add 100 ml acid-methanol, stopper, and shake well for 5 min. Centrifuge 15 min. at 2000 rpm. Remove 20 ml supernatant and adjust to pH 4.5 with 1*N* NaOH. Dilute adjusted solution with enough pH 4.5 buffer to obtain estimated concentration of 0.20 μg/ml.

For hypochlorite-inactivated OTC extracts, adjust pH of 20 ml extract as above, add 40 ml pH 4.5 buffer and 0.5 ml 4–6% sodium hypochlorite solution, and heat in beaker or open Erlenmeyer flask for 30 min. in boiling water bath. Mix thoroughly every 10 min. while heating. Cool to room temperature in running tap water, add 20 ml methanol, and dilute with pH 4.5 buffer to give same final volume as sample. Use this treated extract as diluent to prepare compensating OTC standard curve and reference curve.

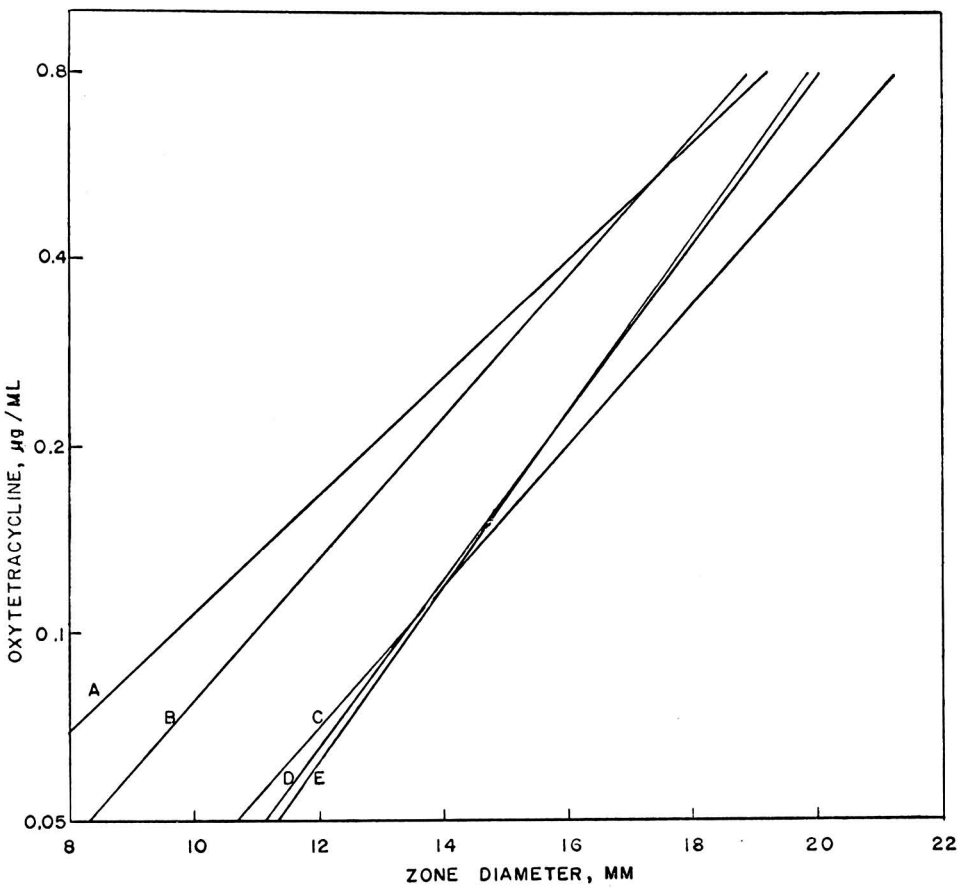

Fig. 1—**Standard curves for oxytetracycline (OTC): *A*, Inactivated mineral feed sample. *B*, Blank mineral feed. *C*, Plain buffer. *D*, Blank, nonmineral feed. *E*, Inactivated nonmineral feed.**

Table 2. Microbiological assay comparisons for nonmineral feed containing oxytetracycline (OTC)

Curve	Assay Solution Treatment	Av., (g/ton)	% Recovery	Std Dev.	Coeff. of Var., %
Plain buffer	M[a]	7.87	78.7	1.81	23
Std curve	F[b]	8.44	84.4	1.68	20
Blank feed	M[a]	8.64	86.4	2.23	26
Ext. curve	F[b]	8.94	89.4	2.20	25
NaOCl-inactivated	M[a]	7.64	76.4	1.50	20
Feed ext. curve	F[b]	7.94	79.4	1.52	19

[a] Final assay solution was mixed immediately prior to filling cylinders.

[b] Final assay solution was filtered prior to filling cylinders.

Results and Discussion

Results (Table 2) show that nonmineral feeds at a level of 10 g per ton of oxytetracycline can be assayed under carefully controlled conditions. The average recovery ranged from 76.4 to 89.4%. Since the negative feed extract curve gave the best recovery, a blank feed should be incorporated in the standard. Using the sodium hypochlorite heat method to prepare a diluent for the standard gave the lowest recovery. Filtering the assay solution before filling the cylinders gave the highest recovery.

Test variation for the mineral feed was so great that it was not evaluated statistically. Ranges for each curve were as follows (g/ton): 1.5–5.2 for the plain buffer curve, 2.3–11.4 for the blank feed curve, and 2.3–11.7 for the inactivated sample curve; *see* Fig. 1.

When blank mineral feed and sodium hypochlorite-inactivated diluent extracts were used in standard curves, a response different from the buffer curves was obtained. This different response improved the recovery of oxytetracycline in feed. The assay reproducibility of mineral feed containing 10 g per ton was very poor. None of the methods investigated resulted in an assay procedure in mineral feeds at this level.

Recommendations

It is recommended—

(1) That collaborative study of oxytetracycline in a nonmineral feed at 10 g per ton be initiated.[2]

(2) That analytical methodology be investigated in our laboratories to develop a method to assay mineral feeds containing oxytetracycline at a level of 50 g per ton. (We have had considerable success with the reproducibility of the AOAC method in mineral feeds at this level.)

[2] Subcommittee B did not concur in this recommendation; they recommended further study. See *This Journal*, **49**, 165–167 (1966).

This report of the Associate Referee was presented at the Seventy-ninth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 11–14, 1965, at Washington, D.C.

Collaborative Study of an Ultraviolet Spectrophotometric Method for Griseofulvin in Animal Feed

By PETER KABASAKALIAN (Animal Health Division, Schering Corp., Bloomfield, N.J. 07003)

Collaborative studies were made of an ultraviolet spectrophotometric method for determining griseofulvin in animal feed. Prior to measurement, the antibiotic is isolated from the feed by extraction with chloroform and separated from ultraviolet-absorbing interferences in the extract by column chromatographic fractionation. The average recovery of griseofulvin by 9 collaborators was 103 ± 8.7% from fortified meal and 102 ± 9.7% from fortified pellets. The method is recommended for adoption as official, first action.

Griseofulvin, an antibiotic obtained from cultures of *Penicillium griseofulum*, has been used for the treatment of superficial fungus infections of man and animals. Recently this antibiotic has been found effective in the

treatment and prevention of trichophyton infections of chinchilla when incorporated in animal feeds.

This report summarizes the results obtained from a collaborative study of an ultraviolet spectrophotometric method for the determination of griseofulvin in animal feeds.

METHOD

Reagents

(a) *Activated alumina.*—Alcoa grade F-20.

(b) *Solvent mixture.*—Mix 65 parts petr. ether with 35 parts $CHCl_3$ by vol.

(c) *Griseofulvin standard soln.*—Accurately weigh ca 25 mg Griseofulvin Reference Standard (available from USP Reference Standards, 46 Park Ave., New York, N.Y. 10016), into 250 ml vol. flask, dissolve, and dil. to mark with solvent mixt. Dil. 10 ml of this soln to 100 ml in vol. flask (1 ml = 10 μg).

Apparatus

(a) *Chromatographic column.*—20 × 400 mm, with fritted disk and stopcock.

(b) *High speed blender.*—Waring type, or equiv., 1 L capacity.

(c) *Spectrophotometer.*—Capable of accurate readings at 290 and 320 mμ.

Preparation of Sample

Grind 250 g feed pellets or mash in high speed blender 5 min. Accurately weigh ca 14 g finely powdered feed into fat-free thimble and ext. in Soxhlet app. 2 hr with 100 ml $CHCl_3$. Evap. ext. to 10 ml on steam bath, dil. with 100 ml petr. ether, and chromatograph.

Preparation of Chromatographic Column

Place 50 ml solvent mixt., (b), in column and add 45 ml activated alumina portionwise, with tapping to ensure uniform packing. Place small glass wool plug on top of alumina and drain solvent to just below top of plug.

Chromatography

Add $CHCl_3$-petr. ether sample ext. to column. As last of ext. passes thru glass wool plug, rinse out sample flask with solvent mixt., add to column, and begin elution with solvent mixt. Adjust liquid head to give flow rate of 15–20 ml/min. Start collecting 25 ml fractions when green eluate first appears (discard yellow and almost colorless eluates which precede). When absorbance of fractions at 290 mμ exceeds absorbance at 320 mμ, stop fractionating, and collect next 700 ml eluate. Dil. eluate to mark in 1 L vol. flask with solvent mixt. Det. absorbance of this soln and of griseofulvin std soln at 290 and 320 mμ against solvent mixt. blank.

Calculations

$$\text{Mg griseofulvin/oz} = \frac{(A_{290} - A_{320})(W_s)(10)(28.35)}{(A'_{290} - A'_{320})(25)(\text{g sample})},$$

where A = absorbance of sample eluate, A' = absorbance of griseofulvin std soln, and W_s = mg reference std griseofulvin used to prep. std soln.

Collaborative Study

Collaborators were supplied with a reference sample of griseofulvin, a sample of nonfortified meal, and samples of fortified meal and pellets each containing 10 mg of griseofulvin per ounce. Collaborators were asked to familiarize themselves with the assay procedure by working through the assay with the nonfortified feed after adding a known quantity of griseofulvin to the chloroform extract. They were requested to perform duplicate assays of the fortified meal and of the pellets by the described method on two different days.

Results and Recommendation

Results obtained by the 10 collaborators are summarized in Table 1. The average recovery of griseofulvin from fortified meal was 103% (coefficient of variation 8.7%) and from fortified pellets, 102% (coefficient of variation 9.7%). Six of 9 collaborators reported a range of 0.5 mg/oz or less for duplicate analyses for fortified meal, while 7 collaborators reported a range of 0.5 mg/oz or less for duplicate analyses for the pellets. Five collaborators reported average recoveries within 5% of the overall average recovery for meal, but only 3 of 9 collaborative results were within the same limits for the pellets.

Collaborators 4 and 5 found it difficult to determine when to start collecting the eluate containing the griseofulvin in the chromatographic separation.

Collaborators 3 and 8 suggested that a smaller volume of eluate would simplify the method.

Table 1. Collaborative results for the determination of griseofulvin in animal feed

	Fortified Meal[a]				Fortified Pellets[a]			
	Griseofulvin Found, mg/oz				Griseofulvin Found, mg/oz			
Coll.	Day 1	Day 2	Range, mg/oz	% Recovery	Day 1	Day 2	Range, mg/oz	% Recovery
1	10.8	11.9	1.1	113	10.2	10.3	0.1	102
2	10.3	10.2	0.1	103	9.5	9.2	0.3	94
3	9.0	11.1	2.1	100	9.8	8.9	0.9	93
4	10.7	10.4	0.3	106	10.7	10.5	0.2	106
5	11.9	11.6	0.3	117	10.8	11.2	0.4	110
6	9.9	9.7	0.2	98	9.1	9.5	0.4	93
7	9.2	9.4	0.2	93	10.2	9.0	1.2	96
8	10.3	10.0	0.3	102	12.3	12.2	0.1	123
9	10.3	9.0	0.7	96	10.0	10.0	0.0	100
10[b]	—	—	—	—	—	—	—	—
Av.[c]	10.32			103	10.19			102
Std dev.	0.90				0.99			
Coeff. of var.	8.7				9.7			

[a] Griseofulvin-labeled strength: 10 mg/oz.

[b] Collaborator 10 apparently could not separate griseofulvin from interfering substances; no numerical data reported.

[c] Summary data do not include results of Collaborator 10.

Collaborator 10 apparently could not separate griseofulvin from interfering substances, and no numerical data were reported.

It is recommended that the ultraviolet spectrophotometric method for the determination of griseofulvin in animal feed be adopted as official, first action.

Acknowledgments

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Frank Herget and Chester A. Luhman, California State Department of Agriculture, Sacramento, Calif.

E. E. Martin, Ralston Purina Company, St. Louis, Mo.

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H. S. Ragheb, Purdue University, Lafayette, Ind.

M. L. Schreiber and N. H. Holm, Kansas State Board of Agriculture, Topeka, Kan.

George Selzer, Division of Antibiotics and Insulin Certification, Food and Drug Administration, Washington, D.C.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee B, and was adopted by the Association. See *This Journal*, **49**, 165–167 (1966).

This report of the Associate Referee was presented at the Seventy-ninth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 11–14, 1965, at Washington, D.C.

Note on Modified Extraction Procedure for the Colorimetric Determination of Diethylstilbestrol in Mixed Feeds

By FRED H. HOSKINS (Department of Food Science and Technology, Louisiana State University, Baton Rouge, La. 70803)

Banes (1) described chemical procedures for the analysis of diethylstilbestrol (DES), and Umberger, *et al.* (2) adapted them for use in meat products, using a chromatographic extraction. The Soxhlet extraction as outlined in the official AOAC method (3) for mixed feeds, however, requires 16 hours of continuous extraction, during which time samples are usually left unattended. To extract and analyze samples on the same day, a modified extraction procedure was developed in this laboratory which yielded results comparable to those of the conventional Soxhlet method.

In this procedure, a 5–20 g feed sample and 2–3 g diatomaceous silica (Celite, Johns-Manville) were weighed directly into a 250 ml Erlenmeyer flask. To this flask, 75 ml 7% ethyl alcohol in chloroform was added, and the sample stirred magnetically over low heat (40°C) for 60 minutes. The extract was then filtered through Whatman #41 paper into a 100 ml volumetric flask, and the residue washed twice and made to volume with chloroform. Careful washing of the residue was the most important factor in high recovery values. Two washes, as compared to three, were sufficient. The remainder of the procedure was identical to the official AOAC method.

Commercial feed samples with known diethylstilbestrol guarantees, plus a series of blank feed samples to which pure drug had been added, comprised the test groups. Pure diethylstilbestrol (Eli Lilly) was added to blank feed and mixed by use of a small laboratory blender to simulate commercial premix conditions. Additional blendings were made with blank feed to obtain samples with the desired DES content. A standard curve was established by plotting recovery against absorbance in the conventional manner.

Sample irradiation in this procedure is especially critical, and several test runs are required to locate optimum conditions for an individual laboratory. In this work, a 15 watt germicidal lamp at a fixed distance of 5.5 inches for a period of 7 minutes was used for irradiation. All cells were standardized corex.

Results are summarized in Table 1. Averages of duplicate determinations were used to calculate results. Diethylstilbestrol recovery by this extraction procedure ranged from 86 to 106%, with an average recovery of 96% from a total of 45 samples. Concentrations of diethylstilbestrol in the test samples ranged from 0.0005 to 0.0050%. All commercial feed samples contained 0.00088% diethylstilbestrol, guaranteed analysis. Results were obtained from a standard curve, taking appropriate dilution factors into consideration.

As expected, commercial feed samples showed more variation than laboratory-prepared samples. These variations may be explained by differences in source, mixing, and sampling procedures on commercial feed. Increasing extraction time from one to two hours might have increased the per cent recovery above these values. In general, higher concentrations seemed to favor higher recovery.

This modified extraction procedure for the colorimetric determination of diethylstilbestrol in mixed feeds is sufficiently accurate and reproducible to warrant its use in routine check and control analyses.

References

(1) Banes, D., *This Journal*, **44**, 323–328 (1961).

(2) Umberger, E. J., Banes, D., Kunze, Frieda M., and Colson, Sylvia H., *ibid.*, **46**, 471–479 (1963).

(3) *Official Methods of Analysis*, 10th Ed., Association of Official Agricultural Chemists, Washington, D.C., 1965, sec. 33.042.

Table 1. Diethylstilbestrol recovery from feeds by a modified extraction procedure

No. Samples	% DES in Sample	% DES[a] Recovery
5	0.00050	88–98
5	0.00060	92–98
10	0.00088[b]	86–106
5	0.00100	87–101
5	0.00200	96–101
5	0.00300	96–100
5	0.00400	97–101
5	0.00500	97–100

[a] Averages of duplicate determinations used to calculate results.

[b] Commercial feed samples, DES guaranteed analysis, 0.00088%.

ALCOHOLIC BEVERAGES

Potentiometric Determination of Chlorides in Wine, Distilled Spirits, and Wine Vinegar

By CLIFFORD E. HUBACH[1] (135 Del Centro Street, Millbrae, Calif. 94030)

Collaborative study of the Pro method for determining chlorides in wines and distilled spirits by direct titration with silver nitrate followed by potentiometric reading of the end point gave accurate and reproducible results. The procedure is suitable for repetitious analyses in control laboratories, and it is also adaptable to determinations requiring a high degree of precision. Recoveries of chloride added to samples averaged 99.7%. The method is recommended for adoption as official, first action.

The chloride content of wines and distilled spirits can readily be determined by direct titration with silver nitrate when a potentiometer is used to indicate the end point. The results are accurate and reproducible. Such a method is ideal for routine analysis, since no preliminary sample treatment is needed. The voltage change at the equivalence point is measured independently of lighting, color, or turbidity; substances normally occurring in the product do not interfere. The procedure is similar to potentiometric acid-base titrations after the equivalence potential for the instrument and electrode system has been established. Subsequently, samples are titrated in the usual manner to this predetermined end point. A typical titration curve for a silver electrode in combination with a silver chloride reference electrode is shown in Fig. 1.

Applications of the potentiometer for the estimation of chlorides in tobacco (1), water (2), and insecticides (3) have been reported. The Beckman Automatic Titrator (2), the Fisher Titrimeter (1, 4), and pH meters (3) have been used for this purpose.

The potentiometer used in this study was a Beckman Zeromatic pH meter. Electrode systems tested included the Beckman #39187 combination electrode, consisting of a silver billet reference electrode and silver-silver chloride indicating electrode, the Beckman #39261 silver billet electrode, and the Beckman #19151 silver-silver chloride pressed billet electrode; both of the latter were used with a Beckman #41263 glass electrode as a reference electrode. Silver billet electrodes were used with and without coatings of silver chloride. The pressed silver-silver chloride electrode is more suitable than the coated silver billet, since the coating of the latter wears off and must frequently be renewed. The silver billet operates satisfactorily but is less specific for the chloride ion than the pressed silver-silver chloride billet. The glass electrode is recommended as the reference electrode because it is stable and because the sample cannot be contaminated through leakage of chloride ions from the electrode. The Fisher Titrimeter employs a silver-silver chloride

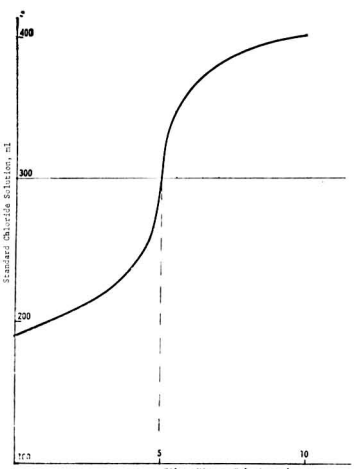

Fig. 1—**Standard titration curve of 5 ml standard chloride solution (1 ml = 1 mg Cl^-) and standard silver nitrate solution (1 ml = 1 mg Cl^-). Equivalence potential = 300 mv.**

[1] Presently associated with The Spice Islands Co., 100 E. Grand Avenue, South San Francisco, Calif. 94082.

reference electrode with a silver billet indicating electrode.

METHOD FOR WINE

Apparatus

(a) *pH meter.*—With millivolt scale, Beckman Zeromatic, or equiv.

(b) *Electrodes.*—Beckman general purpose glass electrode No. 41263 as reference electrode and Beckman No. 19151 Ag-AgCl pressed billet electrode with 30″ lead and pin connector or Beckman No. 39261 Ag billet electrode as indicating electrode. Other electrode combinations such as Beckman No. 39187 Ag billet combination electrode may be used.

(c) *Magnetic stirrer.*—With glass or plastic coated stirring bar.

(d) *Buret.*—10 ml with 0.05 ml subdivisions.

Reagents

(a) *Potassium chloride.* — Reagent grade contg not $> 0.005\%$ Br. Dry in desiccator several days before use.

(b) *Distilled water.*—Cl-free. Use wherever H_2O is specified.

(c) *Chloride standard soln.*—Weigh 2.1027 g KCl, transfer to 1 L vol. flask, and dil. to vol. with H_2O. 1 ml = 1 mg Cl.

(d) *Silver nitrate standard soln.*—Weigh 4.7912 g reagent grade $AgNO_3$, transfer to 1 L vol. flask, and dil. to vol. with H_2O. 1 ml = 1 mg Cl.

Determination

Connect glass electrode to the input terminal and indicating electrode to reference terminal of pH meter set to read on ± 700 millivolt (mv) scale. Warm up at least 30 min. Pipet 5.0 ml std Cl soln into 250 ml beaker. Adjust vol. to ca 100 ml with H_2O and add by pipet 1.0 ml HNO_3. Insert electrodes so that billet is completely covered, add stirring bar, and titr. with std $AgNO_3$ soln, stirring moderately. Add in 1.00 ml increments until 4.0 ml have been added, then 0.20 ml increments until 2.0 ml more have been added, then 1.00 ml increments to total of 10.00 ml. Read buret to 0.01 ml and millivolt scale to 1 mv after addn of each increment. Record readings. Allow at least 30 sec. for pH meter to stabilize before each reading. Plot observed millivolts against ml soln added and det. equivalence point (inflection) voltage from resulting curve. This value will vary with electrode system used.

Use of glass electrode as reference electrode is reverse of usual function; hence curve obtained will be reverse of those produced by other electrode combinations. Rinse electrodes before each use.

Pipet 5.0 ml std Cl soln into 250 ml beaker, and add 95 ml H_2O and 1.0 ml HNO_3. Insert electrodes, stir, and titr. with std $AgNO_3$ soln to predetermined equivalence voltage, adding titrant dropwise as end point is reached. Repeat until results are in close agreement. Conduct all titrations within 5° of temp. of equivalence point detn. Repeat this detn at least once a day or before each group of samples.

Pipet 50.0 ml wine into 250 ml beaker, add 50 ml H_2O and 1.0 ml HNO_3, and titr. as above. $\text{Ppm} = (V_W/V_S) \times C \times 2 \times 10$, where V_W = ml std $AgNO_3$ used by sample, V_S = ml std $AgNO_3$ used by std Cl soln, and $C = 5.0$ = mg Cl in 5 ml std Cl soln used.

METHOD FOR DISTILLED SPIRITS

Apparatus

See Chapter 11, Beverages: Wines.

Reagents

See Chapter 11, Beverages: Wines; and in addn:

(a) *Dilute chloride standard soln.*—Dil. 50 ml std soln used for wines to 500 ml with H_2O. Alternatively, dissolve 0.2103 g KCl in H_2O and dil. to 1 L. 1 ml = 0.1 mg Cl.

(b) *Dilute silver nitrate standard soln.*—Dil. 50 ml std soln used for wines to 500 ml with H_2O. Alternatively, dissolve 0.4791 g $AgNO_3$ in H_2O and dil. to 1 L. 1 ml = 0.1 mg Cl.

(c) *Alcohol soln.*—Place 500 ml alcohol in 1 L vol. flask, add ca 475 ml H_2O, mix, cool to room temp., and dil. to 1 L with H_2O.

Determination

Det. equivalence point voltage as for wine except use alcohol soln instead of H_2O to adjust vol. to 100 ml. Use same std solns as for wine.

Pipet 5 ml dil. std Cl soln into 250 ml beaker, and add 95 ml alcohol soln and 1.0 ml HNO_3. Titr. with dil. std $AgNO_3$ soln to predetd equivalence voltage as for wines.

Pipet 100 ml distd spirits sample into 250 ml beaker, add 1.0 ml HNO_3, and titr. with dil. std $AgNO_3$ soln as above. Ppm Cl in sample = $(V_W/V_S) \times C \times 10$, where V_W = ml std $AgNO_3$ used by sample, V_S = ml std $AgNO_3$ used by

This report of the Associate Referee was presented at the Seventy-ninth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 11–14, 1965, at Washington, D.C.

std Cl soln, and $C = 0.5$ mg Cl in 5 ml std Cl soln used.

Results and Discussion

Preliminary Studies

The chloride content of several varieties of wine, distilled spirits, and vinegar was determined by the method. A standard chloride solution was added to each sample, and the chlorides were again determined. The results of these experiments (Table 1) indicate that the chloride content of wine and vinegar can be determined with an accuracy of about 99%. In distilled spirits the accuracy is slightly less because the quantities of chloride are small in such products.

Since wine and distilled spirits normally contain large quantities of ethanol and various amounts of other substances, the effect of these compounds on the determination of chlorides was studied. Aqueous solutions containing measured quantities of KCl and various concentrations of each of the compounds were tested by the method. The compounds tested had the following range of concentration (%): carbon dioxide, 0–0.25; phosphate, 0–0.50; sulfate, 0–0.25; sulfur dioxide, 0–0.25; tartaric acid, 0–1.00; acetic acid, 0.–10.00; ethanol, 0–75.0; dextrose, 0–10.0; glycerol, 0–2.0; tannin, 0–2.0; potassium, 0–0.5; and sodium, 0–2.0. Chloride can be accurately determined in solution with any of them.

Iron was also tested and found to interfere when present in abnormal amounts. The iron content of finished wine is normally less than 7.0 ppm. The effect of 10 ppm was negligible, but the recovery of chloride was 97.2 and 80.0% at 20 and 100 ppm, respectively.

The effect of copper was not tested since finished wine contains not over 0.2 ppm of this ion.

The procedure for determining chlorides in distilled spirits differs in several respects from that for wine and vinegar. Because of the very low concentrations of chloride in these products, a more dilute standard solution is used in the titration. Furthermore, in solutions of high alcohol content the equivalence point voltage does not coincide with that for wine and must be determined in 50% alcohol.

Table 1. Chloride content of several wines, distilled spirits, and vinegars by the described potentiometric determination

	Chloride			
Sample	Found, ppm	Added, mg	Recovered, mg	% Recovery
Dry white wine	33.7	5.0	5.02	100.4
Dry red wine	50.2	5.0	4.94	98.8
Dry sherry, baked	65.0	5.0	5.02	100.4
Flor sherry, solera	50.2	5.0	4.96	99.2
Tipo red	24.4	5.0	5.02	100.4
Red port	53.0	5.0	5.00	100.0
White port	30.0	5.0	5.00	100.0
Muscatel	53.0	5.0	4.98	99.6
Sweet vermouth	44.0	5.0	4.96	99.2
Red wine vinegar	23.6	5.0	4.96	99.2
Red wine vinegar	53.8	5.0	4.96	99.2
Scotch whisky	2.1	5.0	4.92	98.4
Bourbon whisky	1.3	5.0	4.86	97.2
Tequila	12.4	5.0	4.90	98.0

Collaborative Results

Identical sets of samples were sent to 10 collaborators in 10 different laboratories. Each set contained four wine and four distilled spirits samples to be analyzed by the methods. The wines were prepared to contain approximately 16.0, 36.0, 56.0, and 76.0 ppm of Cl ion and the distilled spirits to contain 1.0, 3.0, 5.0, and 7.0 ppm of Cl. These concentrations cover the range of Cl concentration in normal wine and distilled spirits. The data resulting from this study are given in Table 2.

Conclusions and Recommendations

Collaborative results (Table 2) confirm results of other tests (Table 1) and both lead to the conclusion that the methods are capable of a satisfactory degree of precision. Comments of the collaborators, generally favorable, were as follows: "We used the silver billet electrode, and it appeared to work well; I had difficulty in determining the equivalence point but believe the method can be useful as a routine procedure; the method is simple, yet fast, and should be well suited for routine analysis; basically the method appears sound and rapid; the method appears straightforward for wine and gives results that are easily reproducible, but it is difficult to reproduce results with spirits." The best results were

Table 2. Collaborative results on wine and distilled spirits, ppm Cl

	Wine Sample				Distilled Spirits Sample				
Coll.	1	2	3	4	1	2	3	4	Equipment[b]
A	15.4	35.8	55.6	75.8	5.2	7.1	1.0	3.1	Zeromatic 1
B	15.2	35.4	55.6	75.4	4.9	6.8	0.8	2.9	Zeromatic 2
C	15.2	35.5	55.6	75.8	5.1	7.3	1.0	3.1	Zeromatic 1
D	15.0	35.2	55.6	75.0	4.9	6.9	0.8	2.9	Zeromatic 1
E	16.5	37.5	57.5	77.5	5.0	7.0	1.0	3.0	Zeromatic 1
F	17.2	36.4	55.6	75.8	5.0	8.5[a]	1.4	3.1	Zeromatic 2
G	19.1[a]	37.7	57.4	76.2	4.3	6.2	0.4	2.3	Titrimeter 3
H	16.0	35.0	56.0	76.0	4.3	6.7	2.0	3.4	H-2 Beckman 2
I	14.0	35.0	55.0	75.0	3.7[a]	4.4[a]	0.2[a]	1.9[a]	Zeromatic 2
Av.	15.6	35.9	56.0	75.8	4.8	6.9	1.1	3.0	
Std Dev.	0.99	1.04	0.87	0.75	0.35	0.35	0.49	0.32	

[a] Values omitted from averages and standard deviations.
[b] 1 = Ag:AgCl billet vs. glass electrodes; 2 = Ag billet vs. glass electrode; 3 = Ag billet vs. Ag:AgCl electrode.

obtained by those who had the most experience with potentiometric titrations.

It is recommended that both the method for wine and the method for distilled spirits be adopted as official, first action. It is also recommended that the method for wine be adopted as official, first action for the determination of chlorides in wine vinegar.[2] Samples of vinegar were not sent to collaborators but wine vinegar is merely acetified wine and the presence of acetic acid in the quantities found in vinegar has been determined in this study to have no effect on the chloride determination.

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E. A. Rossi, Jr., United Vintners, Inc., San Francisco, Calif.

R. J. Joyce, Beckman Instruments, Inc., Fullerton, Calif.

J. F. Guymon, University of California, Davis, Calif.

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A. J. Pezzi, Alcohol and Tobacco Tax Laboratory, San Francisco, Calif.

R. L. Brunelle, Alcohol and Tobacco Tax Laboratory, Washington, D.C.

C. H. Riley, Alcohol and Tobacco Tax Laboratory, Cincinnati, Ohio

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(4) Pro, M. J., Internal Revenue Service Document #5142 (2-59).

[2] This recommendation was not approved by the General Referee nor by Subcommittee D and was not adopted as an official, first action method.

The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee D, and were adopted by the Association. See *This Journal*, **49**, 172–175 (1966).

Malt Beverages and Brewing Materials

By IRWIN STONE (Wallerstein Company, Division of Baxter Laboratories, Staten Island, N.Y. 10303)

One new method, Total Haze of Beer After Chilling, is recommended for adoption as official, first action. Other new methods of the American Society of Brewing Chemists, which will be eventually submitted to the AOAC, are Diacetyl in Beer, Microbiological Controls, Aphids in Hops, and Physical Stability of Beer. The ASBC now has nine technical subcommittees investigating various analytical methods which may be of eventual interest to the AOAC.

This is a combined report of the Associate Referee for Malt Beverages and the liaison representative with the American Society of Brewing Chemists (ASBC).

Since last year's report, only one new beer method has been published by the ASBC, Total Haze After Chilling. This method on an important and difficult subject covers visual and nephelometric measurement and standardization and was adopted by the ASBC after 5 years of subcommittee work and collaborative testing (*see* Table 1). The original work was begun in 1951. Formazin turbidity standards were found practical and reproducible in 1956. Collaborative work was initiated in 1957 to standardize the many conditions involved in chilling the beer samples and in measuring the turbidity by both visual and nephelometric methods. Collaborative work on chill haze is extremely difficult because of the inherent sensitivity of the beer samples to factors such as time, slight variations in the air content of the individual bottles, vibration due to length of travel to the collaborator, temperature history during shipping, etc. The mass of data collected in these latter years is difficult to summarize adequately in the limited space of this report. The 1962 report recommended adoption of the methods, and Table 1 summarizes the essential data on the two sets of beer test samples sent to the 15 collaborators.

Other new ASBC methods which are in various stages of preparation but are not yet ready for submission to the AOAC comprise Diacetyl in Beer, Microbiological Controls, Aphids in Hops, Physical Stability of Beer, and Analysis of Filter Aids.

The ASBC now has nine active technical subcommittees investigating various analytical methods of interest to the brewing industry. These subcommittees are in addition to the permanent Subcommittee on Malt and Adjuncts Analysis Check Service,

Table 1. Summary of essential data on two sets of beer samples by 15 collaborators

		Chill Haze in Formazin Turbidity Units (FTU)							
		Tested after 30 Days at 72±2°F				Tested after 60 Days at 72°F			
		Visual Method				Visual Method			
Miles Traveled	Air Content	Entire Bottle	Entire Bottle Reread	10 oz Glasses	Nephelometric Method	Entire Bottle	Entire Bottle Reread	10 oz Glasses	Nephelometric Method
					Set I				
Av. – –	1.06	82.8	85.0	72.1	78.7	126.7	123.9	121.7	114.7
Max. 3100	1.4	140	140	130	112	170	170	170	142
Min. 2	0.5	25	25	25	55	55	55	55	74
					Set II				
Av. – –	0.98	64.4	66.3	68.6	92.8	99.4	97.1	100	132.1
Max. 2300	1.2	135	130	130	130	155	155	155	186
Min. 0	0.5	40	40	40	42	70	70	55	102

which operates a service by which an analyst can check his analytical results against those obtained by over 60 other analysts in the industry on samples of malt and cereal adjuncts.

Other subcommittees are investigating topics such as the analysis of filter aids, beer flavor analysis, aphids in hops, evaluation of hop concentrates, and the oxidation state of beer.

Three new subcommittees were instituted this year: Detection of Enzymatic Chill-proofing Agents in Beer, Microbiological Controls, and a Subcommittee for the Evaluation of Packages and Packaging Materials. The Subcommittee on Microbiological Controls has been assigned to prepare the new methods for publication.

Two reports were presented at this meeting on topics related to the brewing area by Associate Referees in other divisions: aphids in hops, and sodium and potassium in wines; the Associate Referee on Sodium and Potassium reported collaborative tests on the flame photometric determination of these metals in beer.

Since the Food and Drug Administration now permits trace addition of cobalt to beer as a foam adjunct, AOAC is interested in developing an official method for the determination of traces of cobalt in beer. Several new methods have been described in recent publications. The ASBC's Subcommittee on New and Alternate Methods of Analysis has been asked to propose to the Technical Committee that a Subcommittee for the Determination of Cobalt in Beer be formed to study and test available methods with the object of recommending a suitable test procedure. A decision on whether this work will be instituted by the ASBC should be forthcoming shortly.

METHODS

Visual Method

Reagents

Formazin turbidity working standards.—See **10.010(e)**. Prep. daily appropriate series of working stds, in 10 FTU increments for estimating turbidities up to 100 FTU; in 20 FTU increments for 100–200; and in 50 FTU increments for > 200 FTU.

Apparatus

(a) *Clark Turbidimeter, Model T.—See* **10.012**.

(b) *Red Plexiglas sheet.*—¼" thick, ca 1 sq. ft.

(c) *Constant-temperature bath.*—0 ± 0.2°.

(d) *Ice-water bath.*—Contg few drops of wetting agent.

(e) *Flint glass bottles.*—Of same dimensions as the flint glass bottles contg beer test samples; or clear drinking glasses (shells), 10 oz capacity, approx. o.d. 66 mm at bottom and 67 mm at top.

Determination

(Make comparisons with samples at 0°. Keep test samples in 0° bath when not matching turbidities.)

Place container of beer to be tested in upright position in 0° bath and hold 24 hr.

Prep. series of formazin turbidity working stds covering range of expected turbidities of test samples. Fill into flint glass bottles of same dimensions as those holding beer test samples.

If beer is in flint glass bottles, carefully remove bottle from constant temp. bath without disturbing sediment. Dip bottle into ice-H_2O bath contg few drops wetting agent to prevent fogging or accumulation of H_2O droplets on bottle while in viewing box. Place bottle of beer in viewing box between two bottles of formazin turbidity working stds. Compare turbidities by viewing thru red Plexiglas sheet placed 2" in front of bottles. Change formazin stds until that working std is found which most closely matches turbidity of test sample.

If beer is not in flint glass bottles, carefully remove container from constant temp. bath and, without disturbing sediment, pour beer into clear 10 oz drinking glass (shell) which has been pre-chilled by standing (external contact only) in ice-H_2O bath contg wetting agent. Degassing is not necessary. Use formazin turbidity working stds in identical 10 oz glasses to match turbidities as above for bottles.

Report as total haze of the beer after chilling, formazin turbidity units (FTU) of working std giving closest match. In the range up to 100 FTU, report to nearest 10 FTU; 100–200, 20; > 200, 50.

This report of the Associate Referee was presented at the Seventy-ninth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 11-14, 1965, at Washington, D.C.

Nephelometric Method

Apparatus

Nephelometer.—Prep. calibration curve for instrument by use of series of working stds or dilns of 1000 Turbidity Standard. If readout device of nephelometer is 0–100 scale of arbitrary units, set needle to indicate 0 units when cuvette is filled with turbidity-free distd H_2O and 100 units when it is filled with formazin 1000 Turbidity Standard.

Determination

Place containers of beer to be tested in upright position in 0° bath and hold 24 hr.

Pre-chill nephelometer cuvette in small ice-H_2O bath contg wetting agent (external contact only). Carefully remove container of beer from bath and, without disturbing sediment, rinse and fill cuvette with test sample. Place cuvette in ice-H_2O bath contg wetting agent, and degas beer by stirring with thermometer. When beer temp. is 0°, place cuvette in sample chamber of nephelometer and det. reading. (Beer must be at 0° when taking reading.)

Calculations

Calc. FTU corresponding to total haze in the beer after chilling: Turbidity, in FTU = $R \times S/100$, where R = nephelometer (galvanometer scale) reading; S = FTU (usually 1000) of formazin turbidity std used for calibration of nephelometer.

Recommendation

It is recommended that the ASBC method for Total Haze After Chilling be adopted as official, first action.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee D, and was adopted by the Association. See *This Journal*, **49**, 172–175 (1966).

Quantitative Determination of Benzaldehyde in Flavors and Cordials by Ultraviolet Spectrophotometry, 2,4-Dinitrophenylhydrazine Precipitation, and the Official AOAC Procedure

By RICHARD L. BRUNELLE (Alcohol and Tobacco Tax Division Laboratory, Internal Revenue Service, Washington, D.C. 20224)

The present official AOAC method was compared with other gravimetric and spectrophotometric procedures for the determination of benzaldehyde in flavors, cordials, and other distilled spirits products. Collaborative results indicate that the ultraviolet spectrophotometric and the 2,4-dinitrophenylhydrazine gravimetric methods are superior to the present official AOAC procedure, and it is recommended that both be adopted as official, first action as alternative methods.

Woodman and Davis (1), and Shriner and Fuson (2) used phenylhydrazine and 2,4-dinitrophenylhydrazine, respectively, to form the insoluble hydrazone derivatives and determined benzaldehyde gravimetrically. Bohme and Winkler (3) described a colorimetric procedure employing hydrostrychnine as the complexing agent. Since the presence of other carbonyl groups interferes, these methods lack specificity. Mathers and Schoeneman (4) described a method for determining benzaldehyde in which the aldehyde is steam distilled from the fermentation media into a solution buffered at pH 7 and is determined quantitatively by a polarographic procedure. Brunelle and Martin (5) determined benzaldehyde quantitatively by gas-liquid chromatography on a column of 25% apiezon M as liquid phase on Chromport XXX (60–80 mesh). The partitioning of benzaldehyde by this technique permits analysis in the presence of many interfering substances.

In this investigation, cherry and almond flavors were distilled to reduce interference from benzoic acid prior to determining the absorbance maximum at 249 mμ and the minima at 222 and 350 mμ. An average was obtained for the minima, and this value was subtracted from the absorbance maximum.

In the analysis of cordial-type products, only the absorbance at 249 mμ is needed since there is no apparent interference from benzoic acid after distillation.

In the gravimetric determination of benzaldehyde, with 2,4-dinitrophenylhydrazine as the precipitating agent, the procedure of Shriner and Fuson (2) was modified as follows: The 2,4-dinitrophenylhydrazine was solubilized by mixing the reagent with alcohol, followed by the slow addition of sulfuric acid to the alcohol-reagent mixture. (The heat of solution causes the reagent to dissolve more rapidly.)

It was found that the 2,4-dinitrophenylhydrazone precipitate is more heat-stable (m.p. 237°) than the phenylhydrazone precipitate (m.p. 156°) and can be dried simply by heating in a 100° oven for a short period of time (*see* Table 1). To keep the 2,4-dinitrophenylhydrazine reagent soluble in the sample distillate, the alcohol and H_2SO_4 concentrations of the distillate were adjusted prior to precipitation.

Table 1. Relative decomposition of the hydrazone precipitates when heated

Time Heated at 100°C, hr	2,4-Dinitrophenylhydrazone, g	Phenylhydrazone, g
0	0.1410	0.1550
1	0.1412	0.1531
2	0.1409	0.1498
3	0.1408	0.1408
4	0.1410	—
7	0.1409	0.1213

Tables 2 and 3 show the comparative results of the ultraviolet, 2,4-dinitrophenylhydrazine, and official AOAC (6) procedures on a variety of distilled spirits products. Since the results were in excellent agreement, a collaborative study was performed.

Collaborative Study

The following samples were sent to collaborators for analysis:

Cordial A.—(Prepared in this laboratory.) Benzaldehyde, sugar, alcohol, and water was added to a mixture of creme de almond and cherry-flavored vodka, to give about 70 proof cordial.

Cordial B.—(Obtained from a local rectifying plant.)

Flavor C.—(Prepared in this laboratory.) Known quantities of benzaldehyde, vanillin, and benzoic acid were added to an alcohol-water solution.

Each collaborator was asked to analyze the above samples by the proposed and

Table 2. Comparison of three methods for benzaldehyde

Sample No.	Type Sample	Ultraviolet Method, g/100 ml	2,4-Dinitrophenylhydrazine Method, g/100 ml	AOAC Phenylhydrazine Method, g/100 ml	Av. of Methods, g/100 ml	% Deviation from Mean Value of Methods		
						U.V.	2,4-Dinitro	AOAC
38738	Sloe gin	none detected	none detected	none detected	—	–	–	–
33775	Pistachio extract	1.340	1.312	1.112	1.255	6.77	4.54	11.39
38592	Apricot-flavored brandy	0.00045	0.00046	none	0.000455	1.10	1.10	–
37621	Sloe gin	0.0047	0.0048	0.0041	0.0045	4.44	6.67	8.89
38354	Creme de almond	0.0224	0.0229	0.0235	0.0229	2.23	0.00	2.62
38286	Almond extract	1.015	1.025	1.038	1.026	1.07	0.10	1.17
38552	Cherry-flavored brandy	0.0184	0.0190	0.0174	0.0183	0.55	3.83	3.83
38791	Cherry-flavored vodka	0.0201	0.0211	0.0204	0.0205	1.99	2.93	0.49

present official AOAC procedures (6). These collaborators were also instructed to make appropriate comments on these methods.

METHODS

Ultraviolet Spectrophotometric Method

Reagents and Apparatus

(a) *Spectrophotometer.* — Quartz spectrophotometer, Beckman Model DU, or equiv., with ultraviolet sensitive phototube and H lamp.

(b) *Benzaldehyde.*—Redistd; sp.gr. 1.041–1.046.

(c) *Alcohol.*—Reagent grade alcohol or MeOH.

(d) *Benzaldehyde standard soln.*—Weigh 1 g benzaldehyde into 100 ml vol. flask and dil. with alcohol. Transfer 1 ml of this soln to 100 ml vol. flask, using 10% alcohol. Dilute 1, 2, 4, 6, 8, 10 ml aliquots to 100 ml with 10% alcohol (1, 2, 4, 6, 8, 10 ppm benzaldehyde).

Determination

Pipet sample (usually ca 5 ml flavor or 25 ml cordial) into distn flask. Add enough alcohol to ensure min. of 10% alcohol in distillate. Add ca 110 ml H_2O to flavor or 200 ml H_2O to cordial and distill, collecting 100 ml or 200 ml, resp. If necessary, dil. aliquot of distillate with 10% alcohol to produce absorbance of ca 0.5 at 249 mμ, using 10% alcohol blank.

Det. absorbances of std benzaldehyde solns at 249 mμ against blank of 10% alcohol, and plot std curve.

Det. benzaldehyde concn from absorbance, A, of sample at 249 mμ and std curve, or calc. av. absorbance of 1 ppm benzaldehyde (A'). Concn of benzaldehyde in ppm $= (A/A') \times F$, where F is diln factor. (For most accurate work conduct 5 ppm std with each detn.)

For pure cherry exts and imitation cherry flavors, subtract av. of absorbances for min. at 222 and 350 mμ from absorbance max. at 249 mμ to calc. A.

2,4-Dinitrophenylhydrazine Gravimetric Method

Reagent

2,4-Dinitrophenylhydrazine soln.—Add 50 ml alcohol to 3.0 g 2,4-dinitrophenylhydrazine. Slowly add 10.0 ml H_2SO_4 while stirring. After reagent dissolves, add addnl 50 ml alcohol and filter thru Whatman No. 12 paper.

Determination

Measure sample contg ca 10–50 mg benzaldehyde (ca 5 ml flavors, 100–200 ml cordial) into distn flask. Add enough alcohol to ensure at least 10% by vol. in distillate and dil. to ca 350 ml with H_2O. Collect ca 300 ml distillate in 600 ml beaker.

Add 100 ml alcohol and 25 ml H_2SO_4, mix thoroly, and cool to room temp. Add 25 ml 2,4-dinitrophenylhydrazine soln with stirring and stir ca 2 min.

Let ppt settle and filter by decanting most of supernatant thru gooch prepd with thin asbestos mat before transferring bulk of ppt. Wash ppt with H_2O followed by 10 ml alcohol, both at not more than room temp.

Dry at 100° to constant wt (ca 2 hr). Wt ppt $\times$ 0.3707 = wt benzaldehyde.

Results and Discussion

Nine laboratories took part in this collaborative study. Two laboratories reported results by more than one analyst, and these results are shown in Table 4 by Collaborators 8 and 9 of one laboratory and Collaborators 10, 11, and 12 of another.

Most collaborators stated that the ultraviolet procedure was superior to the other

Table 3. Recovery experiment[a] for benzaldehyde

Benzaldehyde Added, g	Ultraviolet Method		2,4-Dinitrophenylhydrazine Method		AOAC Phenylhydrazine Method	
	Detected, g	Recovered, %	Detected, g	Recovered, %	Detected, g	Recovered, %
0.0104	0.0104	100.00	0.0101	97.12	N.D.	
0.0209	0.0209	100.00	0.0216	103.34	N.D.	
0.0522	0.0520	99.62	0.0525	100.57	N.D.	
0.1043	0.1043	100.00	0.1052	100.86	0.1040	99.71
0.2086	0.2084	99.90	N.D.		0.2070	99.23
0.5217	0.5219	100.04	N.D.		0.4998	95.80
1.0430	1.0435	100.05	N.D.		1.0120	97.03

[a] N.D. = Not determined.

Table 4. Collaborative results for benzaldehyde in flavors and cordials, g/100 ml

	Ultraviolet Method			2,4-Dinitrophenylhydrazine Method			AOAC Phenylhydrazine Method		
Coll.	A	B	C	A	B	C	A	B	C
1	0.023	0.002	1.08	0.020	0.002	0.998	0.020	0	0.990
2	0.0235	0.0029	1.04	0.0261	0.0025	0.850	0	0	1.003
3	0.0119	0.0025	1.34	0.0193	0.0008[a]	0.860	0.0157	0	0.865
4	0.0278	0.0026	0.998	0.0232	0.0028	1.070	0.0183	0	0.832
5	0.018	0.0015	1.02	0.020	0.002	0.870	0.020	0.0019	0.940
6	0.0203	0.0021	1.003	0.0201	0.0027	0.8306	0.0212	0.0023	1.502
7	0.0230	0.0025	0.9283	0.0219	0.0018	0.8334	0.0201	0.0028	0.5209
8	0.025	0.0031	1.139	0.018	0.0020	0.504[a]	0.019	0.001	1.018
9	0.0215	0.0022	0.964	0.0200	0.0021	0.884	0.0195	0.0037	0.943
10	0.0217	0.0025	1.006	0.0208	0.0025	1.005	0.0202	0.0025	1.006
11	0.0215	0.0029	1.011	0.0200	0.0025	1.010	0.0200	0.0029	1.005
12	0.0200	0.0027	1.005	0.0200	0.0020	0.998	0.0200	0.0038	1.010
13	0.0202	0.0022	0.9474	0.0202	0.0022	0.9542	0.0207	0.0024	0.9567
Mean	0.0214	0.0024	1.037	0.0208	0.0023	0.9303	0.0196	0.0018	0.9686
Av. deviation from mean value	0.0027	0.0003	0.0690	0.0013	0.0003	0.0740	0.0024	0.0028	0.1161

[a] Not used in computation of averages, since these figures are undoubtedly in error.

two methods because it offers greater accuracy, sensitivity, and speed. Many collaborators agreed that the AOAC method was not sufficiently sensitive to detect very small quantities of benzaldehyde.

Two collaborators mentioned that the filtering process was time-consuming in both gravimetric methods. Centrifuging the precipitate prior to filtering was suggested. However, the Associate Referee believes that the method described (decanting the liquid before filtering the precipitate) solves this problem.

The high alcohol content of some cordials prevents precipitation by the AOAC method, which explains why Collaborators 1, 2, 3, and 4 (*see* Table 4) failed to obtain a precipitate.

One collaborator suggested that the alcohol concentration of the sample be adjusted prior to distillation in both proposed methods so that the distillate would contain at least 10% alcohol. This collaborator noticed that the benzaldehyde concentration in the distillate decreased on standing in aqueous solution. This observation has been investigated, and since it is apparent that benzaldehyde converts to benzoic acid in solutions of less than 10% alcohol, this precaution was written into the method.

Recommendation

It is generally believed that the ultraviolet spectrophotometric and the 2,4-dinitrophenylhydrazine precipitation methods are superior to the present official AOAC procedure; therefore, the Associate Referee recommends that both proposed methods be adopted as official, first action as alternative methods.

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MAPLE PRODUCTS

Conductivity Value of Maple Sirups

By ARTHUR S. WENDT and EARL J. BENJAMIN (Fred Fear & Co., 360 Furman St., Brooklyn, N.Y. 11201)

Collaborative studies were continued on a modified version of the Conlin method for conductivity values of maple sirup. Data show the method to be rapid, precise, and accurate. The method is recommended for adoption as official, first action.

The Referee on Maple Products, C. O. Willits, recommended that collaborative studies be continued on conductivity values for maple sirup. The Associate Referee reviewed the previous work on conductivities (1–4) and the wide variations in conductivity values reported by the various participating laboratories.

The official AOAC method for conductivity involves weighing the sample and transferring it to a volumetric flask. The wide divergence in conductivity values observed in earlier work is due in part to inherent error in weighing and transfer. It is possible to eliminate this potential error by the rapid method of Conlin (5).

Collaborative studies of conductivity values were undertaken in 1964 and 1965 on a modified version of the Conlin method. Previous studies indicated that results comparable to those by the official method were obtained with the alternative rapid method (2, 4). The 1964 collaborative study gave very precise results; low standard deviations (0.5–2.5) were obtained for specific laboratories. The grouped data for 1964 showed interlaboratory deviations to be considerably lower than results obtained in previous studies (1, 2, 4). The better agreement in the 1964 study is due in part to the determinations being run at 20°C. All collaborators used the Leeds and Northrop Conductivity Bridge No. 4961, equipped with a temperature compensator which corrects all readings to 20°C. Consequently, temperature was eliminated as a matter of close control and a source of error.

The 1965 study gave values which were in fairly good agreement; standard deviations ranged from 11.7 to 12.9. These values were approximately halved when the results of one collaborator were not considered. (The particularly low results of this collaborator will be discussed with the collaborator.) One major source of potential error reported by one collaborator, which had been previously noted by others but not emphasized, was the method of immersing the cell into the solution under test. This collaborator and the Associate Referee found that the cell must be shaken free of any adhering liquid from a previous measurement, or distilled water, which is usually used to store the cell when not in use. An error of 30–50 ohms was observed when the measured solution was not "pure". A portion of the measured solution should be used to rinse the cell and electrodes to insure "pure" solution for measurement.

As expected, different conductivity instruments introduced no significant variation in conductivity measurement. Two collaborators measured the solutions on different instruments with the same cell. The instruments used were the Leeds and Northrop No. 4961 and the RC-type manufactured by Industrial Instruments. Results with the different instruments varied with a maximum of 8 conductance units, which falls within the interlaboratory deviations.

Tables 1 and 2 give the results obtained in the 1964 and 1965 studies, respectively. In neither case are there the two distinct populations obtained in earlier collaborative studies by the official weighing method.

For the studies in both these years, three samples were sent to the six participating collaborators. Sample A was a U.S. Grade A + AA combined; Sample B was a U.S. Grade B; and Sample C was a U.S. Grade unclassified. In 1964 Samples A and B were both near maximum values to preclude any

Table 1. Collaborative values for conductivity, 1964[a]

Coll.	Sample A			Sample B			Sample C		
	n	$\bar{x}$	*s*	*n*	$\bar{x}$	*s*	*n*	$\bar{x}$	*s*
1	4	166.0	0.8	4	184.5	1.0	4	152.5	0.6
3	4	166.3	1.9	4	177.9	1.9	4	141.9	0
5	4	164.3	0.5	5	178.0	1.5	5	150.4	2.5
7	4	157.4	0.5	4	172.4	0.5	4	144.5	0.6
12	4	163.0	0	4	178.4	0.3	4	151.0	0
14	3	164.7	1.2	3	184.3	0.6	3	152.0	1.7
$\bar{\bar{x}}$	23	163.5		24	178.9		24	148.7	
S_m		3.2			4.2			4.2	
Range, max.		167.9			185.0			155.0	
Range, min.		157.0			172.0			141.9	

[a] Where *n* = number of analyses reported, $\bar{x}$ = mean of individual laboratories, *s* = standard deviation of individual laboratories, $\bar{\bar{x}}$ = mean of the $\bar{x}$'s, S_m = interlaboratory standard deviations.

Table 2. Collaborative values for conductivity, 1965[a]

Coll.	Sample A			Sample B			Sample C		
	n	$\bar{x}$	*s*	*n*	$\bar{x}$	*s*	*n*	$\bar{x}$	*s*
1	4	125.8	0.5	4	115.7	0.0	4	125.6	0.2
3	3	159.7	0.0	3	153.9	4.3	3	165.3	0.0
	3	168.4	3.1	3	158.8	2.6	3	167.9	0.8
5	3	141.7	0.6	4	132.9	0.2	3	148.7	0.5
7	4	151.4	0.4	4	141.9	0.4	4	157.5	0.8
12	4	153.8	0.5	4	151.5	0.6	4	161.8	0.5
14	3	156.1	0.9	3	145.2	0.4	3	164.2	0.4
	3	150.1	1.1	3	140.2	1.1	3	157.6	0.9
	3	148.5	0.4	3	140.3	2.0	3	155.9	0.4
$\bar{\bar{x}}$		149.9			141.4			155.3	
S_m		11.7			12.7			12.9	
Range, max.		171.9			161.8			168.7	
Range, min.		125.61			115.6			125.36	
Data without Collaborator 1									
$\bar{\bar{x}}$		153.6			145.2			159.8	
S_m		7.4			8.3			5.7	
Range, max.		171.9			161.8			168.7	
Range, min.		141.0			132.6			148.0	

[a] See footnote, Table 1.

preconceived notions of what the samples should be. In 1965 these samples were nearer the middle range. The samples were all adjusted to approximately 65.5 Brix, the density of most table sirups. The sample chosen was 30 ml, since no appreciable difference was observed from 28 to 30 ml. The size sample also takes into account samples from 64 to 67 Brix.

From the data obtained in collaborative study (Table 2), it can be concluded that the method is rapid, precise, and accurate.

METHOD

Apparatus

(a) *Conductivity bridge.*—Use any commercially available conductivity bridges, which are usually self-contained instruments with 2 external connections, one for connection to power source, usually 110 volt A.C., and other to conductivity cell. In addn, some models have means for making adjustment for temp. and cell constant. Leeds and Northrup Bridge No. 4961 and cell No. 4924 are commonly used and conductivity read directly, corrected to 20° when in "SC" position. "RC" type instru-

ments, manufactured by Industrial Instruments Inc., Cedar Grove, N.J., do not have compensator circuit which can be used to adjust instrument for temp. Consequently sirup soln must be read at exactly temp. specified to be comparative. RD-15 conductivity meter, manufactured by Industrial Instruments, corrects all readings to conductivity at 25°.

Calibrate scale (slide wire) of conductivity bridge by use of external fixed resistor with external leads. This should have resistance of 1000 ohms which will approximate that of conductivity cell. Resistor is attached to bridge connections for conductivity cell and slide wire set to same value as that of resistor. Bridge should give zero reading or response.

(b) *Conductivity cell.*—Made of resistance glass with platinized electrodes firmly fixed and adequately protected from displacement. Cell may be of dipping type, for immersing cell into test soln, or of vessel type, into which soln may be run and subsequently drained.

(c) *Constant temperature bath.*—Constant temp. bath that will maintain or supply water at 25 ± 0.1° for controlling temp. of test soln and cell.

Determination of Cell Constant

Dry 2–3 g KCl at 110° to constant wt. Weigh 2 portions dried KCl, one 0.3728 g ± 0.0002 and other 0.7456 g ± 0.0002 g, transfer to two 500 ml flasks, and dil. to vol. with H_2O at 20°. These solns will be 0.01 and 0.02*M* KCl, resp. Transfer portion of 0.01*M* KCl soln to beaker and adjust temp. to 25 ± 0.1°.

If conductivity bridge has temp. compensating device, set at 25°, temp. of KCl solns. With leads of conductivity cell attached to conductivity bridge, place dipping conductivity cell in 0.01*M* KCl, taking care to completely immerse electrodes. Adjust slide wire of bridge to give null-point reading. Repeat until 3 successive and concordant slide wire values (ohms resistance) are obtained. Replace KCl soln with fresh portion before making next measurement, taking care to shake adhering drops of liquid from electrodes before immersing them.

Repeat, using 0.2*M* KCl.

Calc. cell constant by multiplying observed resistance (scale reading in ohms) by 141.2 (specific conductivity of 0.01*M* KCl), and by 276.1 (specific conductivity of 0.02*M* KCl). Average the 2 results.

Determination

Add 70 ml H_2O to 100 ml g-s. graduated cylinder and fill to vol. with maple sirup to be tested. Stopper, mix thoroly, adjust temp. to 25 ± 0.1°, and measure resistance of dil. sirup with conductivity cell in same manner as used to calibrate cell. Repeat until 3 concordant observed scale readings (ohms) are obtained. Conductivity value

= cell constant × 100,000/ohms.

Recommendation

It is recommended that the modified rapid method for determination of electrical conductivity of maple sirups be adopted as official, first action.

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Iman Schurman, Food and Drug Administration, Chicago, Ill.

Jacques Tardif, Department of Agriculture, Quebec, Canada

C. O. Willits, U.S. Department of Agriculture, Philadelphia, Pa.

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DAIRY PRODUCTS

Cryoscopy of Milk: Effect of Variations in the Method

By R. W. HENNINGSON (Department of Dairy Science, Clemson University, Clemson, S.C. 29631)

Bath level, sample temperature, rate of stirring, degree of supercooling, sample size, sample isolation, and refreezing of the sample were the variables in the thermistor cryoscopic method for the determination of the freezing point value of milk chosen for study. Freezing point values were determined for two samples of milk and two secondary salt standards utilizing eight combinations of the seven variables in two test patterns. The freezing point value of the salt standards ranged from −0.413 to −0.433°C and from −0.431 to −0.642°C. The freezing point values of the milk samples ranged from −0.502 to −0.544°C and from −0.518 to −0.550°C. Statistical analysis of the data showed that sample isolation was a poor procedure and that other variables produced changes in the freezing point value ranging from 0.001 to 0.011°C. It is recommended that specific directions be instituted for the thermistor cryoscopic method, 15.040–15.041, and that the method be subjected to a collaborative study.

The cryoscopic method has been the official method for detecting added water in milk since 1923 (1). A screening method was generally used to detect suspicious samples prior to cryoscopy. Thus, the official method was a second or confirmatory test for the presence of added water. The upper limit, −0.533°C, for the freezing point value of unadulterated milk samples was established with a limited number of "authentic" samples (2). The official method was used for over 30 years without change except for modifications of the Hortvet cryoscope (3) and revision of the upper limit to −0.530°C as a result of numerous reports (4–7).

The thermistor cryoscope, introduced in 1956, made the method much faster and easier, and the cryoscopic method became "routine" for the detection of added water in milk in both industrial and regulatory laboratories. The official method immediately became a screening, rather than a confirmatory, test. Thermistor cryoscopes were given official recognition (8) as a result of the work of Shipe. Several collaborative studies of the cryoscopic method (9) showed more variation among laboratories than was desirable. No valid or recent estimate of the upper limit for the freezing point value of "authentic" milk samples has been undertaken. The official method, **15.040–15.041** (8), states that the thermistor cryoscope should be operated in accordance with the manufacturer's instructions. No data have been published which document the effects of minor variations in the method, although England and Neff (10) have qualitatively enumerated factors affecting the reported value for the freezing point.

This study attempts to quantitatively assess the effect of minor variations likely to be found among laboratories on the freezing point value of both secondary salt standards and milk samples. These ruggedness tests are necessary before the thermistor cryoscope procedure can be specified in detail for the official method and before a collaborative study of the thermistor cryoscopic method can be undertaken. Both areas of research must be completed before a collaborative study of the upper limit for the freezing point value of "authentic" milk samples can be initiated.

Experimental

An Advanced Instruments thermistor cryoscope (Advanced Instruments, Inc., 45 Kenneth St., Newton Highlands, Mass. 02160, Standard Laboratory Model 30L) was employed in this investigation. This cryoscope was calibrated with the bath temperature set at −7.0 to −7.5°C, with the bath charged with 430 ml of coolant by a moderate rate of stirring and supercooling

corresponding to 20 mm left of galvanometer zero, a 2 ml sample, and no isolation of the sample. Secondary salt standards equivalent to 7 and 10% sucrose standards with observed freezing point values of -0.422 and -0.621°C, respectively, were used.

Bath level, sample temperature, rate of stirring, degree of supercooling, sample size, sample isolation, and sample refreezing were the seven variables selected as most likely to be different among laboratories and operators. Bath temperature is preset at the factory and less likely to be a common variable in the method, although it does affect the freezing point value of standards and samples. If the bath temperature is other than the preset value, it should not vary from day to day or sample to sample, assuming no malfunction of the instrument.

The effect of the seven chosen variables was determined on the -0.422°C secondary salt standard, the -0.621°C secondary salt standard, and two samples of homogenized, pasteurized milk. Triplicate determinations were averaged for each sample. The seven variables and their values, believed to constitute reasonable variation among laboratories, are shown in test patterns A and B of Table 1. Sixteen combinations were evaluated for the secondary salt standard freezing at -0.422°C, the secondary salt standard freezing at -0.621°C, and two samples of homogenized, pasteurized milk; a total of 64 samples were analyzed.

The experimental design and statistical analysis were those of Youden (11), using two test patterns involving eight combinations of seven factors.

Results and Discussion

The freezing point values for the 16 combinations employed with the four samples are given in Table 2. The freezing point value of the -0.422°C secondary salt standard ranged from -0.413°C to -0.433°C (Table 2), while the freezing point value of the -0.621°C secondary salt standard ranged from -0.431°C to -0.642°C (Table 2). The freezing point value of milk sample 1 ranged from -0.502°C to -0.544°C, while the freezing point value of milk sample 2 ranged from -0.518°C to -0.550°C. With combinations 2, 3, and 6 (Table 2), it was often impossible to obtain a freezing point value because the sample melted after seeding rather than reaching the plateau and then freezing. In such cases a value of -0.001°C was arbitrarily assigned as the freezing point value.

Combinations 2, 3, 6, and 7 (Table 1) included isolation of the sample in the air above the cooling bath of the cryoscope. Although freezing point values were obtained

Table 1. Factor values for 8 combinations of 7 variables used to test the ruggedness of the thermistor cryoscopic method for determining freezing point values of milk

Factor	Combination No. and Factor Value							
	1	2	3	4	5	6	7	8
Test Pattern A								
Bath level, ml	430	430	430	430	380	380	380	380
Sample temp., −°C	20	20	0	0	20	20	0	0
Sample size, ml	2.0	1.5	2.0	1.5	2.0	1.5	2.0	1.5
Rate of stir	med	med	low	low	low	low	med	med
Seeding point, mm left of gal. zero	20	30	20	30	30	20	30	20
Sample isolated	no	yes	yes	no	no	yes	yes	no
Sample refrozen	no	yes	yes	no	yes	no	no	yes
Test Pattern B								
Bath level, ml	430	430	430	430	480	480	480	480
Sample temp., −°C	20	20	0	0	20	20	0	0
Sample size, ml	2.0	2.5	2.0	2.5	2.0	2.5	2.0	2.5
Rate of stir	med	med	high	high	high	high	med	med
Seeding point, mm left of gal. zero	20	10	20	10	10	20	10	20
Sample isolated	no	yes	yes	no	no	yes	yes	no
Sample refrozen	no	yes	yes	no	yes	no	no	yes

Table 2. Freezing point values for 8 combinations of 7 variables used to test the ruggedness of the thermistor cryoscopic method for determining freezing point values of milk

Sample[a]	Combination No. and Freezing Point (−°C)							
	1	2	3	4	5	6	7	8
Test Pattern A								
−0.422°C SSS	0.420	0.001	0.421	0.429	0.433	0.001	0.419	0.417
Milk No. 1	0.523	0.001	0.502	0.544	0.532	0.507	0.511	0.519
−0.621°C SSS	0.622	0.001	0.001	0.634	0.642	0.001	0.431	0.617
Milk No. 1	0.538	0.001	0.001	0.550	0.518	0.001	0.524	0.526
Test Pattern B								
−0.422°C SSS	0.421	0.001	0.001	0.413	0.416	0.422	0.416	0.425
Milk No. 2	0.520	0.001	0.001	0.515	0.518	0.514	0.507	0.530
−0.621°C SSS	0.625	0.001	0.001	0.614	0.620	0.626	0.625	0.636
Milk No. 2	0.528	0.001	0.001	0.520	0.523	0.520	0.530	0.547

[a] SSS = Secondary salt standard.

for all samples with combination 7, the variation among the triplicate samples was greater than with any other combination. This variation with combination 7 indicated that sample isolation was poor; poor isolation also caused the variations for combinations 2, 3, and 6. The lack of freezing point data for these four combinations of variables influenced the analysis of the data for effect of bath level and refreezing because in the test pattern combinations 2 and 3 were both at one bath level while combinations 6 and 7 were at the second bath level. Combinations 2 and 3 were both refrozen, while combinations 6 and 7 were not. The best estimates of the effect of bath level and refreezing are found with the −0.422°C secondary salt standard frozen in test pattern A (Table 2), where freezing point values were obtained for combinations 3 and 7 which cancelled the influence of isolation of the sample on these two factors.

Statistical analysis of the data is presented in Table 3. The greater the number assigned to the variable, the more important the influence of the variable on the result. According to Youden (11), the relative magnitude of the numbers within samples is an accurate index of the importance of the factor.

Data in Table 3 clearly indicate that isolation of the sample should not be a part of the procedure for the thermistor cryoscopic method. The effect of refreezing samples was greatly influenced by sample isolation (Table 3). Only slight changes in the freezing point values were observed for refrozen samples; thus this variable is relatively unimportant. Samples in test pattern A (Table 2) show that bath level is a relatively unimportant variable, although freezing point values were not obtained for combinations 2 and 6. However, data for bath level in test pattern B were influenced by the isolation factor; there were no freezing point values for combinations 2 and 3.

Similarly, the data in test pattern B indicate that sample size and sample temperature were relatively unimportant variables. The data in test pattern A were influenced by similar values in this pattern for these two variables with combination 2 and 6, for which no freezing point values were obtained because of the isolation factor. Data in Table 3 also indicate that rate of stirring and supercooling (seeding point) were relatively unimportant variables in test pattern B. In test pattern A, these variables were also influenced by the lack of freezing point values for combinations 2, 3, and 6, which unbalanced the data for the type of analysis used.

All variables tested are relatively unimportant when compared with the effect of isolation of the sample in the air above the cooling bath. However, these six variables have about equal importance (*see* Table 3). Four of these variables are individually of such magnitude that approximately 1% added water would be indicated under the

Table 3. Statistical analyses of the effect of seven variables on the freezing point value of secondary salt standards and milk samples[a]

Variable	TPA Milk No. 1 1A	TPA Milk No. 2 2A	TPA 422 3A	TPA 621 4A	TPB Milk No. 1 1B	TPB Milk No. 2 2B	TPB 422 3B	TPB 621 4B
	Thousandths °C							
Bath level	124	119	000	108	258	267	211	317
Sample temp.	128	135	208	104	000	007	000	000
Sample size	124	125	211	111	003	000	000	000
Rate of stir	132	129	007	098	003	011	003	007
Seeding point	116	131	006	117	006	005	005	007
Sample isolation	150	401	214	520	265	267	209	311
Sample refrozen	132	141	000	107	251	257	207	308

[a] TPA and TPB = Test Patterns A and B.

conditions tested. Depending on the various combinations, the net effect on the freezing point value may be additive or balancing in nature. The effect of these variables is the basis for Shipe's remark (12) that, in collaborative studies, "the analysts are as variable as the cows." The freezing point values (Table 2) obtained for the same sample with various combinations of seven variables likely to occur in the average laboratory illustrate the range of values that may be found in collaborative studies. Thus, specific directions for the thermistor cryoscopic method are mandatory in the official method for the freezing point value of milk, replacing the present "follow the manufacturer's directions." Such specific directions would provide uniform procedures for standardizing the cryoscope and determining the freezing point value of samples by eliminating the deviations now permissible while following the present directions.

Conclusions and Recommendations

The thermistor cryoscopic method was studied to assess the effect of combinations of variables, assumed likely to occur in the average laboratory, on the freezing point value of milk. The experimental design and statistical analysis of Youden (11) were employed.

Wide ranges for the freezing point value of milk samples and secondary salt standards were found with the combinations of variables. The sample should not be isolated in the air above the cooling bath while seeding the sample and waiting for the plateau to develop. Other variables produced changes in the freezing point value of such magnitude that results indicate a minimum of 1% added water.

It was recommended that specific directions for determining the freezing point value of milk by the thermistor cryoscopic method be included in the official method, **15.040–15.041** and that the method be subjected to collaborative study.

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Comparison of the FAO Schmid-Bondzynski-Ratzloff and Official AOAC Methods for Determining the Fat Content of Cheese

By R. W. WEIK and WILLIAM HORWITZ (Division of Food Standards and Additives and Office of the Commissioner, Food and Drug Administration, Washington, D.C. 20204)

The official AOAC method, 15.164, for determining the fat content of cheese was compared in six laboratories with the Schmid - Bondzynski - Ratzloff method, as adopted by the Food and Agriculture Organization of the United Nations. The results of analysis by the two methods are not significantly different; thus, the two methods can be used interchangeably.

The Food and Agriculture Organization (FAO) of the United Nations has adopted a revised Schmid-Bondzynski-Ratzloff (SBR) procedure as the standard method for determining the fat content of cheese (1). The AOAC method (2) for this determination is based upon the SBR procedure but differs in a few details.

The major differences between the two methods are as follows:

(*1*) In the SBR method adopted by the FAO, the cheese sample is digested directly with HCl; in the AOAC method, the sample is predigested with NH_4OH and then hydrolyzed with HCl.

(*2*) The SBR method submitted to FAO (3) specified 25 ml portions each of ethyl and petroleum ether throughout for the three extractions, while the AOAC method requires 25 ml portions of each ether for the first extraction only; the two subsequent extractions require 15 ml portions of each ether. The revised FAO procedure requires 15–25 ml of each ether for the second and third extractions.

This study compares the two methods for determining the fat content of cheese. Seven Food and Drug Administration District laboratories were requested to analyze ten samples of as many varieties of cheese as possible by both the AOAC and SBR methods. The moisture content, necessary to calculate the fat on a dry basis, was determined by the AOAC official, final action vacuum oven method (4).

Although only single determinations were requested for the AOAC-SBR comparisons, a number of laboratories reported duplicates by each method. The analyses were made by one or more analysts in each laboratory. The results from one laboratory were discarded without regard to the reported results when it was learned that rubber stoppers yielding high blank values were used in the determinations.

Overall, 96 samples of cheese were analyzed. The cheeses were distributed by varieties as follows: Cheddar-type, 28; Swiss, 8; semisoft (42–50% moisture), 21; Italian-type, 11; pasteurized processed products, 9; and creamed cottage cheese, 19.

Results

The data were grouped according to the moisture content of the cheeses, with a dividing line of 75% moisture. The results obtained from cheeses containing less than 75% moisture were analyzed statistically on

This report of the Associate Referee, R. W. Weik, was presented at the Seventy-ninth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 11–14, 1965, at Washington, D.C.

both "as is" and a dry matter basis. The data from cheeses containing more than 75% moisture (creamed cottage cheese) were analyzed on an "as is" basis.

Scatter diagrams, showing the fat content of the two groups of cheeses as determined by both methods, are presented in Figs. 1 and 2. The fat content determined by one method is plotted against the results obtained by the other method according to the Youden procedure (5).

The fat content determined by both methods and expressed on a dry matter basis for the lower moisture group of cheeses (below 75%) is shown in Fig. 1. The even distribution of points on either side of the 45° line indicates that the two methods yield comparable results.

The results of analysis of the fat content of the higher moisture group of cheeses, plotted on an "as is" basis, are shown in Fig. 2. The general distribution of points for this group of cheeses also demonstrates the agreement between the two methods. This overall agreement was confirmed by an analysis of variance of both cheese groups.

The average fat value obtained by each laboratory for their different cheeses, using the AOAC and SBR methods, is shown on an "as is" basis in Table 1. The per cent fat on a dry basis for the cheeses containing less than 75% moisture is shown in Table 2.

These results were first analyzed with respect to each individual laboratory. There was no significant difference ($P > 0.05$) between the method means (average fat determination) for laboratories 2–6 for either the lower or higher moisture group of cheeses

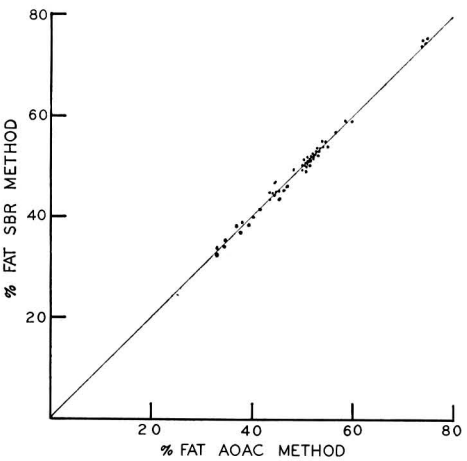

Fig. 1—**Fat content of cheese containing less than 75% moisture (dry matter basis).**

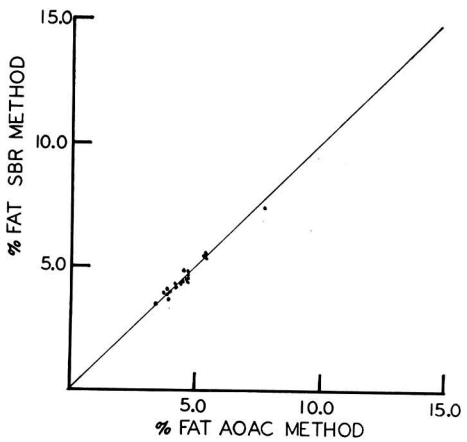

Fig. 2—**Fat content of cheese containing more than 75% moisture ("as is" basis).**

Table 1. Average fat determination by laboratory of the cheese samples analyzed by the AOAC and SBR methods (per cent fat, "as-is" basis)

	Cheeses with Less Than 75% Moisture				Cheeses with Greater Than 75% Moisture			
Lab.	No. Samples	AOAC	SBR	Difference (AOAC − SBR)	No. Samples	AOAC	SBR	Difference (AOAC − SBR)
1[a]	24	27.02	27.20	−0.18	3	3.84	4.09	−0.25
2	7	27.99	28.12	−0.13	3	5.00	4.88	0.12
3	9	30.66	30.64	0.02	1	4.19	4.34	−0.15
4	11	29.49	29.38	0.11	8	4.86	4.88	−0.02
5	16	30.10	30.16	−0.06	4	4.42	4.39	0.03
6	10	28.19	28.07	0.12	–	–	–	—
Av.		28.91	28.93	−0.02		4.46	4.52	−0.06

[a] Significant difference ($P < 0.05$) between method means.

(Tables 1 and 2). There was a significant difference ($P < 0.05$) between the method means for laboratory 1 for both groups of cheeses. However, the analysis of the method means for all laboratories combined, expressed on both an "as is" and dry matter basis, indicated no significant difference in their mean values.

Table 2. Average fat determination by laboratory of the cheese samples analyzed by the AOAC and SBR methods (per cent fat, dry basis)

	Cheese with Less Than 75% Moisture			
Lab.	No. Samples	AOAC	SBR	Difference (AOAC – SBR)
1[a]	24	49.07	49.51	−0.44
2	7	51.55	51.81	−0.26
3	9	49.78	49.75	0.03
4	11	48.73	48.54	0.19
5	16	49.91	50.02	−0.11
6	10	50.54	50.30	0.24
Av.		49.93	49.99	−0.06

[a] Significant difference ($P < 0.05$) between method means.

While one laboratory showed a significant difference between method means, the results suggest that a review is needed of the techniques used by this laboratory. This conclusion becomes more evident when the combined results for all laboratories are considered. The results from the AOAC method and SBR method did not differ significantly from a one-to-one relationship, which would exist if the methods were exactly comparable (Fig. 3).

This study demonstrates that the differences between the AOAC and SBR methods, specifically the use of NH_4OH and different volumes of the ethers for extraction, do not exert any influence upon the results obtained by either method.

Conclusions and Recommendation

The results obtained during this study demonstrate that the AOAC and SBR methods for determining the fat in cheese do not differ significantly and that the two methods are interchangeable.

It is recommended that the official method, **15.164**, for determining fat in cheese be reworded to include the optional omission of ammonia predigestion, and to permit the use of 15–25 ml of both ethyl and petroleum ethers in the second and third extraction steps.

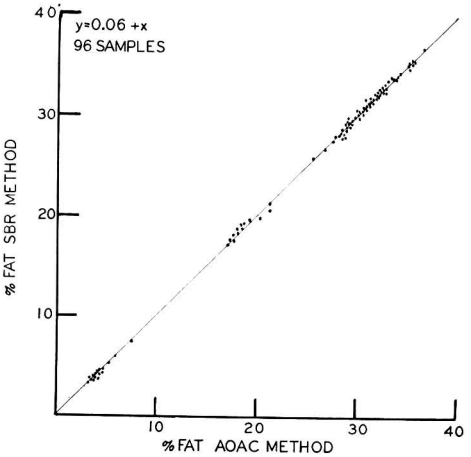

Fig. 3—**Fat content of all cheeses ("as is" basis).**

Acknowledgments

The cooperation of the Buffalo, Chicago, Cincinnati, Denver, Kansas City, Minneapolis, and St. Louis laboratories of the Food and Drug Administration in performing the analyses, and Mr. James S. Winbush in performing the statistical calculations is gratefully acknowledged.

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The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee C, and was accepted by the Association. See *This Journal*, **49**, 167–172 (1966).

Determination of Salt in Butter

By ROBERT W. WEIK (Division of Food Standards and Additives, Food and Drug Administration, Washington, D.C. 20204)

The official AOAC, 15.135, and International Dairy Federation (IDF) methods for determining the salt content of butter have been collaboratively studied. Results indicated that there was no significant difference ($P > 0.05$) between the method means. A highly significant difference ($P < 0.001$) was found between laboratory means which reflected individual differences in performing titration procedures and slight deviations from the prescribed procedure. The overall results indicated that the IDF method as studied was as accurate as the longer present official method, and the IDF method is recommended for adoption as official, first action.

The official AOAC method for determining the salt content of butter (1) is a long and laborious operation which has never been subjected to a collaborative study. Recently, the International Dairy Federation (IDF) submitted a different method for the determination of salt in butter (2) to the FAO/WHO Committee of Government Experts for Dairy Products for their consideration and possible inclusion in the Code of Principles for Milk and Milk Products (3).

Both the AOAC and IDF methods are based upon Mohr's method of determining the chloride content in the sample by titration with standard silver nitrate in the presence of potassium chromate. However, the procedures for preparation of the samples are quite different, as are the normalities of the standard silver nitrate solutions. The AOAC method involves separating the fat and aqueous portion of the butter by repeated washings with hot water and determining the amount of salt in the combined washings or in an aliquot of the washings by titration with 0.03*N* $AgNO_3$ at room temperature, while the IDF method consists of titrating, in the presence of the fat, a hot butter-water mixture with 0.1*N* $AgNO_3$.

Theoretically the Mohr titration should be performed at room temperature (4) since the solubility product of silver chromate increases with an increase in temperature and results in an altered end point. Investigations at the Netherlands Government Dairy Station at Lieden (5) have since demonstrated that the "temperature error" is positive and is opposed by a negative "butter error". The "butter error" is due to performing the titration in the presence of a finely dispersed fat phase and a small quantity of proteins. These investigations demonstrated that the magnitude of these errors was small and the resulting influence was of no significant importance.

After a preliminary study revealed the proposed IDF method to be easier, faster, and apparently as accurate as the official AOAC method, **15.135**, a collaborative study was initiated. Seven butter samples, each containing a known and different amount of salt, were prepared and sent to 7 collaborators with the following instructions:

Prepare the 7 samples for analysis by AOAC method, **15.130**. Determine, in duplicate, the salt content of each sample by AOAC method, **15.135**, and by the following IDF method.

METHOD

Weigh accurately (±10 mg) ca 5 g sample into 250 ml erlenmeyer and add 100 ml boiling H_2O. Let stand, swirling occasionally, 5–10 min. while cooling to 50–55°. Add 2 ml K_2CrO_4 indicator, **31.009(a)**, and titr. with 0.1*N* $AgNO_3$, stdzd as in **42.027**, until orange-brown color persists 30 sec.

ml 0.1*N* $AgNO_3 \times 0.585$/g sample = % NaCl.

Results

The results obtained by the different collaborators are shown in Table 1. Collaborator 5 had no explanation for the discrepancy obtained in results for Samples C and D by the AOAC method. Also, Collaborator 7 used the disappearance of yellow as the end point for the titration rather than the presence of the orange-brown specified in the instructions. The results obtained by Collaborator 7 were included in the statisti-

Table 1. Collaborative results obtained by the AOAC and IDF methods on % salt added to butter

Sample	Salt Added, %	Collaborators 1 AOAC	1 IDF	2 AOAC	2 IDF	3 AOAC	3 IDF	4 AOAC	4 IDF	5 AOAC	5 IDF	6 AOAC	6 IDF	7 AOAC	7 IDF
A	2.00	2.03	2.01	2.08	2.05	2.02	2.01	2.10	2.07	2.06	2.07	2.00	2.07	2.26	2.03
		2.04	2.00	2.08	2.06	2.16	2.02	2.11	2.06	2.09	2.07	2.05	2.08	2.18	2.03
B	1.00	1.04	1.02	0.96	0.97	0.97	0.95	0.97	0.98	0.87	1.02	0.94	1.03	1.07	0.96
		1.02	1.02	0.96	0.96	0.98	0.99	1.06	1.06	1.01	1.01	0.92	1.02	1.08	0.97
C	2.25	2.27	2.27	2.28	2.25	2.22	2.13	2.34	2.30	1.94	2.29	2.05	2.25	2.45	2.25
		2.27	2.27	2.29	2.27	2.17	2.15	2.44	2.22	2.28	2.29	2.18	2.26	2.40	2.26
D	2.50	2.40	2.40	2.41	2.45	2.39	2.35	2.42	2.50	1.97	2.45	2.12	2.40	2.56	2.37
		2.41	2.39	2.42	2.45	2.40	2.33	2.43	2.40	2.40	2.44	2.14	2.38	2.53	2.38
E	1.25	1.28	1.28	1.28	1.28	1.30	1.31	1.34	1.27	1.27	1.27	1.18	1.37	1.38	1.28
		1.28	1.28	1.28	1.28	1.28	1.28	1.29	1.24	1.30	1.29	1.19	1.36	1.37	1.29
F	0.00	0.03	0.02	0.04	0.04	0.02	0.02	0.01	0.03	0.01	0.03	0.01	0.02	0.02	0.04
		0.04	0.02	0.04	0.04	0.01	0.02	0.01	0.01	0.03	0.04	0.01	0.01	0.02	0.04
G	1.50	1.52	1.51	1.55	1.56	1.52	1.47	1.57	1.55	1.44	1.51	1.42	1.46	1.61	1.49
		1.53	1.51	1.56	1.55	1.51	1.50	1.58	1.53	1.52	1.51	1.43	1.47	1.62	1.50

cal analysis, but a statistical test for aberrant observations rejected the two values reported by Collaborator 5. These two values were replaced with the aid of a replacement technique for the statistical analysis (6).

The agreement between the two methods is readily apparent (Tables 1 and 2) and is confirmed by the analysis of variance. There was no significant difference ($P > 0.05$) between the method means. Thus, there was no reason to suspect that the two methods differ in their mean values.

However, there was a highly significant difference ($P < 0.001$) between the laboratory means. The average results from Laboratory 6 by the AOAC method were consistently lower than those of the other laboratories for all seven samples, while in Laboratory 7 the averages by the AOAC method were consistently higher than those of the other laboratories.

Results of the analysis of variance (Table 3) also revealed that the lab × sample interaction was significant, as was the lab × method and the three factor interaction. Laboratories 6 and 7 probably were large contributors to the significance of these interactions. A possible reason for the differences between laboratories, in addition to Collaborator 7 not titrating to the specified end point, is the inherent individual differences in performing color end point titrations. Slight differences in the individual samples following the working of the salt into the butter may also have occurred.

These differences between laboratories were considered in the analysis of the two methods and were not sufficient to produce a significant difference between the method means. The collaborators who included comments with their results favored the IDF method for determining the salt content of butter.

Table 2. Laboratory and method mean values

Lab.	AOAC Method	IDF Method
1	1.51	1.50
2	1.52	1.52
3	1.50	1.47
4	1.55	1.52
5	1.51	1.52
6	1.40	1.51
7	1.61	1.49
Method Mean	1.51	1.50

Table 3. Analysis of variance

Source of Variation	Degrees of Freedom	Mean Square
Total variance	193[a]	
Between samples	6	19.5995[b]
Between methods	1	0.0054
Between labs	6	0.0270[b]
Method × sample	6	0.0041
Lab × sample	36	0.0044[b]
Lab × method	6	0.0322[b]
Lab × method × sample	36	0.0024[b]
Error	96[a]	0.0008

[a] Degrees of freedom adjusted for two replaced values.
[b] $p < 0.001$.

Conclusions and Recommendation

Results obtained from this study show that the proposed IDF method for determining the salt content of butter is as accurate as the present official AOAC method. In addition, the IDF method is faster and simpler.

It is recommended that the IDF method, as collaboratively studied, 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 C, and was adopted by the Association. See *This Journal*, **49**, 167–172 (1966).

This report of the Associate Referee was presented at the Seventy-ninth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 11–14, 1965, at Washington, D.C.

and Harold M. Windlan, C. W. England Laboratories, Washington, D.C.

J. S. Windbush, Bureau of Scientific Standards and Evaluation, Food and Drug Administration, kindly supplied the statistical analyses.

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TOBACCO

Determination of Potassium and Calcium in Tobacco

By ROBERT H. CUNDIFF and J. T. DOBBINS, JR. (Research Department, R. J. Reynolds Tobacco Company, Winston-Salem, N.C. 27102)

The methods for potassium and calcium in tobacco, based on column elution with dilute HCl followed by flame photometry of the extract, were studied collaboratively by 12 laboratories. Potassium results were sufficiently precise to recommend that the potassium procedure be adopted as an official, first action method. Results for calcium indicate that additional study is necessary. Atomic absorption spectrophotometry offers considerable promise in overcoming many of the hindrances experienced with flame photometry, particularly the calcium measurement.

The column elution and flame photometric techniques for determination of potassium and calcium in tobacco (1, 2) appear to offer comparatively simple and rapid means of assaying these cations. A collaborative study of the potassium method was conducted in 1962 (3) with inconclusive results. Dobbins (4) recently studied the interferences in the flame photometric analysis of tobacco eluates and showed that major interferences which were encountered in the calcium analysis could be overcome for the most part by proper use of a radiation buffer and a strontium-releasing agent.

These revisions were incorporated into the procedures, and a collaborative study was conducted on both methods under the auspices of the Analytical Methods Committee of the Tobacco Chemists' Research Conference. Initially, a synthetic solution, similar to that which would have resulted from elutive preparation of 0.5 gram tobacco and containing representative amounts of calcium and potassium, the requisite amount of 3*N* HCl plus 0.5 ppm aluminum and 5 ppm phosphate, was sent to 12 collaborators for analysis. This test was designed to check the reliability of the flame photometric techniques. After evaluation of these data, six tobacco samples were sent to the same 12 collaborators for potassium and calcium analysis. The samples were flue-cured, burley, Maryland and Turkish leaf, and flue-cured and burley stems. All samples were analyzed in duplicate on an "as received" basis, and all data were reported.

Method for Potassium

Reagents

(a) *Potassium stock soln.*—1000 ppm K. *See* **6.016(a)**.

(b) *Potassium standard solns.*—Place 0, 5, 10, 15, 20, 25, and 30 ml KCl stock soln in seven 1 L vol. flasks, add 40 ml 3*N* HCl to each, and dil. to vol. with H_2O.

(c) *Celite 545.*—Acid-washed (Johns-Manville).

Apparatus

(a) *Flame photometer.* — Natural gas-air fuel, or equiv., adequate for K analysis.

(b) *Chromatographic column.*—20 × 150 mm with coarse fritted disk.

Preparation of Sample Solution

Weigh accurately ca 0.5 g tobacco dust into weighing dish, ca 40 ml capacity. Add ca 1 g acid-washed Celite 545 and mix intimately with spatula. Transfer quantitatively thru powder funnel into chromatographic tube. Add addnl Celite thru funnel into tube until 1″ layer accumulates on top sample-Celite mixt. Compact sample and Celite by tapping tip of tube on table top, and insert tip of tube into neck of 1 L vol. flask. Add 40 ml 3*N* HCl into tube by pipet or dispenser, washing down sides of tube, and let elute into vol. flask. When liquid level reaches top of Celite, add 25 ml H_2O to tube and let elute. Add second 25 ml portion H_2O, let elute normally, or force thru rapidly with compressed air. Rinse tip of tube into vol. flask, dil. to vol. with H_2O, and mix well.

Determination

Det. % T for sample eluate and K stds as specified in instruction manual of flame photometer used. *See* also **6.019**.

Prep. calibration curve and det. ppm K of unknown sample from curve.

% K = ppm K × 0.1/g sample.

% K_2O = ppm K × 0.1205/g sample.

Method for Calcium

Reagents and Apparatus

(a) *Calcium stock soln.*—1000 ppm Ca. Dissolve 2.497 g $CaCO_3$ in 16 ml 3*N* HCl and dilute to 1 L with water.

(b) *Calcium standard solns.*—Place 0, 5, 10, 15, and 20 ml Ca stock soln in five 1 L vol. flasks. Add 300 ml KCl stock soln, 20 ml 3*N* HCl, and 100 ml Sr stock soln to each, and dilute to vol. with water.

(c) *Strontium stock soln.*—2000 ppm Sr. Dissolve 6.0856 g $SrCl_2.6H_2O$ in water and dilute to 1 L.

(d) *Flame photometer.*—Acetylene-air or equivalent flame necessary for Ca analysis.

Procedure

Transfer 50.0 ml eluate to 100 ml vol. flask. Add 30 ml potassium stock soln (1000 ppm) and 10 ml strontium stock soln (2000 ppm), and dilute to vol. with water.

Measure emission of unknown solution and std calcium solns at 554 mμ, using procedures specified in instruction manual of flame photometer used.

Prepare calibration curve and determine ppm Ca from calibration curve.

% Ca = ppm Ca × 0.2/g sample.

(*Note:* If any eluate requires further dilution to fall in range of standards, add proportionate amounts of 3*N* HCl, potassium, and strontium solns. Adjust calculations accordingly.)

Results and Discussion

Table 1 lists the means of duplicate values reported by each collaborator on the synthetic potassium-calcium solution, along with accuracy and precision data.

Table 1. Collaborative results on the flame photometric determination of potassium and calcium in a synthetic solution

	K_2O Amount Present: 5.54%	Calcium Amount Present: 3.50%
Coll.	Amount Found, %	Amount Found, %
1	4.22[a]	3.87
2	5.46	3.44
4	5.54	3.73
9	5.6	5.8[a]
11	6.04[a]	3.51
12	5.41	3.92
13	5.55	3.48
15	5.49	3.44
16	5.63	3.47
22	5.29	—
23	5.71	—
27	5.34	3.56
Mean	5.50	3.60
Std Dev.	0.13	0.19
Rel. Std Dev.	2.4	5.3
Range	0.42	0.48
Mean Error	0.04	0.10
Relative Error	0.72	2.78

[a] Values not utilized in calculation of precision and accuracy.

Table 2 gives the means of duplicate determinations for potassium on each tobacco sample, the overall means, and the intra- and interlaboratory precision.

Calcium data were based on results by 10 collaborators, since two collaborators did not have the proper instrumentation for the calcium determination (Table 3). The values obviously inconsistent with the respective mean values were deleted, and the inter-laboratory precision recalculated; these data are also listed in the tables.

The calcium values indicate that additional study of the method is necessary. It was believed, and partially demonstrated by the calcium results from the synthetic eluate, that much of the variance comes from the flame photometric determination itself rather than from the elution of the calcium from tobacco. Each collaborator was asked to report the instrument and fuel used in the analysis for each cation (Table 4).

The low calcium values of Collaborator 4 could have partially resulted from too low a temperature flame in the analysis.

All results by Collaborator 2, who also

Table 2. Collaborative results for determination of % potash in tobacco[a]

Coll.	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Std Dev., %
1	3.48	5.78	4.95	3.12	8.50	12.96	0.05
2	3.49	5.79	4.92	3.01	8.86	13.09	0.02
4	3.47	5.65	4.70	3.08	8.19	12.40	0.04
9	3.53	6.08[b]	4.91	3.09	8.81	13.43	0.13
11	3.29	5.15[b]	4.50[b]	—	7.22[b]	11.04[b]	0.03
12	3.69	5.76	4.90	3.17	9.07	13.09	0.06
13	3.48	5.74	4.90	3.06	8.76	13.00	0.00
15	3.49	5.73	4.92	3.03	8.64	12.90	0.06
16	3.53	5.73	4.90	3.09	8.89	13.10	0.02
22	3.49	5.47	4.76	3.03	9.40[b]	13.74	0.01
23	3.35	5.79	4.98	3.04	8.76	12.95	0.04
27	3.53	5.82	4.93	3.17	8.98	13.32	0.04
Mean	3.49	5.71	4.86	3.08	8.67	12.92	
Std Dev.	0.10	0.22	0.14	0.05	0.55	0.67	
Rel. Std Dev.	2.9	3.9	2.9	1.6	6.3	5.2	
Mean[b]	3.49	5.73	4.89	3.08	8.75	13.07	
Std Dev.[b]	0.10	0.10	0.08	0.05	0.25	0.28	
Rel. Std Dev.[b]	2.9	1.7	1.6	1.6	2.9	2.1	

[a] Sample 1, Flue-cured leaf; Sample 2, Burley leaf; Sample 3, Maryland leaf; Sample 4, Turkish leaf; Sample 5, Flue-cured stems; Sample 6, Burley stems.
[b] Eliminated in calculations.

Table 3. Collaborative results for determination of % calcium in tobacco[a]

Coll.	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Std Dev., %
1	1.92	4.16	3.53	3.09	1.48	2.65	0.11
2	1.69	3.88	3.49	2.98	1.51	2.61	0.03
4	1.59[b]	3.40[b]	2.70[b]	3.20	1.14[b]	2.28[b]	0.04
9	2.00	5.09[b]	3.75	3.18	2.22[b]	3.74	0.68
11	1.94	4.04	3.80	—	1.76	2.78	0.18
12	2.37[b]	4.29	3.86	3.16	1.97	3.10	0.07
13	2.10	4.15	3.99	3.38	1.70	3.00	0.01
15	1.72	3.81	3.48	2.87	1.35	2.55	0.03
16	2.20	4.16	3.61	3.21	1.61	2.70	0.05
27	1.76	3.84	3.63	2.80	1.48	2.61	0.03
Mean	1.93	4.08	3.58	3.10	1.62	2.80	
Std Dev.	0.25	0.44	0.35	0.18	0.31	0.40	
Rel. Std Dev.	13.0	10.8	9.8	5.8	19.1	14.3	
Mean[b]	1.92	4.04	3.68	3.10	1.61	2.75	
Std Dev.[b]	0.21	0.18	0.18	0.18	0.20	0.20	
Rel. Std Dev.[b]	10.9	4.5	4.9	5.8	12.4	7.3	

[a], [b] See footnotes, Table 2.

Table 4. Flame photometer and fuel used for potassium and calcium analyses

Coll.	Flame Photometer	Fuel	
		Potassium	Calcium
1	Beckman DU	Acetylene	Acetylene
2	Coleman 121	Natural gas	Natural gas
4	E. E. L.	Coal gas-air	Coal gas-air
9	Beckman DU	Hydrogen	Hydrogen
11	Beckman DU	Hydrogen-oxygen	Hydrogen-oxygen
12	Beckman DU	Acetylene-oxygen	Acetylene-oxygen
13	Perkin-Elmer 146	Natural gas-air	Acetylene-air
15	Beckman DU	Hydrogen	Hydrogen
16	Beckman DU	Oxygen-acetylene	Oxygen-acetylene
22	E. E. L.	Coal gas-air	—
23	Beckman DU	Hydrogen-oxygen	—
27	Beckman DK	Oxygen-hydrogen	Oxygen-hydrogen

Table 5. Calcium values obtained by four methods

Method	% Calcium					
	1	2	3	4	5	6
Column elution, flame photometry[a]	1.92	4.04	3.68	3.10	1.61	2.75
Column elution, EDTA titration (5)	2.25	4.39	3.78	3.27	1.68	2.82
Column elution, atomic absorption	1.94	4.29	3.80	3.19	1.63	2.86
Acid digestion, atomic absorption	1.86	4.28	3.73	3.17	1.56	2.89

[a] Corrected mean values for this study.

used a lower temperature flame, are slightly lower than the means.

Additional studies have indicated that atomic absorption spectrophotometry is a much preferred tool for both potassium and calcium determinations. Lanthanum is preferred to strontium as a releasing agent for the calcium determination with this instrumentation. Table 5 compares analysis of the tobacco eluates by atomic absorption and by the EDTA titration procedure (5) with atomic absorption analysis of digested tobacco samples. The corrected mean flame photometric values obtained in this collaborative study are also included. These data further demonstrate that the calcium is eluted from the tobacco, and the anomalies appear from flame measurement.

The potassium values are sufficiently precise to recommend the method, although there is a tendency toward increased variance with the increase in potash content of the tobacco.

Recommendations

It is recommended—

(1) That the proposed method for potassium in tobacco be adopted as official, first action.

(2) That the method for calcium in tobacco be further studied.

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Determination of Oven Moisture in Tobacco

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A comparison of mechanical convection and gravity convection oven-drying of tobacco was made on 12 tobacco samples, six with high and six with low moisture content. Data show that better interlaboratory precision was obtained by drying in a mechanical convection oven. The term "apparent moisture" is used because oven drying also causes loss of volatile organic material. The magnitude of this loss is shown to be from 0.25 to 1.5%, depending on the type and treatment of the tobacco. The forced draft oven method is recommended for adoption as official, first action.

When moisture is determined in tobacco by oven methods, volatiles other than water are lost, and this loss causes the "moisture" figures to be higher than the true values. This fact is recognized by the tobacco industry, and although the data obtained are not accurate moisture values they can be used to correct other analytical data to a reasonably uniform moisture-free basis. In this report "moisture" refers to the loss in weight of tobacco when dried in an oven or over desiccant.

The methods used in this study, conducted several years ago by the Analytical Methods Committee of the Tobacco Chemists' Research Conference, were designed for ground analytical samples and not for bulk leaf, strip, or scrap material.

Twelve samples of tobacco were prepared by the American Tobacco Company for this study. Six different tobaccos were ground to pass a 1 mm screen, and each was divided into two lots; then one lot was conditioned to produce a high moisture sample and the other a low. The tobacco samples and designations were as follows:

	Moisture Level	
	Low	High
Lug, flue-cured	1A	1B
Leaf, flue-cured	2A	2B
Lug, burley	3A	3B
Leaf, burley	4A	4B
Cased, blended, cigarette	5A	5B
Cigar, filler	6A	6B

Three methods were tested. In addition each collaborator was asked to determine the moisture by his own procedure if it differed from those under test.

This report of the Associate Referee, C. L. Ogg, was presented at the Seventy-ninth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 11–14, 1965, at Washington, D.C.

This investigation (No. 66-3-39) relates to a project of the Kentucky Agricultural Experiment Station; published with approval of the Director.

Method I

Apparatus

(a) *Drying oven.*—Forced-draft, regulated to 99.5 ± 0.5°. Suggested dimensions: 19 × 19 × 19″. Approx. oven settings: fresh air intake vent ⅕ open; air control damper ¼ open; air exhaust vent ⅓ open.

(b) *Moisture dish.*—Al, diam. 45–65 mm, depth 20–45 mm, with tight fitting cover.

Determination

Weigh accurately ca 5 g sample (ground to pass 1 mm or finer screen) into weighed moisture dish and place uncovered dish in oven.

Do not exceed 1 sample/10 sq. in. shelf space, and use only 1 shelf. Dry 3 hr at 99.5 ± 0.5°; remove from oven, cover, and cool in desiccator to room temp. (ca 30 min.). Reweigh to nearest 1 mg and calc. % moisture = (wt before − wt after drying) × 100/wt sample.

Method II

Proceed as in method I, except use convection oven. Limit number of dishes to keep 50% shelf space free.

Method III

Apparatus

(a) *Desiccator.*—Standard 10–12″ laboratory desiccator containing not < 300 ml fresh 95% H_2SO_4.

(b) *Moisture dish.*—*See* method I.

Determination

Weigh 2 g sample to nearest mg in moisture dish, place in desiccator with cover removed, and let stand 9 days at 30°C. Do not exceed 8 samples/desiccator and do not stack dishes. After 9 days remove from desiccator, cover, and reweigh immediately to nearest mg. Calculate % moisture.

Method IV

If collaborator's usual procedure differed from methods I, II, or III, he was asked to determine moisture by his method and report the results.

Results and Recommendation

The results obtained by the various collaborators and methods are shown in Table 1. Since the method is empirical and because we recognize that the results are higher than the true value, the main consideration is the between-laboratory precision. This precision is shown by the variance figure in the last two columns. The next to last column shows the variance for all data; the last column shows the variance for method I (B series), with the data from Collaborator 102 excluded. The data from this collaborator for the first 6 samples were randomly distributed about the average, the value for sample 1B was slightly higher than the average for this sample, and all values were low by 0.5–1% for the last 5 samples, indicating a probable malfunction of apparatus. Since such a distribution of values would occur normally less than 10% of the time, the second set of variances was calculated and is believed to be a truer representation of the precision of method I. Comparison of variances for methods I and II showed that in all but one case the variance for method I was lower than that for method II. For 5 of the 12 samples the difference was critical (F test) at the 10% level.

Based on interlaboratory precision, method I is the method of choice. This is in general agreement with laboratory practice since most tobacco research laboratories now use a mechanical convection oven method with only slight, if any, differences from method I.

Method III, the desiccator procedure, was included in the study to provide some indication as to the magnitude of the difference between the oven "moisture" figure and the true value. The between-laboratory agreement for this method was very poor. However, three laboratories (103, 104, and 108) were in reasonably close agreement and obtained higher values than the other collaborators. The differences between the median value for each sample in these three sets of data and the median value obtained by method I for the same sample give an indication of the amount of volatiles other than water lost on oven drying (Table 2). Table 2 also shows that the difference for the A and B samples of each tobacco was

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee A, and was adopted by the Association. See *This Journal*, **49**, 172–175 (1966).

Table 1. Collaborative results for moisture in tobacco

Sample	Legend	Code Numbers of Collaborators 101	102	103	104	105	106	107	108	Av. % Moisture	Variance	
						Method I						
1A	Lug, flue	5.57	5.30[b]		5.60			5.67	5.67	5.56	0.0234[c]	
2A	Leaf, flue	5.31	5.45		5.45			5.21	5.44	5.37	0.0117	
3A	Lug, burley	5.49	5.29		5.51			5.29	5.59	5.43	0.0187	
4A	Leaf, burley	4.69	4.64		4.64			4.66	4.72	4.67	0.0073	
5A	Cased, blended	4.26	4.36		4.36			4.38	4.50	4.37	0.0012[d]	
6A	Cigar, tobacco	5.24	5.41		5.30			5.34	5.35	5.33	0.0143[d]	
1B	Lug, flue	11.55	11.75		11.79			11.51	11.73	11.67	0.0159	0.0151
2B	Leaf, flue	11.00	10.50		11.06			11.01	11.17	10.95	0.0673	0.0061[d]
3B	Lug, burley	9.97	9.44		10.39			9.95	10.21	9.99	0.1215	0.0452
4B	Leaf, burley	10.33	9.67		10.67			10.53	10.63	10.37	0.1687	0.0231
5B	Cased, blended[a]	12.60	12.16		12.77			12.75	12.99	12.65	0.0956	0.0258[d]
6B	Cigar, tobacco	10.56	9.91		10.82			10.55	10.77	10.52	0.1318	0.0191[d]
						Method II						
1A	Lug, flue	5.20	5.50	5.47		5.3[b]	5.47	5.66	5.75	5.48	0.0361	
2A	Leaf, flue	5.09	5.26	5.21		5.2	5.56	5.36	5.07	5.25	0.0284	
3A	Lug, burley	5.26	5.32	5.39		5.4	5.57	5.15	5.59	5.38	0.0253	
4A	Leaf, burley	4.52	4.56	4.45		4.5	4.74	4.73	4.26	4.54	0.0277	
5A	Cased, blended	3.80	4.20	3.92		4.1	4.10	4.33	5.11	4.22	0.0275	
6A	Cigar, tobacco	5.04	5.30	5.17		5.2	5.27	5.57	5.89	5.35	0.0687	
1B	Lug, flue	11.09	11.41	11.34		11.2	11.68	11.59	11.76	11.43	0.0623	
2B	Leaf, flue	10.80	10.79	10.83		10.8	11.27	11.11	10.81	10.92	0.0374	
3B	Lug, burley	9.98	9.76	9.79		9.8	10.08	10.19	10.19	9.97	0.0375	
4B	Leaf, burley	9.84	10.11	10.19		10.1	10.18	10.59	10.14	10.16	0.0490	
5B	Cased, blended[a]	12.47	12.21	11.65		12.6	12.42	12.61	13.62	12.51	0.3483	
6B	Cigar, tobacco	10.25	10.29	10.42		10.6	10.75	11.02	11.27	10.66	0.1460	
						Method III						
1A	Lug, flue	3.47	3.15	4.78	4.66	4.2		4.10	4.49	4.12		
2A	Leaf, flue	3.33	3.18	4.84	4.51	4.2		3.70	4.42	4.03		
3A	Lug, burley	3.74	3.22	5.43	4.94	4.3		3.88	5.01	4.36		
4A	Leaf, burley	3.19	2.34	4.46	3.92	3.6		3.12	3.82	3.49		
5A	Cased, blended	2.13	1.87	3.08	2.90	2.6		2.25	2.79	2.52		
6A	Cigar, tobacco	3.98	2.61	5.41	4.02	4.2		3.70	4.88	4.11		
1B	Lug, flue	9.39	8.66	10.76	10.32	10.2		9.82	10.66	9.97		
2B	Leaf, flue	9.03	8.37	10.24	10.03	9.6		9.48	10.18	9.63		
3B	Lug, burley	8.70	7.22	9.50	9.16	9.0		8.63	9.59	8.83		
4B	Leaf, burley	8.67	7.41	9.68	9.38	9.3		8.80	9.74	9.00		
5B	Cased, blended[a]	10.54	9.74	11.27	10.93	10.8		10.65	11.19	10.73		
6B	Cigar, tobacco	9.40	7.16	10.30	9.34	9.4		8.75	10.31	9.24		
						Method IV						
1A	Lug, flue	6.45		4.26	5.58		5.89	6.73	5.64	2.47		
2A	Leaf, flue	6.22		4.02	5.42		5.69	5.89	5.25	2.20		
3A	Lug, burley	6.06		4.37	5.47		5.83	6.41	5.34	2.04		
4A	Leaf, burley	5.26		3.36	4.59		4.93	5.78	4.65	2.42		
5A	Cased, blended	5.47		2.57	4.36		4.98	5.49	4.39	2.92		
6A	Cigar, tobacco	5.84		4.51	5.29		5.56	6.58	5.25	2.07		
1B	Lug, flue	12.46		10.38	11.64		11.79	12.53	11.60	2.15		
2B	Leaf, flue	11.88		9.97	11.13		11.36	11.62	10.95	1.91		
3B	Lug, burley	10.57		9.19	10.13		10.38	10.97	9.82	1.78		
4B	Leaf, burley	11.02		9.25	10.49		10.62	11.43	10.33	2.18		
5B	Cased, blended[a]	13.81		10.84	12.76		13.39	14.14	12.66	3.30		
6B	Cigar, tobacco	11.15		9.79	10.70		10.79	11.66	10.44	1.87		

[a] Cigarette tobacco.
[b] Regular method for this collaborator.
[c] Data from collaborator 102 omitted.
[d] Variances for Method I significantly less than those for Method II (10% level).

reasonably consistent. Comparison of the values by method I with those obtained by GLC (gas-liquid chromatography) would probably give a truer measure of volatile

Table 2. Comparison of median moisture values for methods I and III

Sample	Medians I[a]		III		Differences	Δ_B
1A	5.60	–	4.66	=	0.94	1.07
2A	5.44	–	4.51	=	0.93	0.83
3A	5.49	–	5.01	=	0.48	0.45
4A	4.66	–	3.92	=	0.74	0.95
5A	4.36	–	2.90	=	1.46	1.56
6A	5.34	–	4.88	=	0.46	0.26
1B	11.73	–	10.66	=	1.07	
2B	11.01	–	10.18	=	0.83	
3B	9.95	–	9.50	=	0.45	
4B	10.63	–	9.68	=	0.95	
5B	12.75	–	11.19	=	1.56	
6B	10.56	–	10.30	=	0.26	

[a] Median of 3 highest sets of values.

organic material lost during oven drying. However, GLC methods for moisture in tobacco were not developed when this study was made.

Because method I gave good precision on all types of tobacco tested at both high and low levels of moisture and was more precise than method II, and because it is the method most used in the industry today, it is recommended that method I, the forced draft oven method, be adopted as official, first action.

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GELATIN AND GELATIN PRODUCTS

Dessert Gel Strength Testing

By EMANUEL BORKER, ARTHUR STEFANUCCI, and ALFRED A. LEWIS (General Foods Technical Center, White Plains, N.Y. 10601)

The history of gelatin and gelatin dessert strength testing is reviewed. The current AOAC method for dessert gels using the Bloom Gelometer was found too variable for control of gelatin dessert production in a multi-plant operation. Variability can be reduced with a lower shot flow rate, rigid control of sample preparation, and frequent maintenance in gelometer alignment and adjustment. Detailed instructions for dessert gel strength testing are given.

Gel strength testing is critically important to quality control and research on gelatin and gelatin dessert products. Despite this importance, there has been little substantial change in the testing method for more than a quarter century. Because of the wide acceptance of the Bloom Gelometer method (1) by the trade and in government and industry specifications, it would be extremely difficult to gain general approval of a radically new procedure. However, improvements in the Bloom method are definitely required in order to meet the demands of current production practices.

Historically, evaluation of gel strength has been a rather unsophisticated "art." Prior to 1920 a common test was a highly subjective "finger test" which compared the test gel with arbitrary standards prepared

under the same conditions (2). Another test involved the preparation of solutions of a gelatin at varying concentrations to establish the lowest concentration which would not flow out of a test tube inverted after the solution was permitted to set (3). A variation of this method still serves as a rapid, "go-no go" technique for gel dessert batch mix control in certain areas.

Many mechanical devices have been designed to objectively measure the gel strength of gelatin and glue (i.e., nonedible gelatin). The literature on these devices prior to 1923 has been reviewed comprehensively by Bogue (2), Alexander (4), and Sheppard and Sweet (5). Sheppard and Sweet (6) designed a balanced beam plunger test and studied the effects of plunger shape, gel penetration depth, and container to plunger diameter ratio. This test development followed their study of a torsion dyanamometer method which did not give a simple correlation between breaking point of the gel and gelatin concentration (7).

The basic instrument now commonly used in testing gel strength of gelatin, glues, and the like was designed by O. T. Bloom and patented in 1925 (1). The plunger, suspended on a spring, is lowered in the prepared gel a fixed distance by the force of lead shot delivered to a receiver. Gel strength is then defined as the weight of the shot and receiver needed to move the plunger the fixed distance against the resistance of the gel.

In 1924 the Bloom Gelometer was adopted as standard by the National Association of Glue Manufacturers (8). Detailed procedures were established which included gelatin concentration, chill time, and instrument adjustments such as shot flow rate, plummet size, and depth of gel depression. A glue concentration of 12.5% (w/v) was set as the standard. The Edible Gelatin Manufacturer's Society adopted these procedures with a 7.5% gelatin concentration instead of 12.5%, and substituted the word "gelatin" for "glue" (9). Later, the Glue Association modified the recommended sampling procedures (10).

Since its introduction in 1924, there have been at least four commercial models of the Bloom Gelometer (11). Initially these instruments were made and marketed by Swift and Company. The Precision Scientific Company introduced the most recent design around 1945; construction was substantially different although basic elements remainded the same.

The procedures used for weak gels such as gelatin dessert gels have been based on the procedure for glue and gelatin. Tourtellotte and Kind (12) reported an AOAC collaborative study on gelatin dessert testing by one inch and one-half inch diameter plummets. Except for the one inch plummet and a light weight shot receiver, the conditions for sample preparation, chilling, and gelometer adjustments were those adopted by the Glue Manufacturers in 1924. An adapter for the gelometer was designed in 1945 to substantially reduce the shot flow; this adapter was designed to reduce errors in measuring the strength of weak gels (13). However, an AOAC study showed that precision with this adapter was poor because it did not regulate the shot flow uniformly from one instrument to another or give duplicate results on the same instrument. For weak gels, the AOAC adopted the one inch plummet with a 200 g/5 sec. shot flow rate (14). Coefficients of variation obtained at three gelatin concentrations under these conditions were about 0.1.

For control of the great volume of gelatin dessert mix production in multi-plant operations the AOAC procedure was found inadequate to assure uniform gel characteristics and to conserve the costly functional raw material, gelatin, by close-level control. Many control chemists devised ingenious variations of the basic procedure to permit better performance in their individual plants.

This paper reports a study of the key elements involved in dessert gel strength testing. Rigid conformance to exact sample preparation instructions and frequent maintenance in gelometer alignment and adjustment are important to all procedures; otherwise, high variability can be expected in any procedure.

Two sets of conditions appeared promising for reducing variability in gel strength

Table 1. Basic precision of the methods (coefficients of variation)

Variable	Method	
	75 g/⅝″	45 g/1″
Among punches on same jar	0.043	0.019
Among readings on several jars from same sample when single punch/jar is taken	0.048	0.023
Among jar averages from same sample when two punches are taken/jar (usual procedure)	0.037	0.019

measurement. One procedure used the one inch plummet and a 45 g/5 sec. shot flow rate; the other, a ⅝″ plummet and 75 g/5 sec. shot flow rate. The 45 g shot flow rate was obtained by soldering an adapter to the shot spouts; a height adjustment of the hoppers was made for the 75 g flow rate. An experiment was designed to compare these procedures; variables included operator difference, time of test difference, gelatin concentration, and container. Data (Table 1) showed that the lower shot flow rate procedure was superior. These variables (Table 1) were chosen since the weights, i.e., gel strengths, are unique to the combination of shot flow rate and plunger used. Consistent differences in gel strength were found among operators in both methods, but they were greater in the higher shot flow method. Operator averages for both methods are shown in Table 2.

A further test of these two procedures was made under normal conditions. Half of the routine control tests over a 20-day production period were made by each procedure. Two analysts tested a total of 240 samples by each procedure. For the ⅝″ plummet/75 g shot rate, the average gel strength was 17.2 ± 1.05, with a coefficient of variation of 0.061; for the 1″ plummet/45 g shot rate, the average was 45.0 ± 2.36, with a coefficient of variation of 0.053. The data (Table 3) confirmed the earlier evaluation.

It was concluded that the method using a 45 g shot flow rate would meet the needs of modern plant operations and the procedure described below has been used for routine control of commercial gelatin dessert production. The procedure has successfully met the needs of a multi-plant organization (including foreign affiliates) for more than 5 years.

Gel Strength Test Method

Principle

The gel strength of prepared gelatin dessert is determined with a Bloom Gelometer using a 1″ plummet, a shot flow rate of 45 g/5 sec., and soldered adapter.

Apparatus

(a) *Bloom Gelometer.*—See *Adjustment and Maintenance Schedule.*

(b) *Bloom jars.*—Specimen Jar No. 1016 (T. C. Wheaton Co., Millville, N.J.).

(c) *Chill bath.*—10 ± 0.1°C. (The rack on which the jars are placed must be perfectly level.)

(d) *Glassware.*—Volumetric flasks calibrated to deliver 474 ml H_2O at 150°F; and Erlenmeyer flasks, 750 ml, narrow neck.

(e) *Magnetic stirrer and magnetic stirring bars.*—Teflon-coated, overall length ca 1⅝″.

(f) *Stoppers.*—Rubber, Nos. 7 and 9, one-hole.

Recommended Procedure

Weigh 85 g sample, or quantitatively transfer contents of package of gelatin dessert into dry 750 ml erlenmeyer. Add exactly 474 ml H_2O at 148–152°F from calibrated volumetric flask. Place magnetic stirring bar in erlenmeyer and stopper with No. 7 one-hole rubber stopper. Mix on magnetic stirrer for 5 min., making certain that entire sample dissolves. Remove from stirrer and let sit on benchtop at room temperature. One half hour after adding H_2O, place finger over hole in stopper and gently invert 2 times. Pour 120 ml of solution into clean, dry Bloom jar, (b). Stopper with No. 9 one-hole stopper and place Bloom jar in 10 ± 0.1°C chill bath, making certain water level in bath covers liquid level in jars. Let gels stand in chill bath 17 hr.

Determine gel strength with a Bloom Gelometer which has been adjusted according to the *Adjustment and Maintenance Schedule.* Before each series of runs, check level of gelometer and platform, plumbness of plummet, contact points and gap, spring tension, shot flow rate, and shot and contact setting.

Turn on gelometer circuit switch and turn knurled coil-spring screw until contact disc makes first perceptible electrical contact with lower contact. Tighten coil-spring lock nut.

Table 2. Average gel strengths for operators and days

Day	75 g/⅝″ Operator I	Operator II	Operator III	Av.	45 g/1″ Operator I	Operator II	Operator III	Av.
1	17.8	18.6	17.3	17.9	42.1	44.8	42.5	43.1
2	17.6	18.4	17.2	17.7	43.0	42.4	42.7	42.7
3	17.6	19.1	17.9	18.2	43.1	43.3	41.5	42.7
4	17.9	18.5	18.3	18.2	43.3	42.3	43.6	43.1
5	18.2	18.8	17.3	18.1	41.2	41.7	39.3	40.8
6	18.0	18.9	17.8	18.2	40.8	40.5	41.4	40.9
7	18.6	18.3	18.1	18.3	43.4	44.2	43.0	43.5
8	18.5	18.3	17.8	18.2	43.3	41.8	41.1	42.1
Av.	18.0	18.6	17.7	18.1	42.5	42.6	41.9	42.4

Remove Bloom jar from chill bath and place on racking platform, which is in lowered position. Center the jar under plummet. Raise platform with coarse elevating knob until contact disc is ca 1 mm from upper contact. Then, with fine elevating knob, slowly rack up gel until contact disc makes first perceptible electrical contact with upper contact. (Contact disc should touch upper contact squarely. If this does not occur, instrument is out of plumb and should be releveled according to adjustment instructions.) Place shot receiver on pan; this will open circuit and chattering will stop. Gently raise hopper spout arm and engage it in dog of electromagnetic system, thus causing shot to flow into pan. (The arm should sit on extreme end of dog, reducing chance of delay when arm is released.)

When amount of shot necessary to depress plummet 4 mm into gel falls into shot receiver, contact disc touches lower contact, energizing the electromagnet, pulling in the dog, releasing the hopper arm, and stopping shot flow. Then close circuit switch and weigh shot receiver and shot to nearest 0.1 g.

Take two "punches" on each jar, average, and record as gel strength. Do not take second reading until shot used from first punch has been put back into hopper. (If a delay occurs in dropping of arm, or there is other instrument failure, return shot to hopper and take another punch.)

Adjustment and Maintenance Schedule

(Make all adjustments before each series of readings or at any time the instrument is not in proper working order.)

Shot.—Keep total of 800 g shot (size No. 12, Precision Scientific Catalog No. 68707) in hopper at all times. Check shot for uniformity of size by screening on U.S. Standard Screens No. 12 and No. 14; use shot that passes thru No. 12 and remains on No. 14. Replace old or worn shot by new shot or reclaim by screening.

Table 3. Variance components (g^2)

Component Source	⅝″ Plummet/ 75 g Shot Rate	1″ Plummet/ 45 g Shot Rate
Punches on same jar (av. of 2)	0.06	0.20
Jars from same sample	0.06	0.64
Among samples by same analyst on same day	0.61	2.01
Day-to-day differences	0.17	0.68
Analyst differences (consistent)	0.08	1.73
Analyst differences (inconsistent)	0.12	0.46
Total	1.10	5.72
Total Std Dev.	1.05 g	2.39 g
Av. Gel Strength	17.2 g	45.0 g

Leveling.—Adjust two leveling screws located at bottom of base until spirit level built into base of instrument is centered. Place spirit level on racking platform and check level in three directions: right to left across front, front to back diagonally from left to right, and front to back diagonally from right to left. (It should be level for all heights of platform.)

Turn knurled coil-spring adjustment knob located on top of gelometer until plummet assembly hangs free on coil spring. (*Note:* Contact disc should not touch either of contact points in spark gap.)

Place perfectly squared block on racking platform and place spirit level on block. Check level in same three directions as above. Carefully move racking platform upward until plummet plunging surface barely touches squared block. Sighting at eye level, note if

plummet surface rests squarely on surface of block when plummet rod is centered in bakelite guide plate. If these conditions are not met, carefully bend pan assembly.

Spark gap.—Turn circuit switch on. Turn knurled coil-spring adjustment knob located on top of gelometer until contact disc makes first perceptible electrical contact with lower contact point. (When perfectly set, the slightest motion of the pan system will cause a succession of "makes and breaks" in the circuit, producing a sound much like that of a telegraph sounder.) Tighten spring adjustment nut.

Place squared block on racking platform. Slowly raise platform until block just meets plummet plunging surface and contact disc barely touches lower contact. Check gap between upper face of contact with a 4 mm feeler gauge. When moving gauge in and out it should just touch upper face of contact disc and upper contact point. If out of adjustment, loosen upper contact locking screw and adjust upper contact to distance of exactly 4 mm. After securing locking screw, check again with gauge. Clean contact points with crocus cloth daily, or more frequently, depending upon response. To prevent beveling of upper contact point, wrap small piece of cloth around feeler gauge. Rest one side of gauge on upper surface of contact disc and pass gauge in and out until surfaces are polished. Re-adjust gap to 4 mm. Upper contact point should be perfectly square; if beveled, replace with new contact.

Spring tension.—Turn circuit switch on. Turn knurled coil-spring adjustment knob until contact disc makes first perceptible electrical contact with upper contact. Place light weight shot receiver on pan assembly and add shot by hand until contact disc makes first perceptible electrical contact with lower contact. Weight (receiver and shot) required to lower system 4 mm is called "spring tension" and it must be 2.1 ± 0.2 g. If spring tension is too great or too small, replace with new spring that meets specification. (Since this work was done, it has been found difficult to obtain springs of this tension. The Gelatin Manufacturers recommend a spring with 1.8 ± 0.2 g tension.)

Contact setting.—For this test contact disc should be riding on lower contact. Turn knurled coil-spring screw until contact disc is making just perceptible electrical contact with lower contact. Tighten coil-spring lock nut.

Shot receiver.—Use an aluminum foil dish. (Cenco Cat. No. 12720 is recommended since these dishes are light, i.e., ca 1.3 g.) Replace receivers that develop distorted bottom surfaces.

Shot flow reducing adapter.—Solder the Shot Flow Reducing Adapter (Precision Scientific Cat. No. 68708) to hopper. Position adapter so that shot can only pour through orifice made by adapter and hopper edge. If shot pours out sides, re-adjust adapter. Orifice should be large enough so that shot flow is uniform and continuous.

Shot flow rates.—Adjust instrument to give shot flow rate of 45 ± 1.5 g/5 sec., as follows: Turn circuit switch off; place shot receiver on pan; with stopwatch in one hand, grasp hopper spout arm and engage in dog with quick but uniform motion; after 5 sec., stop shot flow by turning circuit switch on; obtain net weights of shot delivered for 5 determinations. If instrument is not delivering shot flow rate of 45 ± 1.5 g/5 sec., raise or lower electromagnetic system (depending on whether net weight was too high or too low) by loosening electromagnetic system locking nut and turning system to adjust nut. Determine shot flow rate for new position as above. Repeat procedure to attain desired shot flow rate.

If all adjustments have been made properly and if adapter has been soldered on correctly, all shot in the hopper should empty out in continuous flow and following measurements should hold:

Distance from edge of adapter to pan assembly resting on lower contact:	48– 52 mm
Distance from bottom of gap assembly to top of electromagnetic assembly:	8– 15 mm
Distance from base of instrument to bottom of electromagnetic assembly on rear left post:	185–190 mm[1]
Distance from base of instrument to bottom of gap assembly right rear post:	250–255 mm[1]
Distance from top of gap assembly to bottom of edge of hopper:	192–198 mm[1]

Lower relay (for instruments with additional relays).—Remove plate on bottom of instrument to expose lower relay. Adjust gap between contact points to attain maximum in-

[1] Applicable only to new Gelometers, i.e., 1940 designs.

strument sensitivity. Clean contact points with crocus cloth periodically.

Chill bath.—Keep platform rack in chill bath perfectly level; check with spirit level. Rack should not sag when loaded with jars; heavy perforated metal plate at least ¼" thick is recommended. Water level of bath should cover liquid level in Bloom jars. To insure proper circulation of water in chill bath around each jar, place no more than 54 (6 × 9) jars on 21 × 30" platform. Maintain bath temperature at 10 ± 0.1°C and record automatically. (Thermometer with 0.1°C subdivisions should also be used. *Note:* A recorder may reproduce to ± 0.3°C (width of ink band) but the record will show any gross changes in temperature during the 17-hour period.)

Keep chill bath covered at all times. If cover is hinged on one side, it can be opened and closed easily by a pulley and weight system.

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DRUGS

Determination of Acetaminophen in Combination with Other Drugs

By JOSEPH LEVINE and JOHN R. HOHMANN (Division of Pharmaceutical Chemistry, Food and Drug Administration, Washington, D.C. 20204)

Acetaminophen can be separated from acidic, basic, and neutral solutions on a sodium carbonate-sodium bicarbonate column. Salicylamides and barbiturates are eluted in chloroform; acetaminophen is eluted in ether and measured spectrophotometrically.

Acetaminophen is widely used as an analgesic and antipyretic, both alone and in combination with a large variety of drugs.

In the NF XI assay for acetaminophen tablets (1) the active ingredient is extracted from the tablet with methanol, while in the

NF XII assay (2) it is extracted from a water suspension with chloroform-alcohol. The quantity of the extracted material is measured in methanolic solution by UV absorbance. Brown and Gwilt (3) measure absorbance in sodium hydroxide solution. Aftalion, *et al.* (4) determine acetaminophen by nonaqueous titration with sodium methoxide in dimethylformamide and by potentiometric titration with sodium nitrite after hydrolysis to *p*-aminophenol.

Koshy and Lach (5) isolated acetaminophen from tablets and sirups by ion exchange chromatography, separating it from *p*-aminophenol. They also used this procedure for combinations with several other drugs but could not successfully separate acetaminophen from aspirin (6). Using partition chromatography, Koshy (7) separated acetaminophen from combination with caffeine and aspirin. Aspirin was trapped ionically on a sodium bicarbonate layer, caffeine on a sulfuric acid layer; acetaminophen, which is not ionized under these conditions, passed through the column. In this partition system, neutral or weakly acidic drugs are not separated from acetaminophen.

In the method reported here, acetaminophen is separated from acidic, basic, and neutral substances by a buffered partition chromatographic system. Appreciable differences between the degree of dissociation of acetaminophen and those of the accompanying substances can be achieved by proper selection of the buffer pH. By selecting solvents to provide differential solubilities among these compounds, acetaminophen is sharply separated from all the pharmaceuticals with which it is combined in commercial formulations, including salicylamide and barbiturates, which, like acetaminophen, are very weak acids.

A column segment containing sodium carbonate-sodium bicarbonate buffer at pH 10.1 as immobile phase will quantitatively trap acetaminophen from chloroform solution. Salicylamide, barbiturates, and other substances with great solubility in chloroform and/or weaker acid strength will be eluted from the column, together with neutral or basic compounds. Acetaminophen is then eluted from the column with ether and measured spectrophotometrically. In using this technique for combinations of acetaminophen and sulfonamides, a small amount of sulfonamide will accompany the acetaminophen. This is readily removed by passing the ether eluate from the buffered column over a Celite:1*N* HCl column which will retain only the sulfonamide.

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Experimental

Preparations

(a) *Chromatographic column.* — See sec. 32.013(a) and (b) (8).

(b) *Preparation of sample.*—Transfer an accurately weighed representative portion of sample containing about 60 mg acetaminophen to 200 ml volumetric flask. Add about 25 ml water and 2 ml 1*N* NaOH to dissolve acetaminophen, and dilute to volume with water.

(c) *Preparation of standard solution.* — Transfer about 0.6 mg acetaminophen, accurately weighed, into 100 ml flask. Dilute to volume with methanol containing 1 ml 0.1*N* HCl.

(d) *Preparation of columns.—Column I.*— Mix 4 g acid-washed Celite 545[1] with 3 ml pH 10.1 buffer[2] (7 volumes 0.5*M* Na_2CO_3 + 1 volume freshly prepared 1*N* $NaHCO_3$). Transfer to column and tamp to a uniform mass, using gentle pressure. Cover with a pad of glass wool.

Column II.—Transfer 2.0 ml of sample preparation to 100 ml beaker. Acidify with 2 drops concentrated HCl and add 3 g Celite. Mix and transfer to column. "Dry-wash" beaker with about 1 g Celite to insure quantitative transfer, and tamp as above.

Mount Column II directly above Column I.

Procedure

(Use water-saturated solvents.)

Pass 150 ml $CHCl_3$ over both columns. Discard Column II and pass an additional 50 ml $CHCl_3$ over Column I. Discard eluate. Elute acetaminophen from Column I with 200 ml ether. (For samples containing sulfonamides, place below Column I, immediately prior to

[1] Johns-Manville Corp.

[2] The value of pH 10.1 is obtained on a pH meter with glass + calomel electrodes. Because of the effect of the high sodium concentrations, the accuracy of this value is uncertain.

Table 1. Recovery of acetaminophen from simulated commercial preparations

Acetaminophen			
mg Added	mg Found	% Recovery	Other Ingredients
150.0	148.7	99.1	Pheniramine maleate, 10 mg Phenylpropanolamine HCl, 25 mg Chlorpheniramine maleate, 6 mg Phenylephrine HCl, 15 mg Amphetamine sulfate, 6 mg Phenyltoloxamine HCl, 25 mg
150.0	150.0	100	Sulfadiazine, 150 mg Sulfamethazine, 150 mg Sulfamerazine, 150 mg
150	147.0	98	Aspirin, 300 mg Phenacetin, 90 mg Caffeine, 30 mg
150	147.3	98.2	Salicylamide, 200 mg
125	122.8	98.2	Phenobarbital, 12 mg Butabarbital sodium, 10 mg Secobarbital sodium, 9 mg
150	151.9	101.3	Mephenesin, 200 mg Terpin hydrate, 135 mg Glyceryl guaiacolate, 70 mg

elution with ether, a separate column prepared with 3 g Celite and 2 ml 1*N* HCl.) Evaporate eluate to dryness. Dissolve residue in about 40 ml methanol and add 1 ml 0.1*N* HCl. Transfer to 100 ml volumetric flask and dilute to volume with methanol. Determine absorbance of sample and standard solution at 249 mμ.

Results and Discussion

Recovery studies were made on control samples in which weighed quantities of acetaminophen were combined with drugs with which it is formulated in commercial products. Assay values are shown in Table 1.

Analyses of commercial acetaminophen tablets assayed by the NF XI method and by the proposed method are reported in Table 2.

Commercial samples of various combinations were assayed by the proposed procedure without complications. Results are given in Table 3.

To check the efficacy of the procedure for the separation of acetaminophen from the drugs with which it is combined in commercial preparations, the behavior of each of these drugs under the conditions of the analysis was determined. The combinations were those listed in such standard reference publications as the *American Drug Index* and the *Physicians' Desk Reference*. In the chromatographic separation, neutral compounds, such as phenacetin, glyceryl guaiacolate, and mephenesin, are carried through the column with the chloroform wash, as is caffeine. The salts of organic bases either

Table 2. Analysis of three commercial brands o acetaminophen tablets

Brand	Labeled (mg/tab)	Found, mg: NF XI Assay	Found, mg: Proposed Method
A	300	309	296
		306	302
B	300	300	297
		301	301
C	324	323	318
		322	322

Table 3. Analysis of various commercial preparations for acetaminophen

Acetaminophen		
Labeled (mg)	Found (mg)	Accompanying Material
125	121	Aspirin, 230 mg
	121	Caffeine, 30 mg
150	143	Demerol, 25 mg
	146	Dihydrocodeinone bitartrate, 5 mg
120	119	Salicylamide, 3.5 g
	117	Phenacetin, 2.5 g Phenylephrine HCl, 5 mg

(*a*) remain in the sample column (Column II) during the elution of the acetaminophen from this column with chloroform or (*b*) are eluted from this column as their hydrochlorides (*see* (9)); they then pass through the buffered column (Column I) as the free base. Pheniramine maleate, chlorpheniramine maleate, phenylpropanolamine hydrochloride, phenylephrine hydrochloride, and amphetamine sulfate are included in group (*a*). In group (*b*) are phenindamine tartrate, demerol hydrochloride, dihydrocodeinone bitartrate, dextromethorphan hydrobromide, isometheptene mucate, and dimethoxamate hydrochloride. Faintly acidic compounds, such as salicylamide and barbiturates, are eluted from the buffered column with chloroform, while the more strongly acidic compounds, salicylic acid and aspirin, are retained on this column during elution of acetaminophen with ether.

Since the sulfonamides, which are amphoteric compounds, are partially eluted from both columns with chloroform, a small amount of sulfonamide will accompany the acetaminophen during elution of the buffered column with ether. When this eluate is passed over in acid column segment, only the sulfonamides are retained.

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Chromatographic Separation and Spectrophotometric Determination of Phenylephrine Hydrochloride, Codeine Phosphate, and Some Other Pharmaceuticals in a Mixture

By DONALD J. SMITH (Food and Drug Administration, 50 Fulton St., San Francisco, Calif. 94102)

Codeine phosphate, chlorpheniramine maleate, pyrilamine maleate, phenylpropanolamine hydrochloride, and hydrocortisone acetate were analyzed in samples containing phenylephrine hydrochloride. A series of four columns is used to separate the various pharmaceutical components prior to analysis. Assays of commercial samples ranged from 87.2 to 118% of declared content.

The determination of phenylephrine hydrochloride in complex drug mixtures is a familiar problem to the drug analyst. Because of the unfavorable partitioning behavior of phenylephrine hydrochloride, it is usually isolated by removing interfering substances from the original aqueous solution rather than by column or separatory funnel extraction techniques. The present investigation describes a generally applicable procedure for separation of phenylephrine hydrochloride from mixtures and its subsequent determination. The other constituents of these mixtures are separated and determined by methods found in the literature.

A number of methods for phenylephrine have appeared in the literature. Hiskey and Levin (1) and Koshy and Witchner (2)

described colorimetric procedures in which 4-aminoantipyrine is used as the chromogenic reagent, a reaction specific for phenols which have the *para* position available for coupling. An ion exchange chromatographic separation of phenylephrine hydrochloride from other drugs has been published by Kelly and Auerbach (3). The isolated compound was determined colorimetrically either with Millon's reagent or by coupling with diazotized *p*-nitrophenol. In the Clark and Rosenberg procedure (4), phenylephrine was acetylated on a column and eluted quantitatively with chloroform. A Celite column separation procedure for phenylephrine, chlorpheniramine maleate, and codeine phosphate or dihydrocodeinone bitartrate in elixirs was described by Hyatt (5, 6).

Earlier work in our laboratory showed that with an aqueous phase:Celite column, large volumes of ammonia-saturated chloroform were needed to quantitatively remove the phenylephrine hydrochloride. However, with a relatively dry sample:Celite column (1 ml: 4 g), other drugs would be eluted with small amounts of organic solvent and the relatively pure phenylephrine remaining on the column could then be eluted with a small quantity of ethanol.

In the method to be described, the sample column is eluted first with chloroform, which is then passed through a Celite: sodium hydroxide column to remove certain excipients (such as citric acid) found in sirups. If chlorpheniramine or pyrilamine maleates are present, they are trapped on a nitric acid column, described by Levine (7), which is placed below the sodium hydroxide column. Codeine phosphate, slightly soluble in chloroform (8), is quantitatively removed from the "dry" column by a small volume of this solvent. The codeine is trapped by Levine's sulfamic acid column (7) placed below the nitric acid column. If both chlorpheniramine and pyrilamine maleates are present, pyrilamine can be determined in the presence of chlorpheniramine by ultraviolet spectrophotometry; chlorpheniramine can be quantitatively analyzed in the mixture by the rapid colorimetric procedure of Hudanick (9). Phenylpropanolamine hydrochloride, if present, remains on the sample column during the chloroform elution and can be removed with chloroform which was passed through an ammonia:Celite column (1 + 1) as described by Healton and Hopes (10). Finally, the phenylephrine is quantitatively removed with ethanol and determined by ultraviolet spectrophotometry.

METHOD

Reagents and Apparatus

(a) *Chloroform.*—ACS reagent grade, dry and water-saturated.

(b) *Sulfuric acid.*—About 0.1*N*.

(c) *Hydrochloric acid.*—About 2.0% (1 + 49 HCl and water).

(d) *Celite 545.*—Acid washed, Johns-Manville.

(e) *Sodium hydroxide.*—About 1*N*.

(f) *Nitric acid.*—About 1*N*.

(g) *Sulfamic acid.*—About 0.5*N*.

(h) *Cyanogen bromide solution.*—Dissolve 2.0 g in 50 ml water (keep refrigerated).

(i) *Buffered sulfanilic acid solution.*—Dissolve 2.5 g sulfanilic acid and 4.0 g anhydrous sodium acetate in 40 ml water, and dilute to 175 ml with 95% ethanol.

(j) *Phenylephrine HCl standard solution.*—Dissolve 2.5 mg in 10 ml 1*N* NaOH and 20 ml water in 100 ml volumetric flask. Dilute to volume with 95% ethanol. Prepare within 8 hr of use.

(k) *Chlorpheniramine maleate standard.*—(*1*) Ultraviolet determination: dissolve 2.0 mg in 2% HCl and bring to volume in 100 ml volumetric flask. (*2*) Colorimetric determination: prepare solutions containing 0.01, 0.02, 0.04, and 0.08 mg/ml with 2% HCl.

(l) *Pyrilamine maleate standard solution.*—Dissolve 2.5 mg in 2% HCl and dilute to 100 ml with 2% HCl.

(m) *Codeine phosphate and phenylpropanolamine HCl standard solutions.*—Dissolve 10.0 mg and 40.0 mg with 0.1*N* H_2SO_4 in separate flasks and dilute to 100 ml with H_2SO_4.

(n) *Chromatographic column.*—25 × 300 mm, with close-fitting tamping rod.

(o) *Recording spectrophotometer.* — Beckman DK 2-A or similar instrument.

Sample Preparation

(a) *Tablets, capsules, sirups, and other liquids.*—Accurately weigh sample containing the optimum amounts of the active ingredients (Table 1) and transfer to beaker. For a more concentrated liquid sample, make appropriate dilution with water, and take 2 ml aliquot for

Table 1. Optimum concentrations for standards[a]

Standards	Solvent	Concentration, mg/100 ml	Wavelength of Max. Used, mμ	Approx. Absorbance at Max.
Chlorpheniramine maleate	2% HCl	2.0	262	0.441
		4.0	480	0.390
Codeine phosphate	0.1*N* H_2SO_4	10.0	280	0.434
Phenylephrine HCl	see Reagent (j)	2.5	290	0.387
Phenylpropanolamine HCl	0.1*N* H_2SO_4	40.0	257	0.365
Pyrilamine maleate	2% HCl	2.5	310	0.551

[a] Prepared in 1 cm cells.

analysis. Add 2 ml water to powdered samples, warm on steam bath to dissolve, and cool.

(b) *Sustained release tablets or capsules.*— Prepare sample as above. Add 50 ml of 95% ethanol and heat on steam bath to dissolve. Evaporate alcohol solution just to dryness and cool. Add 2 ml water to the sample residue.

Place glass wool plugs in four columns and prepare each as follows:

Column I—Add 4 g Celite/ml sample solution and mix thoroughly. Transfer mixture to column and dry-rinse beaker with Celite (ca 1 g).

Column II—Mix 3 g Celite and 2 ml 1*N* NaOH, transfer to column, tamp moderately, and place a layer of glass wool on top.

Column III—Mix 3 g Celite and 2 ml 1*N* nitric acid. Transfer to the column as before.

Column IV—Mix 3 g Celite and 2 ml 0.5*N* sulfamic acid. Transfer to the column as before.

Arrange columns so effluent from I flows through II, III, and IV in sequence. Pass 150 ml $CHCl_3$ through the columns. Complete the elution of $CHCl_3$ from Column I with air pressure and allow effluent to flow into other columns. Column I retains phenylephrine HCl and phenylpropanolamine HCl.

Remove Column I. Pass 50 ml $CHCl_3$ through Column II (which retains citric acid frequently found as excipient in sirups), III, and IV in sequence; then pass an additional 25 ml $CHCl_3$ through Column III (which retains chlorpheniramine and pyrilamine maleate), and Column IV (which retains codeine phosphate) in sequence. Separate the columns and discard Column II.

Mix three portions of 5 ml NH_4OH and 5 g Celite. Pack one portion into each of the three columns (I, III, and IV). Place a layer of glass wool on top. Elute each of these columns separately with 150 ml water-saturated $CHCl_3$, removing $CHCl_3$ retained on Column I with air pressure. Collect the eluate from Column I in a 250 ml separator containing 10 ml 1*N* NaOH. (Note: This eluate contains a small amount of the phenylephrine HCl and all of the phenylpropanolamine HCl. The eluate from Column III contains chlorpheniramine and pyrilamine, and the eluate from Column IV contains codeine.) Evaporate the eluates from Columns III and IV on the steam bath just to dryness with an air stream. Transfer resulting residues to volumetric flasks with 2% HCl and 0.1*N* H_2SO_4, respectively, and make to appropriate concentration.

Phenylpropanolamine HCl procedure. — Shake separator vigorously for about 1 min. and let layers separate. Drain $CHCl_3$ layer into a beaker. Extract the aqueous layer with 3 additional 10 ml portions of $CHCl_3$. Save aqueous layer for phenylephrine determination. Evaporate combined extracts on steam bath to about half the volume, add 20 ml 0.1*N* H_2SO_4, and continue heating until all the $CHCl_3$ has been removed. Dilute solution to the optimum concentration with 0.1*N* H_2SO_4, and scan the spectrum of the sample and the standard between 400 and 200 mμ, using 0.1*N* H_2SO_4 as the reference solution. Determine the absorbance of phenylpropanolamine at the maximum at approximately 257 mμ, and calculate the concentration of the sample from the standard spectrum.

Phenylephrine HCl determination.—Quantitatively transfer the 10 ml NaOH solution from the separator to a 100 ml volumetric flask with about 5 ml water. Place flask beneath Column I and elute with portions of ethanol. (Pressure may be necessary to achieve a reasonable flow rate.) Collect a total of 100 ml in a volumetric flask. (Cloudy solution at this point is acceptable.)

Dilute phenylephrine to optimum concentration with ethanol. Pipet 20 ml standard solution, 20 ml diluted sample, and 20 ml of a "blank", prepared by diluting 10 ml 1*N* NaOH and 5 ml water to 100 ml with ethanol, into separate 50 ml Erlenmeyer flasks. Add 5.0 ml water to each flask, mix, and scan the spec-

trum of each solution from 400 to 220 mμ. Determine phenylephrine at the maximum of about 290 mμ. The phenylephrine alcoholic NaOH solution must be read the same day as prepared.

Chlorpheniramine maleate determination in the absence of pyrilamine maleate.—Dilute chlorpheniramine to its optimum concentration with 2% HCl for ultraviolet determination. Scan the spectrum of the sample and the standard solutions between 400 and 220 mμ, using 2% HCl as the reference solution. Determine the absorbance of chlorpheniramine at the maximum at about 272 mμ, and calculate the concentration of the sample solution from the standard spectrum.

Chlorpheniramine maleate determination in the presence of pyrilamine maleate.—Follow the method described by Hudanick (9), with the following modification: Immediately after the addition of CNBr, scan the spectrum of the solution between 700 and 480 mμ. Stop chart and wavelength drive at the absorbance maximum (ca 480 mμ) and wait until the maximum absorbance is obtained (about 9 min. after CNBr addition). Measure absorbance, using the 700 mμ reading as the baseline.

Determine pyrilamine maleate by making a suitable dilution with 2% HCl of a portion of the same solution used for determining chlorpheniramine maleate. Scan the spectrum of the sample and the standard solutions between 400 and 220 mμ, using 2% HCl as the reference. Determine the absorbance of pyrilamine maleate at the maximum (about 310 mμ), and calculate the concentration of the sample solution.

Dilute codeine phosphate to its optimum concentration with 0.1*N* H_2SO_4. Scan the spectrum of sample and standard solutions between 400 and 220 mμ, using 0.1*N* H_2SO_4 as the reference. Determine the absorbance of codeine phosphate at the maximum (about 280 mμ), and calculate the concentration of this sample solution from the standard spectrum.

Results and Discussion

Recovery data for mixtures of standard substances assayed by this procedure are presented in Table 2. Results of recovery experiments performed by intralaboratory collaborators by adding known amounts of standard substance directly to samples are shown in Table 3. Table 4 contains results obtained by the author and intralaboratory collaborators from analysis of commercial tablets, sirups, and other liquid dosage forms containing some or all of the active ingredients mentioned in this procedure.

In this method, the sample column is

Table 2. Recoveries of mixed standards in aqueous solution in the absence of sample excipients

Substance	Added, mg	Found, mg	Recovery, %	Other Substance Present
Phenylephrine HCl	3.36	3.26	97.0	Chlorpheniramine maleate (1.60 mg)
		3.29	98.0	
Codeine	6.105	5.96	97.5	
Chlorpheniramine maleate	1.60	1.66	104	
Phenylephrine	3.36	3.31	98.4	
Codeine phosphate std	6.105	6.105	100.0	Phenylephrine HCl (3.36 mg)
Chlorpheniramine maleate std	1.60	1.58	98.6	

Table 3. Recoveries of compounds added to the samples

Compounds	Added, mg	Found, mg	Recovery, %	Other Substance Determined
Pyrilamine maleate	2.397	2.36	98.4	See Sample 7, Table 4
	2.415	2.38	98.6	
Phenylpropanolamine HCl	45.5	44.8	98.4	
	46.3	46.0	99.4	
Codeine phosphate	10.8	10.9	101	See Sample 9, Table 4
	11.1	11.1	100	
Phenylpropanolamine HCl	42.6	42.6	100	
	41.3	39.5	95.6	
Chlorpheniramine maleate	1.05	1.02	97.0	See Sample 11, Table 4

Table 4. Results of sample analysis of phenylephrine hydrochloride in drug combinations

Sample[a]	Component	Declared, mg/unit	Found, mg/unit 1	2	3	Av.	% of Declared
1-SRC[b]	Chlorpheniramine maleate[c]	8/cap	7.36	7.47	7.33	7.38	92.3
	Phenylephrine HCl	20/cap	17.2	17.2	17.9	17.4	87.2
	Pyrilamine maleate	6.25/5 ml		5.89		5.89	94.2
2-S	Phenylephrine HCl	10.0/5 ml	10.8	10.5		10.6	106
	Chlorpheniramine maleate[c]	2.0/5 ml	2.19	2.15		2.17	109
	Pyrilamine maleate	6.25/5 ml	6.08	5.80		5.94	95.0
3-S	Codeine phosphate	10.80/5 ml	11.4	11.5		11.5	106
	Phenylephrine HCl	10.0/5 ml	10.1	9.70		9.90	99.0
	Chlorpheniramine maleate[c]	2/5 ml	2.28	2.18		2.23	112
	Pyrilamine maleate	25.0/tab	24.0	23.9		24.0	96.0
	Phenylephrine HCl	10.0/tab	11.0	11.1		11.0	110
4-SRT	Chlorpheniramine maleate[c]	2.5/tab	2.97	2.89		2.93	118
	Phenylpropanolamine HCl	30.0/tab	30.9	30.0		30.4	102
	Phenyltoloxamine citrate[d]	12.5/tab	1.08	1.06		1.07	8.56
5-L	Phenylephrine HCl	1.25/ml	1.23	1.24		1.24	99.2
	Hydrocortisone acetate	5/ml	4.68	4.73		4.70	94.0
6-L	Phenylephrine HCl	1.25/ml	1.29	1.30		1.30	104
	Chlorpheniramine maleate	1.0/ml	1.04	1.04		1.04	104
	Codeine phosphate	5.0/5 ml	4.81	4.81	4.88	4.83	96.6
7-S	Chlorpheniramine maleate	0.75/5 ml	0.715	0.700		0.708	94.3
	Phenylephrine HCl[e]	2.5/5 ml	——	——		——	——
	Codeine phosphate	10.80/5 ml	11.2	11.1		11.2	103
8-S	Phenylephrine HCl	10.0/5 ml	8.64	8.96		8.80	88.0
	Chlorpheniramine maleate	2.0/5 ml	2.09	2.08		2.08	104
	Pyrilamine maleate	6.25/5 ml	6.13	6.07		6.10	97.6
	Phenylephrine HCl	10.0/5 ml	9.37	9.30		9.34	93.4
9-S	Chlorpheniramine maleate	2.0/5 ml	2.07	2.07		2.07	104
	Pyrilamine maleate	6.25/5 ml	5.98	5.92		5.95	95.2
10-S	Codeine phosphate	59.4/29.57 ml	59.6	59.6		59.6	100
	Pyrilamine maleate	25.0/29.57 ml	23.4	23.5		23.4	94.0
11-S	Codeine phosphate	59.4/29.5 ml	61.2	60.6		60.9	102
	Pyrilamine maleate	25.0/29.5 ml	22.0	22.8		22.4	90.0

[a] SRC = sustained release capsule, S = sirup, SRT = sustained release tablet, L = liquid.
[b] These capsules contained pelletized contents and were analyzed on an individual capsule basis.
[c] Colorimetric determination of chlorpheniramine maleate was made directly on the sample.
[d] Standard phenyltoloxamine citrate passed easily through the four columns. Low recovery of phenyltoloxamine citrate was obtained, however, from a combination of standards.
[e] The procedure for phenylephrine HCl is not applicable in the presence of sodium benzoate.

prepared by using a large ratio of Celite to aqueous phase. The use of dry chloroform for the first elution further dehydrates the sample preparation, leaving phenylpropanolamine and phenylephrine hydrochlorides on the column. The relatively dry column permits the elution of chlorpheniramine maleate, pyrilamine maleate, and codeine phosphate quantitatively with only 150 ml of dry chloroform. By comparison, columns prepared with a 1:1 aqueous phase: Celite ratio require from 400 to 800 ml of water-saturated chloroform for quantitative elution of chlorpheniramine maleate (10).

Some samples analyzed by the author exhibited interference from one excipient or another (e.g., potassium guaiacolsulfate and sodium benzoate). Since the interfering excipients were the exception in the author's experience, this problem was reserved for future study. Some other items to be undertaken in further investigations include prewashing Column I with acetic acid-chloroform before addition of the ammonium hydroxide-Celite layer to eliminate interference from any organic-soluble acids trapped as salts, and water washing and sodium sulfate drying of the eluting chloroform to remove the ethanol preservative (about 0.75–1.5%), used in commercially

prepared chloroform. This ethanol may be a factor in the phenylephrine loss from Column I. Other possibilities are the addition of a dry Celite base layer in Column I or a separate water:Celite column which may eliminate leaching of phenylephrine.

Sample ingredients are more efficiently extracted on the dry column. The eluate can be passed through a number of possible combinations of Celite columns described in the literature. Other chloroform-soluble antihistamines retained by the nitric acid column (7) may be amenable to analysis by the described procedure. Compounds in combination with phenylephrine and other drugs which we have analyzed with little or no modification include aspirin, hydrocortisone acetate, dextromethorphan hydrobromide, caffeine, thenyldiamine, naphazoline hydrochloride, tetracaine hydrochloride, amphetamine phosphate, methapyrilene fumarate, phenobarbital, and salicylic acid. Insufficient recovery data were available to include these in the present work.

Both the sulfuric and sulfamic acid columns of Levine (7) were used in the analysis of the same sirup sample. The sulfuric acid column in this case apparently retained an interfering excipient, in addition to the codeine, which produced a distorted ultraviolet spectrum of codeine. When a sulfamic acid column was used for the same sample, however, an undistorted codeine spectrum was obtained. For this reason, the sulfamic acid column was specified in this method.

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A Rapid Method for the Quantitative Determination of Antihistamines and Some Related Compounds by Gas Chromatography

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Laboratory-prepared and commercial samples of antihistamine mixtures are analyzed by gas chromatography with a Carbowax 20M-SE 30 phase on Anakrom ABS column. Recoveries for the simulated tablet preparations range from 96.9 to 102.5%. Recoveries from commercial preparations range from 90.7 to 113.2% of label declarations. Retention times for 12 antihistamines, dextromethorphan HBr, and phenylpropanolamine HCl relative to diphenylpyraline HCl, the internal standard, are given.

Multi-component antihistamine preparations present a formidable analytical problem because the similar molecular structure of many of the antihistamines make their separation by the usual chemical and physical methods difficult. With gas chromatography, many of the separations thought

impossible a few years ago can be accomplished with ease, speed, and accuracy.

MacDonald and Pflaum (1) and Kazyak and Knoblock (2) first used gas chromatography to separate antihistamines in the free base form with an SE-30 liquid phase. Fontain, *et al.* (3) separated an ethanolic solution of antihistamine salts on a 2% Carbowax 20M column pretreated with 10% KOH. Celeste and Turczan (4) separated and quantitatively estimated a number of antihistamines in their free base form, using a 10% DC High Vacuum silicone grease column.

A new gas chromatographic procedure has been devised for the direct determination of antihistamines by injection of a water solution of their salts. The procedure involves dissolving the samples in water, adding an internal standard, and injecting the solutions into the gas chromatograph.

Satisfactory separation, response, and peak symmetry could not be obtained when either Carbowax 20M or SE-30 was used alone as the liquid phase. When the two were used together as the liquid phase, these difficulties were overcome.

The column chosen for this work is a mixture of the polar and nonpolar phases, 2% Carbowax 20M and 2% SE-30, on Anakrom ABS 80–90 mesh. This column need only be conditioned overnight at 200°C with a nitrogen flow of 200 ml/min. before use.

Diphenylpyraline HCl was used as the internal standard to obtain a detector response factor and determine relative retention times. The detector response factor eliminates error from variations in injection volumes when used in the calculations and is also an indication of column reproducibility. This antihistamine was selected as the internal standard because it is not commonly found in combination with other antihistamines, and its retention time falls approximately in the middle of the substances which were chromatographed. Any antihistamine not being determined can be conveniently used as the internal standard.

METHOD

Reagents

(a) *Internal standard.*—2.5 mg/ml diphenylpyraline HCl in water.

(b) *Standard solution.*—Prepare solution in water of each of the antihistamines to be determined in the same concentration as expected in the sample solution plus a final concentration of 0.25 mg/ml of internal standard.

Apparatus

(a) *Gas chromatograph.*—With a hydrogen flame detector. The instrument was equipped with a 1 mv recorder and all determinations were made with an electrometer setting of 1000. The attenuation was varied from 5 to 20×, depending on the size of sample injected; 1 μg of antihistamine at a setting of 10× would give a recorder response from 20 to 80% of full scale deflection depending on the retention time of the antihistamine.

Conditions were as follows: column temperature, 185°C; flash heater temperature, 250°C; cell temperature, 250°C; carrier gas, prepurified nitrogen; flow rates, carrier gas—20 psi, 200 ml/min.; hydrogen—16 psi; air—35 psi, 425 ml/min.

(b) *Column.*—4 × 4 mm i.d., 2% SE-30, 2% Carbowax 20M on Anakrom ABS, 80–90 mesh. Conditioned overnight at 200°C with a nitrogen flow of 200 ml/min.

Procedure

Weigh and finely powder 20 tablets to pass through a 60-mesh sieve. Weigh a portion containing about 25 mg of each of the antihistamines to be determined. If the concentrations vary in a mixture, weigh an amount of sample equivalent to 0.1 mg/ml of the antihistamine present in the smallest quantity. Transfer sample to 100 ml volumetric flask, add 10 ml internal standard solution and about 50 ml water, and place on the shaking machine for 15 minutes. Dilute to volume, mix, and filter. Inject 4–6 μl standard and sample solution into the gas chromatograph. Compare the retention times of sample and standard peaks relative to the internal standard for qualitative identification. The concentration of sample may be calculated as follows.

Determine the peak area by triangulation:

$$R = (A_{Std} \times C_{IS}) / (A_{IS} \times C_{Std})$$

$$C_{Sa} = (A_{Sa} \times C_{IS}) / (A_{IS'} \times R_{Sa})$$

where R = relative response (5), A = area, C = concentration, Std = standard, IS = internal standard, IS′ = internal standard in sample, and Sa = sample. Since the concentration of the internal standard is the same in both standard and sample solutions,

$$mg_{Sa}/\text{tablet} = (A_{Sa} \times C_{std} \times A_{IS} \times \text{av. wt tablet}) / (A_{Std} \times A_{IS}' \times wt_{Sa})$$

Table 1. Relative retention times of 12 antihistamines and 2 related compounds relative to diphenylpyraline HCl

Compound	Relative Retention Time
Phenylpropanolamine HCl	0.13
Pheniramine maleate	0.31
Phenyltoloxamine hydrogen citrate	0.53
Cyclizine HCl	0.64
Pyribenzamine citrate	0.67
Chlorpheniramine maleate	0.76
Methapyrilene HCl	0.77
Diphenylpyraline HCl	1.000[a]
Dextromethorphan HBr	1.05
Dexbrompheniramine maleate	1.21
Chlorothen citrate	1.45
Phenindamine tartrate	1.49
Chlorcyclizine HCl	1.66
Thonzylamine HCl	2.08
Pyrilamine maleate	2.51

[a] Internal standard.

Results and Discussion

The retention times of 12 antihistamines, dextromethorphan HBr, and phenylpropanolamine HCl relative to diphenylpyraline HCl, the internal standard, are listed in Table 1. The two non-antihistamine compounds are usually found in combination with antihistamines.

Table 2. Analysis of laboratory-prepared mixtures simulating tablet formulation

Compound	mg Added	mg Recovered	Recovery, %
Phenylpropanolamine HCl	97.4	97.4	100.0
Chlorothen citrate	23.0	22.6	98.4
Methapyrilene HCl	16.0	16.4	102.5
Thonyzlamine HCl	48.0	47.6	99.2
Pheniramine maleate	11.27	11.32	100.4
Cyclizine HCl	10.48	10.55	100.7
Phenindamine tartrate	28.85	27.96	96.9
Pyrilamine maleate	39.78	40.42	101.6
Chlorpheniramine maleate	4.16	4.10	98.6

Figure 1 is a chromatogram of a mixture of 10 antihistamines combined. Known mixtures of the antihistamines were prepared to simulate tablet granulation. Table 2 shows the results of analysis of these mixtures, and Table 3 gives the results of the analysis of various commercial mixtures. Figure 2 is a chromatogram of five antihistamines at different concentrations. Figure 3 is a typical curve for a sample with the internal standard added.

We use this procedure routinely for a variety of tablet and nasal preparations. When interferences from flavoring material

Table 3. Analysis of 6 commercial tablet preparations

Sample	Ingredients	mg Declared	mg Found	% of Declared
(1)	Pheniramine maleate	6.25	6.89	110.2
	Phenyltoloxamine dihydrogen citrate	6.25	5.73	91.6
	Methapyrilene HCl	6.25	5.80	92.8
	Pyrilamine maleate	6.25	5.80	92.8
(2)	Dextromethorphan HBr	5	5.42	108.4
	Chlorpheniramine maleate	1	1.03	103.0
	Glyceryl guaiacolate	20	——	——
(3)	Tetracycline HCl	125	——	——
	Acetophenetidin	120	——	——
	Caffeine	30	——	——
	Salicylamide	150	——	——
	Chlorothen citrate	25	28.3	113.2
(4)	Pheniramine maleate	6.25	5.9	94.4
	Phenyltoloxamine dihydrogen citrate	6.25	6.26	100.2
	Methapyrilene HCl	6.25	5.67	90.7
	Pyrilamine maleate	6.25	6.39	102.2
(5)	Chlorpheniramine maleate	8.00	7.99	99.9
	Phenylephrine HCl	20		
	Methscopolamine NO_3	2.5		
(6)	Tripelennamine HCl	25	24.0	96.0
	Antazoline HCl	50		No response

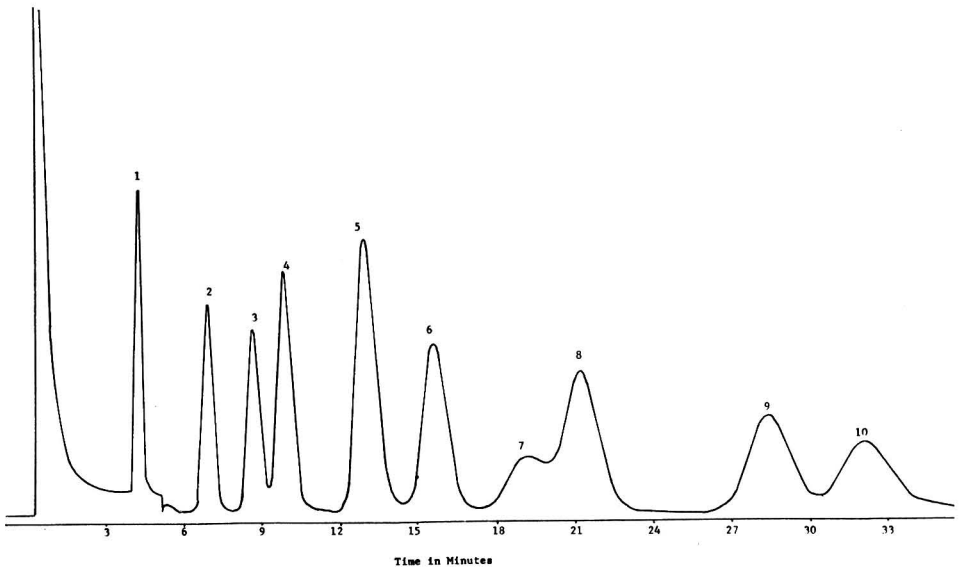

Fig. 1—**Chromatogram of a standard mixture of 10 antihistamines. 1. Pheniramine maleate; 2. phenyltoloxamine dihydrogen citrate; 3. cyclizine HCl; 4. chlorpheniramine maleate; 5. diphenylpyraline HCl; 6. dexbrompheniramine maleate; 7. chlorcyclizine HCl; 8. phenindamine tartrate; 9. thonzylamine HCl; 10. pyrilamine maleate. Concentrations injected: peaks 1–7 = 1 μg; 8–10 = 2 μg.**

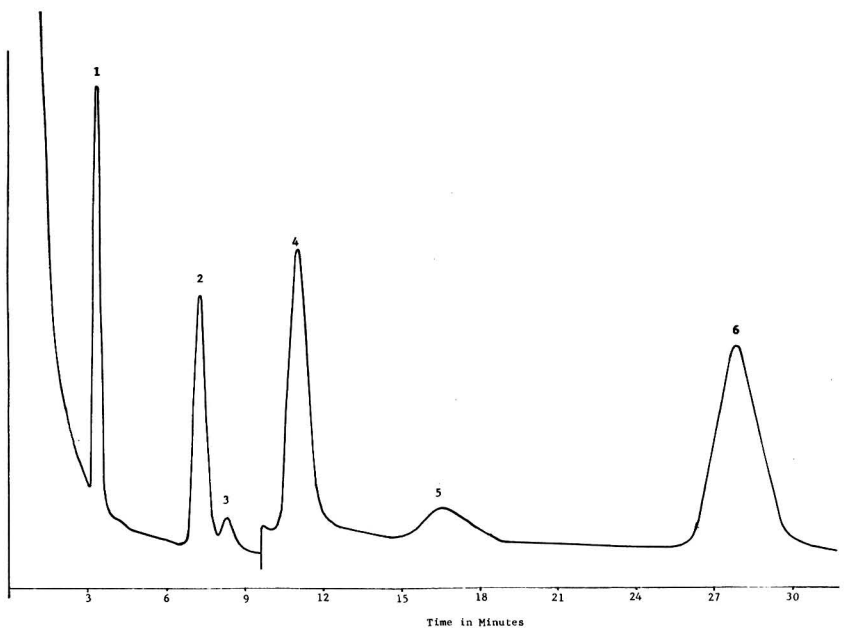

Fig. 2—**Chromatogram of a mixture of 5 antihistamines and internal standard. 1. Pheniramine maleate; 2. cyclizine HCl; 3. chlorpheniramine maleate; 4. diphenylpyraline HCl (internal standard); 5. phenindamine tartrate; 6. pyrilamine maleate. Concentrations injected: 1 and 2, 0.5 μg, 3, 0.2 μg, 4, 1.0 μg, 5, 1.5 μg, and 6, 2.0 μg.**

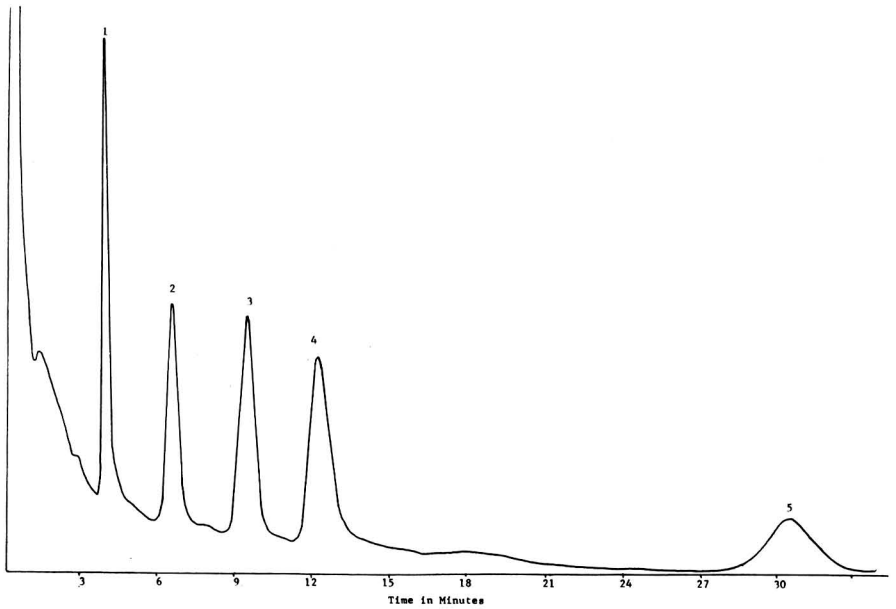

Fig. 3—**Typical sample curve. 1. Pheniramine maleate; 2. phenyltoloxamine dihydrogen citrate; 3. methapyrilene HCl; 4. diphenylpyraline HCl; 5. pyrilamine maleate. Concentrations injected: peaks 1–5 = 1 μg.**

(e.g., sirups) or other constituents are present, the sample is first cleaned up by the procedure described by Celeste and Turczan (4) and then analyzed by the gas chromatographic procedure described here. This method is straightforward and involves a minimum of manipulations. It is sensitive, rapid, accurate, and reproducible when applied to a variety of products.

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FEEDS

Nonnutritive Residues: A System of Analysis for the Replacement of Crude Fiber

By P. J. VAN SOEST (U.S. Department of Agriculture, Agricultural Research Service, Beltsville, Md. 20705)

A literature review of the use of crude fiber for the estimation of the poorly digestible part of feedstuffs, together with a discussion of the factors that affect nutritive availability of different chemical entities in feeds, shows that crude fiber may be unsuitable for the evaluation of feeds. The new system described, utilizing detergents, divides dry matter of feeds along lines consistent with modern views in nutrition and emphasizes the cell-wall and lignin contents in feeding materials of plant origin and the acid-detergent insoluble fraction in high protein feeds of plant and animal origin.

The need for a more comprehensive system of analysis to distinguish between the digestible and indigestible parts of feedstuffs has been recognized by the creation of an associate refereeship. This move represents a new approach to the old problem of feedstuff evaluation. In the past, feed quality has been estimated by crude fiber content, a procedure known to have many defects. This paper presents a review of the factors which affect the availability of nutrients in feedstuffs and outlines a new system of analysis consistent with modern views in nutrition.

Crude fiber analysis was invented more than 150 years ago to represent the supposedly indigestible part of vegetable foods and feedstuffs (1, 2). It has been standardized in essentially its present form since 1860 (1, 3). The discovery of the digestibility of cellulose in 1854 (4) ruined the conceptual basis on which the method was originally advanced. The retention of crude fiber since that time has been related partly to misconceptions about its nature and partly to a lack of understanding of the biochemical factors influencing the nutritive availability of different chemical fractions in feeds (5).

The original concept of crude fiber—the characterization of the indigestible part of a feedstuff—remains to challenge prospective replacements for crude fiber. There are many technical difficulties in meeting this challenge, and it is not easy to develop suitable methods of comparable laboratory convenience and economy (6, 7).

The present use of crude fiber is based on the assumption that fiber content is related to quality. While this is generally true, it is not statistically precise (8). Crude fiber can be unsatisfactory because of its variable composition and digestibility (9). It is sometimes more digestible than the nitrogen-free extract (NFE), which is supposed to represent the highly digestible carbohydrates (10); *see* Table 1. The low digestibility of NFE results partially from extraction of indigestible lignin (11) and partially from digestible hemicellulose (12, 13) in the fiber determination. The xylan fractions of hemicellulose can be less digestible than cellulose, which tends to remain in the crude fiber

Table 1. Relative digestibility of crude fiber (by the Weende method) and NFE[a]

Feed	No. of Digestibility Trials	Av. Digestibility		% Cases Where Digestibility is Equal to or Higher than NFE
		Crude Fiber	NFE	
Dry feed	110	52.4	59.5	30
Succulant feed	61	63.5	76.3	20
Silage	25	58.2	64.6	28
Concentrates	88	53.3	78.5	10

[a] Data of Crampton and Maynard (10).

This report of the Associate Referee was presented at the Seventy-ninth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 11–14, 1965, at Washington, D.C.

(14, 15). Losses of hemicellulose (as pentose) and lignin from crude fiber are shown in Table 2 for oat straw and clover-grass hay (16). Lignin, cellulose, and pentosans account for almost all of the NFE in oat straw.

In addition to these variables, there are important plant species differences in the composition of crude fiber and the degree of loss of lignin from the crude fiber fraction. Lignin of grasses is more alkali-soluble than that of legumes (2, 17). The lower lignin content of grasses is consistent with a higher fiber digestibility relative to alfalfa (2, 18). Fiber digestibility also varies within species because of the increase in lignification with maturity (8, 19, 20).

These variables combine to form a difficult problem in finding a method that would be biologically realistic. The problem would be easier if some standard criteria could be established to compare fiber methods. Primary standards as such have no meaning for crude fiber. Because of these difficulties, past collaborative studies have been more concerned with reproducibility and mechanics than with the meaning and significance of crude fiber analysis.

However, an experimental basis for a standard might be established on the digestibilities of individual chemical entities of feeds and the classification of these entities according to their relative nutritional availability and the factors determining this availability. This could be accomplished if samples of feed and feces from total collection feeding trials were analyzed for their respective fractions, and the digestibilities of these fractions were calculated. A prospective estimation of the nonnutritive residue or indigestible portion would then be based on a proper separation of the chemical fractions according to their nutritive value. Nonnutritive residue could be defined as chemical components of feedstuffs that cannot be completely digested. Few components of feedstuffs are not degraded to some extent on passage through the digestive tract. However, it is possible to estimate digestibility of partially available fractions if the factors involved are known.

Table 2. Composition of crude fiber and NFE[a]

	% Crude Fiber	% NFE	Crude Fiber, % of Total	NFE, % of Total
Straw (Oat)				
Lignin	7.0	19.0	28.5	71.5
Cellulose	83.8	31.0	74.2	25.8
Pentosans	11.6	45.6	21.2	78.8
Total	102.4	95.6		
Hay (Clover and Grass)				
Lignin	11.5	9.8	40.6	59.4
Cellulose	80.1	20.5	69.3	30.7
Pentosans	11.0	29.4	17.6	82.3
Total	102.6	59.7		

[a] Data of Nordfeldt, Svanberg, and Claesson (16).

Much experimental data have accumulated in the recent nutrition literature—largely ruminant—on the digestibility of various feedstuffs, particularly forages. Samples of feed and feces have been available to laboratories for detailed analysis. *In vitro* rumen digestion studies have also contributed valuable information. A summary of these studies is given below.

A distinction between two categories of vegetable substances, i.e., those contained in the plant cell wall and those contained within the metabolic part of the cell, is of fundamental nutritional importance (21–23). The digestive tracts of higher animals do not secrete enzymes capable of degrading hemicellulose and cellulose of the cell wall, and these carbohydrates are indigestible unless opportunity for microbial fermentation exists in a part of the digestive tract (24, 25). Because of their digestive physiology, ruminants utilize cell-wall carbohydrates more efficiently than do other species (26). The carbohydrates of the cell wall are not completely digestible because of lignification, which causes digestibility to decline as the plant matures (19, 20, 27, 28). A critical factor is the ratio of lignin to cellulose (19, 22, 28, 29).

Not all components in the cell wall are lignified to the same extent. Some such as pectin are not lignified at all. Pectin is easily soluble and highly digestible (14, 15) and can be classed on a nutritional basis with the more available cellular contents.

The cellular contents contain lipids, proteins, and amino acids (up to 90% of crude protein), sugars, starches, and other easily digestible carbohydrates, in addition to other water-soluble matter, all of which, apart from a few relatively unimportant exceptions (e.g., tannins, waxes), are completely digestible by animals without the aid of fermentation. The availability of the cellular protein and carbohydrates is unaffected by the degree of lignification (23). Apparent protein digestibility is primarily a function of protein intake and metabolic fecal loss (30, 31). Further evidence of the easy availability of the cellular contents lies in the methods of isolating and determining cell-wall constituents, which usually involve extraction with alcohol-benzene and water solubles followed by enzymic digestion of proteins and starches (32). These procedures and the more recent improved techniques for *in vitro* rumen digestion (33) bear a resemblance to the biological digestion processes.

The nitrogenous constituents of feedstuffs fall into several categories: up to one-third of the total nitrogen may be nonprotein (34), and about 5–10% of the total nitrogen is bound with lignin in the cell wall and is largely indigestible (35). In addition, commercial processes of preparing or handling feeds (during which steaming, pelleting, or other heating may occur) may induce the nonenzymic browning (Maillard) reaction (36), which can render large parts of the true protein unavailable. This reaction affects the quality of feedstuffs of animal origin, which largely serve as high-quality protein supplements. The essential amino acid, lysine, is one of the most critical in this regard (37).

The problem of damaged protein quality is more serious for nonruminants, which have definite amino acid requirements, than for ruminants, for which total available nitrogen is the critical factor (26). The heating of proteins, in addition to the possibility of creating indigestible residues, tends to slow the digestion rate. This effect exerts a beneficial influence on protein utilization in ruminants by reducing the bacterial rate of deamination in the rumen and the subsequent loss of urea in the urine (38, 39). Conversely, excessive heating of proteins is detrimental to utilization by nonruminants (37). The degree to which seed proteins, such as soy, are heated to inactivate an inhibitory factor is lower than that required to induce nonenzymic browning (40).

Recently, the AOAC method for determining digestible protein (**22.025**) has been criticized because it tends to measure extent and not rate of digestion (41). Both criteria are needed for adequate appraisal of proteinaceous feedstuffs for all classes of livestock. For nonnutritive residues, only extent of digestion is pertinent.

Any prospective replacement for crude fiber, and the proximate system that is founded on it, must be comprehensive in scope. Feedstuffs of both plant and animal origin must be dealt with and evaluated for all species of livestock, both ruminant and nonruminant. The problem can be solved if feed nutrients are classified according to the known factors affecting their availability. This leads to a division of feed dry matter as outlined in Table 3.

The dry matter of feedstuffs is divided into two major fractions: The first fraction, easily soluble and digestible, contains substances which are capable of digestion by enzymes secreted in the digestive tracts of all animals; the second (insoluble) fraction contains substances which can be digested only by microorganisms. Animal species which possess an important gastrointestinal fermentation capability will utilize significant amounts of cellulose and hemicellulose (24, 25). Carbohydrates in this second group are not completely available because of lignification. In general, increasing maturity of the plant leads to greater lignification and decreased digestibility. In addition to the carbohydrates in the second group, lignin, keratin proteins, and heat-damaged protein are included and are essentially indigestible (35, 42).

Suitable procedures for convenient separation (outlined in Table 3) have been developed with solutions of detergents. Cell walls of forages can be prepared as neutral-detergent fiber by boiling for 1 hour with

a 3% solution of sodium lauryl sulfate buffered at pH 7.0 (43). Lignocellulose can be prepared by the acid-detergent fiber procedure (44). The difference between the neutral-detergent fiber and acid-detergent fiber is an estimate of hemicellulose, although this difference does include some protein attached to cell walls (23). The acid-detergent fiber may be used as a preparatory step for lignin determination (44). A procedure for the preparation of cell-wall constituents (44) has not been successfully applied to concentrates and feedstuffs containing starch because of interference in filtration. This difficulty has been overcome by modifying the procedure with the neutral detergent reagent (45).

Some preliminary analyses which demonstrate the importance of the division of feed constituents are shown in Table 4. Comparison of the digestibility data with values of lignin and crude fiber show inconsistencies. With feather samples, for example, acid-detergent fiber will properly classify these, relative to vegetable feeds.

The problem is more complex for grasses, straw, and alfalfa. However the combination of the data—the amount of cell-wall constituents relative to the lignin content—do account for the nutritional differences in

Table 3. Division of forage organic matter by system of analysis, using detergents

Fraction	Components	Nutritional Availability	
		Ruminant	Nonruminant
	Category A		
Cell contents (soluble in neutral detergent)	Lipids Sugars, organic acids, and water-soluble matter Starch Nonprotein nitrogen Soluble protein Pectin	Virtually complete	Highly available
	Category B		
Cell-wall constituents (fiber insoluble in neutral detergent)	Attached protein	Complete	High
Soluble in acid detergent	Hemicellulose	Partial	Very low
Insoluble in acid detergent (acid-detergent fiber)	Cellulose	Partial	Very low
	Lignin	Indigestible	Indigestible
	Lignified nitrogen compounds	Indigestible	Indigestible
	Heat-damaged protein	Indigestible	Indigestible
	Keratin	Indigestible	Indigestible
	Silica	Indigestible	Indigestible

Table 4. Composition of some feedstuffs[a]

Feedstuff	Crude Fiber, %	Neutral-Detergent Fiber, %	Acid-Detergent Fiber, %	Lignin, %	Dry Matter Digest, %
Corn-soybean meal mixture	5.0	11.0	7.1	0.6	79
Cooked feathers	1.5	—	20.0	—	83[b]
Raw feathers	1.3	—	68.2	—	17[b]
Alfalfa hay (*Medicago sativa*)	23.5	40.2	25.1	5.3	62
Alfalfa hay, late cut	38.6	55.2	39.5	8.7	53
Orchardgrass (*Dactylis glomerata*)	24.1	52.3	27.1	2.7	72
Orchardgrass, late cut	35.0	70.4	40.1	4.7	57
Wheat straw	42.4	81.8	53.3	7.6	21

[a] Original data.
[b] Digestibility by pepsin digestion.

these materials. Alfalfa has a relatively low cell-wall content, which is highly lignified, while cell walls of grass and straw form a higher proportion of the dry matter (2). Thus, a compensation occurs whereby a smaller amount of lignin has a greater effect on the reduction of overall dry-matter digestibility for grass and straw. These results illustrate that no one measure can adequately classify the nonnutritive portion of feedstuffs.

It should be further pointed out that with concentrate feeds, lignin is of lesser importance because the proportion of the dry matter which is lignified, viz., cell walls, does not form a large part of the feed. Using *in vitro* rumen fermentation to evaluate forages and hays (33, 46, 47) is not appropriate for concentrates because it essentially measures cellulose and cell-wall digestibility. The success of *in vitro* rumen methods depends upon the proportion of cell walls in the feedstuff being evaluated. Similarly, digestibility-predicting regression equations based on lignin, crude fiber, protein, and other constituents (48, 49) have limited application because most such equations use ruminant digestion data and are not applicable to nonruminants. Again, it is the cell-wall fraction where the different capabilities of ruminants and nonruminants are important.

Nevertheless, it should be feasible to develop separate prediction equations for different species of livestock based on a division of feed nutrients as outlined in Table 3. Prediction equations for ruminants have been developed (23) based on cellular contents, cell-wall constituents, acid-detergent fiber, and lignin. Digestion trials are needed to establish the ability of nonruminants to digest cell-wall carbohydrates of varying degrees of lignification before similar equations can be calculated for these species.

In concentrates, cell-wall content is important as a measure of total fiber content plus other insoluble residues, such as sand, products of the Maillard reaction, or insoluble animal proteins, e.g., hair or leather. In these cases, the ash and nitrogen contents of acid-detergent fiber will be important in assessing the contribution of these residues to the value of cell-wall constituents and lignin content as determined in the 72% acid procedure (43). The value of acid-detergent insoluble nitrogen provides an estimate of the availability of crude protein (35).

If one considers that the detergent methods determine not specific chemical entities, but rather distinguish the less available parts of feedstuffs along lines that allow a classification according to mode of availability, then a common boundary, meaningful for all livestock, can be drawn around roughages, forages, and concentrates of both plant and animal origin.

It is recommended that studies continue.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee A, and was accepted by the Association. See *This Journal*, **49**, 161–165 (1966).

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Determination of Water in Molasses by the Karl Fischer Method

By E. A. EPPS, JR. (Louisiana Agricultural Experiment Station, Baton Rouge, La. 70803)

Collaborative studies were conducted in 1963, 1964, and 1965 to compare the Karl Fischer method with the vacuum drying method (29.008) for determining water in molasses. Results by the Karl Fischer method were in good agreement with known water contents in all studies, and the method is recommended for adoption as official, first action.

Accurate determination of water should be a simple matter; yet often it is one of the most troublesome procedures for the analyst. Water in molasses has been difficult to estimate because sugars and other constituents decompose, and vacuum drying is time-consuming.

The Karl Fischer method (1) has proved

a versatile and accurate means of determining water in a wide variety of substances. This method, based upon the oxidation of sulfur dioxide by iodine in the presence of water, is highly specific and very rapid. The Associate Referee investigated the possibility of using the method for determining water in molasses. Zerban and Sattler (2) and others have used the method for molasses, but it has not previously been studied by AOAC.

This study has been conducted for three years; for the first two years, there were not enough collaborators to justify any recommendations and no specific procedural details were given. Collaborators were asked to make duplicate determinations on different days by titration with the Karl Fischer reagent and by drying with vacuum on sand as in AOAC method **29.008** (3). (More detailed instructions were given for the Karl Fischer method (1) in 1965.)

METHOD

Apparatus

Buret with automatic zero; reservoir for reagent; magnetic stirring device; titrn vessel (300 ml Berzelius beaker with stopcock attached to side at bottom for withdrawing excess soln is recommended), electrodes, and circuitry for deadstop end point detection. All openings must be tightly closed or protected with drying tubes to prevent contamination from atmospheric H_2O. Various titrn assemblies may be obtained from laboratory supply houses or one may be assembled.

Assemble titrn app. and follow manufacturer's instructions, set for direct titrn. Set timer to give 30 sec. end point. Add enough dry MeOH to cover electrodes on electrode probes and turn on stirrer. Adjust speed to obtain good stirring without splashing. Do not let stirrer bar contact electrodes. Titr. until satisfactory end point is reached. App. newly assembled or not recently used may require repetition of this step to dry out system.

Reagents

(a) *Karl Fischer reagent.*—Available from laboratory supply houses or prep. as follows: Dissolve 133 g I in 425 ml dry pyridine in dry g-s. bottle. Add 425 ml dry MeOH or ethylene glycol monomethyl ether. Cool to $< 4°$ in ice bath and bubble in 102–105 g SO_2. Mix well and let stand 12 hr. (Less trouble with stopcock leakage is obtained with ethylene glycol monomethyl ether.) Reagent is reasonably stable, but restdze for each series of detns.

(b) *Anhydrous methanol.*—Reagent grade MeOH contg $< 0.1\%$ H_2O. Prep. by distg over Mg.

Determination

Add ca 120 mg H_2O from weighing pipet or other suitable device and titr. with Karl Fischer reagent.

Calc. C = mg H_2O/ml reagent.

For titrn of molasses, C = ca 5 mg/ml. Weigh molasses estimated to give 20–40 ml titer into titrn app. and titr. % $H_2O = (C \times \text{ml reagent}) / (\text{g sample} \times 10)$.

Drain excess liquid and repeat with succeeding samples. If time lapse occurs between titrn of samples, adjust liquid in titrn vessel to end point by titrn with reagent before adding next sample.

Results and Recommendation

The Karl Fischer method is so well known and has been so widely applied that detailed discussion is not needed; the book, *Aquametry* (4) by Mitchell and Smith, is recommended as a source of detailed information. Most collaborators used titration equipment which is commercially available. The important features are complete protection of the system from atmospheric moisture and a means of detecting the end point. Visual detection of the end point is possible but the color change is not sharp enough to be easily perceived by most analysts. Therefore the deadstop method is used for end point detection. The analyst can easily make his own equipment if he wishes.

The results obtained in 1963 are shown in Table 1. The three collaborators obtained good agreement between the methods.

Table 2 gives the results from the 1964 study. Again, agreement between the methods is good, with somewhat less variation shown by the Karl Fischer method.

The 1965 study (Table 3) included some collaborators who were not experienced in

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Table 1. Comparison of Karl Fischer and vacuum-drying methods for % water in molasses, 1963

Sample	Karl Fischer Method				Vacuum-Drying Method			
	Coll. A	Coll. B	Coll. C	Av.	Coll. A	Coll. B	Coll. C	Av.
Cane molasses A	25.68	25.86	26.30	25.95	24.64	26.13	26.50	25.76
Cane molasses B	29.22	28.99	29.30	29.14	29.67	30.07	29.50	29.75
Sucrose (35.86% water)	35.86	36.82	36.50	36.39	35.90	35.31	35.90	35.70

the Karl Fischer or the vacuum drying methods, and the range of results is wider than in the earlier years.

The trend in all three years is for the average results to be higher by the Karl Fischer method than by vacuum drying, but this was not true in all cases. Samples made from known amounts of sugar and water were included in all years; such standards are subject to preparative errors but do give a good reference point for evaluation of the method. In 1963 and 1964 averages for both methods were in good agreement with the "known" water contents. Vacuum drying gave a lower value and Karl Fischer a higher value. The agreement in 1965 was not nearly so good; the calculated value for water in the levulose solution may not have been correct. Both methods showed less water than "known".

The samples sent out in 1965 were levulose-water 30.0%, corn sugar molasses, hemicellulose extract (a product of wood hydrolysis), cane molasses, and mixtures of equal parts of levulose and each of the molasses samples. The value for the mixtures can be calculated by taking the average value for moisture in the levulose solution and the molasses. The close agreement of calculated and found values (Table 4) is evidence of the soundness of both methods.

It is concluded that the Karl Fischer method is suitable for determination of water in molasses.

It is recommended that the method be adopted as official, first action.

Table 2. Comparison of Karl Fischer and vacuum drying methods for water in molasses, 1964

Coll.	Vacuum Drying		Karl Fischer	
Sample I: Heavy Cane Molasses				
A	18.43	18.27	19.18	18.81
B	18.38	18.06	19.57	19.71
C	17.10	17.25	20.04	21.04
D	19.27	19.61	19.53	19.89
Av.	18.30		19.72	
Std dev.	0.87		0.65	
Sample II: Standard Cane Molasses				
A	28.46	29.25	28.57	28.29
B	29.29	29.40	29.37	29.14
C	28.64	29.49	30.34	30.56
D	30.28	30.60	29.53	29.28
Av.	29.43		29.47	
Std dev.	0.73		0.78	
Sample III: Levulose Solution (23.80% Water)				
A	23.60	23.37	23.76	23.93
B	24.01	23.94	24.19	24.25
C	23.00	23.19	24.51	25.23
D	24.39	23.92	24.21	24.07
Av.	23.68		24.27	
Std dev.	0.47		0.45	
Sample IV: Sucrose Solution (30.23% Water)				
A	29.64	29.70	30.22	30.05
B	29.63	29.79	30.82	30.65
C	29.93	30.17	32.67[a]	32.78[a]
D	30.09	30.81	30.21	30.42
Av.	29.97		30.39	
Std dev.	0.39		0.29	

[a] Eliminated from average; collaborator reported sugar crystallized out.

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Table 3. Comparison of Karl Fischer (K) and vacuum-drying (O) methods for water in molasses, 1965

	Levulose 30% Water		Corn Sugar Molasses		Levulose CSM		Hemicellulose Extract		Levulose HE		Cane Molasses		Levulose CM	
Coll.	K	O	K	O	K	O	K	O	K	O	K	O	K	O
A	29.46	29.01	23.74	21.98	26.76	25.40	42.52	43.95	35.83	36.72	30.25	30.40	29.93	29.37
B	29.72	29.20	24.34	25.81	27.22	26.62	42.95	44.18	36.42	36.93	30.99	31.41	30.18	30.22
C	29.93	27.36	24.45	21.49	27.14	27.36	45.53	41.87	36.67	34.26	31.25	29.34	30.53	28.54
D	28.90	29.06	23.90	23.71	26.90	26.74	42.80	43.89	36.00	36.93	30.00	31.31	30.00	30.53
E	27.60	29.40	23.90	23.40	25.40	26.40	40.60	43.60	35.60	37.00	30.00	31.30	28.80	30.30
F	29.07	28.45	23.57	23.35	26.33	26.22	41.96	42.66	35.43	36.32	29.94	31.06	28.95	29.39
Av.	29.11	28.75	23.98	23.29	26.63	26.46	42.39	43.36	35.99	36.36	30.41	30.80	29.73	29.73
Std dev.	0.70	0.75	0.76	1.51	0.68	0.64	1.15	0.90	0.48	1.06	0.57	0.80	0.70	0.76

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Table 4. Calculated and found water content % of mixtures of levulose solution and molasses

	Karl Fischer Method		Vacuum Drying Method	
Sample	Calcd	Found	Calcd	Found
Levulose-corn sugar molasses	26.55	26.63	26.02	26.46
Levulose-hemicellulose extract	35.75	35.99	36.06	36.36
Levulose-cane molasses	29.76	29.73	29.77	29.73

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AFLATOXINS

Determination of Aflatoxins in Agricultural Products: Use of Aqueous Acetone for Extraction

By W. A. PONS, JR., A. F. CUCULLU, L. S. LEE, J. A. ROBERTSON, A. O. FRANZ, and L. A. GOLDBLATT (Southern Regional Research Laboratory, New Orleans, La. 70119)[1]

An analytical procedure originally developed for the determination of aflatoxins in cottonseed products has been modified for application to many agricultural materials. Aflatoxins are rapidly extracted free of lipid contamination with 70% acetone. Many interfering pigments are removed from the crude extract by precipitation as insoluble lead derivatives, transfer of aflatoxins into chloroform, and further purification of the chloroform extract with silica gel.

The procedure was compared with four

[1] One of the laboratories of the Southern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

recently proposed methods for the analysis of peanut products. This technique is capable of detecting as little as 0.3 ppb of aflatoxin B_1 in peanuts and peanut butters. Adequate recovery of added aflatoxins was obtained when this method was applied to numerous different agricultural products.

Recently a rapid, simple, and sensitive method for the extraction of aflatoxins from cottonseed products was proposed by Pons and Goldblatt (1). An acetone:water solvent (70:30 v/v) was used to yield extracts essentially free of lipids, followed by treatment of the extract with lead acetate to remove gossypol pigments as insoluble lead derivatives. The aflatoxins were then transferred into chloroform and were determined by thin layer chromatography (TLC) on silica gel. Estimation of aflatoxins at levels as low as 1 ppb (μg/kg) in cottonseed meats and 4 ppb (μg/kg) in meals was possible with this method.

In applying this technique to peanut products and a number of other agricultural materials, some difficulties were experienced with incomplete removal of residual pigmentation. Moreover, when extracts were concentrated to detect aflatoxins present at low levels (1–2 ppb), pigments and streaking components interfered with the determination. Addition of a further cleanup step to the procedure removed most of these interferences. When the final chloroform extract from the original procedure was absorbed on a small silica gel column and washed with diethyl ether, extraneous fluorescent materials and streaking components were removed while the aflatoxins remained on the silica gel column.

Subsequent elution with methanol:chloroform (3:97 v/v) quantitatively removed aflatoxins and produced extracts for TLC which were substantially lower in total solids and residual pigmentation. This was especially helpful in extracts containing low levels of aflatoxin, when the extract must be concentrated to a small volume (0.25 ml) prior to spotting on a TLC plate.

For these reasons, a silica gel purification step was incorporated into a modified aqueous acetone procedure for analysis of aflatoxins in cottonseed and peanut products and in a number of other agricultural products including cereal grains, beans, potatoes, leafy green and root vegetables, tobacco, and mixed feeds. Details of the modified procedure and experimental data on application of the technique to these products are presented in this communication.

METHOD

Reagents

(a) *Acetone.*—ACS grade. (*1*) 70% Acetone: Mix 700 ml acetone and 300 ml distilled water. (*2*) 20% Acetone: Mix 200 ml and 800 ml distilled water.

(b) *Lead acetate solution (20%).*—Dissolve 200 g ACS neutral lead acetate in distilled water, add 3 ml glacial acetic acid, and dilute to 1 L.

(c) *Chloroform.*—ACS grade.

(d) *Methanol.*—ACS grade.

(e) *Diethyl ether.*—ACS grade.

(f) *Methanol:chloroform (3%).*—Mix 30 ml MeOH and 970 ml $CHCl_3$. Store in a glass-stoppered bottle.

(g) *Hexane.*—Purified; boiling range 60–70°C.

(h) *Sodium sulfate.*—Anhydrous granular.

(i) *Silica gel G-HR.*—No. 72-G-HR, Brinkmann Instruments, Inc., Westbury, N.Y.

(j) *Silica gel for column chromatography.*—0.05–0.20 mm. No. 7734, Brinkmann Instruments, Inc.

(k) *Quantitative aflatoxin standard.*—Prepare from pure crystalline aflatoxins B_1 and G_1. Accurately weigh 1.5 mg B_1 and 1.0 mg G_1 and combine in 500 ml volumetric flask. Dissolve in $CHCl_3$ and dilute to volume. (This stock solution contains 3.0 μg B_1/ml and 2.0 μg G_1/ml.) Using an accurate volumetric pipet, pipet 2 ml stock solution into 10 ml glass-stoppered volumetric flask and dilute to volume with chloroform. (The diluted standard contains 0.6 μg/ml B_1 and 0.4 μg/ml G_1.) When not in use, the standard solution may be preserved by placing the 10 ml volumetric flask in a suitable screw cap jar containing several ml $CHCl_3$ and storing at about 0°F (−18°C) in a freezer. Before use, the jar and flask should be equilibrated to room temperature. Storage under these conditions minimizes evaporation of the standard, oxidation or destruction of aflatoxins, and moisture condensation in the chloroform solution. The unused portion of the aflatoxin stock solution may be preserved by storage under similar conditions.

(1) *Qualitative aflatoxin standard.*—May be prepared from semipurified aflatoxin concentrates or from purified sample extract containing aflatoxins B_1, B_2, G_1, and G_2. The qualitative standard should contain approximately 0.8 μg/ml B_1, 0.3 μg/ml B_2, 0.8 μg/ml G_1, and 0.3 μg/ml G_2.

Apparatus and Equipment

(a) *Mechanical shaker.* — Burrell "Wrist Action" shaker, or equivalent, for holding 500 ml capacity Erlenmeyer flasks.

(b) *Waring Blendor.*—Standard laboratory model with 1 L glass container, preferably fitted with Central Scientific No. 17248 Cenco-Pinto 6-blade cutter assembly for the comminution of dry sample material.

(c) *Centrifuge.*—International Size 1, Type SB, or equivalent, equipped with head for holding 250 ml centrifuge bottles.

(d) *Wiley Mill.*—Standard Model No. 3, equipped with 1 and 2 mm mesh screens, or equivalent.

(e) *Riffler-Sampler.* — Any standard riffler such as a Jones Sampler, or equivalent, is satisfactory.

(f) *Extraction flasks.*—500 ml ⅁ Erlenmeyer flasks fitted with polyethylene ⅁ stoppers, size 32 (Kimble No. 28160). If these are not available, ⅁ ground glass stoppers may be used.

(g) *Separatory funnels.*—Squibb pear shape, 250 ml, preferably with Teflon stopcocks.

(h) *Funnels.*—Fluted, 60°, long stem, ca 100 mm diameter.

(i) *Filter paper.*—Whatman No. 4, or equivalent, 18.5 cm circles.

(j) *Vials.*—2 dram, with polyethylene friction-fit covers (Kimble 60975-L, or equivalent).

(k) *Chromatographic column.* — (*1*) Use standard Butt extraction tubes (Corning Glass No. 92195). Place small glass wool plug in constriction of the tube, add 2 cm anhydrous Na_2SO_4 to just cover glass wool plug, and then add about 10 g granular silica gel for column chromatography (*Reagent* (j)). A 20 ml beaker loosely filled with the gel is a convenient measure. Cover silica gel with a small layer of anhydrous Na_2SO_4 (ca 2 cm); or (*2*) Construct from pyrex tubing, 35 mm o.d. and about 300 mm long with the end drawn to a 10 mm constriction. Entire elution volumes of ether and of $CHCl_3$:MeOH can be added to the sample on the column.

TLC Equipment

(a) *Glass plates.*—20 × 20 cm (8 × 8″), mounting board; applicator adjustable for 0.5 mm thickness; storage racks for plates; desiccating storage cabinet (Brinkmann Instruments, Inc., or equivalent).

(b) *Microliter syringe.*—10 μl Hamilton style 701-SN, fitted with a special 26 gauge tip, 2 cm long, or any other micropipet suitable for accurate delivery of 1–20 μl.

(c) *Spotting template.*—Construct from glass or other suitable material a support designed to hold the microliter syringe in an upright position with the tip just touching the surface of the TLC-coated plate.

(d) *Development tank.*—8.5″ wide × 8.5″ high × 4.5″ deep, fitted with ground glass edges on tank and cover (Brinkmann Instruments No. 25-10-20). Line all four sides of the tank with heavy filter paper or blotting paper.

(e) *Fluorescent viewer.*—Ultraviolet Products, Inc., San Gabriel, Calif., Model C-4 or C-6, equipped with four 15 watt long wave UV lamps for reflected UV, and a Blak-Ray Transilluminator and contrast filter for viewing plates by transmitted UV is recommended. Any UV system with 15 watt long wave fluorescent bulbs is satisfactory. Best results are obtained when plates are viewed under both reflected and transmitted UV with a suitable filter to screen out long wave UV from the observer's eyes.

A 100 watt long wave flood blub, similar to GE H-100 PSL 38-4, fitted in a suitable lamp such as Ultraviolet Products Blak-Ray B-100A is useful for observing low aflatoxin levels.

Sample Preparation

Cottonseed and cottonseed meats.—Riffle or split sample to provide an analytical sample about 1 kg and grind in Wiley Mill to pass a 2 mm screen. Screen ground sample on 4/64 screen to remove the coarse lint. Mix the ground sample thoroughly. For optimum sampling, the ground sample should be riffled down to about 50 g to provide sample for analysis.

Cottonseed and peanut meals.—Grind in a Wiley Mill to pass a 1 mm screen.

Shelled peanuts.—Examine sample and discard any extraneous material. Pass large samples (over 2 kg) successively through a

Use of a company and/or product named by the Department does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

Boerner Sampler, or equivalent, and reduce to about 2 kg. Grind 2 kg sample in Waring Blendor in 100 g batches, running the Blendor at high speed for 10–20 sec. for each batch. Combine entire ground sample and riffle to final sample size of about 50 g.

Peanut butter.—If refrigerated, warm sample to room temperature; mix well with a spatula.

Mixed feeds.—Grind in a Wiley Mill to pass a 1 mm screen.

Other agricultural products.—Use an approved sampling technique for the particular commodity. Grind the sample either in a Wiley Mill, using a 1 or 2 mm screen, or in a Waring Blendor. Dry grinding in a Waring Blendor will be suitable for most products.

Crude oils.—Mix well, warming to above room temperature if necessary to obtain adequate mixing.

Sample Extraction

(a) *Dry, ground samples.*—Weigh 50 g sample[2] into a 500 ml Erlenmeyer flask, and cover bottom of flask with 6 mm solid glass beads. Add 250 ml 70% acetone (graduated cylinder may be used for measurement), and fit flask with a leakproof polyethylene or glass stopper. Shake on mechanical shaker for 30 min. so that the sample material collecting in the neck of the flask is constantly washed back into the solvent. Filter through filter paper (*Apparatus* (i)) folded to fit a 100 mm i.d. funnel; cover the funnel with a watch glass to minimize evaporation. Collect the clear filtrate (at least 150 ml) in a stoppered 250 ml flask.

Proceed as directed below under *Extract Purification.*

(b) *Peanut butter.*—Weigh a 50 g sample into tared 250 ml beaker. Measure 250 ml 70% acetone, add about 100 ml solvent to sample in the 250 ml beaker, mix well with a spatula, and transfer to 500 ml ₹ Erlenmeyer flask. Wash sample into Erlenmeyer flask in two washes with rest of the solvent. Cover bottom of the flask with 6 mm solid glass beads, stopper with a leakproof polyethylene or ground glass stopper, and shake on a mechanical shaker for 30 min. Filter as in (a) and proceed as directed under *Extract Purification.*

(c) *Oils.*—Weigh a 30 g sample into a 250 ml beaker. Dissolve or disperse in 100 ml hexane and transfer to 250 ml separatory funnel. Wash hexane dispersion or solution three times with 50 ml 70% acetone with gentle agitation. Withdraw each successive lower aqueous acetone phase into 400 ml beaker, and proceed as directed under *Extract Purification,* using the entire 70% acetone extract.

Extract Purification

Measure 150 ml from *Sample Extraction,* (a) or (b) or entire extract from (c), into 400 ml beaker marked at 150 ml. Add 60 ml distilled water, 20 ml lead acetate solution (*Reagent* (b)), and several carborundum boiling chips. Boil on steam bath until volume is reduced to 150 ml. Cool to about 20°C in an ice-water bath.

Transfer the contents to 250 ml centrifuge bottle and centrifuge at 2000 rpm for 10 min. The supernatant solution should be clear and free from suspended material. Decant supernatant into 250 ml separatory funnel. Disperse precipitate in the centrifuge bottle in 60 ml 20% acetone, mix well, and recentrifuge at 2000 rpm for 10 min. Add wash solution to original supernatant in the separatory funnel.

(Note: If a centrifuge is not available, the following steps may be substituted: To the boiled and cooled crude extract, add about 3–4 g Celite analytical filter aid (about 2 level teaspoons), stir well, and filter under reduced pressure on a small Büchner funnel fitted with a 5.5 cm circle of Whatman No. 4, or equivalent, filter paper. Wash filter cake with 60 ml 20% acetone. Combine filtrate and wash solution; pour into 250 ml separatory funnel.)

Extract the combined supernatant and wash solution with 50 ml $CHCl_3$, shaking funnel vigorously for about 1 min. Let phases separate and transfer the lower ($CHCl_3$) phase to 250 ml beaker.

Repeat extraction with additional 50 ml $CHCl_3$ and transfer lower phase to the 250 ml beaker.

Evaporate on steam bath until the volume is about 2–3 ml. Quantitatively transfer $CHCl_3$ extract to chromatographic column (*Apparatus* (k)) with no more than 10 ml $CHCl_3$. A small stirring rod and a fine stream of $CHCl_3$ directed from a wash bottle will facilitate transfer. Let 100 ml ethyl ether percolate through column, either in increments or in one portion, depending on the length of the column. Discard the ether eluate. After the ether has percolated through the column, add 150 ml 3% MeOH-

[2] Use of 50 g sample and 150/250 ml aliquot of extract for analysis yields a final extract equivalent to 3 g original sample.

$CHCl_3$ and collect eluate in 250 ml beaker. Evaporate to about 2 ml. (Note: With extracts containing high aflatoxin levels or low amounts of interferences, the silica gel column may be eliminated and the following substituted: Percolate chloroform extracts through a small column of anhydrous Na_2SO_4 (ca 4 cm in a Butt tube) to remove traces of water. Wash Na_2SO_4 with ca 20 ml $CHCl_3$, evaporate combined $CHCl_3$ extracts to 2–3 ml, and continue as outlined below.)

Transfer quantitatively to small vial and evaporate to dryness on a steam bath, preferably under a stream of nitrogen. Avoid overheating the dry sample extract. Cool and dry sample extract to room temperature.

Preliminary TLC Analysis

Prepare TLC plates as follows: Shake 45 g Silica Gel G-HR with 90 ml distilled water for 30 sec. in stoppered flask. Pour into applicator set for a coating 0.5 mm (500 μ) thick, and immediately coat five 20×20 cm glass plates. Let plates air-dry for about 60 min.; then dry in a forced-draft oven for 2 hr at 103–105°C. Store in desiccator cabinet over silica gel or Drierite desiccant.

Pour 150 ml 3% MeOH-$CHCl_3$ solvent into lined development tank, replace the cover, and shake vigorously to saturate the filter paper liner. Let equilibrate for 30 minutes.

Add exactly 500 μl $CHCl_3$ to vial containing the dry sample extract, stopper, and shake gently to dissolve the sample extract.

Spot 2, 5, and 10 μl aliquots of sample extract and 2, 4, and 6 μl of quantitative aflatoxin standard (B_1 and G_1) on TLC plate, placing the spots about 1.5 cm apart on a line about 4 cm from the bottom of the plate. Also, spot 5 μl of qualitative standard alone on the same plate as a guide for establishing adequate resolution.

For optimum quantitative analysis, spot extract in 1–2 μl increments and blow on the spot to hasten solvent evaporation. This will aid in obtaining small sample and standard spots (no larger than 0.5 cm and about the same size). The microliter syringe should be held in an upright position to obtain circular spots on TLC development.

Draw a line about 2 cm from top of plate and one about 0.5 cm from the edge along each side of the plate. Place the plate in the equilibrated chamber and let develop until the solvent front reaches top line (about 14 cm solvent travel). Withdraw plate and air-dry for 5–10 min.

When dry, observe the TLC plate under long wave UV light in a dark room or in a suitable dark viewing cabinet. Observe first the chromatogram of the qualitative standard to confirm adequate resolution of all four aflatoxins. Four clearly identifiable fluorescent spots should be visible. In order of decreasing R_f, they are B_1, B_2, G_1, and G_2.

Since TLC conditions cannot be exactly duplicated, R_f values may vary slightly from plate-to-plate. However, when standards and samples are spotted on the same plate, this variation is not significant. An internal standard is also useful for positive identification of aflatoxins in sample extracts.

If aflatoxins B_1 or G_1 are present in sample spots, compare with fluorescence intensity of quantitative standard. Select spots which most nearly match in intensity. If necessary, interpolate when the intensity of the sample spot is between two of the standard spots.

Calculate the approximate concentration of aflatoxins B_1 or G_1, if present in the sample, as outlined under *Calculations*.

Quantitative TLC Analysis

Dry remaining sample extract under a slow stream of nitrogen and dilute for quantitative analysis. The dilution volumes below for estimated B_1 content may be used as a guide.

Approx. B_1 Content, ppm	*Dilution Volume, ml*
0–10	0.25
10–20	0.50– 1.0
20–50	1.0– 2.5
50–100	2.5– 5.0
100–150	5.0– 7.5
150–200	7.5–10.0
200–300	10.0–15.0

Spot 3, 5, and 7 μl of the suitably diluted sample extract and 2, 3, 4, and 5 μl of the quantitative aflatoxin standard on a TLC plate as previously outlined. On the same plate, spot another 5 μl of sample extract and over it spot 5 μl of qualitative standard as internal standard. Also spot 5 μl of the qualitative aflatoxin standard on the plate as a guide for establishing adequate resolution. (Note: If preliminary TLC analysis indicates only traces of aflatoxins, i.e., 1–5 ppb, 5, 10, and 20 μl of the sample extract are spotted on the TLC plate instead of the 3, 5, and 7 μl aliquots outlined above.)

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Develop plate, dry, and observe under long wave UV light as above. Examine first patterns of the 5 μl sample aliquot and the 5 μl sample aliquot with the internal standard to verify the presence or absence of individual aflatoxins. If aflatoxins B_1 and B_2 are present in the sample pattern, compare their intensities with that of the B_1 spot (quantitative standard). Similarly, compare aflatoxins G_1 and G_2 intensities, if present in the sample pattern, with G_1 in the quantitative standard. In all cases, better matching of fluorescent intensity will be possible if sample and standard spots for evaluation are selected at low fluorescent intensities.

Calculations

ppb aflatoxin $B_1 = V_S \times C_S \times SD \times 1000/W \times X \times 0.6$

where V_S = μl of aflatoxin standard in which B_1 spot matches the sample spot B_1, C_S = concentration of aflatoxin B_1 in the standard aflatoxin solution (μg/μl), SD = volume to which sample extract is diluted for TLC analysis, in μl, W = g sample weight, and X = μl sample extract spotted. (The same procedure is used to calculate aflatoxins B_2, G_1, and G_2.)

Application to Peanut Products

Most current procedures have been designed for the determination of aflatoxin in peanut products (2–8), although increasing attention will undoubtedly be given to aflatoxin determination in other agricultural materials. To demonstrate applicability of the proposed procedure to peanut materials, three samples each of raw ground peanuts and peanut meals comprising high, medium, and low aflatoxin levels were analyzed by the aqueous acetone method and by four other methods proposed for analysis of peanut products. The methods selected are outlined in Table 1 and comprise a procedure (Method A) judged to be typical of older "classical" procedures involving prolonged methanol extraction, and three methods (B, C, D) judged typical of newer improved analytical procedures for peanuts. Analyses were performed in duplicate on different days, and all aflatoxin values reported represent the average of independent observation of TLC plates by two observers. All analytical values were rounded to two significant figures, since present evidence indicates that visual TLC analysis does not warrant any higher degree of precision (5).

Average values obtained from application of the five methods to raw peanuts and peanut meals are listed in Table 2. Consistently low values obtained by Method A may reflect loss of aflatoxins during prolonged methanol extraction as suggested by Trager, *et al.* (3) and Nabney and Nesbitt (9); inconsistent results obtained with Method C may be due to suboptimal water concentration in the extraction solvent (7). It would seem that values obtained with the proposed aqueous acetone procedure and with Methods B and D are all in essential agreement for these peanut products.

A convenient indication of the effectiveness of a given procedure for providing clean extracts for TLC analysis is the level of total solids remaining in the final purified extract. Accordingly, final extracts from each method were dried under nitrogen, weighed, and calculated to a common basis of mg total solids/50 g sample equivalent, since the final extracts from the five procedures represented a range of sample equivalents from 10 g (Method B) to 200 g (Method A).

Table 1. Description of aflatoxin methods used for analysis of peanut products

Method	Basic Method Conditions				
	Type Extrn	Time	Solvent Used[a]	Extract Purification	Ref.
A	Soxhlet	6 hr	MeOH	Liq.:liq. extraction	(3)
B	Blendor	3.5 min.	MeOH:W	Chromatography-Celite	(5)
C	Blendor	5 min.	A:H:W	Chromatography-Florisil	(6)
D	Blendor	4 min.	A:H:W	Liq.:liq.-sep. funnel	(7)
Aqueous acetone	Shaker	30 min.	A:W	Pb ppt-liq.:liq.-Silica gel column	Proposed method

[a] MeOH = methanol; A = acetone; H = hexane; W = water.

Table 2. Comparison of five methods for determination of aflatoxins in ground raw peanuts and peanut meals

Method	Aflatoxins, ppb[a, b]					
	B_1	G_1	B_1	G_1	B_1	G_1
	Ground raw peanuts					
	1		2		3	
A	730	380	100	ND	38	5
B	1000	440	160	16	57	ND
C	650	110	110	15	25	ND
D	720	300	210	16	32	4
Aqueous acetone	980	470	250	37	43	17
	Peanut meals					
	1		2		3	
A	300	ND	150	32	19	ND
B	760	190	350	110	29	Tr
C	360	60	110	27	11	ND
D	780	200	320	91	40	10
Aqueous acetone	760	200	430	69	29	Tr

[a] Average values from duplicate determinations on different days, ppb = μg/kg.
[b] ND = None detected.

Average values from six determinations by each method are listed in Table 3. The aqueous acetone procedure yields final extracts quite low in total solids. It should be emphasized that a value for total solids is merely indicative of extract cleanliness and is not an absolute measure of interfering pigmentation. As judged from the resolution of aflatoxins on TLC plates and the absence of interfering pigmentation at the R_f of aflatoxins B_1 and G_1, final extracts from satisfactory TLC analysis of the peanut products listed in Table 2 could be made on final extracts from Methods B, C, D, and the aqueous acetone procedure.

Table 3. Total solids in final TLC extracts from five aflatoxin methods[a]

Method	Av. for Ground Peanuts[b]	Av. for Peanut Meal[b]
A	290	250
B	58	50
C	98	32
D	88	22
Aqueous acetone	7.4	10

[a] Calculated to common basis, mg/50 g sample equivalent for each method.
[b] Average values represent 6 determinations/sample type for each method.

Table 4. Recovery of aflatoxins added to agricultural products by the aqueous acetone method[a]

Type of Sample	B_1		G_1	
	Added, ppb	Found, ppb	Added, ppb	Found, ppb
Cottonseed meats	152	152	128	128
Cottonseed meats	76	78	64	67
Cottonseed meats	38	32	32	25
Crude cottonseed oil	125	121	64	70
Ground peanuts	50	54	—[b]	—[b]
Peanut meal	38	37	32	31
Peanut butter	76	64	64	55
Peanut butter	38	32	32	28

[a] 50 g samples of each product plus purified aflatoxins added during extraction step in calculated amounts shown.
[b] Not estimated.

Recovery of Aflatoxins—Detection Limits

Purified aflatoxins added to both cottonseed and peanut products were adequately recovered by application of the proposed procedure, as may be judged from the data assembled in Table 4. However, such data are not indicative of the sensitivity of the procedure and, in particular, the accuracy at trace aflatoxin levels. To explore these factors, purified aflatoxin B_1 was added to 50 g samples of aflatoxin-free ground peanuts and peanut butter at the beginning of the extraction step at levels ranging from 6 to 0.1 ppb. The recovery of aflatoxins at these levels, Table 5, indicates that aflatoxin levels as low as 0.5 ppb can be estimated in peanut butter and raw peanuts

Table 5. Detection limits for peanut products: aqueous acetone procedure

Raw Peanuts[a]		Peanut Butter[a]	
Aflatoxin B_1, ppb		Aflatoxin B_1, ppb	
Added	Found[b]	Added	Found[b]
6.0	6.0	6.0	5.0
4.0	4.3	3.0	3.0
3.0	3.0	1.5	1.5
2.0	2.2	0.75	1.0
1.5	1.4	0.5	0.5
1.0	1.3	0.38	Tr
0.5	0.6		
0.25	Tr		
0.10	ND		

[a] 50 g samples of designated aflatoxin-free peanut product plus added purified aflatoxin B_1 at calculated levels indicated.
[b] ND = None detected.

Table 6. Recovery of aflatoxins added to low moisture agricultural products — aqueous acetone method

Type of Sample	Aflatoxin in 50 g Control, ppb[a]	Total Solids-TLC Extract (mg/50 g)	Aflatoxins, ppm	
			B_1	G_1
Calculated value	—	—	50[b]	20[b]
Soybeans	Tr G_1	13.3	50	15–20
Rough rice	3–B_1	10.6	50	10–15
White rice	ND	7.0	50	10–15
Wheat	ND	5.2	50	15–20
Oats	3–B_1	10.3	45–50	15–20
Barley	ND	7.7	50	10–20
Sorghums	ND	4.2	50	15–20
Yellow corn	ND	2.5	50	15–20
Red beans	ND	2.7	45–50	15
Green peas, dried	ND	4.3	50	10–15
Alfalfa seed	1–B_1	6.2	25	10
Leaf tobacco	ND	6.9	45–50	—[c]
Mixed chick feed	ND	3.0	35–45	15
Dehydrated sweet potato	ND	4.8	50	15

[a] ND = None detected.
[b] Calculated from mixture of 50 g each agricultural material plus 2.0 g meal containing 1260 ppb B_1 and 530 ppb G_1.
[c] Interfering fluorescent spot.

with reasonable accuracy. During our work on analysis of peanut products, we noted that peanut meal and peanut butter extracts consistently contained higher levels of pigmentation than did raw peanut samples. In addition, samples of peanut meal and butters varied considerably in the amounts and types of residual interfering pigmentation. These variables make it somewhat difficult to translate sensitivity data, such as those reported in Table 5, to all types of peanut samples, and such data should be considered indicative of the general sensitivity of the technique, not the absolute sensitivity for all types of peanut samples.

Application to Other Agricultural Products

To demonstrate applicability of the proposed method to low moisture agricultural products, the materials listed in Table 6 were comminuted by dry grinding in a Waring Blendor, and 50 g portions of each were then mixed with accurately weighed 2 g portions of a finely ground homogenous peanut meal which contained 1260 ppb B_1 and 533 ppb G_1. The aflatoxin content of the mixtures was calculated on the basis of the 50 g portions of agricultural products used. Duplicate analyses were conducted on each mixture, and the range of recovery values is also tabulated in Table 6. From these data it appears that adequate recoveries of aflatoxins were obtained except for alfalfa seed. It may also be noted that the levels of residual total solids are quite low. No extensive research has been done on application of the procedure to low moisture commodities such as those listed in Table 6; the results merely indicate that the procedure should be readily adaptable to assays for aflatoxin in such materials.

Table 7. Recovery of aflatoxin B_1 added to high moisture agricultural products

Type of Sample	Total Solids-TLC Extract (mg/50 g)	Aflatoxin B_1, ppb	
		Control	Mixture[a, b]
Calculated value	—	—	50
Carrots	6.2	Tr	40
Red potato	0.3	3	40
Red cabbage	1.5	2	40
Spinach	2.2	ND	40
Beets	0.8	1	40
Onion	0.3	0.5	40

[a] Calculated from mixture of 50 g each commodity plus 2.0 g aflatoxin-contaminated meal containing 1260 μg/kg of aflatoxin B_1.
[b] Uncorrected for water contributed by sample. When corrected for moisture in samples, values are 48 ppb of B_1.
ND = None detected.

Analogous recovery experiments were conducted with high moisture products as outlined in Table 7. Since all these materials contained more than 80% moisture, dry

grinding was impractical. They were sliced, and representative 50 g portions were mixed with 2 g of the peanut meal in a blender jar and extracted with aqueous acetone by 4 min. homogenization in an explosion-proof blender. Extracts were filtered through paper and analyzed as outlined previously in the analytical procedure. No correction was made for the water contributed by the sample. The recovery values, 40 ppb (Table 7), as compared to a calculated value, 50 ppb, would seem to be low. However, since an aliquot is used, correction must be made for the water contributed by the sample, 40–45 ml. When such correction is made, the values range from 47 to 48 ppb. This emphasizes that in analysis of high mositure samples it is necessary to correct the analysis for water contributed by the sample.

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Note on Occurrence of Giant Cells in *Aspergillus flavus* Link

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The presence in the mold, *Mucor*, of spherical cells with yeast-like growth patterns has been known for many years, as indicated by Bartnicki-Garcia (1), who provided a brief history of work on this phenomenon. Similar dimorphic growth forms including larger bodies, termed giant cells, are not uncommon in other molds, according to Hesseltine (2). Bartnicki-Garcia (1) states that most species of *Mucor* studied required CO_2 for induction of the yeast-like stage. It appears, therefore, that in *Mucor* it is possible to induce the formation of the spherical bodies.

Swellings of the mycelium of *A. flavus*[1] were noted when sterile water was added to cultures of the mold in which the agar had dried. When the water was added, many spores floated out on the surface. Two days after the addition of water, swollen spherical bodies were found in mycelial strands when the floating spore masses were examined. Swollen conidiospores also were encountered. The cultures, from time of original inoculation to observation of spherical bodies, ranged in age from 9 to 20 days. Two younger cultures and an older culture did not show any swellings.

Spherical bodies varying in diameter from 10 to 50 μ were observed in micro colonies of *A. flavus*[2] which grew in the agar surrounding the original central macro colony (Figs. 1–3). They were also noted when agar blocks (ca 5 mm square), cut from agar surrounding a macro colony of *A. flavus*[3], were seeded with *A. flavus*[1] spores. In performing this test the agar blocks were transferred to a sterile petri dish and inoculated lightly with a loop on the upper surface, and the inoculated surface was covered with a cover glass. Examination after 17 hr showed no spore germination in blocks originally 2 mm from the macro colony but

[1] M 93 (NRRL A 13794) isolated by Hodges from moldy corn and identified by Hesseltine.

[2] Several isolates including M 93.

[3] M 60 (1 M 1 15957 i i) obtained from Austwick.

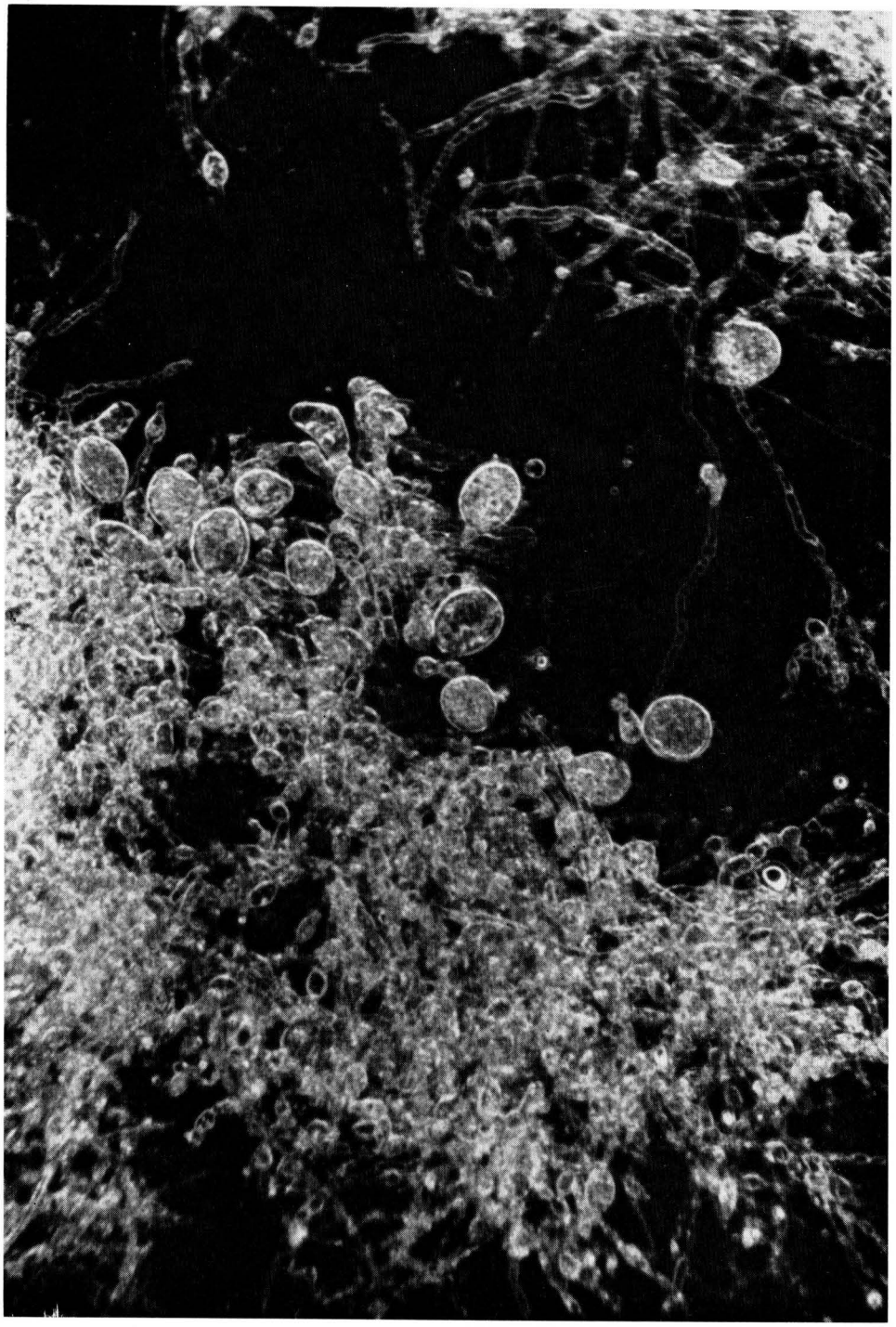

Fig. 1.—Giant cells in mycelium taken from small colonies of *A. flavus* growing near a larger and older colony of *A. flavus* (M 93). 200 ×.

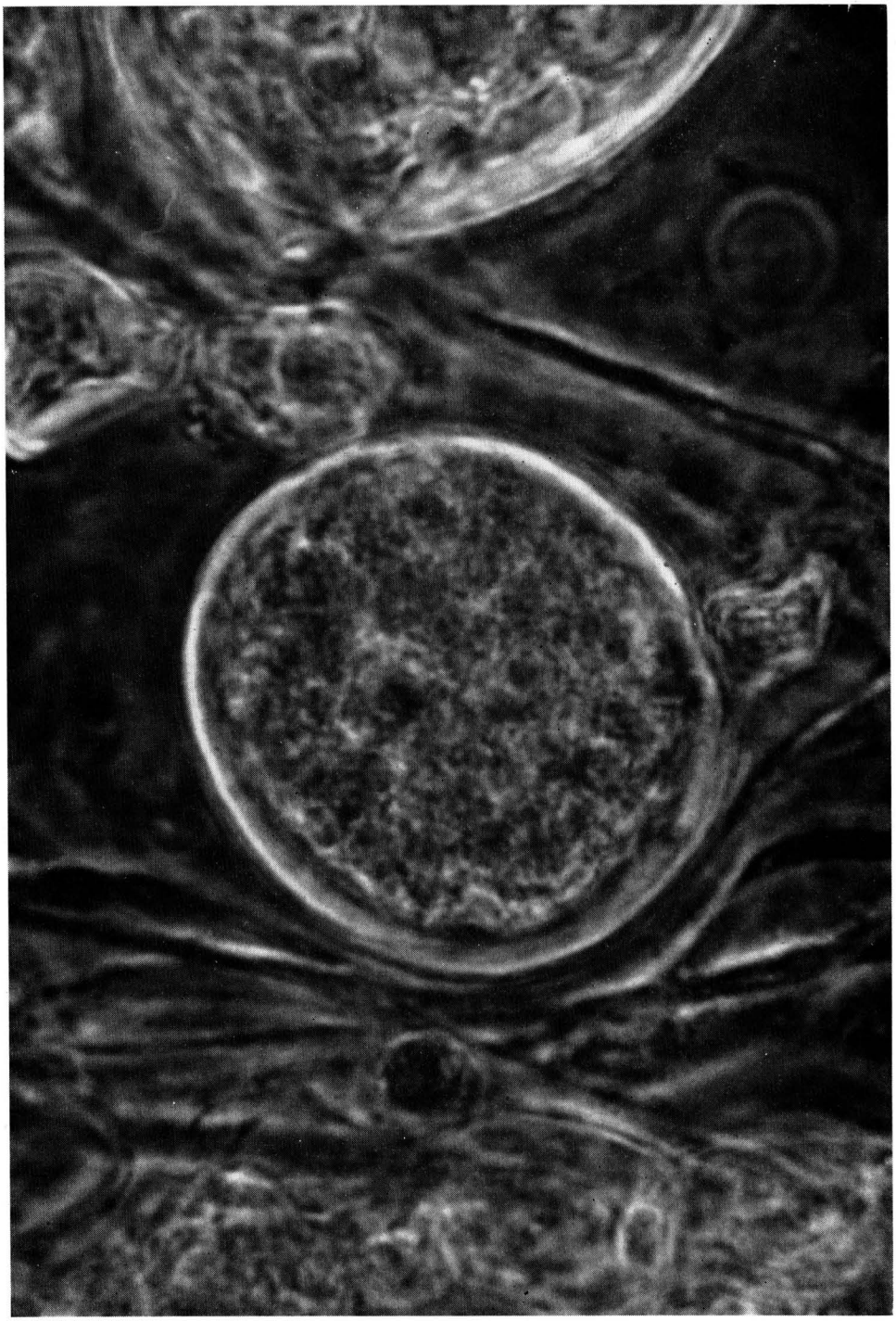

Fig. 2—**Giant cell from hyphal masses shown in Fig. 1. 2000 ×.**

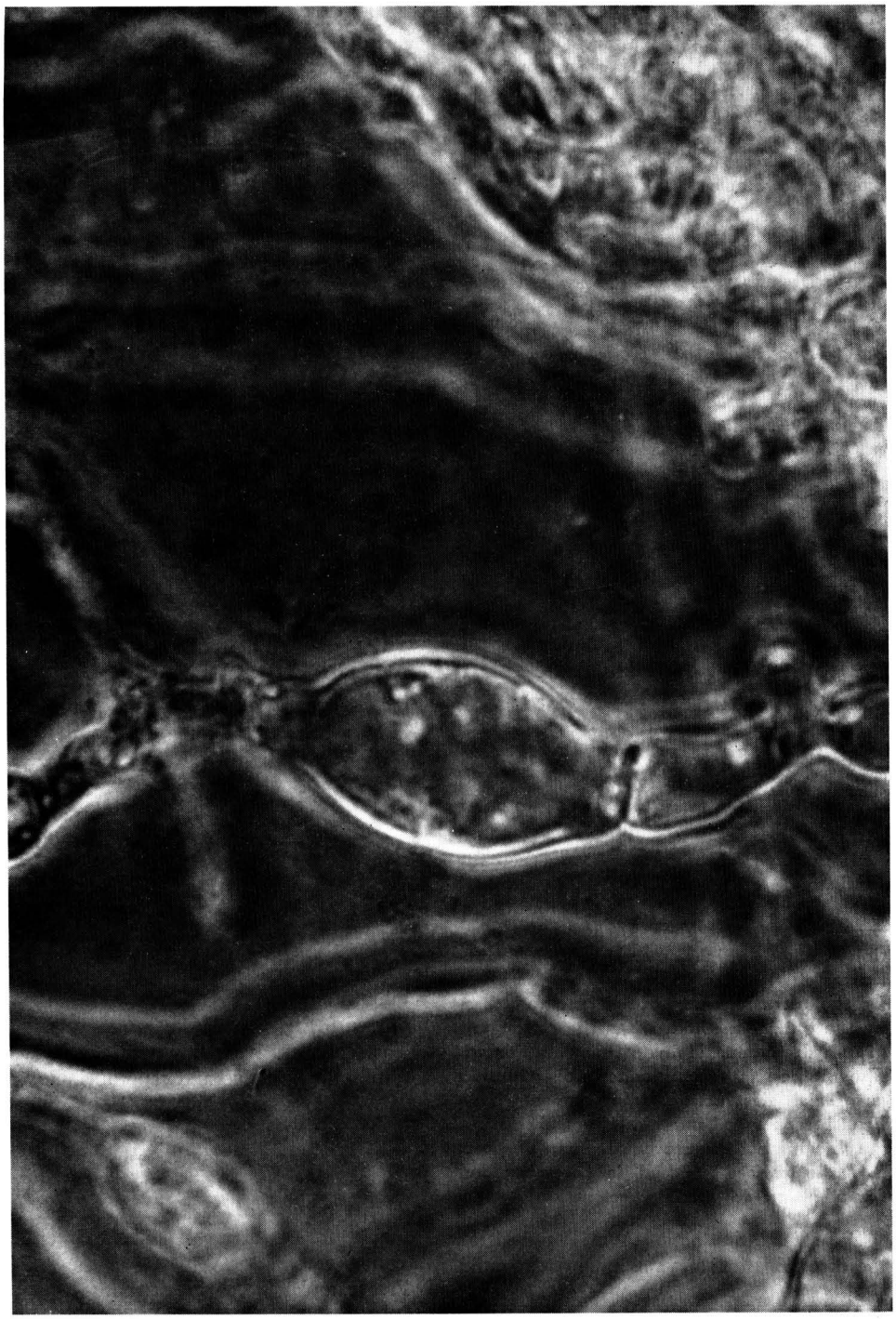

Fig. 3—Swollen hypha from hyphal masses shown in Fig. 1. 2000 ×.

from 5 to 70% germination in blocks which were 6 to 17 mm from the macro colony. No swellings were noted in the germ tubes at this time, but 5 hr later, swellings were abundant in the tubes. No germination had occurred in the blocks 2 mm from the macro colony. Swellings have also been observed in mycelium from macro colonies.

Discussion

It was thought at first that the large, rounded bodies were Hülle cells which might be helpful in the identification of *A. flavus*. However, the thin walls of the spheres ruled out this possibility, and Hesseltine (2) likens the bodies observed to giant cells and swellings noted in other molds, especially *Mucor*. The study is being continued, since the bodies may be found useful in diagnosis. The fact that swellings were obtained in germ tubes on agar blocks cut from agar plates in which *A. flavus* was growing suggests that extrinsic metabolic products of *A. flavus* growth may be the cause of the swellings. On control blocks of agar inoculated and cultured similarly, no formation of swellings had been observed. Whether the phenomenon is similar to that reported by Wragg and Legator (3), in which *E. coli* produces unusual growth forms when exposed to aflatoxins, remains to be determined.

References

(1) Bartnicki-Garcia, S., *Bacteriol. Rev.*, 27, 293–304 (1963).
(2) Hesseltine, C. W., personal communication.
(3) Wragg, J., and Legator, M., personal communication.

FLAVORS

Collaborative Studies of Methods for Determining Vanillin and Ethyl Vanillin in Flavoring Material

By J. FITELSON (Fitelson Laboratories, Inc., 254 W. 31 St., New York, N.Y. 10001)

Collaborative study was conducted on two ultraviolet absorption methods for vanillin and one paper chromatographic method for vanillin and ethyl vanillin. The official, first action screening method for vanillin was compared with another ultraviolet absorption method which determines *p*-hydroxybenzaldehyde. Results were more accurate by the ultraviolet absorption method than by the official method, when the estimation of *p*-hydroxybenzaldehyde was eliminated. The method is recommended for adoption as official, first action. Improvements have been incorporated into the paper chromatographic method to prevent tailing of spots, and the revised method is recommended for adoption as official, first action.

The official, first action AOAC method for the determination of vanillin (1) is a rapid sorting procedure based on the absorption of ultraviolet light in alkaline solution. Another method, also based on alkaline absorption in the ultraviolet, has been published (2) and is claimed to show better accuracy. A paper chromatographic method for the separation and determination of both vanillin and ethyl vanillin has also been proposed (3).

This collaborative study was undertaken to establish the value and reproducibility of these methods. The proposed ultraviolet absorption method (2) has also been used to determine the amount of *p*-hydroxybenzaldehyde naturally present in vanilla extract, and this feature of the method would be valuable for detecting vanilla extract in flavoring material.

Five samples were prepared in this laboratory and submitted to collaborators: Sample A was an authentic Bourbon vanilla extract; Sample B was the same extract with

This report of the Associate Referee was presented at the Seventy-ninth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 11–14, 1965, at Washington, D.C.

0.20 g ethyl vanillin added to each 100 ml; Sample C was a commercial 10-fold vanilla concentrate diluted to one-fold strength; Sample D was an authentic Tahiti extract; and Sample E was an imitation flavor made from water, alcohol, sugar, caramel, and 0.30 g vanillin plus 0.10 g ethyl vanillin in each 100 ml. Each collaborator was also furnished small amounts of pure standards made by repeated recrystallizations of vanillin, ethyl vanillin, and *p*-hydroxybenzaldehyde. Subsequent work by this laboratory and by several of the collaborators showed that identical results were obtained by using standard curves prepared with commercially pure reagents.

Results and Discussion

Ultraviolet Light Absorption Methods

Collaborative results for *p*-hydroxybenzaldehyde by the proposed method (2) were extremely variable and unreliable. Several collaborators reported the presence of *p*-hydroxybenzaldehyde in Sample E, which contains no vanilla extract. Many results showed none of this compound in the vanilla extract samples. The measurement of the *p*-hydroxybenzaldehyde depends on its absorption at 336 mμ in alkaline medium. This measurement is very near the maximum absorption of vanillin and ethyl vanillin (348 mμ) so that extreme care is necessary to obtain correct values. Very slight differences in absorption will cause large changes in the calculated values for *p*-hydroxybenzaldehyde. It is obvious that this part of the proposed method is too sensitive for routine use, and therefore it has been dropped from the method. Since the collaborative results for vanillin by this method were corrected for the apparent *p*-hydroxybenzaldehyde content (by means of equations), these results were recalculated to show the values obtained by using only the readings at 348 mμ. The proposed method now becomes quite simple, since elaborate calculations and standards for the *p*-hydroxybenzaldehyde are not necessary. The vanillin content reported now includes the *p*-hydroxybenzaldehyde, as in the AOAC method (1). Table 1 lists the results obtained by both methods. In these methods, vanillin and ethyl vanillin are not separated; thus the total result includes the ethyl vanillin, calculated as vanillin. Theoretically, the ethyl vanillin in Sample B is equivalent to 0.17 g vanillin in 100 ml, and in Sample E to 0.09 g vanillin.

Both ultraviolet light absorption methods are quite rapid and show about the same type of reproducibility and recovery of added vanillin. However, the official method consistently shows lower vanillin values for vanilla extracts, although the differences are not significantly great. In the official method,

Table 1. Comparison between AOAC and modified ultraviolet absorption methods for vanillin; g vanillin/100 ml

	Official AOAC Method					Proposed Modified Method				
Coll.	Sample A	Sample B	Sample C	Sample D	Sample E	Sample A	Sample B	Sample C	Sample D	Sample E
1	0.16	0.34	0.24	0.06	0.37	0.20	0.36	0.22	0.08	0.39
2	0.17	0.33	0.22	0.07	0.41					
3	0.16	0.34	0.20	0.04	0.41	0.19	0.36	0.23	0.08	0.38
4	0.16	0.33	0.21	0.06	0.39	0.19	0.38	0.25	0.08	0.40
5	0.17	0.36	0.21	0.05	0.39	0.20	0.40	0.25	0.08	0.39
6	0.16	0.35	0.22	0.06	0.42	0.19	0.38	0.22	0.08	0.40
7	0.17	0.34	0.22	0.05	0.38	0.20	0.38	0.24	0.08	0.39
8	0.17	0.35	0.21	0.06	0.39	0.19	0.39	0.24	0.08	0.40
9	0.16	0.34	0.20	0.06	0.38	0.19	0.37	0.23	0.08	0.38
10	0.17	0.35	0.21	0.05	0.39	0.19	0.38	0.23	0.08	0.39
11	0.17	0.34	0.21	0.06	0.39	0.19	0.37	0.23	0.08	0.39
12	0.17	0.35	0.21	0.06	0.39	0.19	0.38	0.23	0.08	0.40
13	0.16	0.33	0.26	0.06	0.39	0.19	0.36	0.23	0.08	0.38
Max.	0.17	0.36	0.26	0.07	0.42	0.20	0.40	0.25	0.08	0.40
Min.	0.16	0.33	0.20	0.04	0.37	0.19	0.36	0.22	0.08	0.38
Av.	0.17	0.34	0.22	0.06	0.39	0.19	0.38	0.23	0.08	0.39

the absorption at 348 mμ, the maximum for vanillin, is corrected for background by taking readings at 270 and 380 mμ. This type of correction is valid for impurities which absorb linearly in the region examined. However, Smith (2) has shown that vanillic acid and *p*-hydroxybenzoic acid, naturally present in vanilla extracts in appreciable amounts, absorb at 270 mμ but not at 348 mμ, so that a false correction will be made. In some cases, this error can be as much as 0.01–0.02 g of vanillin equivalent. In addition, caramel can also cause false background corrections (2, 3). The proposed method of Smith uses the alkaline-neutral difference technique in which a neutral solution with all components is used as reference for the alkaline solution. Under these conditions, the only significant interfering compound is *p*-hydroxybenzaldehyde, since most of the acidic phenolic substances show no absorption at 348 mμ and such compounds as caramel show the same absorption in both neutral and alkaline states. Theoretically, the proposed method should show higher accuracy.

Modified Ultraviolet Absorption Method for Vanillin

Preparation of Standard Curve

Dissolve 0.100 g vanillin in 5 ml alcohol and dil. to 100 ml with H_2O. Transfer 15, 10, and 5 ml, resp., to 250 ml vol. flasks, dil. to vol. with H_2O, and mix (*Solns A*). Pipet 10 ml of each *Soln A* into 100 ml vol. flask, dil. to vol. with H_2O, and mix. Pipet another set of 10 ml *Solns A* into 100 ml vol. flasks, add ca 80 ml H_2O and 2 ml 0.1*N* NaOH, mix, dil. to vol. with H_2O, and mix again. Obtain absorbances of alk. solns at 348 mμ, using neutral solns as reference blanks. Plot std curve.

Determination

If sample contains >0.3 g vanillin/100 ml, dil. with 35% alcohol to below this level. Pipet 10 ml sample (or dild sample) into 100 ml vol. flask, dil. to vol. with H_2O, and mix. Pipet 2 ml dild soln into each of two 100 ml vol. flasks. Dil. one with H_2O and mix. To other flask add 80 ml H_2O and 2 ml 0.1*N* NaOH, mix, dil. to vol. with H_2O, and mix again. Det. absorbance of alk. soln at 348 mμ, using neutral soln as reference blank. Obtain vanillin content from std curve.

Paper Chromatographic Method

Most collaborators had no significant difficulty with the method and agreed that it was simple and useful. Some collaborators felt that part of the wording of the method was not clear, and they made useful suggestions for improving the technique. It was found that if the developed paper was allowed to remain for long periods before extraction of the vanillin, small losses of vanillin occurred. These could be prevented by limiting the air exposure of the developed paper to 1 hour or, if longer periods were necessary, by placing the paper in a refrigerator. Several analysts used a colorimeter that could read at 348 mμ instead of the more widely used spectrophotometers for ultraviolet light absorption measurement; results by these collaborators were about the same as those made with the more elaborate instruments (Table 2).

Several collaborators complained about the tailing of the spots and, in a few cases, about inadequate separation of the vanillin and ethyl vanillin. The solvent system used in this method was restudied systematically, and it was established that the methanol component was the important portion of the mixed solvent. When this alcohol was replaced by other alcohols, no separations could be obtained. Separations were poor and tailing resulted when the methanol vapor in the developing tank was high. By using water in the tank, the methanol vapor concentration was reduced and separations improved. Best separations and sharp spots were found when 100 ml water was placed in the tank and development was allowed to proceed for 2 hours, even though the solvent front usually reached the top before this period. In view of the collaborators' comments and the restudy of the solvents, the method has been slightly modified to yield better results.

Results generally show good reproducibility and accuracy; a few results that differ widely from the averages may have been caused partly by inadequate development. One analyst, who obtained rather high results for most samples, also reported the presence of ethyl vanillin traces in samples

Table 2. Determination of vanillin and ethyl vanillin by paper chromatography

Coll.	Vanillin					Ethyl Vanillin[a]	
	Sample A	Sample B	Sample C	Sample D	Sample E	Sample B[b]	Sample E[b]
1[c]	0.17	0.17	0.21	0.07	0.30	0.18	0.08
2	0.17	0.17	0.20	0.06	0.29	0.19	0.10
3[c]	0.20	0.21	0.26	0.10	0.27	0.21	0.09
4	0.19	0.19	0.24	0.07	0.32	0.21	0.09
5	0.16	0.17	0.20	0.06	0.30	0.16	0.07
6	0.16	0.15	0.22	0.06	0.28	0.18	0.10
7[c]	0.15	0.16	0.22	0.10	0.26	0.17	0.10
8[c]	0.16	0.18	0.20	0.06	0.30	0.18	0.10
9	0.16	0.19	0.21	0.09	0.29	0.16	0.08
10	0.16	0.16	0.20	0.07	0.29	0.19	0.09
11	0.17	0.17	0.22	0.06	0.29	0.20	0.10
12	0.17	0.17	0.22	0.06	0.29	0.19	0.10
13	0.17	0.17	0.21	0.07	0.30	0.19	0.10
14	0.18	0.17	0.21	0.07	0.32	0.18	0.10
Max.	0.20	0.21	0.26	0.10	0.32	0.21	0.10
Min.	0.15	0.15	0.20	0.06	0.26	0.16	0.07
Av.	0.17	0.17	0.22	0.07	0.29	0.19	0.09

[a] Samples A, C, and D contained no ethyl vanillin. One collaborator reported traces of ethyl vanillin in Samples C and D, probably caused by contamination. Other collaborators reported none present.
[b] Sample B contained 0.20 g ethyl vanillin. Sample E contained 0.30 g vanillin and 0.10 g ethyl vanillin.
[c] These collaborators used a Spectronic 20 instrument. All others used ultraviolet spectrophotometers.

not containing this compound, indicating probable contamination of the pipet. In most cases, the results for samples with known amounts of vanillin and ethyl vanillin were slightly low but were within experimental error found in the original study (3).

Paper Chromatographic Method for Vanillin

Reagents and Apparatus

(a) *Mobile solvent.*—Cyclohexane (practical):ethyl acetate:MeOH—100:30:20.

(b) *Immobile solvent.* — 10% dimethylformamide in ether.

(c) *Sodium carbonate soln.*—Dissolve 4 g Na_2CO_3 in H_2O and dil. to 1 L.

(d) *Chromatographic paper.*—Whatman No. 3 MM, 8 × 8″.

(e) *Chromatographic tank.*—Mitchell tank and equipment, **24.093**.

(f) *Spotting pipet.*—10 μl.

(g) *Long wave ultraviolet light.*

Preparation of Standard Curve

Prep. solns of vanillin and Et vanillin in 35% alcohol, contg 0.10, 0.15, 0.20, 0.30, and 0.40 g/100 ml. Draw parallel lines on chromatographic paper 1″ and 1½″ above bottom edge, using hard pencil. Apply one 10 μl spot of each soln on the 1″ line, keeping spots 1″ apart and starting 2″ from left side of paper. Use sep. papers for vanillin and Et vanillin curves. Use same micropipet for all spottings, rinsing thoroly before each application. Let spots air-dry, without heat. Handle paper carefully near edges to avoid high blanks.

Meanwhile place 100 ml H_2O in bottom of chromatographic tank contg one trough. Fill trough with mobile solvent, (a), cover tank, and seal. Let stand 15 min. Dip paper into immobile solvent, (b), from top down to 1½″ line, leaving bottom 1½″ of paper free from immobile solvent. Do not permit solvent to reach spots. (Dipping can readily be done by use of shallow pan contg solvent.) Air-dry paper few min., remove seal from tank, and place paper in tank with bottom edge dipping into mobile solvent. Reseal tank and develop 2 hr, even tho solvent front reaches top before end of this period. Remove paper and air-dry. Do not expose developed paper to air > 1 hr. If delay is necessary, place paper in jar and store in refrigerator.

Expose paper to NH_3 fumes for few min. (This can be done by placing paper in wide-mouth half-gallon jar contg small beaker with NH_3 on bottom, and capping jar.) Examine paper under long wave ultraviolet light and outline dark blue areas with soft pencil. Et vanillin will show higher R_f value than vanillin. Remove marked areas with scissors and cut each into smaller pieces before placing them in 50 ml erlenmeyers. Cut out 2 blanks from side of paper, each approx. equal in area to developed spots. Use side area for these

blanks, away from spotted areas and their developed rise.

Pipet 10 ml Na_2CO_3 soln, (c), into each flask, swirl, and let stand 10–15 min., with frequent swirling. Centrifuge or filter thru rapid paper, discarding first portion of filtrate. Det. absorbance at 348 mμ, using Na_2CO_3 soln as reference. Also obtain av. absorbance of the 2 blanks and correct std absorbances before plotting std curve.

Determination

If sample contains > 0.4 g vanillin/100 ml, dil. below this level with 35% alcohol. Make one 10 μl spotting on the 1″ line with same micropipet used to prep. std curves. Proceed as above and det. vanillin and Et vanillin by comparison with appropriate std curves.

Recommendations

The proposed modified ultraviolet absorption method is as rapid as the present official method and shows about the same precision.

It is recommended—

(1) That the modified absorption method for vanillin described in this paper be adopted as official, first action.

(2) That the modified paper chromatographic method for determination of vanillin and ethyl vanillin, when occurring together, be adopted as official, first action.

(3) That further collaborative studies be made on the modified ultraviolet absorption method for vanillin.

(4) That the modified paper chromatographic method described in this report be expanded to determine coumarin, and that it be further studied collaboratively.

The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee D, and were accepted by the Association. See *This Journal*, **49**, 172–175 (1966).

Acknowledgments

The Associate Referee wishes to express his appreciation to the collaborators for their participation in this study and for their useful comments and suggestions.

The following members of the Food and Drug Administration served as collaborators:

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The following members of other government agencies also participated: F. J. Feeny, Internal Revenue Service, Washington, D.C. and D. M. Smith, Food and Drug Directorate, Ottawa, Canada.

The following members of industrial concerns participated:

K. Schoen, David Michael & Co., Inc., Philadelphia, Pa.

J. D. Ingle, Food Materials Corp., Chicago, Ill.

R. M. Way, Crescent Mfg. Co., Seattle, Wash.

A. Filandro, Virginia Dare Extract Co., Inc., Brooklyn, N.Y.

J. L. Laughlin, Foote & Jenks, Inc., Jackson, Mich.

C. Wayo, Gerber Products Co., Fremont, Mich.

E. J. Merwin, Givaudan Corp., New York, N.Y.

W. H. Stahl, McCormick & Co., Inc., Baltimore, Md.

References

(1) "Changes in Methods," *This Journal*, **47**, 183 (1964); **48**, 213 (1965).

(2) Smith, D. M., *ibid.*, **48**, 509–514 (1965).

(3) Fitelson, J., *ibid.*, **47**, 1161–1165 (1964).

Sorting Procedure for the Determination of Vanillin in Vanilla Extracts by Ultraviolet Absorption

By FRANK J. FEENY (Alcohol and Tobacco Tax Division Laboratory, Internal Revenue Service, Washington, D.C. 20224)

Further collaborative study was conducted on the ultraviolet absorption method for vanillin, 19.008. Results indicated that the method should be adopted as official, final action as a sorting procedure for vanillin.

Five samples, described below, were submitted to eight collaborators in seven laboratories for determination of the vanillin content by the official, first action ultraviolet absorption method, **19.008**.

Sample A: 100% Bourbon (1X vanilla)

Sample B: 100% Tahiti (1X vanilla)

Sample C: 80% Bourbon, 20% Java (1X vanilla)

Sample D: Reconstituted from commercial Bourbon (2X)

Sample E: Commercial (2X vanilla)

Table 1 shows the results of the collaborative study. Sample E was analyzed in accordance with the clarification procedure outlined in the method. Several collaborators also analyzed Sample E without clarification, and although the photometric readings were considerably higher, they found no significant difference in the calculated amount of vanillin. These results indicate that the clarification procedure is not needed for pure vanillas. However, since the isopropyl clarification procedure appeared very desirable when caramel color was present in imitation vanillas, we recommend its retention.

Each collaborator prepared his own standard vanilla solution, and with one exception the corrected absorbance for 1 μg/ml, based on the standard, was between 0.144 and 0.152 with an average of 0.148. One laboratory found a value of 0.138 for the vanilla standard; this laboratory reported difficulty in dissolving vanillin in water to prepare the standard. The method does not specify a procedure for preparing a standard, and the Associate Referee assumed that all standards would be made from an alcoholic stock solution of vanillin. Since this assumption proved not valid in at least one instance where no alcohol was used, it is recommended that the following procedure be incorporated in the sorting procedure, method **19.008**:

Table 1 Collaborative results on the ultraviolet absorption method for vanillin content of vanilla extracts

	Sample				
Coll.	A	B	C	D	E
1	0.202	0.094	0.117	0.184	0.456
2	0.209	0.099	0.119	0.188	0.474
3	0.202	0.098	0.116	0.186	0.458
4	0.197	0.100	0.116	0.188	0.473
5	0.201	0.097	0.111	0.178	0.462
6	0.201	0.098	0.120	0.186	0.469
7	0.198	0.092	0.109	0.185	0.455
8	0.20	0.10	0.11	0.18	0.45
Max.	0.209	0.100	0.120	0.188	0.474
Min.	0.197	0.092	0.109	0.178	0.450
Av.	0.201	0.097	0.114	0.184	0.462
Std dev.	0.007	0.007	0.011	0.009	0.026

METHOD

Prep. std vanillin soln by dissolving 0.1000 g vanillin in 3 ml alcohol in 100 ml vol. flask, and dil. to mark with H_2O (1 ml = 1 mg). Pipet 3 ml into 100 ml vol. flask, add 2 ml 0.1*N* NaOH, and dil. to vol. with H_2O. Det. absorbance at 270, 348, and 380 mμ against H_2O contg 2 ml 0.1*N* NaOH dild to 100 ml with H_2O. Calc. corrected absorbance as $A_{348}(0.29A_{270} + 0.71A_{380})$, where A_{348}, A_{270}, and A_{380} are observed absorbances at these wavelengths. Divide this value by 3 to obtain corrected absorbance of 1 ppm vanillin.

Recommendation

Since no difficulties were reported for the method, it is recommended that the ultraviolet absorption method be adopted as official, final action as a sorting procedure.

This report of the Associate Referee was presented at the Seventy-ninth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 11–14, 1965, at Washington, D.C.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee D, and was adopted by the Association. See *This Journal*, **49**, 172–175 (1966).

EXTRANEOUS MATERIALS IN FOODS AND DRUGS

Dilution Method for Mold Count of Catsup

By JOHN D. WILDMAN (Division of Microbiology, Food and Drug Administration, Washington, D.C. 20204)

The method of diluting catsup with an equal volume of stabilizer solution previous to the application of the official method for mold count has been tried by twelve different analysts in eleven District laboratories of the Food and Drug Administration. The analysts were in general agreement that the dilution method was easier and more accurate than the official method. The method, with the exception of the method of preparation of stabilizer solution, is recommended for adoption as official, first action.

At the 1963 AOAC Meeting, Troy (*This Journal*, **47**, 529–530 (1964)) reported on work in which catsup was diluted with a stabilizer solution prior to application of the official method for mold, **36.060**. In 1964, Wildman and Troy (*ibid.*, **48**, 549–551 (1965)) reported differences between the dilution method and the official method by means of a theoretical dilution curve calculated from the first member, e^{-m}, of the Poisson equation. The study showed that counting was easier on the diluted sample than on the undiluted sample, and, from theoretical considerations, that some mold fragments in the undiluted specimen were hidden by the insoluble solids of the tomato, causing counts on the undiluted sample to be lower than counts calculated from the theoretical dilution curve.

This year's study was conducted to evaluate the dilution method when applied to market samples of catsup. Twelve analysts in eleven Food and Drug Administration laboratories collaborated in the study. Counts were made on 46 different samples of catsup obtained locally and representative of commercial brands in the respective areas. Although two analysts examined more than 100 fields each per sample, in general, each count was based on the examination of 100 fields from each sample. One analyst made three 100-field counts on each of four samples, and in another instance, two analysts made separate 100-field counts on each of four samples. In both cases each 100-field count was entered in the record. Table 1 gives a total of 59 such counts. Tables 2 and 3 show average counts and a comparison of actual and theoretical results, respectively.

Table 1. Mold counts on catsup samples examined before and after dilution

Lab.	Analyst	Mold Count: Before Dilution	Mold Count: After Dilution	Sample No.	Count No.
1	1	19	20	1	1
		20	19	2	2
		14	14	3	3
		22	21	4	4
2	2	11.5	10	5	5
		15.0	13.5	6	6
		7.0	6.5	7	7
		10.5	11.8	8	8
		13.0	10.0	9	9
3	3	24.0	16.0	10	10
		20.0	22.0	11	11
		15.0	12.0	12	12
		14.0	12.0	13	13
4	4	6	12	14	14
		15	20	15	15
		15	11	16	16
		1	3	17	17
		25	23	18	18
4	5	8	14	14	19
		14	15	15	20
		11	13	16	21
		2	2	17	22
		26	16	18	23
5	6	37	34	19	24
		32	28	20	25
		37	32	21	26
		39	37	22	27
6	7	3	2	23	28
		9	6	24	29
		15	7	25	30
		13	9	26	31
7	8	12	9	27	32
		30	17	28	33
		36	30	29	34
		38	15	30	35

(*Continued*)

Table 1. (*Continued*)

Lab.	Analyst	Mold Count: Before Dilution	Mold Count: After Dilution	Sample No.	Count No.
8	9	35	19	31	36
		30	22	31	37
		25	18	31	38
		25	18	32	39
		23	17	32	40
		31	16	32	41
		37	25	33	42
		28	23	33	43
		26	19	33	44
		19	17	34	45
		36	24	34	46
		30	20	34	47
9	10	24	29	35	48
		30	24	36	49
		11	11	37	50
		16	10	38	51
10	11	24	20	39	52
		17.5	19	40	53
		7	12	41	54
		11	6	42	55
11	12	31	26	43	56
		14	11	44	57
		16	10	45	58
		32	17	46	59

Table 2. Averages of counts segregated on basis of magnitude of count on undiluted sample

Range of Counts, %	Av. Count on Undiluted Sample, %	Av. Count on Diluted Sample, %
0–4.99	2.0	2.3
5.0–9.99	7.4	10.1
10.0–14.99	12.4	11.0
15.0–19.99	16.3	14.0
20.0–24.99	22.4	20.6
25.0–29.99	25.7	19.5
30.0–34.99	30.8	21.3
35.0–39.99	36.9	27.0

Discussion

Results of the present study show that counts on the undiluted samples are lower than would be expected on the basis of the dilution curve derived from the Poisson expansion. The consistently lower average counts on the undiluted samples are probably caused by the analysts' failure to see all of the mold present on such samples. This possibility in itself is a reason for adopting the dilution method. The analysts were in general agreement that the dilution method was easier to use than the official method. They also agreed that the methods of stabilizer solution preparation described in method **36.060** should be retained.

Table 3. Relation of average mold count on undiluted sample and a predicted count based on count on diluted sample

Av. Count on Diluted Sample, %	Predicted Mold Count, %	Differences Between Predicted Count and Actual Count on Undiluted Sample, %
2.3	4.75	−2.75
10.1	20.5	−13.1
11.0	21.25	−8.85
14.0	26.8	−10.5
20.6	37.5	−15.1
19.5	35.6	−9.9
21.3	38.2	−7.4
27.0	46.7	−9.8

Recommendation

It is recommended that the dilution method be adopted as official, first action, except the procedures for preparation of stabilizer solution and for centrifuged sediment volume.

Acknowledgments

The writer is indebted to the following members of the Food and Drug Administration for their participation in the counting work described above: Anthony W. Daly, San Francisco District; Mathew L. Dow, St. Louis District; Martha J. Hall, Dallas District; Joyce G. Hundley, Baltimore District; Joseph L. Leone, Buffalo District; Evelyn M. Osman, Cincinnati District; Wallace M. Ribbron, Detroit District; J. Phyllis Skyrme, Boston District; Ted Smith, Baltimore District; Barbara M. Spirko, Philadelphia District; and Lee TerBush, Microanalytical Branch, Washington, D.C.

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Extraneous Materials in Flour

By GARLAND L. REED (Food and Drug Administration, 1009 Cherry St., Kansas City, Mo. 64106)

Collaborative results are presented comparing method 36.025(a) for extraneous materials in flour with method 36.029. Last fall method 36.029 was adopted as official, first action based on a collaborative study by the AACC. The present study was conducted to obtain additional data and to clarify the exact wording to be used in 36.029. The data, although limited, tend to show that the two methods are equal.

In 1964, Subcommittee E recommended that the acid hydrolysis method reported by the American Association of Cereal Chemists (AACC) be adopted as official, first action, and that the method be subjected to additional collaborative study.

Collaborative Study

Collaborative Samples and Instructions

Collaborative samples were prepared in the Associate Referee's laboratory and sent to seven collaborators. Each collaborator received four 50 g portions of flour: two portions contained 1 g of a well-mixed "high count" flour, and two portions contained two g of this "high count" flour, with the following instructions:

Examine two of the four weighed portions of flour (as marked) by AOAC method **36.025(a)** and the other two by the acid hydrolysis method (*This Journal*, **48**, 555–558 (1955)). Transfer as completely as possible entire contents of jar to beaker. Rinse jar and cap with portions of the first aqueous portion to be added in the method, i.e., pancreatin solution or HCl (5 + 95). Follow methods exactly as written.

Count fragments showing any of the following characteristics as of insect origin: (*1*) Shape of a whole or part of a specific insect organ or structure; (*2*) articulation point (various types); (*3*) one or more body hairs (setae); (*4*) one or more setal scars; (*5*) surface pattern characteristic of a specific insect; (*6*) one or more sutures (various types).

Return filter papers for comparison of all plates; 4 plastic petri dishes are included for this purpose.

Collaborative Results

The filter papers returned by the collaborators were examined by Alberto W. Vazquez (Division of Microbiology, Food and Drug Administration, Washington, D.C.) so that the fragment count could be placed on a common criterion. The counts reported by the collaborators differed only slightly (Table 1) from those obtained by Mr. Vazquez.

Collaborative study showed that the acid hydrolysis method, **36.025(a)**, needed clarification as follows:

Change sentence 4 to read; "Rinse beaker and rod with hot H_2O (not in excess of 50 ml), transfer rinsings to extraction vessel, and retain beaker and rod."

Change sentence 8 to read; "If excessive starchy material has separated with oil layer, hydrolyze with 100–200 ml HCl (5 + 95) before continuing."

The collaborative study did not reveal any advantage of one method over the other, once an analyst has become familiar with the technique of the procedure. Some difficulty may be encountered in either method if the analyst does not recognize certain "pitfalls", i.e., complete digestion, complete settling in extraction vessel prior to trapping off, complete separation of oil layer, and need to hydrolyze separated material before filtration. These "pitfalls" can easily be avoided by visual means as the analyst proceeds with the method.

The analyst cannot expect to find microanalytical methods written so explicitly that they may be followed blindly without thought. Food materials vary widely in composition, and if methods were to be written so that the human factor is not considered, a method would be required for each individual product. For example, in the two

Table 1. Number of insect fragments counted by collaborators

	1 g Flour		2 g Flour	
Coll.	Acid Hydrolysis	AOAC 36.025(a)	Acid Hydrolysis	AOAC 36.025(a)
1	18 + 1 RH	14	37	31 + 1 RH
2	23	37	54	29
3	14	17 + 1 RH	25	33
4	30 + 1 RH	15	34 + 1 RH	45
5	15	13	34	28
6	21	18	37 + 2 RH	17
7	17	20	39	33
Av.	19.7	19.1	37.1	30.9

methods described, the analyst must consider minor modification in digestion time, settling time, etc., depending on the grade and type of flour under study.

Data in Table 1 were submitted to Jacob N. Eisen (Statistician, Bureau of Scientific Research, Food and Drug Administration, Washington, D.C.) to answer three specific questions:

(*1*) Do the data support the conclusion that the two methods give comparable results?

Yes. Data show that both methods give comparable results. The difference between means for higher count flour is not significant since it is small compared to ranges of 25–54 by acid hydrolysis and ranges of 17–45 by AOAC method **36.025(a)**.

(*2*) Is there any indication that one method gives more uniform results than the other?

No. Variability is smaller for the higher count flour by both methods with standard deviations of 8.71 by acid hydrolysis and 8.30 by AOAC method **36.025(a)**. There is no significant difference in variability for the lower count flour, although the standard deviations were 5.53 by acid hydrolysis and 8.23 by AOAC method **36.025(a)**.

(*3*) Can any other conclusions be derived from the limited data?

There may be a trend for collaborators to obtain higher counts by the acid hydrolysis method, i.e., 4 of 7 collaborators obtained higher counts on the lower count flour and 5 of 7 obtained higher counts on the higher count flour by the acid hydrolysis method than by AOAC method **36.025(a)**. The total of 9 increases out of 14 determinations which, although not significantly different from ½, may indicate a trend with additional data.

Insect fragment counts increased from + 4 to + 31 by the acid hydrolysis method with an average increase of 17, whereas with AOAC method **36.025(a)**, the count changed from − 8 to + 30 with an average increase of 12 as the amount of "high count" flour increased. Collaborator 4 had the highest increase of 30 for the AOAC method and lowest increase of 4 for the acid hydrolysis method, indicating he is out of line with the other collaborators.

Although Mr. Eisen did not comment, results with AOAC method **36.025(a)** by Collaborators 2 and 6 are also out of line since their count decreased rather than increased as the amount of "high count" flour increased. This variability may be due to the methods. However, we cannot rule out variability in the actual number of fragments contained in such small samples, i.e., 1 or 2 g of a well-mixed flour.

Conclusions and Recommendations

Although data are limited, both methods are comparable for the isolation and quantitative estimation of extraneous materials. The variability obtained by either method may be due to variation in recovery or to variation of insect fragments in individual portions of well-mixed flour. This problem cannot be resolved because added insect fragments cannot be compared to milled fragments for a recovery study. Thus a single determination cannot be considered as representative of a lot or batch of flour since the result obtained by a single determination could be either much higher or lower than the true average from multiple determinations.

It is recommended that the acid hydrolysis method, **36.029**, be revised by changing the wording in sentences 4 and 8 as suggested in this report, and that the entire acid hydrolysis procedure, **36.028–36.029**,

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee E, and was adopted by the Association. See *This Journal*, **49**, 175–179 (1966).

so modified be adopted as official, final action.

Acknowledgments

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Improved Method for the Determination of Maggots in Canned Mushrooms

By ROBERT F. BROWN and ARTHUR C. AHO (Division of Microbiology, Food and Drug Administration, Washington, D.C. 20204)

Collaborative studies were conducted on a new sieving method, using both a nested combination of Nos. 20, 40, and 140 sieves and a crystal violet stain, for determining maggots and other arthropod filth in canned mushrooms. The method greatly improves the yield, accuracy, speed, and facility with which this determination can be accomplished. It is recommended for adoption as official, first action.

Two methods exist for the determination of maggots and other insect contamination in canned mushrooms: Microanalytical Manual Method M18F.2 (1) and AOAC method **36.077** (2). However, these methods do not give good recoveries of maggots in mushrooms. The butter stirrer in M18F.2 did not adequately separate maggots from mushroom tissue. Likewise, method **36.077**, which involves the hand-rubbing of mushrooms on a No. 8 sieve, was laborious, time-consuming, and inefficient.

Maggots can be satisfactorily separated from mushroom tissue in a Waring Blendor (Standard Model) with the new-style standard blade assembly, operated for 30–45 seconds at 3000–3500 rpm by a Powerstat, Type 2PF136. These conditions were chosen after many trials showed that they gave optimum results.

In method M18F.2, a No. 16 sieve nested in a No. 80 sieve is used to separate maggots from mushroom tissue. During this study, a large number of Cecid maggots (Family *Cecidomyiidae*) were found to pass through the No. 80 sieve. Investigation with various sizes of sieves revealed that a nested combination of Nos. 20, 40, and 140 sieves greatly improved the recovery of maggots and reduced the residue of mushroom tissue from the extraction.

Maggots were stained to make them more discernible. However, examination of large residues of mushroom tissue on the dark bolting cloths used in existing methods was found to be inefficient, laborious, and time-consuming. In the method described, both mushroom tissue and maggots are stained and the mushroom tissue is subsequently destained. The bleaching agent, a commercial hypochlorite preparation, destains the mushroom tissue almost completely, while the maggots and other forms of arthropod filth remain vividly stained. Adult insect cuticle does not stain readily but is easily recognized because its dark color is not readily bleached. Crystal violet was the best stain. Because maggots are stained and easily discernible and the amount of mushroom residue is reduced, ruled filter

Table 1. Results of first collaborative test of new proposed method for the determination of maggots in canned mushrooms; number of maggots/100 g[a]

Coll.	Samples 1	2	3	4	5	6	7	8	9	10	11	Av.
						Proposed Method						
1	11	37	91	4	39	21						34
2	45	43	9	65	41	96						49.8
3	8.5	17.5	6	17.5	39.5	42						21.8
4	28	14	14	8	6	0						11.7
5	32	34	52	44	29	25	9	25	22	34	13	29
						Method M18F.2						
2	9.4	11.7	11.1	1.9	8.0	7.7						8.3
5	5	21[b]	22[c]	35[c]	36[c]							23.8

[a] 100 g drained wt of canned mushrooms plus maggots from proportionate amount of drained liquid.
[b] Sample used was Can Code: 0723.
[c] Sample used was Can Code: 0723; also No. 100 sieve substituted for No. 80.

paper (9 cm, No. 8) can be used instead of the dark bolting cloth.

METHODS

Reagents and Apparatus

(a) *Crystal violet.*—Satd aq. soln.

(b) *Sodium hypochlorite soln.*—Commercial product contg ca 5.25%.

(c) *Aerator.*—*See* **36.007(c)**.

(d) *High speed blender.*—To measure speed, attach 1-hole No. 8 rubber stopper to square rotor shaft and insert tachometer. Det. variable transformer setting for 3000–3500 rpm. (Attachment of blender jar does not alter speed significantly.)

(e) *Variable transformer.*—Output voltage 0–140; max. amperes 7.5.

Determination

Determine wt of contents of can. Drain mushrooms 2 min. on No. 20 sieve. For containers of <3 lb net wt use 8″ sieve; for larger containers use 12″ sieve. Measure vol. of liquid and subtract (allowing 1 g per ml) from wt of contents of can to get drained wt of mushrooms.

Place 100 g drained mushrooms into blender. Mix drained liquid well and remove proportionate quantity while agitating liquid. Add liquid and ca 300 ml H_2O to blender. Operate blender 30–45 sec. at 3000–3500 rpm. Attain proper speed quickly by boosting setting to 1.5–2 times the desired setting on variable transformer for few sec. at start. Fragments of mushroom after blending should not be >3–5 mm in length. Pour mixt. into a nested set of 8″ Nos. 20, 40, and 140 sieves. Rinse tissue 2–3 min. with spray of tap H_2O from aerator. *See* **36.007(c)**. Discard material on No. 20 sieve. Transfer residue from No. 40 sieve to 600 ml beaker with H_2O and bring total vol. to ca 100 ml. Add 5 ml crystal violet soln and heat just to boiling. Pour stained mixt. into the No. 40 sieve. Wash mushroom tissue and maggots, if any, to edge of sieve and remove excess stain with tap H_2O from aerator. Using wash bottle contg NaOCl soln and gentle spray of tap H_2O from aerator, alternately spray tissue with H_2O and NaOCl soln until stain has been removed from mushroom tissue. Wash tissue into 600 ml beaker and transfer to ruled paper with suction. Avoid obscuring maggots with mushroom tissue. Not more than 2–3 papers should be necessary.

Transfer residue from No. 140 sieve to 600 ml beaker with H_2O and repeat staining, bleaching, and filtering as above.

Examine papers for maggots and other extraneous materials at 10–20×. Maggots are stained dark violet.

Results

Collaborative Study

Two series of collaborative tests were made on the described method. The first series on six cans of mushrooms was primarily for preliminary evaluation of the procedure. Earlier results on the same sample by method M18F.2 were compared with those by the described procedure (Table 1).

The second series provided direct collaborative comparison between method M18F.2 and the described method from cans of the

same code and from the same can (Table 2). Each collaborator was sent three 16 oz cans: the first 100 g of each can was analyzed by the proposed method; 100 g of the remaining mushrooms were analyzed by method M18F.2.

Comments of Collaborators

Collaborative data indicate a substantially greater recovery of Cecid maggots by the described method than by method M18F.2.

Staining and bleaching problems experienced during the first collaborative test were largely overcome during the second test. During the first test one collaborator obtained only partially stained or unstained maggots.

Proper operation of the Waring Blendor was somewhat difficult to achieve. During the first test, one collaborator found that his Waring Blendor and rheostat did not grind mushroom tissue to the proper dimensions when run at the specified speed. Although the operating speed and duration of grinding were determined after considerable experimentation, these specifications proved optimal for one specific machine. Since differences may occur among various blenders, a definite size for the ground mushroom tissue was specified in the described procedure. Some trial and error was undoubtedly necessary with different rheostats and blenders to achieve this size. An experiment to compare grinding action by two Waring Blendors revealed no significant difference in the particle dimensions.

Cut or broken maggots raised another problem. Because of the extensive comminution of the mushrooms necessary to dislodge the maggots, a few cut maggots are inevitable. However, the number of cut maggots is kept to a minimum by strict adherence to the procedure. One collaborator reported a few cut maggots in the first collaborative test; two collaborators reported fewer cut maggots after the second collaborative test.

The second collaborative test included a revised procedure in which a proportionate aliquot of the packing medium was added to 100 g of drained mushrooms. This procedure eliminated the need, inherent in the original method, for filtering the drained juice and separately counting the recovered filth. Although one collaborator commented that this would not accomplish the proper distribution of maggots, results (Table 3) indicate no significant difference between maggot counts based on an added proportional aliquot and those derived by counting the filth in the entire sample of packing medium and then calculating that proportion which corresponds to 100 g of drained mushrooms.

Table 2. Results of second collaborative test; comparison of new method with M18F.2 on the same sample

Sample	Method M18F.2	Proposed Method
	Collaborator 1	
1	2	2
2	0.4 (sciarid)	3
3	2	13
Av.	0.8	6
	Collaborator 2	
1	5	44
2	2	45
3	14	49
Av.	7	46
	Collaborator 3	
1	28	21
2	8	31
3	12.5	14.5
Av.	16.2	22.2
	Collaborator 4	
1	10	5
2	4	25
3	6	18
Av.	6.6	16
Grand total	93.9	270.5
Av.	7.8	22.5

The principal contaminant was the Cecid fly maggot. One collaborator reported possible fragmented parts of a Sciarid maggot (Family *Sciaridae*). A few mites, unidentified eggs, and insect fragments were also found.

The Cecid maggots ranged in size from 1 to 2 mm. Larger maggots were never observed in the samples used in these tests.

In the second collaborative test, mushroom tissue was examined on the No. 40

Table 3. Number of maggots calculated for the total drained liquid from a quantity of drained liquid proportionate to 100 g of the total drained wt of mushrooms compared with the no. of maggots calculated for the total drained liquid based on the remaining liquid and with the actual total count

	Samples [a]						
	MYPS-8	MYPS-9	MYPS-10	MYPS-11	MYPS-12	Total	Av.
Drained wt, g	469.5	455.5	501.5	459.0	466.5	2352.0	
Total liquid, ml	260	260	197	295	280	1292	
Liquid equiv. to 100 g drained wt, ml	55.4	57.1	39.3	64.3	60.0	276.1	
No. of maggots in ml liquid equiv. to 100 g drained wt	1	1	0	2	0	4	
No. of maggots calcd for total liquid based on no. of maggots in ml liquid equiv. to 100 g drained wt mushrooms	4.7	4.5	0	9.2	0	18.4	3.6
Remaining liquid, ml	204.6	202.9	157.7	230.7	220.0	1015.9	
No. of maggots in remaining liquid	1.5	1	2	4	6.5	15.0	
No. of maggots calcd for entire liquid based on no. of maggots in remaining liquid	1.9	1.3	2.5	5.1	8.3	19.1	3.8
Total no. of maggots in total liquid	2.5	2	2	6	6.5	19.0	3.8

[a] Boston code (can code MSW-NIKKO 4027).

sieve to provide for recovery of larger maggots. The No. 20 sieve eliminated large quantities of mushroom tissue. During the second test, very few maggots were observed in the material from the No. 40 sieve.

From the collaborator's comments, the improved effectiveness, speed, and ease of the described method mark it as a distinct improvement over the existing methods.

Discussion

The described method gives a three-fold increase in recovery of maggots compared to method M18F.2 on the same can. Maggot recovery was increased in practically every sample where this method was compared with existing methodology.

The high recovery by this method is undoubtedly due to change in sieve sizes. Data in Table 1 show that a decrease in sieve size from No. 80 to No. 100 increased the number of maggots found.

The method with the differential staining procedure also increases the accuracy of counting.

This staining procedure has improved the recognition of the maggots and has speeded the counting. Mites, springtails, and other arthropod filth are also more readily identifiable.

Sieve sizes should be adjusted for large maggots; some work has already been undertaken in this area. The size range of maggots recovered during this investigation was 10–0.1 mm.

The fate of animal hairs, rodent pellets, and other types of filth in this method is not completely known. The deleterious effect of the hypochlorite on animal hairs is a problem which requires further study.

Recommendation

It is recommended that the described method be adopted as official, first action to replace method **36.007**.

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OILS, FATS, AND WAXES

Gas Chromatography of Unsaponifiable Matter. IV. Aliphatic Alcohols, Tocopherols, and Triterpenoid Alcohols in Butter and Vegetable Oils

By JEROME EISNER,[1] JOHN L. IVERSON, and DAVID FIRESTONE (Division of Food Chemistry and Division of Food Standards and Additives, Food and Drug Administration, Washington, D.C. 20204)

The unsaponifiable matter of butterfat, castor oil, cocoa butter fat, corn, cottonseed, linseed, milo maize, peanut, rice bran, safflower, soybean, and tung oils was fractionated on a Florisil column and the third fraction (tocopherols, high molecular weight alcohols, and triterpenoid alcohols) was gas chromatographed. This fraction showed three homologous series, tentatively identified as normal, *iso-* and/or *anteiso-*, and multiple branched chain alcohols. Each sample was examined before and after hydrogenation with Adams catalyst. Gas chromatograms from most of the oils displayed characteristic patterns which could be used to identify individual oils.

An earlier paper (1) described how a number of vegetable fats and oils, when present alone, could be identified by their difference in sterol composition. This paper lists aliphatic alcohols, tocopherols, and triterpenoid alcohols present in butter and 11 vegetable oils and demonstrates the different compositions of the oils. These compounds were isolated from extracted unsaponifiable matter by Florisil column chromatography, and the individual components were detected by gas chromatographic analysis.

Capella, *et al.* (2) separated unsaponifiable matter of vegetable oils into sterols and other lipid classes on silicic acid columns. Capella (3) identified cycloartenol (a triterpenoid alcohol) in linseed oil and suggested that triterpenoid alcohols similar to cycloartenol might be present in other animal and vegetable oils. Using silicic acid and alumina thin layer chromatography, Capella, *et al.* (4) fractionated unsaponifiable matter into various classes. Triterpenoid alcohol and sterol fractions of several oils were studied by gas chromatography, and the following triterpenoid alcohols were identified: β-amyrin, cycloartenol, butyrospermol, tirucallol, and cyclolaudenol.

Ohta and Shimizu (5) established the presence of 24-methylenecycloartanol in rice bran oil. Eisner, *et al.* (6) used triterpenoid alcohols to distinguish between pressed olive oil and olive pomace oils and also suggested that the high molecular weight aliphatic alcohols present in olive oil consisted of three homologous series: (*a*) straight chain, (*b*) *iso-* and/or *anteiso-*, and (*c*) multiple branched chain. Capella (7) found that ceryl alcohol (C_{26}) was the major alcohol present in many oils, whereas the C_{24} and C_{28} homologues were present in lesser amounts.

In addition, much work has been done on the determination of tocopherols present in oils by paper chromatography, IR spectrophotometry, thin layer chromatography, gas chromatography, and chemical techniques (8–12). Tocopherol content has been used to detect adulteration of butter with vegetable oils (13).

Experimental

The method of Eisner, *et al.* (14) was followed as modified in 1963 (1). Five g samples were used and the entire unsaponifiable matter from each oil (36–136 mg) was chromatographed on a Florisil[2] column. (Note: Fraction 6 of (1) was omitted.) The third Florisil

[1] Present address: Division of New Drugs, Bureau of Medicine.

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[2] New address for supplier: Floridin Co., 2 Gateway Center, Pittsburgh, Pa. 15222.

fraction (aliphatic alcohols, tocopherols, and triterpenoid alcohols) was weighed, diluted to 90 μg per μl with $CHCl_3$, and gas chromatographed by injecting a 1.0 μl aliquot of sample.

A Barber-Colman Model 10 gas chromatograph equipped with an argon ionization detector was used with a 6′ × ¼″ i.d. glass column containing 1.5% SE-52 on silane-treated Gas Chrom P, 100–120 mesh. The gas chromatographic conditions used were as follows: Column, 210°C; cell, 269°C; flash heater, 300°C; argon flow rate, 12 psi (inlet); cell voltage, 1000 v; recorder range, 50 mv; electrometer gain, 3×10^{-8} amp.

Standards

See (6)—*Reagents* (**b**), (**c**), and (**d**). In addition, docosyl and octacosyl alcohols were obtained from the same source and stock solutions prepared as described.

Preparation of standard curves. — These were prepared as described previously (6). Figure 1 shows a typical gas chromatogram of a number of reference alcohols, tocopherols, and triterpenoid alcohols.

Nature of Samples

Twelve different types of oils collected from commercial sources by Food and Drug inspectors were used: (*1*) butter oil, (*2*) castor oil, (*3*) cocoa butter import sample from New York District, (*4*) corn, (*5*) cottonseed, (*6*) linseed, (*7*) milo maize, (*8*) peanut, (*9*) rice bran, (*10*) safflower, (*11*) soybean, and (*12*) tung. Two samples each of corn, cottonseed, milo maize, peanut, rice bran, safflower, and soybean oils and one sample each of butter, castor, linseed, and tung oils, and cocoa butter were examined individually.

Results and Discussion

Aliphatic Alcohols.—Isothermal gas chromatography of Florisil fraction 3 (alcohol fraction) indicated that three homologous series of aliphatic alcohols were present in the 12 oils: (*a*) normal aliphatic alcohols in the range of C_{23} to C_{30}, (*b*) probably *iso*- and/or *anteiso*-alcohols (i/a) in the range of C_{22} to C_{32}, and (*c*) probably multiple branched chain alcohols (br) in the range of C_{22} to C_{23}. Reference standards for the latter two series of alcohols were not available, and tentative identifications were made after examination of semilog plots of the gas chromatographic data. Similar series of alcohols were previously found in olive oil (6). The C_{25} normal aliphatic alcohol was the major odd-chain aliphatic alcohol in the 12 oils, occurring together with smaller amounts of the C_{23}, C_{27}, C_{29}, and C_{31} normal alcohols. The even-chain normal aliphatic alcohols, C_{24}, C_{26}, C_{28}, C_{30}, and C_{32}, were also present in the oils (see Figs. 1–6 for typical chromatographic patterns and Table 1 for identification of gas chromatographic peaks), but at much lower levels than the odd-chain alcohols.

Iso- and/or *anteiso*-alcohols and multiple branched chain alcohols were present as trace components. In addition, analyses before and after hydrogenation suggested that several unsaturated alcohols were present in the oils.

Tocopherols.—Tocopherols eluted in the following order: δ-tocopherol, β- and/or γ-tocopherol, and α-tocopherol. These components eluted in about 10 to 18 minutes but varied widely in peak areas among the various oils. δ-Tocopherol eluted with the C_{27} br alcohol, and one or the other component was present as a minor constituent in all oils except castor oil where none was detected. β-Tocopherol and/or γ-tocopherol was a major component in the following oils: corn, cottonseed, peanut, rice bran, soybean, and tung. Govind Rao, *et al.* (15) separated α-, β-, γ-, and δ-tocopherol from a number of vegetable oils by thin layer chromatography, estimating the content of each with the Emmerie-Engel reagent. Since these authors found no β-tocopherol in castor, cottonseed, peanut, safflower, and soybean oils, we assumed that β-tocopherol was not present in these oils. Tung oil appeared to contain a relatively large amount of γ-tocopherol (Fig. 5A). It was assumed that no β-tocopherol was present in tung oil since Shone (16) found that only α- and γ-tocopherols were present. The decrease in peak area of γ-tocopherol after hydrogenation (Fig. 5B) may have been due to autoxidation. γ-Tocopherol was the major tocopherol in soybean oil (Fig. 7A), whereas approximately equal amounts of γ- and α-tocopherols were present in cottonseed oil (Fig.

Table 1. Aliphatic alcohols, tocopherols, and triterpenoid alcohols present in oils[a]

Peak No.	Component[b]	Peak No.	Component[b]
1	C_{22}br	24	C_{29}br
2	C_{22}i/a	25	C_{29}i/a
3	C_{22}	26	C_{29}
4	C_{23}br	27	C_{30}br
5	C_{23}i/a	28	X
6	C_{23}	29	β-amyrin and/or C_{30}i/a
7	C_{24}br	29A	X
8	C_{24}i/a	30	Dihydrocycloartenol and/or β-amyrin and/or C_{30}i/a
9	C_{24}		
10	C_{25}br	30A	X
11	C_{25}i/a	31	Cycloartenol and/or C_{30}
12	C_{25}	32	C_{30}
13	C_{26}br	33[c]	X
14	C_{26}i/a	34[c]	X
15	C_{26}	35	C_{31}br
16	δ-tocopherol and/or C_{27}br	36	C_{31}i/a
17	X	37	C_{31}
18	C_{27}i/a	37A	X
19	C_{27}	38	C_{32}br
20	β- and/or γ-tocopherol and/or C_{28}br	38A[c]	X
21	C_{28}i/a	39	C_{32}i/a
22	X	40	C_{32}
23	α-tocopherol and/or C_{28}	41	C_{33}br

[a] Master lists of all peaks in the oils examined. Peaks may contain unidentified material tentatively identified on the basis of gas chromatography.

[b] Numbers refer to chain length of aliphatic alcohols (straight chain unless otherwise indicated); i/a = *iso*- and/or *anteiso*-; br = multiple branched chain; X = unidentified component.

[c] One or two of these peaks may be 24-methylenecycloartenol (unhydrogenated sample) and 24-methylenecycloartanol (hydrogenated sample).

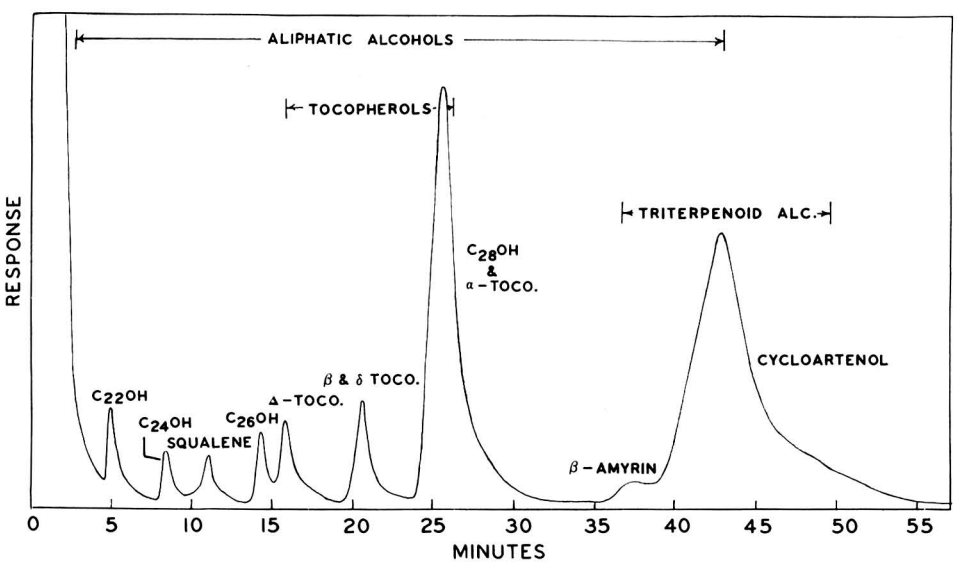

Fig. 1—**Isothermal gas chromatogram of standard alcohols, tocopherols, and triterpenoid alcohols. Column temperature, 210°C.**

7C). α-Tocopherol eluted with the C_{28} normal alcohol, and the gas chromatographic peak due to one or both of these components was a major peak in chromatograms from cottonseed oil, milo maize oil, rice bran oil, and tung oil. Although moderate levels

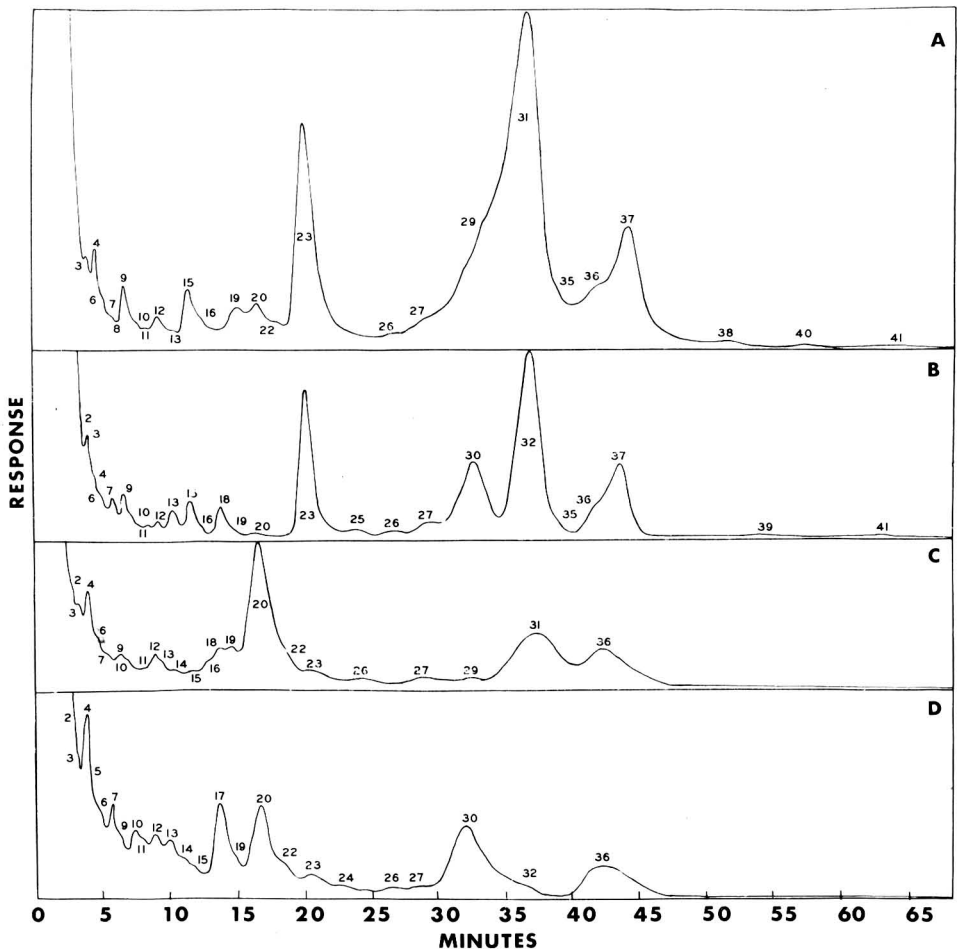

***Fig. 2*—Isothermal gas chromatograms of Florisil fraction 3: A, milo maize oil, unhydrogenated; B, milo maize oil, hydrogenated; C, corn oil, unhydrogenated; D, corn oil, hydrogenated. See Table 1 for identification of numbered peaks.**

of α- and δ-tocopherols were reported to be present in soybean oil (15), these two isomers were not definitely detected in the soybean oils examined here.

Triterpenoid Alcohols.—Triterpenoid alcohols eluted in 35 to 65 minutes. Only a few of these alcohols have been identified in the 12 oils examined. Cycloartenol has been found in linseed oil, peanut oil, and rice bran oil (3, 4, 17). Milo maize oil also contains relatively large amounts of cycloartenol (Fig. 2A). Linseed oil alcohols appear to consist predominately of cycloartenol (Fig. 6C). Capella (4) found β-amyrin in colza, olive, palm, peanut, and sesame oils. β-Amyrin and/or C_{30} i/a was present in all the oils we investigated. Ohta and Shimizu (5) isolated and identified 24-methylenecycloartanol in rice bran oil, and it has also been found in cocoa fat (18). Three unidentified components (Figs. 3A, 3B; peaks 33, 34, and 38 A) were observed in gas chromatograms of the rice bran alcohols we examined, and one or two of these peaks may be from 24-methylenecycloartanol (unhydrogenated alcohols) and methylcycloartanol (hydrogenated alcohols). Shimizu, *et al.* (19) studied the gas chromatographic behavior of a number of triterpenoid alcohols (6′ × ¼″ i.d. column packed with 1% SE-30 sili-

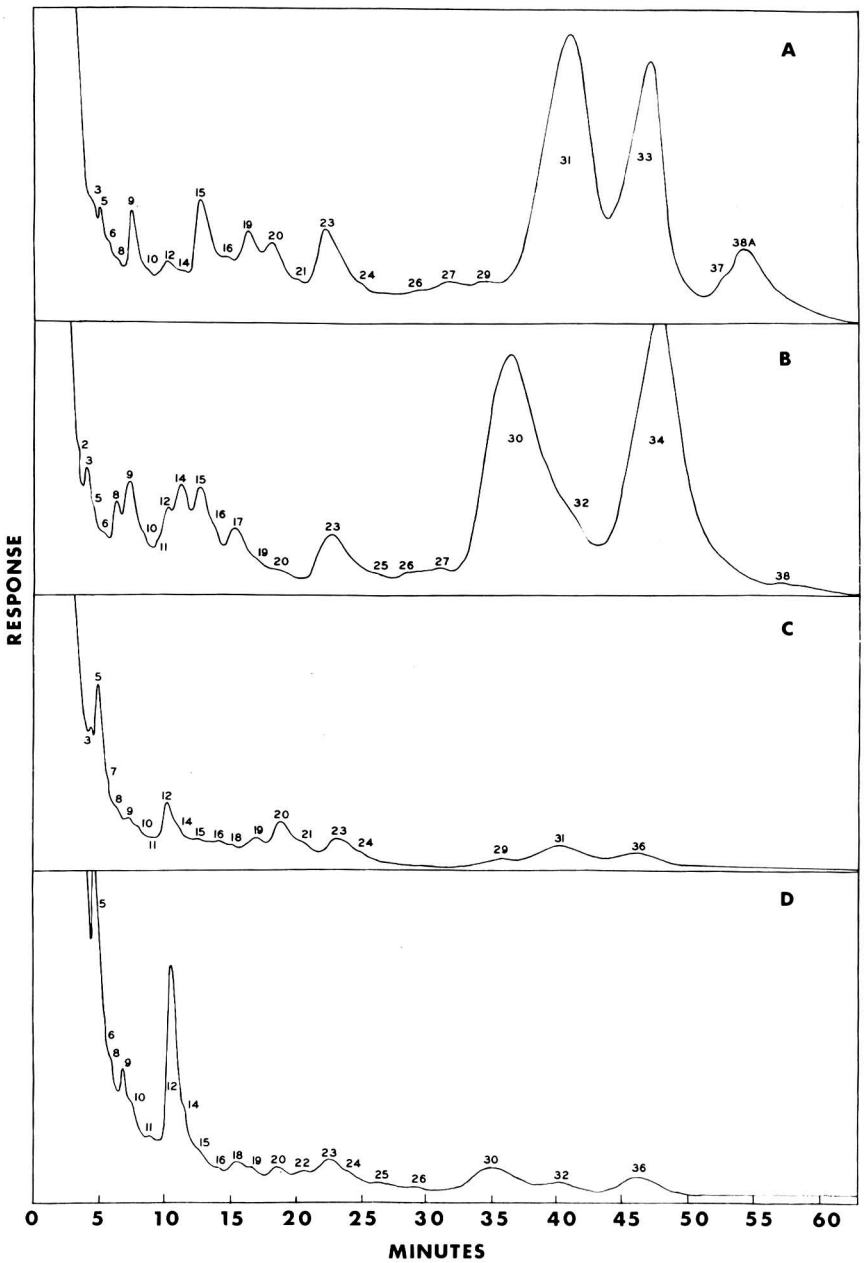

Fig. 3—**Isothermal gas chromatograms of Florisil fraction 3: A, rice bran oil, unhydrogenated; B, rice bran oil, hydrogenated; C, peanut oil, unhydrogenated; D, peanut oil, hydrogenated. See Table 1 for identification of numbered peaks.**

cone gum rubber on Anakrom ABS, 70-80 mesh; column temperature, 240°C), and observed that 24-methylenecycloartanol could not be separated from 24-methylcycloartanol, although the latter had a slightly greater retention time relative to cholestane

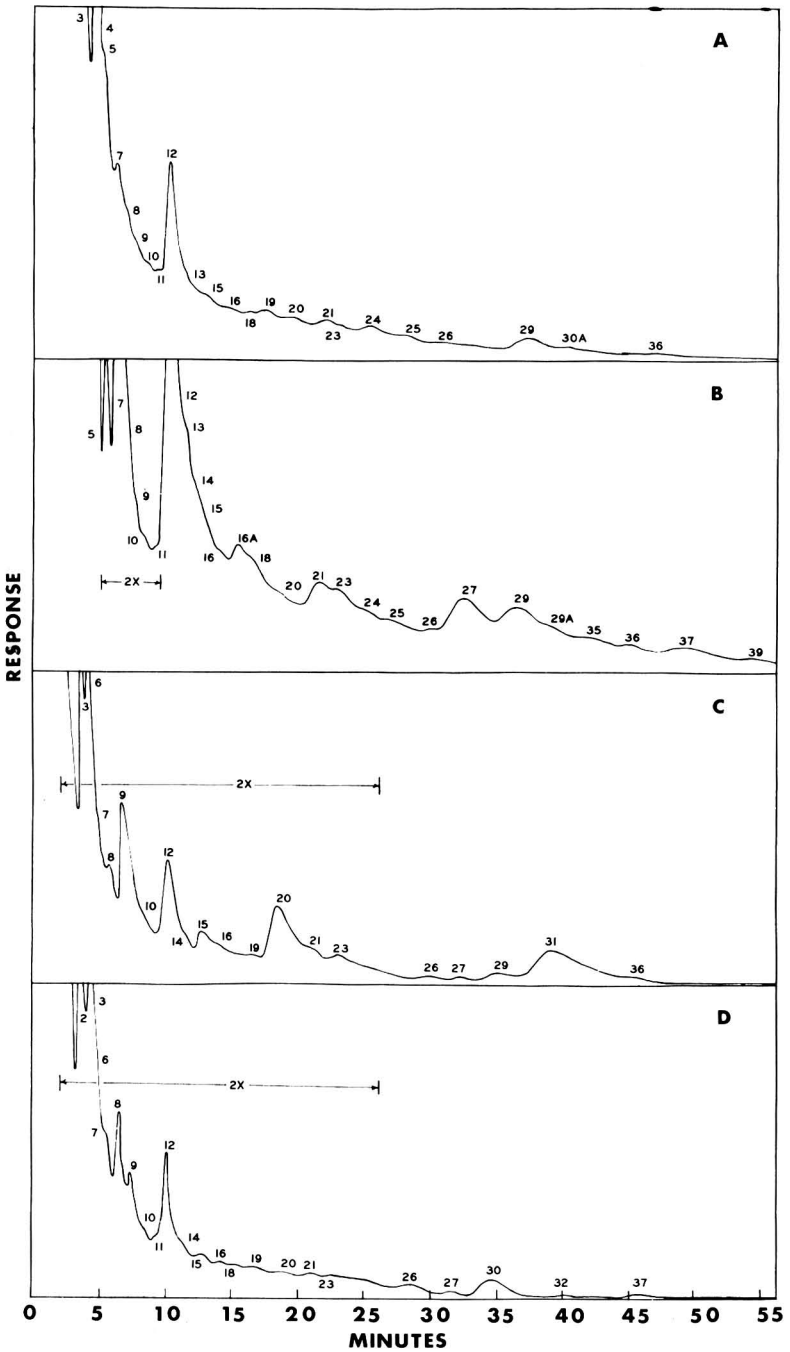

Fig. 4—**Isothermal gas chromatograms of Florisil fraction 3: A, butter oil, unhydrogenated; B, butter oil, hydrogenated; C, cocoa butter fat, unhydrogenated; D, cocoa butter fat, hydrogenated. See Table 1 for identification of numbered peaks.**

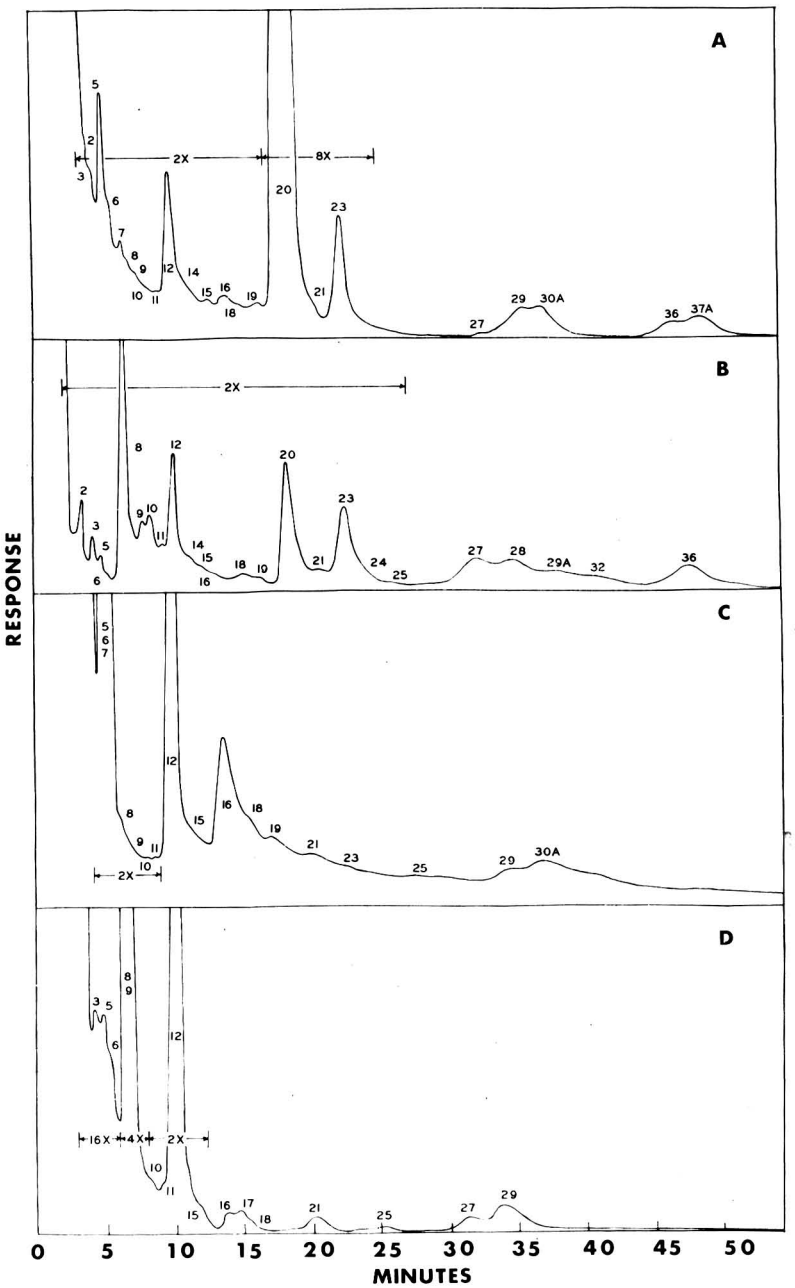

Fig. 5—**Isothermal gas chromatograms of Florisil fraction 3: A, tung oil, unhydrogenated; B, tung oil, hydrogenated; C, castor oil, unhydrogenated; D, castor oil, hydrogenated. See Table 1 for identification of numbered peaks.**

(3.56 vs. 3.53). Isolation and characterization of individual components would be necessary for absolute identification of the triterpenoid alcohols and other alcohol constituents.

Identification of Individual Oils.—The gas

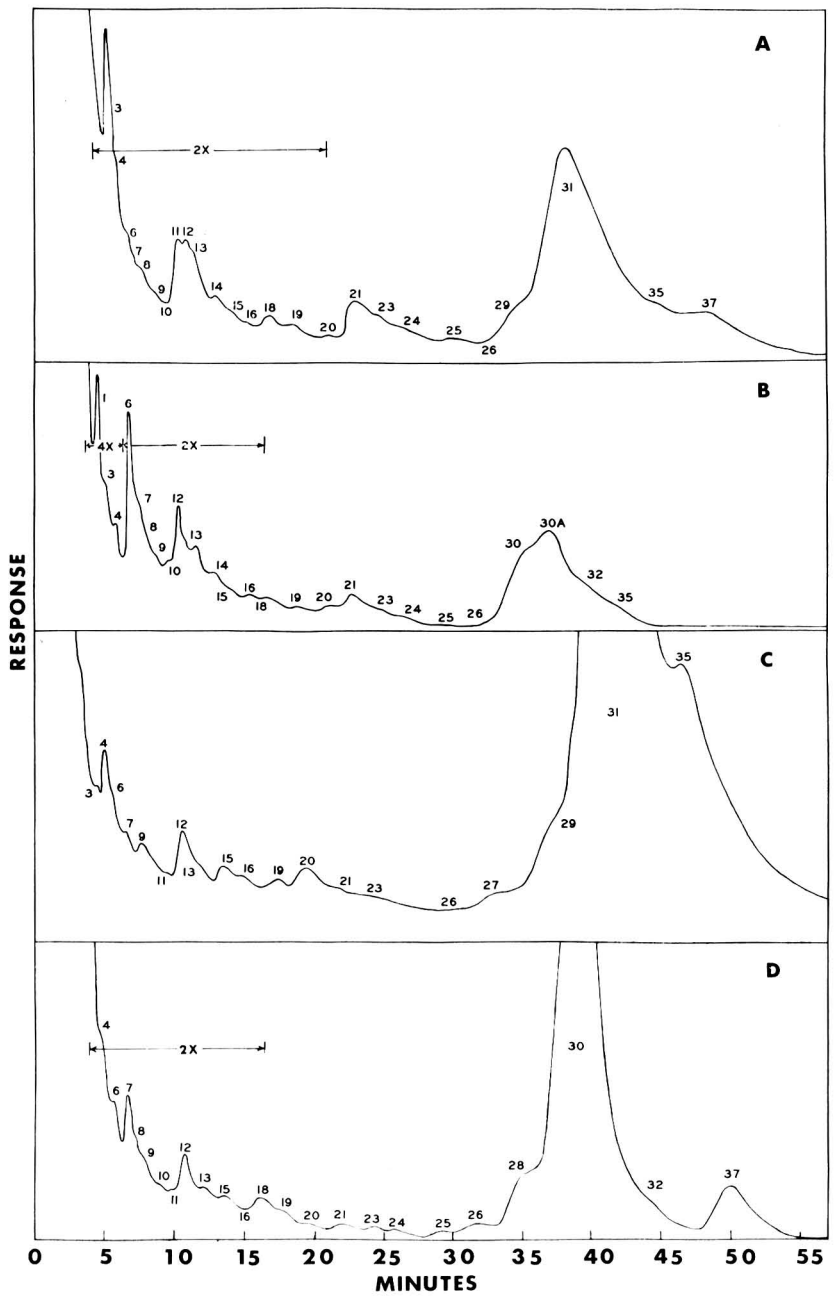

***Fig. 6*—Isothermal gas chromatograms of Florisil fraction 3: A, safflower oil, unhydrogenated; B, safflower oil, hydrogenated; C, linseed oil, unhydrogenated; D, linseed oil, hydrogenated. See Table 1 for identification of numbered peaks.**

chromatographic patterns (Figs. 2–7) of unhydrogenated and hydrogenated castor, corn, cottonseed, linseed, milo maize, peanut, rice bran, safflower, soybean, and tung oils can be used to distinguish individual oils. Although butter oil and cocoa butter did not

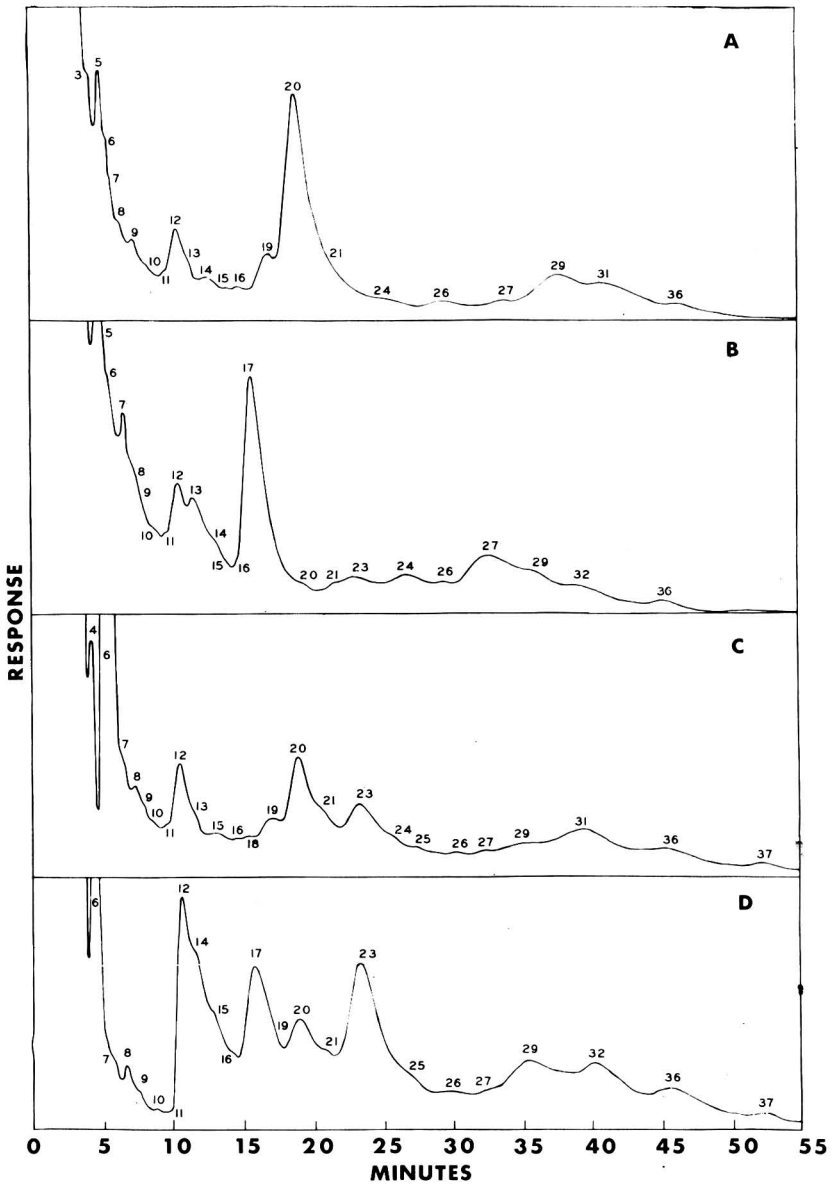

Fig. 7—**Isothermal gas chromatograms of Florisil fraction 3: A, soybean oil, unhydrogenated; B, soybean oil, hydrogenated; C, cottonseed oil, unhydrogenated; D, cottonseed oil, hydrogenated. See Table 1 for identification of numbered peaks.**

give readily identifiable patterns, the sterol composition of cocoa butter (1) was distinctive because of an unusually high level of stigmasterol, and the presence of cholesterol in butter oil readily distinguishes it from vegetable oils.

Most of the oils contained the same series of alcohols, tocopherols, and triterpenoid alcohols; however, the proportion of each component varied considerably so that gas chromatographic patterns served as a visual means of identification. Milo maize oil (Fig. 2A) had more C_{31} normal alcohol (peak 37) than the other oils and also contained rela-

Table 2. Peak areas (cm^2) of several components present in ten vegetable oils

Oil	Components[a]					
	α-Tocopherol (23)	γ-Tocopherol (20)	Cycloartenol (31)	Unidentified (38 A)	C_{31} i/a (36)	C_{31} (37)
Milo maize	2.0	0.2	5.0	—	0.6	1.5
Corn	0.1	1.6	1.1	—	0.7	—
Rice bran	1.0	0.8	4.5	3.5	—	0.3
Peanut	0.4	0.5	0.4	—	0.2	—
Tung	0.9	9.0	—	—	0.2	—
Castor	—	—	—	—	—	—
Linseed	trace	1.0	20.0	—	—	1.0
Safflower	0.1	0.1	5.5	—	—	0.4
Soybean	0.2	4.5	0.5	—	0.2	—
Cottonseed	0.7	1.0	0.6	—	0.2	0.1

[a] Numbers in parentheses are peak numbers indicated in the figs. and in Table 1; subscripts not in parentheses refer to chain length of aliphatic alcohols (straight chain unless otherwise indicated); i/a = *iso*- and/or *anteiso*-.

tively large amounts of cycloartenol (peak 31). Although the fatty acid composition of milo maize and corn oils are similar, the alcohol fractions were sufficiently different to distinguish between them. The sterol composition of each oil is also different (6).

Rice bran oil (Fig. 3A) was distinguished from the other oils by a relatively large amount of cycloartenol and two unidentified (but characteristic) components (peaks 33 and 38 A) in the alcohol fraction.

The tocopherol peaks and the triterpenoid alcohol peaks appeared to be characteristic for tung oil (Fig. 5A). We estimated that tung oil contains about ten times as much γ-tocopherol as α-tocopherol. Shone (16) found that tung oil contains four times more γ- than α-tocopherol.

The peak areas (cm^2) of several components in each of the ten oils were estimated from the gas chromatograms, and the results, shown in Table 2, illustrate differences in composition that can be used to identify individual oils.

Preliminary examination of the alcohol and sterol fractions from several unknown mixtures containing 2, 3, or 4 oils (combinations of butter, corn, cottonseed, peanut, and soybean oils) indicated that gas chromatographic analyses of these fractions were of limited value for detecting individual vegetable oils in mixtures. The presence of cholesterol (characteristic of all animal fats) indicated that butter oil was present in the mixtures.

Work is continuing on development of techniques for detecting individual oils in mixtures.

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FOOD ADDITIVES

Improved Cleanup Method for Determination of Biphenyl in Citrus Fruits and in Biphenyl-Impregnated Kraft Papers by Thin Layer Chromatography

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A simple, rapid, and efficient method has been developed for determining biphenyl in citrus fruit and in biphenyl-impregnated kraft papers. Biphenyl is separated from citrus tissue by steam distillation and from biphenyl-impregnated papers by solution in iso-octane. Plastic sheets coated with silica gel, to which a polyvinyl alcohol binder and a fluorescent indicator have been added, are used for cleanup by thin layer chromatography (TLC). The TLC spots are extracted and measured spectrophotometrically at 248 mμ. Recoveries of added biphenyl ranged from 92 to 98%. The new method is simple and brief; it is more accurate than cleanup by oxidative degradation since the variable background is practically eliminated. The new method is easier and faster than the silicic acid chromatostrip technique, and is just as accurate.

Biphenyl-impregnated kraft papers are used to reduce spoilage of citrus fruits during transport. The amount of biphenyl accumulated in the fruits during transit depends upon type of fruit, temperature, type of carton, and exposure time.

Foreign as well as domestic markets are increasingly concerned with food additives. The maximum quantities of biphenyl permissible in citrus fruits for consumption are fixed by law and vary according to country. A simple, rapid, and accurate method of determining biphenyl residues is therefore desirable.

Quantitative techniques generally require rigorous cleanup. Unless extraneous material is removed before absorbance is measured, spectrophotometric methods give high background readings. Natural citrus constituents, mainly terpene hydrocarbons, interfere by absorbing light of the same wavelength as that used to measure biphenyl. The usefulness of any residue method depends upon the efficiency of cleanup procedures.

A number of procedures for the quantitative determination of biphenyl have been described. Most methods separate biphenyl and natural citrus volatiles from citrus tissue by steam distillation.[1] In several methods the sample is cleaned up by oxidative degradation, and interfering substances are selectively removed by washing (1–4). Another cleanup method uses separation by silicic acid chromatostrips (5). Baxter (6) preferred silicic acid chromatostrip separation for routine analysis and permanganate oxidation for occasional analysis of biphenyl. Winkler (7) compared silicic acid chromatostrips, acetic acid-permanganate oxidation, and alumina column chromatography; he recommended further study of alumina column chromatography.

We have developed an improved thin layer chromatography method suitable for both routine and occasional analyses of bi-

[1] Since this paper was completed, W. J. McCarthy and J. D. Winefordner published an account of their use of ether extraction to separate biphenyl from citrus tissue (*This Journal*, **48**, 915–922 (1965)).

phenyl. The steps involved are as follows: steam distillation, cleanup with Chromagrams (plastic sheets coated with silica gel to which a polyvinyl alcohol binder and a fluorescent indicator have been added), and spectrophotometric measurement of extracted TLC spots at 248 mμ. Selected portions of methods described by Gunther, *et al.* (2) and Stanley, *et al.* (5) are combined with modifications made at this laboratory. Recoveries of added biphenyl, precision of measurement, and background interference are compared with other cleanup methods.

METHOD

Apparatus[2]

(a) *Toledo meat chopper.*—Model 5125.

(b) *Blender.* — Waring Blendor equipped with 1 L jars.

(c) *Heaters.*—Cenco giant cone, 500 watt.

(d) *Clevenger trap.*—Modified according to Gunther, *et al.* (2).

(e) *Spectrophotometer.*—Bausch and Lomb 505 is suitable.

(f) *Ultraviolet lamp.*—2537 Å.

(g) *Chromagrams.* — Poly(ethylene terephthalate) coated with silica gel to which a polyvinyl alcohol binder and lead-manganese-activated calcium silicate fluorescent indicator have been added. (Eastman K301R, 200 × 200 mm).

Reagents

(a) *Biphenyl.*—Eastman No. 721.

(b) *Silica gel.*—Davison Grade 08, 12–28 mesh. Activate 2 hr at 500°C. Use immediately after cooling in a desiccator.

(c) *Iso-octane (2,2,4-trimethylpentane).* — Phillips Petroleum, pure grade. Purify with activated silica gel as follows: Place 600 g newly activated silica gel (Reagent (b)) in a 1 L separatory funnel and cover with redistilled solvent; stopper and shake to displace all the air. Distill solvent and allow distillate to drop directly from the condenser into the separatory funnel containing activated silica gel. Adjust the solvent flow from the separatory funnel to maintain the solvent level above the silica gel. Each 600 g batch of silica gel will purify approximately 6 L solvent. Read the absorbance of the purified solvent against commercial spectral grade solvent from 220 to 300 mμ. Solvents purified by this procedure contain fewer interfering substances than commercial spectral grade solvents.

(d) *Petroleum ether.*—Reagent grade; purify with activated silica gel as described under Reagent (c).

(e) *Ethanol.*—95%, redistill before use.

(f) *Antifoam.*—Dow Corning Antifoam A.

Sampling and Sample Preparation

(A). *Biphenyl-impregnated papers.* — Biphenyl is easily removed from the kraft papers by dissolving in iso-octane. Punch 70 discs (6 mm in diameter) from representative areas of a weighed paper to determine the original biphenyl content. Weigh discs (about 0.3 g) to the nearest 0.01 g; place them in 10.0 ml iso-octane. Store at 7°C. Use these papers, with discs removed, in citrus cartons during storage.

Reweigh each paper after use in citrus cartons. Cut the entire sheet into small pieces, mix thoroughly, place 3.0 g in 25.0 ml iso-octane, and store at 7°C. Wrap the remaining pieces of the biphenyl-impregnated paper in aluminum foil and store at −20°C for future sampling.

(B). *Sampling and preparing citrus fruits.*— To obtain a representative fruit sample, raise one end of the carton and remove 8–10 vertical columns of fruit from the raised end. Biphenyl content is highest in fruit touching the biphenyl paper (8); hence the sample must include a cross-section of the carton contents. Suitable samples might consist of 40 lemons, 30 oranges, 15 grapefruit, or 50 mandarins per carton (2).

Rinse the fruit sample in tap water and drain briefly. Cut large fruits into quarters and grind. Small fruits may be ground whole. Mix ground material thoroughly and weigh two 450 g subsamples into tared blender jars. (Note: Additional subsamples may be frozen in 1 pt jars for later use.) Add 400 ml ice-cold distilled water to blender jar and blend 5 min. at high speed. Quantitatively transfer each sample to a tared 1500 ml beaker and weigh.

Determination

Steam distillation of citrus fruits.—Add three or four 2 mm carborundum chips, 2 ml concentrated H_2SO_4, and 0.5 ml antifoam to 2 L round-bottom flask. Weigh flask, stand, and large funnel. Quantitatively transfer ⅓ by weight of the thoroughly mixed diluted sample to the flask.

Fill the Clevenger trap with distilled water.

[2] Mention of specific brand names does not constitute endorsement by the U.S. Department of Agriculture and is for identification only.

Pipet 15.0 ml iso-octane on top of water. Place flask and contents on wire gauze above a heater previously brought to temperature. Affix trap and Allihn condenser. Reflux the mixture 2 hr from the time boiling begins. Drain the water from the trap and discard. Drain iso-octane layer into a 30 ml storage bottle containing 3 g anhydrous Na_2SO_4. Store at 7°C. Each ml iso-octane distillate represents 10.0 g of the parent ground-fruit sample.

Preparation of standard curve.—Prepare a standard calibration curve by spotting 10–100 μg biphenyl in iso-octane on Chromagram strips and following the procedure described under thin layer chromatography. The absorbance units for 1, 2, 3, 4, and 5 μg biphenyl are 0.112, 0.222, 0.333, 0.438, and 0.538, respectively, as shown in Fig. 1.

Check recovery of biphenyl from citrus fruits by adding 50 mg biphenyl to ground, diluted citrus tissue and carry through the steam distillation, thin layer chromatography, and spectrophotometric measurements as described.

The minimum limit of detection of biphenyl is 5 ppm in citrus fruits and 5 mg on one kraft paper.

Thin layer chromatography.—Cut unwashed 200 × 200 mm Chromagram sheets into strips 13 × 200 and 50 × 200 mm. Use 13 × 200 mm strips for biphenyl-impregnated papers, and 50 mm wide strips for biphenyl from citrus distillates.

Mark two lines 10 cm apart, activate the strips 10 min. at 85°C, and use immediately upon cooling. With a microsyringe, apply portions of iso-octane fractions in small spots on the 13 mm strips and in narrow streaks on the 50 mm strips. Allow only enough time for the iso-octane to evaporate from the spot or streak before developing, since biphenyl is lost upon standing. Spot two strips with iso-octane for background blanks. Suggested sample sizes are as follows: biphenyl-impregnated papers, 10–20 μl; citrus distillates, 30–100 μl; and blanks, 10–100 μl. Develop the strips with petroleum ether.

Remove the strips from the developing chamber one at a time, and examine under ultraviolet light (2537 Å) while still wet to avoid loss of biphenyl. For biphenyl papers, one lavender (biphenyl) spot is visible against a pink fluorescent background approximately 5 cm from the origin. Three lavender spots are visible for fruit; oxygenated hydrocarbons remain at the origin, terpene hydrocarbons (mainly limonene) move in a zone at the solvent front, and biphenyl is about 5 cm from the origin. The blank should have no lavender spots. Mark extremities of biphenyl spot or a representative area on the blank strips. Cut the biphenyl area from the strips and place in a tube (25 × 80 mm) containing 10.0 ml redistilled 95% ethanol. Stopper and let the ethanol mixture stand 15 min. with occasional shaking.

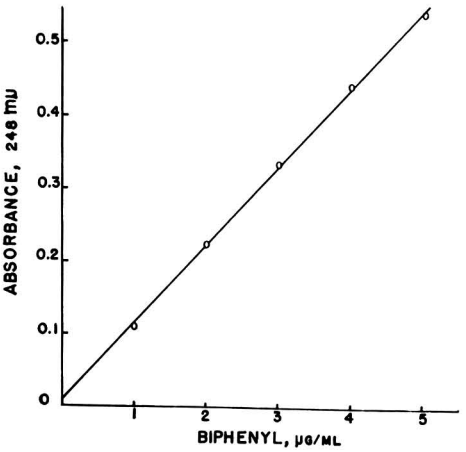

Fig. 1—**Standard curve for biphenyl ultraviolet absorbance readings taken at 248 mμ. Absorbance units for 1, 2, 3, 4, and 5 μg biphenyl are 0.112, 0.222, 0.333, 0.438, and 0.538, respectively.**

Measure the absorbance of the ethanol extract spectrophotometrically at 248 mμ. Correct absorbance of ethanol extract with the average blank absorbance. With matched 1 cm quartz cells, the blank absorbance is usually less than 0.030 units.

Calculations

ppm Biphenyl = $(C/W) \times (V_1/A) \times V_2 \times D$, where C = μg/ml biphenyl from Fig. 1; W = sample weight of fruit or biphenyl-impregnated paper; V_1 = ml iso-octane fraction; A = ml iso-octane applied to the Chromagram; V_2 = ml ethanol; and D = dilution factor.

Results and Discussion

The thin layer chromatography (TLC) method described was compared with other procedures for the determination of biphenyl. Biphenyl was steam distilled from citrus tissue into iso-octane and was separated from biphenyl-impregnated papers by dissolving in iso-octane. The iso-octane extracts were subjected to cleanup by (a)

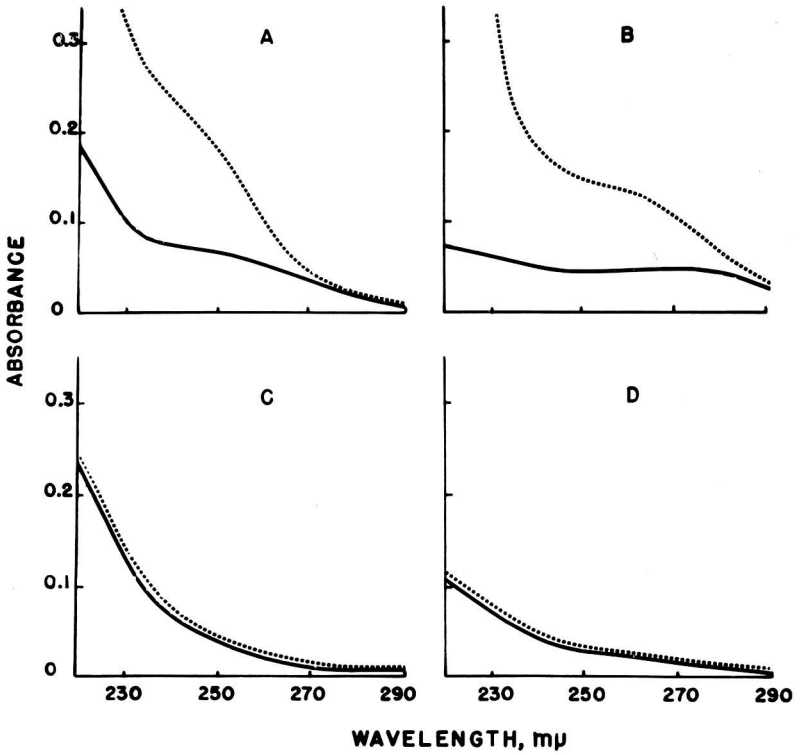

***Fig. 2*—Comparison of interfering ultraviolet-absorbing materials remaining after cleanup of orange distillate without biphenyl (dotted line) and reagent blanks (solid line) by 4 methods: A: oxidative degradation with concentrated H_2SO_4 and selective removal of interfering materials by washing, solvent iso-octane; B: similar to A but with 90% H_2SO_4; C: pre-washed silicic acid chromatostrips, solvent 95% ethanol; and D: silica gel Chromagrams not pre-washed, solvent 95% ethanol. The spectra represent distillate from 0.04 g whole fruit/ml iso-octane in 1 cm matched quartz cells.**

TLC on silica gel Chromagrams; (b) TLC on silicic acid chromatostrips (5); (c) oxidative degradation with concentrated sulfuric acid and selective removal of interfering substances by washing (2); and (d) oxidative degradation with 90% sulfuric acid and selective removal of interfering substances by washing. In all methods biphenyl was measured spectrophotometrically at 248 mμ.

Cleanup of an orange distillate without biphenyl by these four methods produced different background interference (Fig. 2). Both oxidative methods gave high background readings from citrus constituents remaining after cleanup. In both TLC methods, biphenyl was completely separated from the natural citrus constituents. Chromagram thin layer strips which were not prewashed produced less background than prewashed silicic acid chromatostrips.

Oxidation of citrus distillates with concentrated sulfuric acid reduced biphenyl recovery. Portions of a fortified citrus distillate (47.6 ppm biphenyl) were subjected to oxidative degradation (2) with concentrated sulfuric acid from 5 different bottles from the same lot of acid. Loss of biphenyl in these portions ranged from 0 to 18%. The recoveries were 82, 104, 105, 110, and 82%. Biphenyl loss through oxidative degradation with concentrated sulfuric acid also has been reported by Steyn and Rosselet (1) and Rajzman (4). Oxidative degradation of the same fortified citrus distillate with 90% sulfuric acid from the same bottles improved biphenyl recovery but did not eliminate the background interference.

Table 1. Biphenyl analyses of oranges and biphenyl-impregnated kraft papers after cleanup by 2 methods

Cleanup Method	Standards, mg Biphenyl[a]	Kraft Papers, g Biphenyl/Sheet[b]	Orange Distillates, ppm Biphenyl[b]
Oxidative degradation[c]	44.17	1.53	53.0
Silicic acid chromatostrips	43.38	1.55	51.3
t-value	0.655	1.590	−2.049[d]

[a] Average of 12 determinations.
[b] Average of 59 determinations.
[c] 90% H_2SO_4.
[d] Significant at the 5% level.

Silicic acid chromatostrip separations of 12 standards, 59 biphenyl-impregnated papers, and 59 orange distillates were compared with cleanup by oxidative degradation (Table 1). According to the t-test (9), there was no significant difference between these two methods, either for standards or for biphenyl-impregnated papers. Oxidative degradation with 90% sulfuric acid gave significantly higher results than the chromatostrip method for orange distillates; this is attributed to interfering background remaining after cleanup. Absorbance readings may be corrected for reagent background but citrus samples from the same lot with and without biphenyl are rarely available for a citrus background correction.

A citrus distillate was fortified with 47.6 ppm biphenyl (Table 2). Recoveries of biphenyl were as follows: oxidative degradation with concentrated sulfuric acid, 82%; oxidative degradation with 90% sulfuric acid, 115%; silicic acid chromatostrip separation, 94%; and silica gel Chromagram separation, 95%.

Cleanup of portions of two orange distillates by oxidative degradation with 90% sulfuric acid, by thin layer chromatostrip separation, and by Chromagram separation was compared (Table 3). Individual results, standard deviations, and 95% confidence intervals are presented to indicate precision.

The thin layer chromatographic cleanup method described is simpler and faster than any of the three other methods to which it was compared. Biphenyl recoveries with Chromagrams are equivalent to those obtained with silicic acid chromatostrips and are more accurate than those obtained with oxidative degradation by concentrated or 90% sulfuric acid. Precision of biphenyl measurement with thin layer Chromagram

Table 2. Per cent recovery of added biphenyl (47.6 ppm) from a citrus distillate by 4 cleanup methods

	Oxidative Degradation		Silicic Acid Chromatostrips	Silica Gel Chromagrams
	Concd H_2SO_4	90% H_2SO_4		
	89.7	113.7	88.7	95.4
	89.4	114.7	98.5	96.0
	58.9	115.8	101.3	92.2
	76.8	114.4	94.7	97.5
	93.6	115.3	96.2	94.7
	80.9	114.6	87.1	92.4
Av.	81.6	114.8	94.4	94.7

Table 3. Precision of biphenyl analyses of orange distillates after cleanup by 3 methods

	Biphenyl Found, ppm					
	Oxidative Degradation[a]		Silicic Acid Chromatostrips		Silica Gel Chromagrams	
	Sample		Sample		Sample	
	1	2	1	2	1	2
	70.5	16.1	53.0	10.7	57.0	10.0
	71.0	16.1	57.7	12.3	57.3	12.8
	71.5	16.9	59.0	12.3	55.5	14.5
			56.9	11.5	58.0	10.5
			56.6	9.2	56.7	10.5
			52.3	8.8	56.0	11.4
Mean	71.0	16.4	55.9	10.8	56.8	11.6
Std dev.	±0.5	±0.5	±2.7	±1.6	±0.9	±1.7
Confidence interval (95%)	±1.3	±1.0	±2.3	±1.4	±1.0	±1.8

[a] 90% H_2SO_4.

cleanup is as good as that obtained with silicic acid chromatostrips and oxidative degradation.

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Extraction and Estimation of Polycyclic Aromatic Hydrocarbons in Smoked Foods. I. General Method

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A method for the isolation and determination of polycyclic aromatic hydrocarbons in smoked foods has been developed. The compounds are extracted with ethanol, saponified with potassium hydroxide in a Soxhlet apparatus, concentrated, diluted with water, and extracted with a hydrocarbon solvent. The polycyclic hydrocarbons are isolated by partition, paper, and thin layer chromatography and measured by ultraviolet and spectrophotofluorometric procedures. Average recoveries of benzo(a)pyrene, dibenz(a,h)anthracene, benz(a)anthracene, and benzo(g,h,i)perylene, added to 500 g samples of frankfurters, cheese, and fish at levels of 2 ppb, ranged from 70 to 88%. The carcinogen, benzo(a)pyrene, was isolated from smoked fish and ham samples at levels varying from 0.8 to 3.2 ppb.

Smoke for food processing is produced commercially in the United States by three methods: by burning dampened sawdust, by burning dry sawdust, and by friction (1). The most common method currently used is burning dampened sawdust in a batch operation. Liquid smoke materials prepared by burning hardwood or hardwood sawdust are also available; however, they play a minor role in overall usage.

Preservative action, flavor, and color are the three major effects claimed to result from the smoking of food products. The phenolic compounds present in the smoke are generally considered to be of great importance in the production of the first two effects; however, the mechanisms involving surface color formation have not been adequately established.

The composition of smoke is believed to be affected by many factors including the type of wood, type of generator, moisture content of wood, temperature of combustion, and air supply (1). Additional factors which may affect the rate of deposition and composition of smoke constituents include the method of application, the wetness of the product surface, and the smoke temperature. Tilgner and Kazimierz (2) report that curing smoke should be generated at a temperature of not over 425°C to obtain good yield of smoke flavor components and to prevent formation of polycyclic hydrocarbons. However, this temperature specification is not relevant when flame or glow methods are used to generate smoke, since the carcinogenic hydrocarbons in the human environment commonly arise from exposure of

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organic materials to higher temperatures characteristic of combustion processes.

During the last ten years, interest has been increasing in the analyses of curing smoke (2–4) and smoked food products (2, 4–8) for carcinogenic polycyclic aromatic hydrocarbons. This interest has been motivated by the high incidence of carcinoma of the alimentary tract in those countries where smoked foods are consumed in large amounts (7–9). Much of the research has been conducted in Poland and Russia, and specific details of the procedures employed and recovery data are not readily available in the United States. Various investigators (2–4, 7) have reported the presence of benzo(a)pyrene in curing smoke, and Tilgner and Miler (4), in 1963, demonstrated that this compound may also be accompanied by another carcinogen, dibenz(a,h)anthracene.

In smoked foods, Dobes, *et al.* (3) found 1.9–10.5 μg and 1.7–7.5 μg of benzo(a)pyrene per kg of smoked sausage and fish, respectively. Wojtelowicz, *et al.* (7) and Gorelova, *et al.* (10) also reported the presence of this carcinogen in smoked fish, the levels varying from 1.7 to 53 μg/kg. The high amounts in the latter instance were attributed to soot deposited on the food by the use of primitive curing methods (3). In regard to the surface deposition of the smoke constituents, various investigations have shown that the polycyclic compounds will migrate into the interior of the food, the extent of migration depending upon the character of the product and its storage time (2, 6, 11). Bailey and Dungal (8) have analyzed fish and mutton smoked in Iceland, where a high incidence of stomach carcinoma occurs. In this investigation, the only strong carcinogen isolated was benzo(a)pyrene in quantities ranging from 0.3 to 2.1 μg/kg. However, other polycyclic hydrocarbons were also present at higher concentrations. These workers used a method in which the initial extraction was carried out in a Soxhlet extractor followed by a saponification step and column chromatography. The report indicates that heavy emulsions were formed after the saponification step and that centrifugation was necessary for good separations. Recoveries of benzo(a)pyrene added to 1 kg of food products varied from 50 to 56%.

At the present time there is a dearth of information in the literature regarding the presence of polycyclic hydrocarbons in foods smoked in North America with the exception of two recent publications. Genest and Smith (12), in 1964, developed a detection method for benzo(a)pyrene with a reported sensitivity of 10 to 50 ppb, depending on the food product analyzed; smoked fish, frankfurters, and cheese were analyzed, but the aforementioned hydrocarbon was not detected. In 1965, Lijinsky and Shubik (13) reported the presence of trace amounts of benzo(a)pyrene in smoked fish and of other polycyclic compounds in several liquid smoke products and bacon. However, only a general discussion of the procedure used is given, without specific details and recovery data.

The objective of this investigation was to develop a method for the detection and estimation of trace amounts of polycyclic aromatic hydrocarbons in smoked food products. In a previous undertaking (14) we were able to achieve a sensitivity of 2 ppb in the recovery of the hydrocarbons added to milk, and this was our ultimate goal in this study. This report describes such a method in which the polycyclic compounds are extracted from the food product with ethanol in a Soxhlet extractor, and the extractable fat is saponified with potassium hydroxide at the same time. After concentration of the ethanolic extract and dilution with water, the polycyclics are extracted into a hydrocarbon solvent which is filtered through a chromatographic column. A partition step between a hydrocarbon solvent and dimethyl sulfoxide is followed by column chromatography and paper or thin layer chromatography. The separated hydrocarbons are then subjected to ultraviolet and fluorescence spectral analysis.

METHOD

Apparatus

(a) *Separatory funnels.*—125, 500, 1000, and 2000 ml, with Teflon stopcocks (K-63603,

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Kontes Glass Co., Vineland, N.J., or equivalent).

(b) *Paper chromatography tanks and accessories.*—Glass, square, 60 cm deep × 30 cm wide (Scientific Glass Apparatus Co., Inc., Bloomfield, N.J., or equivalent).

(c) *Trough.*—Glass, 20 cm wide × 30 cm long × 5 cm deep.

(d) *Chromatographic paper.*—Whatman No. 1 filter paper for chromatography, or equivalent. Cut large sheets to 20 × 50 cm.

(e) *Ultraviolet equipment.* — (*1*) Lamps: longwave, 3660Å; shortwave, 2537Å; (*2*) Chromato-Vue Cabinet (Ultraviolet Products, Inc., San Gabriel, Calif., or equivalent).

(f) *Recording spectrophotometer.*—Cary 11 (Applied Physics Corp., Monrovia, Calif., or equivalent).

(g) *Spectrophotofluorometer.* — Aminco-Bowman (American Instrument Co., Inc., Silver Spring, Md., or equivalent). A 1P28 photomultiplier tube was used.

(h) *Thin layer chromatography apparatus.*—(*1*) Glass plates, 20 × 20 cm (25-10-11); (*2*) applicator, standard adjustable, model S-II (25-00-12);* (*3*) mounting board, Plexiglas, standard size for plate up to 200 mm wide (25-10-05); (*4*) drying rack, for 20 × 20 cm plates (25-10-31); (*5*) developing tank, standard, rectangular, 22 cm deep, 8.5 cm wide, 20.5 cm long (25-10-22); (*6*) desiccating storage cabinet, stainless steel, 30 cm high, 30 cm wide, and 25 cm thick (25-50-51) (Brinkmann Instruments, Inc., Westbury, N.Y., or equivalent).

(i) *Chromatographic column.*—38 mm i.d. × 230 mm length, with coarse fritted disc (K-42200, Kontes Glass Co., or equivalent).

(j) *Extraction thimbles.*—Double thickness, 90 × 200 mm (E-9790, Scientific Glass Apparatus Co., Inc., Bloomfield, N.J., or equivalent).

(k) *Soxhlet extraction apparatus.*—(*1*) Extractor (K-58600-M), size E, modified with siphon made of 5 mm i.d. tubing throughout and without constriction or bulb; (*2*) condenser, reflux, Allihn (K-45600), size E; and (*3*) flask, boiling, round bottom, 2 L capacity with 29/42 ⧩ joint and pouring lip (K-60200) (Kontes Glass Co., or equivalent).

(l) *Condenser, concentrator.* — Condenser surface height, 200 mm equipped with 24/40 ⧩ receiver joint and 29/42 ⧩ bottom joint (K-44450, Kontes Glass Co., or equivalent).

(m) *"Universal" meat chopper.*—No. 333 (Launders, Frary, and Clark Co., New Britain, Conn., or equivalent).

(n) *Evaporation flasks.*—125, 250, and 1000 ml all-glass flasks (K-61725) equipped with 24/40 ⧩ stopper (K-33175) having inlet and outlet tubes to permit passage of nitrogen across the surface of contained liquid to be evaporated. The inlet tube of the stopper used to convey the nitrogen is cut off 2 cm below the joint, and the outlet tube connected to a vacuum is constricted at the end and bent downward at a 45° angle to prevent flow-back of the condensate into the flask. (Kontes Glass Co., or equivalent.)

(o) *Pressure filter.*—30 ml capacity, fine porosity fritted disc microfunnel (K-95500), modified to include a 24/40 ⧩ outer joint and an adapter equipped with a 24/40 ⧩ inner joint (K-18300) for connection to a tank of nitrogen (Kontes Glass Co., or equivalent).

(p) *Nitrogen cylinder.*—Water-pumped or equivalent purity nitrogen in cylinder equipped with regulator and valve to control flow rate at 5 psig.

(q) *Heating mantle.*—Hemispherical, for 2 L round-bottom flask. (Use with variable transformer heat control.)

(r) *Perforated aluminum or stainless steel tube.*—Approximately 100 mm long × 20 mm diameter, perforated with about sixty 5 mm holes.

Reagents

Reagents purified by distillation were distilled with an air-cooled reflux condenser (ca 300 mm long) between the reservoir and the water-cooled condenser. The solvents were distilled in 2 L lots; the first 200 ml of distillate was discarded and the next 1600 ml collected for use.

Iso-octane, benzene, and methyl alcohol were purified to meet the specifications of the following test:

Add 1 ml of purified *n*-hexadecane to the specified quantity of solvent in a 250 ml evaporation flask, and place on the steam bath. Insert tube assembly, connect inlet tube to nitrogen supply and outlet tube to vacuum line and solvent trap, and evaporate. Stop evaporation when only 1 ml of residue remains. (To the benzene residue add 10 ml purified iso-octane, re-evaporate, and repeat to insure complete removal of benzene.) Dissolve the 1 ml hexadecane residue in iso-octane and adjust to 25 ml. Determine absorbance in the 5 cm path length cells compared to iso-octane as reference. The absorbance of the solution of the solvent residue (except for methanol) should not exceed 0.01 per cm path length between 280 and 400 mμ.

For methanol this absorbance value should not exceed 0.03 per cm path length between 250 and 275 mμ; 0.015 between 275 and 300 mμ; 0.010 between 300 and 350 mμ; and 0.00 between 350 and 400 mμ.

(a) *Iso-octane (2,2,4-trimethylpentane).* — Purify by distillation or by passage through a column of activated silica gel (Grade 12, Davison Chemical Co., Baltimore, Md., or equivalent) about 90 cm long and 5–8 cm diameter. Use 180 ml for the test described in the preceding paragraph.

(b) *Benzene.*—ACS reagent grade. Purify by distillation. Use 150 ml for the test.

(c) *n-Hexadecane.*—99%, olefin-free. Purify by percolation through a column of activated silica gel (Grade 12, Davison Chemical Co., Baltimore, Md., or equivalent). Dilute 1 ml *n*-hexadecane to 25 ml with iso-octane and determine the absorbance in a 5 cm cell compared to iso-octane as reference between 280 and 400 mμ. Absorbance shall not exceed 0.00 per cm path length in this range.

(d) *Methanol.*—ACS reagent grade. Purify as follows: Reflux 2 L alcohol with 10 g potassium hydroxide and 25 g zinc dust for 3 hr. Distill, using the air-cooled reflux condenser connected to a water-cooled condenser. Provide the collection flask with a drying tube to protect the distilled solvent from moisture. Use 50.0 ml for the test.

(e) *Ethanol.*—USP grade. Redistill before use.

(f) *N,N-Dimethylformamide.*—Redistill before use (Matheson Co., Inc., East Rutherford, N.J., or equivalent).

(g) *Ethyl ether.*—Analytical reagent (Mallinckrodt Chemical Works, St. Louis, Mo., or equivalent).

(h) *Toluene.*—ACS reagent grade. Redistill before use.

(i) *Dimethyl sulfoxide (DMSO).*—Spectrophotometric grade (Crown Zellerbach Corp., Camas, Wash., or equivalent). Absorbance in 1 cm cell; distilled water reference, sample completely saturated with nitrogen. There should be no irregularities in the absorbance curve within the following wavelengths:

Wavelength	*Absorbance (Maximum)*
261.5	1.00
270.0	0.20
275.0	0.09
280.0	0.06
300.0	0.015

(Note: Spectrograde DMSO was used in this study because it is the only standardized product available at present. Specifications for ACS reagent grade DMSO are currently under study, and this reagent may be satisfactory for use in this method. Details for the purification of DMSO were published (15).

(j) *Phosphoric acid.* — 85%, ACS reagent grade.

(k) *Acetylated linters powder.*—21% acetylated (No. 124/21 ac, Schleicher and Schuell Co., Keene, N.H., or equivalent).

(l) *Florisil.*—60–100 mesh (F-100, Fisher Scientific Co., Silver Spring, Md., or equivalent). Place 300 g Florisil in a 1 L glass-stoppered Erlenmeyer flask. Add 500 ml redistilled methanol to flask. Stopper and shake for 1 minute, removing the stopper periodically to release pressure. Transfer slurry to 600 ml fritted Büchner funnel of coarse porosity and allow the contents to drain by gravity. Wash the flask with three 35 ml portions of redistilled methanol and pass through the funnel. Wash adsorbent in the funnel with additional 100 ml redistilled methanol and allow to drain. Then, apply vacuum to remove residual methanol. Transfer treated Florisil to a tray lined with aluminum foil (free of rolling oil). Dry in a vacuum oven (with a 24–26″ vacuum) at 50°C overnight. Store the adsorbent in amber bottle.

Test the prepared adsorbent before use as follows: Pour 10 g treated Florisil into a clean 30 ml glass funnel (30 mm diameter) with coarse fritted disc. Place 15 g Na_2SO_4 on top of adsorbent and wash with 50 ml purified iso-octane. Prepare a solution of benzo(a)-pyrene in iso-octane (0.2 μg/ml). Pipet 10 ml of this solution onto the Na_2SO_4, and filter solvent through adsorbent into a 125 ml evaporation flask. Wash adsorbent with four 20 ml portions of iso-octane, allowing the column to drain between washes until the solvent no longer drips from it. Remove and retain first evaporation flask and replace with a second 125 ml evaporation flask. Elute the benzo(a)-pyrene from the column, using four 25 ml portions of redistilled benzene, again allowing the column to drain between washes. Add 1 ml hexadecane to each flask. Evaporate under stream of nitrogen on a steam bath to 1 ml hexadecane residue. Add 5 ml iso-octane to residue in second flask and re-evaporate; repeat to insure complete removal of benzene. Dilute the 1 ml residues to 15 ml with iso-octane, and measure absorbance with a recording spectrophotometer from 350 to 400 mμ, using 5 cm path length cells, with iso-octane in the reference cell. No benzo(a)pyrene

should be present in the first iso-octane eluate, and 95 to 100% of the added benzo(a)pyrene should be found in the benzene eluate as calculated from the absorbance at the 383 mμ maximum. A concentration of 1 μg/ml of benzo(a)pyrene in iso-octane gives an absorbance of 0.120/cm path length at the 383 mμ maximum.

(m) *Polycyclic aromatic hydrocarbons.* — The compounds were obtained from various sources (see *Acknowledgments*). Purity of the compounds was checked by paper and thin layer chromatography before use.

(n) *Sodium sulfate.*—ACS reagent grade, anhydrous, granular.

Procedures

Extraction and saponification.—Grind 500 g smoked food product in meat grinder. (Note: It may be necessary to reduce the sample size accordingly when the fat content exceeds 35%.) Add 500 g Na_2SO_4 and mix well. Pass mixture through grinder to assure adequate mixing. Transfer sample to extraction thimble containing 150–200 g Na_2SO_4. (To obtain complete extraction of polycyclic aromatic hydrocarbons from cheese, place the perforated tube in center of extraction thimble and press lightly into the Na_2SO_4. Pack food and Na_2SO_4 mixture around it.) Place thimble in Soxhlet extractor prepared as follows:

Arrange 5 or 6 glass stoppers in bottom of extractor with penny head of one stopper, preferably a No. 22, protruding over (without touching) the aperture of the siphon tube. Place a thick layer (8–10 g) of pyrex glass wool on top of stoppers.

Add 1 L ethanol, 50 g KOH, and inert boiling chips to 2 L reservoir. Insert the extractor containing sample in the reservoir fitting and add 400 ml more ethanol to extractor. Connect condenser and extract about 8 hr at a rate of at least 4 cycles/hr. (Adjust with variable transformer heat control.) At the end of extraction and saponification step, remove Soxhlet apparatus from reservoir and replace with condenser-concentrator or suitable water-cooled condenser. Distill the ethanol at the same setting used in the extraction until liquid volume in reservoir is approximately 450 ml. (Determine level corresponding to this volume prior to extraction.) Remove condenser and filter saponified material while warm through glass wool plug into 2 L separatory funnel containing 250 ml distilled water. Wash reservoir with three 50 ml portions of ethanol (the distillate obtained above may be used) and filter washes through plug into the funnel. Wash reservoir with two 100 ml portions of iso-octane and again filter into the funnel. Add additional iso-octane to separatory funnel, the amount depending upon the food product being analyzed: 50 ml for fish, 75 ml for frankfurters, and 100 ml for cheese. Let the mixture cool until lukewarm (about 15 minutes). Extract mixture by shaking the separatory funnel for 3 minutes. When the layers separate, draw off lower layer into a second separatory funnel. Allow residual solids in first funnel to settle and separate for an additional minute; then carefully draw them off completely into the second separatory funnel. Repeat the extraction operation with 200 ml iso-octane. After separation of the layers, draw off lower layer and residual solids as previously described into a third separatory funnel and again extract with 200 ml iso-octane. Draw off aqueous layer, again allowing residual solids to settle and carefully removing them as before. Discard aqueous layer and residual solids. Wash each iso-octane extract twice with 500 ml warm distilled water (about 125°F) by gently swirling the funnels. (Note: Vigorous shaking may cause emulsions.) Discard aqueous layer after each wash. Then, wash each extract twice with 500 ml distilled water (room temperature), using a more vigorous swirling technique than before. Draw off aqueous layer and discard.

Pour 60 g treated and tested Florisil (Reagent (1)) into chromatographic column, with gentle tapping to settle the contents. Add 50 g Na_2SO_4 to column, again tapping to even surface layer. Prewet column with about 75 ml iso-octane, allow to drain by gravity, and discard the filtrate. Filter iso-octane extract in first separatory funnel through column, collect 100 ml of the filtrate in graduated container, and discard. Remove graduated container, place 1 L evaporation flask under column, and continue filtration. Let column drain. Wash the first separatory funnel with the extract contained in the second separatory funnel and filter through the column. Wash second and first separatory funnels successively with extract in third separatory funnel and filter through the column into flask. Again let the column drain. (If it is desired to quantitatively determine only benz(a)anthracene, benzo(a)pyrene, dibenz(a,h)anthracene, and the other condensed ring types which are more strongly held by the adsorbent, discard all iso-octane extracts collected prior to elution with benzene.) Wash third, second, and first

separatory funnels in that order in tandem with two successive 50 ml portions of benzene and pass washings individually through column into evaporation flask. Then pass 75 ml benzene through column. Let column drain until solvent no longer drips. Add 2 ml hexadecane to filtrate. Fit tube assembly into evaporation flask and evaporate solvent under nitrogen on steam bath as previously described for purified benzene under *Reagents,* but evaporate to 2 ml residual hexadecane. (A loose aluminum foil jacket around the flask will speed evaporation.) Add 10 ml iso-octane, re-evaporate, and repeat once to insure complete removal of benzene.

Quantitatively transfer 2 ml hexadecane concentrate to a 500 ml separatory funnel with portions of iso-octane (198 ml total). Wash solution with three 100 ml portions of phosphoric acid, shaking 1 minute for each operation. After each wash, let layers separate and discard the lower (acid) layer. After final discard, swirl funnel and let it stand for a few minutes; then carefully draw off any residual acid which may settle out from the iso-octane. Add 50 ml dimethyl sulfoxide (DMSO) pre-equilibrated with iso-octane and shake funnel 3 min. Set up three 125 ml separatory funnels containing 25 ml iso-octane pre-equilibrated with DMSO. After phase separation in 500 ml separatory funnel, draw off lower DMSO layer into first 125 ml separatory funnel and wash in tandem with the iso-octane contained in the three 125 ml separatory funnels, shaking each wash for 1 min. Repeat extraction with two more successive 50 ml portions of DMSO, washing each extract in tandem through the same three portions of iso-octane.

Collect the successive DMSO extracts (150 ml total) in separatory funnel (preferably 1 L) containing 300 ml distilled water and 50 ml iso-octane. Let cool for a few minutes after the last extract has been added, since some heat is generated on the dilution of the sulfoxide with water. Shake funnel vigorously for 2 min. and let layers separate. Draw off lower aqueous phase into a second separatory funnel (preferably 1 L) and repeat extraction with 50 ml iso-octane. Draw off and discard aqueous layer. Wash each 50 ml extract twice with 75 ml distilled water, shaking each wash for about 15 sec. Let phases separate and discard aqueous layers after each wash.

Pour 40 g Florisil (Reagent (1)) into chromatographic column, gently tapping to settle contents. Add 35 g Na_2SO_4, again tapping to even surface layer. Prewet column with 60 ml iso-octane, letting the liquid drain through by gravity. Filter iso-octane extract from first separatory funnel through column. Wash first funnel with iso-octane extract in second separatory funnel and filter through column as before. Then wash second and first separatory funnels successively with two 35 ml portions of iso-octane and filter them individually through column. Discard iso-octane filtrates. Add 25 ml benzene to column, drain, and discard filtrate. Place 250 ml evaporation flask under column and elute the polycyclic hydrocarbons with three 45 ml portions of benzene, letting column drain after each addition.

Evaporate the collected filtrate on the steam bath under nitrogen to about 10 ml as previously described. Transfer solution quantitatively with benzene to a 50 ml glass-stoppered Erlenmeyer flask and concentrate carefully on steam bath under nitrogen to 0.5 ml or less. (Caution: Do not evaporate to dryness, since prolonged heating of the polycyclic hydrocarbons in the dry state will cause losses.) Save concentrated solution for paper chromatography.

Paper chromatography.—For the separation of the hydrocarbons from background material, use the descending paper chromatographic technique. Wash papers before use by letting the mobile solvent, iso-octane, migrate down the length of the paper as in the development of the chromatogram.

Apply entire concentrate of the smoked food extracts, with a micropipet, in a streak about 1 cm wide and 15–17 cm long along a base or starting line drawn 5 cm from the top of the sheet. Wash sides of flask four times with small portions (0.2 ml from a graduated pipet) of benzene and streak the solvent from each wash on the paper. Immerse paper to within 10 mm of starting line in immobile solvent (35% v/v *N,N*-dimethylformamide in ethyl ether) contained in glass trough. (Caution: Do not immerse the starting line where the concentrate was applied in the immobile solvent). Saturate tank with iso-octane by placing the solvent in the bottom of the tank to a depth of about 25 mm about 1 hr before use. Suspend sheet in tank saturated with iso-octane (the mobile solvent). Develop chromatograms in a darkened room, and occasionally examine them by observing the fluorescence briefly under ultraviolet light. In 3–3.5 hr (25°C) the solvent front will have moved to the end of the paper; however, for optimum separation and removal of background material from the compounds of in-

terest, develop for 4 to 4.5 hr. (At this point, benzo(a)pyrene, if present, will have moved approximately 22 cm down the paper.)

When development is complete, remove sheets from tank and suspend them on support rod in a darkened room until *just* dry. Observe final positions of polycyclic compounds by fluorescence under both longwave and shortwave UV light in Chromato-Vue Cabinet. Cut out fluorescent bands or streaks, place in small flask, and elute with 5 to 10 ml portions of hot methanol until fluorescence under ultraviolet light can no longer be seen in the last portion of solvent. Swirl flask repeatedly during extraction, and filter individual extracts successively through pyrex glass wool plug into 50 ml glass-stoppered Erlenmeyer flask to remove small fragments of paper. (Three or four extractions are usually enough to remove the polycyclic compounds from the paper.) Concentrate combined eluate to 0.5 ml or less on the steam bath under nitrogen. (Caution: Do not evaporate to dryness!) Add 5 ml benzene, again concentrate to 0.5 ml or less, and save for thin layer chromatography.

Thin layer chromatography.—Place 50 g cellulose acetate and 275 ml 95% ethanol in glass-stoppered Erlenmeyer flask. (Five plates can be prepared with this amount.) Shake mixture vigorously by hand for 4 min. With applicator, apply adsorbent to the plates to a thickness of 1 mm. Let plates air-dry 4 hr and store in desiccator until needed.

Pour 50 ml mobile solvent (ethanol-toluene-water (17:4:4 v/v/v) in tank and equilibrate for at least 1 hr. Apply the extracts obtained from the paper on plate in a line ca 2 cm in length along the starting line (drawn 2 cm from the bottom of the plate). Wash sides of individual flasks four times with benzene (0.2 ml from a graduated pipet) and transfer solvent from each wash to plate as before. After evaporation of solvent, place plate in the tank and develop chromatogram for 1.5–2.0 hr (25°C).

When development is complete, remove plate from tank and observe the fluorescent bands under both longwave and shortwave UV light in the Chromato-Vue Cabinet. (This operation may be carried out while the plate is still wet.) Outline fluorescent bands, remove plate from the cabinet, scrape off adsorbent around the bands with a spatula, and discard. Collect each outlined band in a 125 ml beaker and elute the polycyclic hydrocarbon from adsorbent by extracting with 5–10 ml portions of hot methanol until fluorescence under ultraviolet light can no longer be seen in the last portion of solvent. Swirl the flask repeatedly during extraction, and successively filter the individual extracts through 50 ml pressure filter under nitrogen into a 50 ml flask. (Three or four extractions are usually enough to remove the compound from the adsorbent.)

Next, add 1 ml hexadecane to the combined extract and evaporate the methanol on the steam bath under a stream of nitrogen. Remove any residual methanol by two successive additions of 5 ml iso-octane and re-evaporation. Dissolve the 1 ml residue in 2 ml iso-octane and record the ultraviolet spectrum in a 1 cm cell, using iso-octane in the reference cell. Compare any maxima observed with those in the spectra of known polycyclic aromatic hydrocarbons. Estimate the quantity of identified hydrocarbons by the baseline technique in conjunction with spectra of these hydrocarbons recorded with standard solutions under the same instrumental conditions.

The solutions of sample extract may be used to confirm the identification by applying the technique of spectrophotofluorometry.

Notes on Method

In preliminary studies, the use of ethyl alcohol as an extracting agent for the polycyclic hydrocarbons and as a medium for the saponification of the fat was advantageous, since both steps could be carried out simultaneously. As found by Prickett, *et al.* (16), dilution of the alcohol concentrate with water is rather critical since the residual soaps can cause emulsions in the subsequent iso-octane extraction if the alcohol-water ratio is not properly adjusted. For example, Bailey and Dungal (8) attributed losses of benzo(a)pyrene to heavy emulsions which were formed after the saponification step in their method. We did not have this difficulty in the present study. While some losses of iso-octane occur in the initial extraction of the aqueous-ethanolic layer, the polycyclic hydrocarbons can be essentially extracted quantitatively by proper adjustment of the volume of the hydrocarbon solvent to compensate for the loss. The amount of iso-octane added in the initial extraction varied with the individual food product and was adjusted so that approximately 200 ml of solvent was recovered for each product.

Two adsorbents, a magnesia-Celite 545

mixture and Florisil, were evaluated for column chromatography. The magnesia column was first studied because of its demonstrated superiority over other adsorbents in both industry and government laboratories for the determination of specific polycyclic hydrocarbons in various petroleum products and resins (17). (Different batches of alumina varied considerably in their retentive properties for polycyclic compounds even when the adsorbent was treated according to available standardization procedures.)

The magnesia column was prepared and standardized as described in our procedure for petroleum waxes (15). A gradient elution system consisting of iso-octane, 10% benzene in iso-octane, and 20% benzene in iso-octane was then used to remove lower aromatic compounds, such as naphthalene or anthracene, and other interfering background material. The more mobile of the 4-ring compounds, such as pyrene, were then eluted from the column with benzene, while the more retentive 4-, 5-, and 6-ring types, such as benz(a)anthracene, benzo(a)pyrene, and benzo(g,h,i)perylene, were eluted with an acetone-benzene-water mixture. In the case of the acetone-benzene-water eluate, the acetone was removed by washing with water and the resulting benzene solution dried by passage through anhydrous sodium sulfate. Separations of the individual compounds in the two benzene fractions were then obtained by paper and thin layer chromatography as described in the present method. Recoveries of four polycyclic compounds added together to 500 g samples of cheese, frankfurters, and fish at the 2 ppb level varied from 55 to 83%. The ultraviolet curves obtained for these compounds indicated that the column used after a dimethyl sulfoxide-iso-octane partition step and subsequent paper and thin layer procedures efficiently removed extraneous material. However, the column chromatographic procedure was lengthy, and some difficulties were encountered in the separation of phases during the extraction of the iso-octane solution with phosphoric acid. Phosphoric acid extractions prior to the dimethyl sulfoxide partition step are needed to remove coloring matter and extraneous material. Therefore, we sought to eliminate these problems by the use of another adsorbent.

Previous incomplete studies of other food products in which a Florisil column was used indicated that this adsorbent efficiently removed interfering background material extracted from the foods. However, different batches of the adsorbent were found to vary widely in their retentive properties. Later investigations demonstrated that Florisil could be easily standardized by treatment with methanol prior to activation by heating. To substantiate these findings, samples from eight different lots of Florisil were standardized with reproducible results. The adsorbent also effectively retained coloring matter extracted from foods as well as other extraneous material which had caused emulsions in the phosphoric acid extractions.

Studies were conducted to investigate the compounds which may be partially lost from the Florisil column when the iso-octane extracts of the aqueous saponified products were filtered through the adsorbent and discarded. These studies were carried out by adding a known amount of a polycyclic compound to the initial iso-octane extract of the foods and collecting fractions of the filtrate as the extracts were passed through the column. Ultraviolet spectral analyses of these fractions indicated that approximately 35% of the pyrene and fluoranthene would be lost if the entire iso-octane filtrate was discarded. In contrast, benz(a)anthracene, benzo(a)pyrene, dibenz(a,h)anthracene, benzo(g,h,i)perylene, and the usually more mobile compounds, such as 4-methyl pyrene, were not found in the discarded filtrate and were recovered quantitatively when the method was carried out in its entirety. The losses are apparently due to the presence of oils or other extraneous materials extracted from the food products which elute the polycyclic hydrocarbons less tenaciously held on the Florisil. This supposition is supported by the observation that standard polycyclic compounds added to the column cannot be eluted from the adsorbent when the same volume of purified iso-octane is passed through the column. Also, all of the iso-

octane extracts obtained after the dimethyl sulfoxide partition step may be filtered through the column and discarded without losses, indicating that the extraneous material was eliminated during either the initial chromatography or the partition step. From the results of the above studies, we concluded that essentially all of the initial iso-octane extracts derived from the foods must be retained. The discarded 100 ml of eluate consists mainly of the iso-octane prewash retained on the adsorbent and the anhydrous salt. It should be emphasized, however, that if it is desired to quantitatively determine only benzo(a)pyrene, benz(a)anthracene, dibenz(a,h)anthracene, and other 5- or 6-condensed ring hydrocarbons, which are strongly held by the adsorbent, all of the iso-octane extracts passed through the column may be discarded prior to elution of the adsorbent with benzene.

As in the determination of polycyclic hydrocarbons in petroleum products (15, 18) and milk (14), a partition step between iso-octane and dimethyl sulfoxide is advantageously used to reduce interfering background material. Phosphoric acid extractions of the iso-octane phase prior to sulfoxide extraction remove any residual coloring matter remaining in the iso-octane.

Various paper and thin layer chromatographic techniques were investigated in an attempt to isolate the polycyclic hydrocarbons without the use of a chromatographic column. Difficulties, however, were encountered in the application of the oily concentrate obtained after the dimethyl sulfoxide partition step. Excessive tailing, streaking, and occlusion of the hydrocarbons with background material were noted in the chromatograms. Preparative thin layer chromatography as an alternative for column chromatography was also unsuccessful, and it was concluded that the latter procedure was necessary.

Both paper and thin layer techniques were evaluated for the initial separation of the polycyclic hydrocarbons following column chromatography. In thin layer chromatography, the best overall results were obtained on cellulose plates 0.5 mm thick with *N,N*-dimethylformamide (applied as a 35% solution in ethyl ether) as the immobile solvent and iso-octane as the mobile solvent. With this system, excellent separations and recoveries were obtained with mixtures of standard solutions of pyrene, 4-methyl pyrene, benz(a)anthracene, benzo(a)pyrene, dibenz(a,h)anthracene, and benzo(g,h,i)-perylene. In the actual isolation of the extracted hydrocarbons from food, however, the bands were close together or overlapping, and problems were encountered in quantitatively recovering the compounds from the plate. These problems were especially acute when the smoked products contained eight or more fluorescent bands, because there was apparently insufficient distance for the migration and separation of the compounds on the 20 cm plate. Another possible factor for incomplete separation was the presence of the residual extraneous material from the food products. Because of the aforementioned problems, the more lengthy paper chromatographic procedure was employed. With the use of long chromatographic sheets, excellent separations and reproducible recoveries were obtainable.

The use of cellulose acetate as substrate and ethanol-toluene-water (17:4:1 v/v/v) as developer for the separation of polycyclic aromatic hydrocarbons has been described in the literature (19, 20). As found by Sawicki, *et al.* (21), however, improved separations with decreased tailing may be obtained with the above solvent system in proportions of 17:4:4. Cellulose acetate was used in this study because it efficiently removed the ultraviolet-absorbing background material from the hydrocarbons isolated from the food products. In addition, it provides a means for the separation of benz(a)-anthracene and chrysene and of most of the individual components of the so-called "benzopyrene" fraction (e.g., benzo(a)-pyrene, benzo(k)fluoranthene, benzo(e)-pyrene, and perylene), whereas most other paper and thin layer chromatographic procedures fail in these respects.

The following observations were made concerning the separations of various polycyclic hydrocarbons by the paper and thin layer techniques used in the present study: Pyrene and fluoranthene cannot be com-

pletely separated by either technique without repeated chromatography. In the authors' experience, this is also true of other available methods. Identification and estimation of the admixed individual compounds can be made, however, from the ultraviolet absorbance spectrum. This can be accomplished either by the baseline technique, using the absorbance maxima at 334 mμ for pyrene and at 287 mμ for fluoranthene, or by the variable reference technique described in detail by Jones, *et al.* (22). The foregoing also holds true for benzo(g,h,i)perylene and dibenz(a,h)-anthracene, in which case the concentrations may be calculated by the baseline technique, using the absorbances at 384 mμ and 297 mμ, respectively. When estimating the dibenz(a,h)anthracene content, however, a correction must be made for the contributing absorbance of the benzo-perylene compound which exhibits a maximum at 299 mμ. Benz(a)anthracene and benzo(g,h,i)perylene are readily separated on paper but cannot be separated on the cellulose acetate adsorbent. Benzo(a)pyrene and benzo(g,h,i)perylene are separable by either paper or thin layer procedures.

Recovery Studies

Most of the recoveries were conducted with smoked food products obtained from local retail markets. The following foods were examined: all meat frankfurters, Gouda cheese, and domestic herring. Unsmoked cheddar cheese was also included. Preliminary analyses for polycyclic hydrocarbons in these products were made to establish that they were free of the compounds used in the recovery studies.

Solutions of polycyclic aromatic hydrocarbons, benz(a)anthracene, benzo(a)pyrene, dibenz(a,h)anthracene, and benzo(g,h,i)perylene, were prepared at concentrations of 1 μg/ml in iso-octane. One ml aliquots of the solutions were pipetted into the thimble containing the 500 g sample of the food product mixed with an equal quantity of the anhydrous salt. In most instances, three of the above hydrocarbons were added to each of the food products, alternating the dibenz(a,h)anthracene and benzo(g,h,i)-perylene in each recovery study. The analysis was then carried out as previously described.

Results and Discussion

The recoveries of representative polycyclic hydrocarbons (including 4-, 5-, and 6-ring types) added at a level of 2 ppb to 500 g of food product are summarized in Table 1. Average recoveries ranged from 70 to 83% for frankfurters, from 75 to 80% for cheese, and from 70 to 88% for fish.

Representative ultraviolet absorption spectra obtained for the polycyclic compounds added to and recovered from smoked fish are presented in Fig. 1. Similar ultraviolet spectra were obtained for frankfurters and cheese. Fluorescence spectra of compounds recovered from cheese are shown in Fig. 2. These spectra demonstrate that the hydrocarbons can be readily identified by either technique and quantitated by ultraviolet spectrophotometry at levels of 2 ppb or less, following chromatography on the cellulose acetate plate. This is particularly true with benzo(a)pyrene, in which case the ultraviolet and fluoresence spectra of the recovered hydrocarbon in all of the food products studied

Table 1 Recoveries of polycyclic hydrocarbons added to 500 g of food products at a level of 2 ppb

Polycyclic Hydrocarbon[a]	No. of Runs	Recovery, %	Av., %
Frankfurters			
Benz(a)anthracene	3	75, 83, 92	83
Benzo(a)pyrene	3	67, 70, 75	71
Dibenz(a,h)anthracene	3	70, 70, 70	70
Benzo(g,h,i)perylene	1	80	80
Cheese			
Benz(a)anthracene	2	75, 83	79
Benzo(a)pyrene	3	73, 75, 83	77
Dibenz(a,h)anthracene	1	80	80
Benzo(g,h,i)perylene	2	70, 80	75
Fish			
Benz(a)anthracene	4	83, 83, 92, 92	88
Benzo(a)pyrene	4	73, 73, 82, 83	78
Dibenz(a,h)anthracene	1	70	70
Benzo(g,h,i)perylene	3	80, 80, 80	80

[a] Nomenclature and numbering of polycyclic aromatic hydrocarbons are in accordance with Chemical Abstracts.

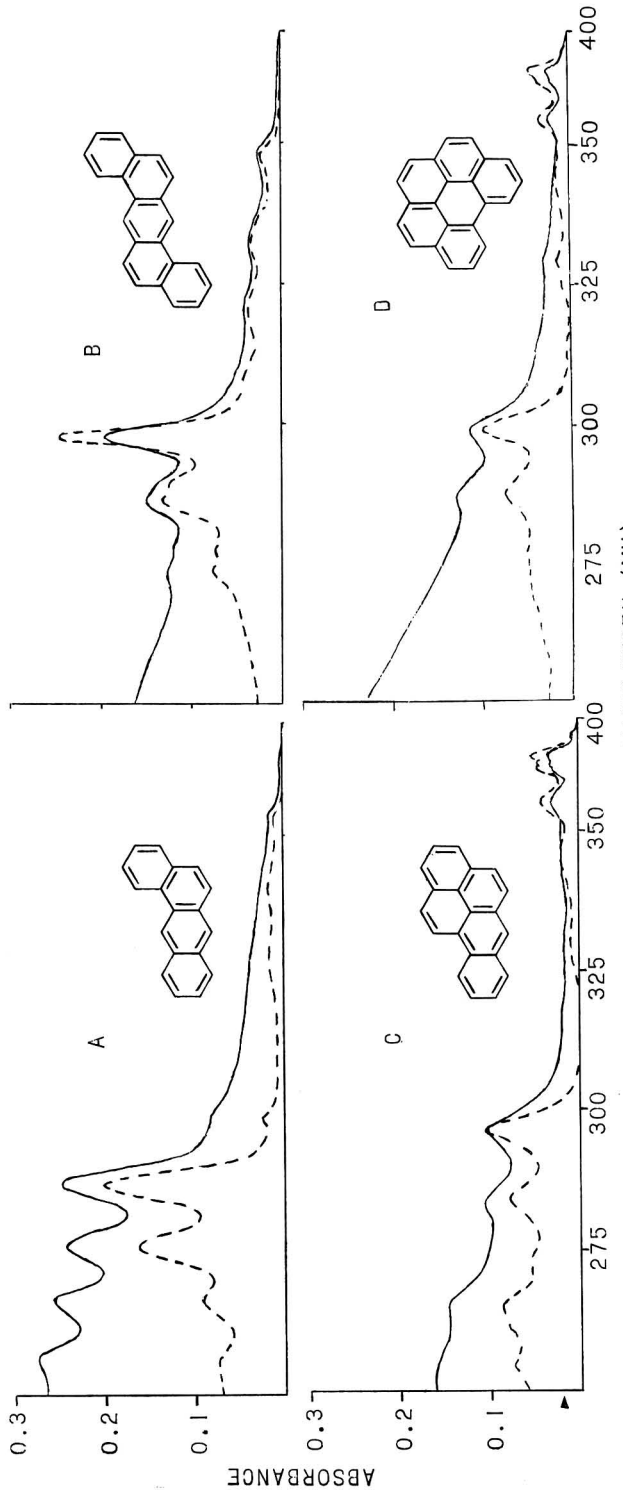

Fig. 1—Ultraviolet absorption spectra of polycyclic hydrocarbons recovered from 500 g fish at the 2 ppb level. Solid line, recovery curve; broken line, reference curve. A. Benz(a)anthracene; B. Dibenz(a,h)anthracene; C. Benzo(a)pyrene; D. Benzo(g,h,i)perylene.

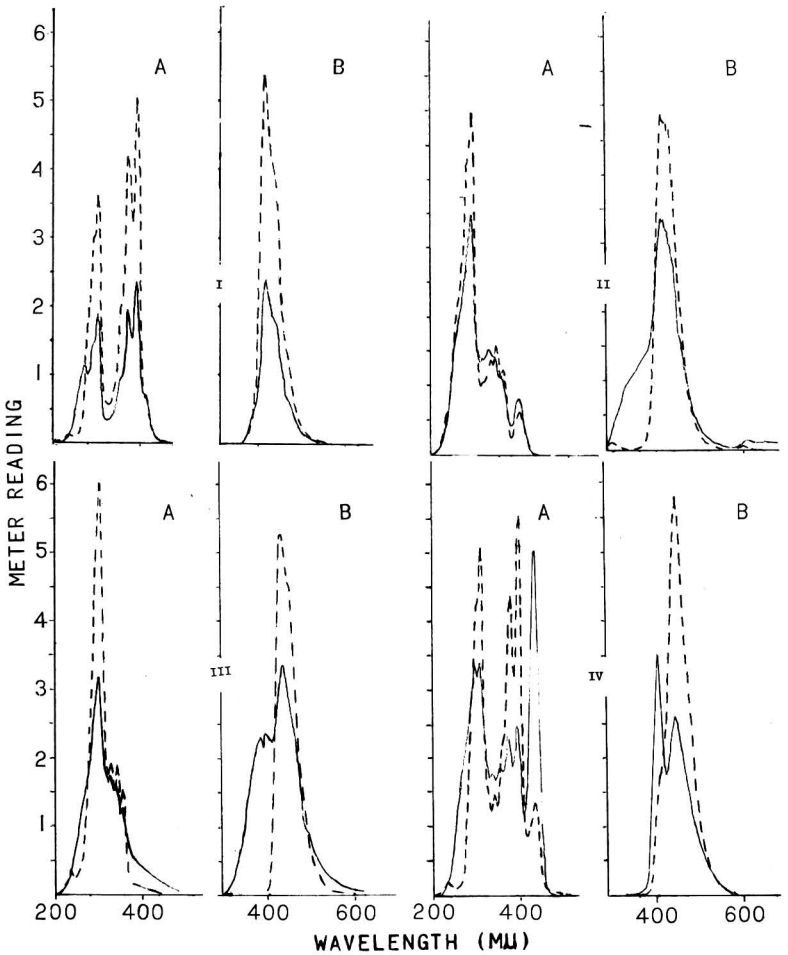

Fig. 2—**Fluorescence spectra of polycyclic hydrocarbons recovered from 500 g cheese at the 2 ppb level. Solid line, recovery curve; broken line, reference curve. I. Benzo(a)pyrene (ref. std, 1.1 mg/L); A. Excitation spectra at emission 415 mμ; sensitivity 50; 0.01 MM; B. Emission spectra at excitation 385 mμ. II. Dibenz(a,h)anthracene (ref. std, 1.0 mg/L); A. Excitation spectra at emission 410 mμ; sensitivity 50; 0.003 MM; B. Emission spectra at excitation 305 mμ. III. Benz(a)anthracene (ref. std, 1.2 mg/L); A. Excitation spectra at emission 405 mμ; sensitivity 50; 0.003 MM; B. Emission spectra at excitation 290 mμ. IV. Benzo(g,h,i)perylene (ref. std, 1.0 mg/L); A. Excitation spectra at emission 425 mμ; sensitivity 50; 0.003 MM; B. Emission spectra at excitation 305 mμ.**

were remarkably comparable to the standards. Because of the relatively intense fluorescence characteristics of this hydrocarbon, it would be possible to isolate and determine quantities much lower than the 2 ppb level employed in these investigations. With other less fluorescent compounds, such as dibenz(a,h)anthracene, isolation at a much lower level may be difficult because the bands are not easily discernible either on paper or on thin layer plates when viewed under ultraviolet light.

Various foods (including both smoked and unsmoked products) have been analyzed for polycyclic hydrocarbons by the above method. The hydrocarbons were identified by comparing R_f values with those of known compounds and by their ultraviolet and fluoresence spectra. The results of these investigations are shown in Tables 2 and 3.

Table 2. Polycyclic aromatic hydrocarbons found in smoked food products

Product	Benz(a)-anthracene (ppb)	Benzo(a)-pyrene (ppb)	Benzo(e)-pyrene (ppb)	Benzo-(g,h,i)-perylene (ppb)	Fluor-anthene (ppb)	Pyrene (ppb)	4-Methyl Pyrene (ppb)
Beef, chipped	0.4				0.6	0.5	
Cheese, Gouda					2.8	2.6	
Fish, herring					3.0	2.2	
Fish, herring (dried)	1.7	1.0	1.2	1.0	1.8	1.8	
Fish, salmon	0.5		0.4		3.2	2.0	
Fish, sturgeon		0.8			2.4	4.4	
Fish, white					4.6	4.0	
Frankfurters					6.4	3.8	
Ham	2.8	3.2	1.2	1.4	14.0	11.2	2.0
Pork roll					3.1	2.5	

The highest total polycyclic aromatic content was found in the smoked ham. The polycyclic hydrocarbons pyrene and fluoranthene were present in all of the foods examined; however, in most instances lesser amounts were found in the unsmoked products. As previously discussed, the variable reference technique (22) can be used to identify these compounds that are difficult to separate. This is accomplished by adding an equal concentration of either pyrene or fluoranthene, calculated by the baseline technique, to the reference cell and thereby "blocking out" the absorbance due to the respective hydrocarbon. Ultraviolet spectra of the admixed pyrene and fluoranthene (20-fold dilution) extracted from smoked ham and pyrene and fluoranthene of the same admixture after use of the variable reference procedure are presented in Fig. 3.

The carcinogen, benzo(a)pyrene, was found in smoked sturgeon, dried smoked herring, and smoked ham samples at levels of 0.8, 1.0, and 3.2 ppb, respectively. The ultraviolet and fluorescence spectra of this hydrocarbon isolated from the ham sample are presented in Figs. 4 and 5. Identification of the carcinogen was also confirmed by its molecular weight (m/e 252) as determined by mass spectrometry. The ultraviolet and fluorescence spectra for benzo(a)anthracene and benzo(g,h,i)perylene also isolated from the ham are shown in Figs. 6–8. Although these compounds were readily identified after their initial chromatography on the cellulose acetate adsorbent, they were rechromatographed on the plates to obtain more definitive spectra. To remove *n*-hexadecane, the iso-octane solutions of the hydrocarbons were placed on 10 g of the Florisil adsorbent and the standardization test for the adsorbent as described under *Reagents* was carried out. The benzene eluate containing the hydrocarbon was then concentrated and applied to the plate as before.

Table 3. Polycyclic aromatic hydrocarbons found in unsmoked food products

Food Product	Fluoranthene (ppb)	Pyrene (ppb)
Cheese, cheddar	0.8	0.7
Fish, haddock	1.6	0.8
Fish, herring (salted)	0.8	1.0
Fish, salmon (canned)	1.8	1.4

In conclusion, benzo(a)pyrene and other polycyclic hydrocarbons in varying concentrations have been isolated from smoked food products. As previously discussed, many factors (i.e., type of generator, temperature of combustion, degree of smoking, etc.) affect the composition of curing smoke (1). The variations in the type and concentrations of the hydrocarbons found in the smoked foods are undoubtedly related to the aforementioned factors. The presence of the noncarcinogenic hydrocarbons pyrene and fluoranthene in unsmoked products suggests another source of contamination; however, no plausible explanation of their origin can be given at the present time.

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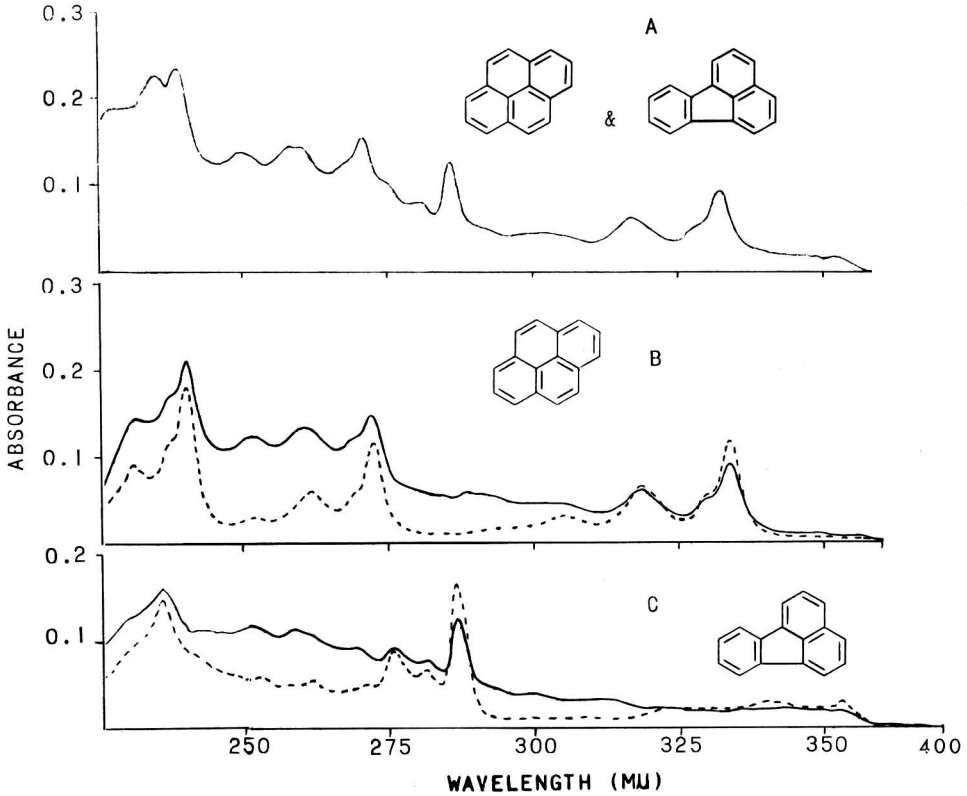

Fig. 3—**Ultraviolet absorption spectra. A. Admixture of pyrene and fluoranthene isolated from smoked ham. B. Pyrene from pyrene-fluoranthene admixture (solid line, unknown; broken line, reference); C. Fluoranthene from pyrene-fluoranthene admixture (solid line, unknown; broken line, reference).**

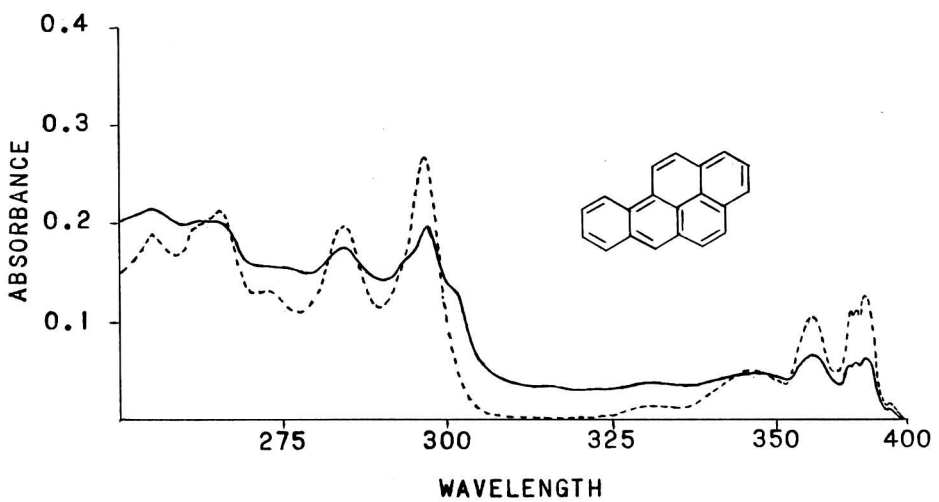

Fig. 4—**Ultraviolet absorption spectra of benzo(a)pyrene isolated from smoked ham. Solid line, unknown; broken line, reference.**

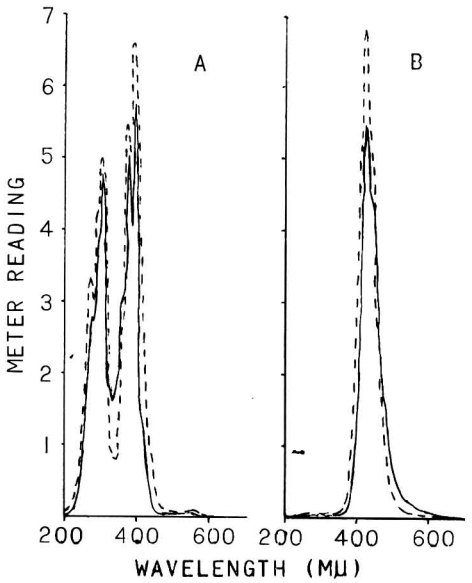

Fig. 5—**Fluorescence spectra of benzo(a)pyrene isolated from smoked ham. Solid line, unknown; broken line, reference. A. Excitation spectra at emission 415 mμ; sensitivity 50; 0.01 MM; B. Emission spectra at excitation 390 mμ.**

tives and Instrumentation Branch, for general guidance and advice and for assistance in preparing the report; Henry Fischbach, Division Director and Chairman of the Trace Substances Commission, IUPAC, for his interest, cogent suggestions, and support; and Myron M. Schachter, for training in the use of the spectrophotofluorometer. We are also glad to acknowledge the cooperation of Virdell E. Munsey, Chief, Food Additives Branch, Division of Food Standards and Additives, and the helpful suggestions of Lessel L. Ramsey, Deputy Director, Bureau of Scientific Standards and Evaluations.

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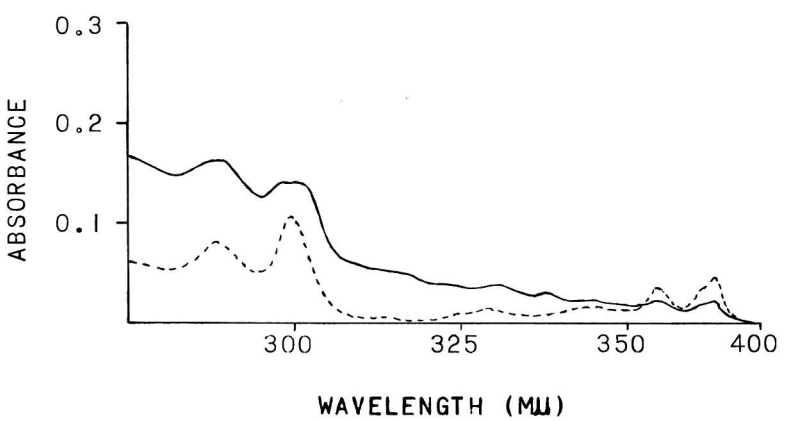

Fig. 6—**Ultraviolet absorption spectra of benz(a)anthracene from smoked ham. Solid line, unknown; broken line, reference.**

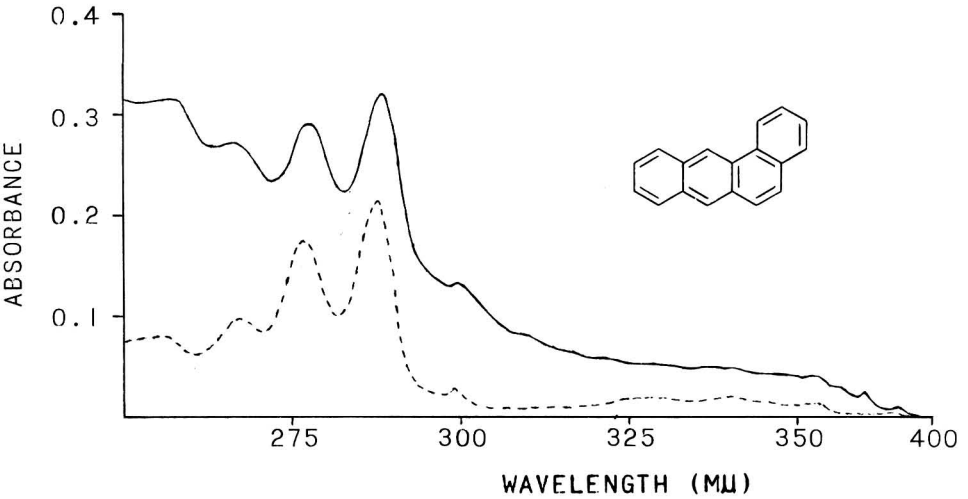

Fig. 7—**Ultraviolet absorption spectra of benzo(g,h,i)perylene isolated from smoked ham. Solid line, unknown; broken line, reference.**

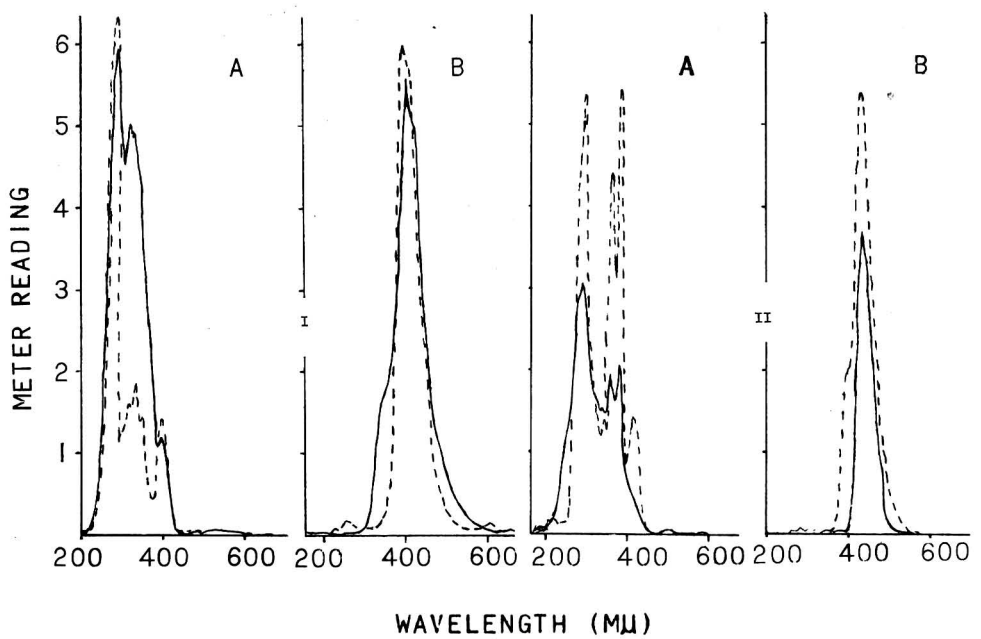

Fig. 8—**Fluorescence spectra of two polycyclic hydrocarbons isolated from smoked ham. Solid line, unknown; broken line, reference. I. Benz(a)anthracene; A. Excitation spectra at emission 400 mμ; sensitivity 50; 0.01 MM; B. Emission spectra at excitation 290 mμ. II. Benzo(g,h,i)perylene; A. Excitation spectra at emission 425 mμ; sensitivity 50; 0.01 MM; B. Emission spectra at excitation 390 mμ.**

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Extraction and Estimation of Polycyclic Aromatic Hydrocarbons in Smoked Foods. II. Benzo(a)pyrene

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The carcinogen benzo(a)pyrene has been isolated from various smoked foods by modification of the general procedure for polycyclic aromatic hydrocarbons described in Part I. Recoveries of benzo(a)pyrene, added to 75, 100, and 150 g samples of frankfurters, fish, and cheese at levels of 1–2 ppb, ranged from 73 to 100%. Positive identification of the hydrocarbon recovered from feeds at levels as low as 0.02 ppb can be made from its fluorescence spectra.

In recent years, benzo(a)pyrene and other polycyclic hydrocarbons have been found to be widely distributed in the human environment (1–16). Such compounds have been identified in curing smoke (10–13) and smoked foods (9, 11, 13–15). The known carcinogenicity of benzo(a)pyrene and its widespread occurrence have led some investigators to conclude that this hydrocarbon is at least in part the agent responsible for some environmental cancers. For example, Bailey and Dungal (9) have isolated benzo(a)pyrene from fish and mutton smoked in Iceland. Since this was the only strong carcinogen found, these workers suggested that its presence might explain the high incidence of stomach carcinoma which occurs in that country. Wojtelowicz, *et al.* (13) and Kaufmann, *et al.* (16), in further evaluations of the role of benzo(a)pyrene in the development of cancer, compared a group of Baltic fishermen, who consume large amounts of smoked fish found to contain benzo(a)pyrene, and a group of citizens living inland. The incidence of gastric carcinoma in the fishermen was approximately four times greater than that in the people who consumed lesser quantities of smoked fish.

Because of this evidence and the lack of information on foods smoked in the United States, interest has increased in the development of a rapid analytical procedure for benzo(a)pyrene. Genest and Smith (17) developed a detection method for the hydro-

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carbon. In brief, the procedure consists of drying the food for an extended period, extracting the dried product in a Soxhlet apparatus, and partitioning between dimethyl sulfoxide and a hydrocarbon solvent, followed by thin layer chromatography and spectrophotofluorometric analysis. The authors indicate that the presence of certain interfering materials limited the sensitivity of the method, which varied from 10 to 50 ppb, depending upon the food product being analyzed.

During the past year, we made a concerted effort to develop sensitive, practical analytical procedures necessary to study the polycyclic hydrocarbons present in foods. Our first objective was to devise a procedure that could be used to determine trace quantities of polycyclic hydrocarbons in the smoked products; this method was described in Part I. During the aforementioned investigation, it was found that the procedure could be modified and considerably shortened if the analyst were concerned only with the determination of benzo(a)pyrene. This report describes such a method in which the food product is digested directly in ethanolic potassium hydroxide. Subsequent steps are essentially as reported in our method for specific hydrocarbons; however, it was not necessary to use the rather lengthy paper chromatographic technique in the modified procedure. An experienced analyst can complete the analysis in one day.

METHOD

Apparatus

The following apparatus listed in Part I (p. 596) was used: (**a**); (**e**); (**g**); (**h**)—Substitute 10 × 20 cm glass plates (25-10-11) for (*1*); (**i**); (**m**); (**n**)—Substitute 125 and 300 ml capacity flasks; (**o**); and (**p**).

In addition, these items should be added.

(**a**) *Flask, boiling.*—1 L capacity, round-bottom, with 24/40 ⅀ joint and pouring lip (Kontes Glass Co. No. K-60200, or equivalent).

(**b**) *Condenser.*—Friedrich type, with 24/40 ⅀ joint (Kontes Glass Co. No. K-43700, or equivalent).

(**c**) *Heating mantle.*—Hemisperical, 1 L capacity.

(**d**) *Recording spectrophotometer and accessories.*—(*1*) Cary 11 (Applied Physics Corp., Monrovia, Calif., or equivalent). (*2*) Cells: (a) fused rectangular quartz cells, optical path length 10 mm ± 0.005 mm, 1.5 ml capacity (5-503 QS); (b) fused quartz cells, optical path length 50 mm ± 0.05 mm (2-228Q), Tolerance A (Optical Cell Co., Inc., Brentwood, Md., or equivalent).

Reagents

Use the same reagents, purification, and testing procedures described in Part I (p. 597), omitting (**f**), (**g**), and (**m**).

Procedure

Sample extraction.—Grind a representative sample of the smoked food product to be analyzed in a meat grinder. Weigh 100 or 150 g ground food and place in 1 L reservoir. (For dried smoked products, use 100 g samples to insure complete digestion of the food.) Add 400 ml ethanol, 15 g KOH, and boiling chips. (*Note*: For cheese, add KOH in 1:10 weight ratio, i.e., 15 g KOH/150 g cheese.) Insert Friedrich condenser into reservoir fitting and reflux mixture at a rapid rate for 1.5 hr. (*Caution*: To prevent foaming, do not apply heat too vigorously to the products initially. After refluxing at a relatively slow rate for about 5–10 min. gradually increase the heat to obtain a rapid reflux rate.) When digestion is complete, remove condenser and transfer material while warm into 2 L separatory funnel containing 250 ml distilled water. Wash the reservoir with two 100 ml portions of ethanol, then 150 ml iso-octane; transfer all the washes to the funnel.

Shake separatory funnel for 3 minutes. Let layers separate and drain lower layer into a second separatory funnel. Let residual solids settle and separate for an additional minute, and carefully drain completely into the second separatory funnel. Repeat extraction with 100 ml iso-octane. After the layers separate, drain aqueous layer and residual solids as previously described into a third separatory funnel and again extract with 100 ml iso-octane. Drain aqueous layer, again let residual solids settle, and carefully remove them as before. Discard aqueous layer and residual solids. Wash each iso-octane extract four times with 250 ml warm distilled water (ca 120°F) by gently swirling funnels. (Note: Vigorous shaking may cause emulsions.) Discard aqueous layer after each wash.

Pour 60 g treated and tested Florisil into chromatographic column with gentle tapping

to settle contents. Add 50 g Na_2SO_4 to column, again tapping to level surface layer. Prewet column with 75 ml iso-octane, let drain by gravity, and discard filtrate. Filter iso-octane extract in first separatory funnel through column and drain. Wash first separatory funnel with extract contained in second separatory funnel, and filter through column as before. Wash second and first separatory funnels successively with extract in third separatory funnel, and filter through column. Wash third, second, and first funnels in that order with 100 ml iso-octane and pass through column as above. Discard all iso-octane eluates. Elute benzo(a)pyrene by passing 175 ml benzene through column into evaporation flask. Drain column until solvent no longer drips. Add 2 ml hexadecane to benzene filtrate. Fit tube assembly into evaporation flask and evaporate solvent under nitrogen on steam bath as for purified benzene (p. 597), but evaporate only to 2 ml residual hexadecane (a loose aluminum foil jacket around the flask will speed evaporation). Add 10 ml iso-octane, re-evaporate, and repeat once to insure complete removal of benzene.

Quantitatively transfer 2 ml hexadecane concentrate to a 500 ml separatory funnel with portions of iso-octane (198 ml total). Wash solution with two 100 ml portions of phosphoric acid, shaking 1 min. each time. After each wash, let layers separate and discard lower (acid) layer. After final discard, swirl funnel and let it stand for a few minutes, then carefully draw off any residual acid which settles out from the iso-octane. Add 50 ml dimethyl sulfoxide (DMSO) pre-equilibrated with iso-octane and shake funnel 3 min. Set up three 125 ml separatory funnels containing 25 ml iso-octane pre-equilibrated with DMSO. After phase separation in 500 ml separatory funnel, drain lower DMSO layer into first 125 ml separatory funnel, and wash in tandem with the iso-octane contained in the three 125 ml separatory funnels, shaking each wash for 1 min. Repeat extraction with 2 more successive 50 ml portions of DMSO, washing each extract in tandem through the same three portions of iso-octane.

Collect successive DMSO extracts (150 ml total) in separatory funnel (preferably 1 L) containing 300 ml distilled water and 50 ml iso-octane. Let mixture cool for few minutes after the last extract has been added since some heat of dilution is generated. Shake funnel vigorously for 2 min. and let layers separate. Drain lower aqueous phase into a second separatory funnel (preferably 1 L) and repeat extraction with 50 ml iso-octane. Wash each 50 ml extract twice with 75 ml distilled water, shaking each wash for ca 15 seconds. Let phases separate, and discard aqueous layers after each wash. Filter iso-octane extract in first separatory funnel through Na_2SO_4 (ca 35 g, held in 30 ml coarse fritted-glass funnel or in 65 ml filter funnel with glass wool plug, and previously washed with iso-octane) into a 250 ml evaporation flask. Rinse first separatory funnel with extract from second funnel and pass through filter. Then wash second and first separatory funnels successively in tandem with two 25 ml portions of iso-octane and pass individual portions through filter. Evaporate collected filtrate on steam bath under nitrogen as previously described to ca 10 ml. Transfer solution quantitatively with benzene to 50 ml glass-stoppered Erlenmeyer flask and concentrate on the steam bath under nitrogen to 0.5 ml or less. (Caution: Do not evaporate to dryness since prolonged heating of the polycyclic hydrocarbons in the dry state will cause losses.) Save concentrated solution for thin layer chromatography.

Thin layer chromatography.—Place 50 g cellulose acetate and 275 ml 95% ethanol in glass-stoppered Erlenmeyer flask. (Ten plates can be prepared with this amount of slurry.) Shake mixture vigorously by hand for 4 min. With applicator, apply adsorbent to the plates (10×20 cm) to a thickness of 1 mm. Let air-dry 4 hr, and store in desiccator until needed.

Pour 50 ml mobile solvent (ethanol-toluene-water (17:4:4 v/v/v)) in tank and equilibrate for at least 1 hr. Apply entire benzene concentrate of food product extract to the plate in a small spot at starting line (drawn 2 cm from bottom of plate). Wash sides of flask four times with 0.2 ml benzene (graduated pipet) and transfer solvent from each wash to plate as before. After solvent has evaporated, place plate in the tank and develop chromatogram for 1.5 hr (25°C).

When development is complete, remove plate from tank and observe under both longwave and shortwave UV light in Chromato-Vue Cabinet. (This operation may be carried out while plate is still wet.) Outline the fluorescent spot of benzo(a)pyrene ca 6 cm from starting line, if present (R_f range 0.30–0.33). Remove plate from cabinet, scrape off adsorbent around spot with spatula, and discard. Transfer outlined spot to a 125 ml beaker and elute benzo(a)pyrene from adsorbent by

extracting with 5–10 ml portions of hot methanol until fluorescence under ultraviolet light can no longer be seen in last portion of solvent. Swirl flask repeatedly during extraction and successively filter individual extracts through 50 ml pressure filter under nitrogen into a 50 ml flask. (Three or four extractions are usually enough to remove compound from adsorbent.) Then, add 1.0 ml hexadecane to combined extract and evaporate methanol on steam bath under stream of nitrogen. Remove any residual methanol by two successive additions of 5 ml iso-octane and re-evaporation under nitrogen.

Carefully transfer the 1.0 ml hexadecane into 1 cm path length cell (total capacity 1.5 ml) and record ultraviolet spectrum in 250–400 mμ range, using iso-octane in reference cell. Compare any maxima observed with those in benzo(a)pyrene spectrum (at levels of 1 or 2 ppb, observable maxima will be at 283, 296, 362, 377, and 383 mμ). Estimate quantity of hydrocarbon by using baseline technique in conjunction with spectra of standard solutions recorded under same instrumental conditions. Carefully transfer the 1.0 ml hexadecane into a fluorometer cell and record excitation spectrum at emission maxima at 415 mμ. Record the emission spectrum at the excitation maxima at 390 mμ. Compare spectra obtained with those of standard solutions of benzo(a)-pyrene. Fluorometer sensitivity controls are normally set to yield 50% full scale deflection with standard containing 1.0 μg/ml.

Recovery Studies

Benzo(a)pyrene solutions were prepared in concentrations from 0.001 to 0.3 μg/ml in ethanol.

An appropriate aliquot of each solution was then placed in the 1 L reservoir containing the food sample previously analyzed and known to be free of benzo(a)pyrene. Ethanol and potassium hydroxide were then added and the procedure carried out as described.

Results and Discussion

Recoveries of benzo(a)pyrene added at levels of 2 ppb (0.15, 0.2, or 0.3 μg) to 75, 100, or 150 g of frankfurters, fish, and cheese are summarized in Table 1. Recoveries ranged from 73 to 100%. Representative ultraviolet and fluorescence spectra obtained for benzo(a)pyrene added to food samples are presented in Figs. 1 and 2. Recoveries at 1 ppb levels (0.1 and 0.15 μg added to 100 and 150 g samples, respectively) also yielded good results consistent with the above data and spectra.

Table 1. Recoveries of benzo(a)pyrene added to various food products at a level of 2 ppb

Food Product	Sample Wt, g	Recovery Range, %
Frankfurters	75, 150	87–100
Cheese	150	73–76
Fish	100, 150	90–100

During the development of this procedure, efforts were made to further simplify it by deleting the dimethyl sulfoxide partition step. Elimination of this step resulted in a significant increase of background absorbance material in the ultraviolet spectrum of benzo(a)pyrene, particularly in the 250–300 mμ region. However, the characteristic maxima in the 360–400 mμ range were still observable. Although confirmation of the compound's presence could be made by its fluorescence spectra, the excitation spectrum possessed less fine peak resolution than that obtained in recovery studies in which the partition step had been employed. In the few investigations conducted in this manner, the emission spectrum was apparently unaffected by the presence of this extraneous material.

Studies have been conducted to determine the ultimate sensitivity limits of the method for benzo(a)pyrene with respect to its visibility on the cellulose acetate plate and subsequent ultraviolet and fluorescence analysis. The lowest level that could be detected on the plate under ultraviolet illumination was 0.001 μg added to and recovered from 100 g of food product. The presence of the hydrocarbon at this level (0.01 ppb) could be detected but not definitely confirmed by its fluorescence spectrum. Positive identification of the hydrocarbon could be made at a level of 0.02 ppb. (Fluorometer sensitivity controls for the above determinations were set to yield 50% of full scale deflection

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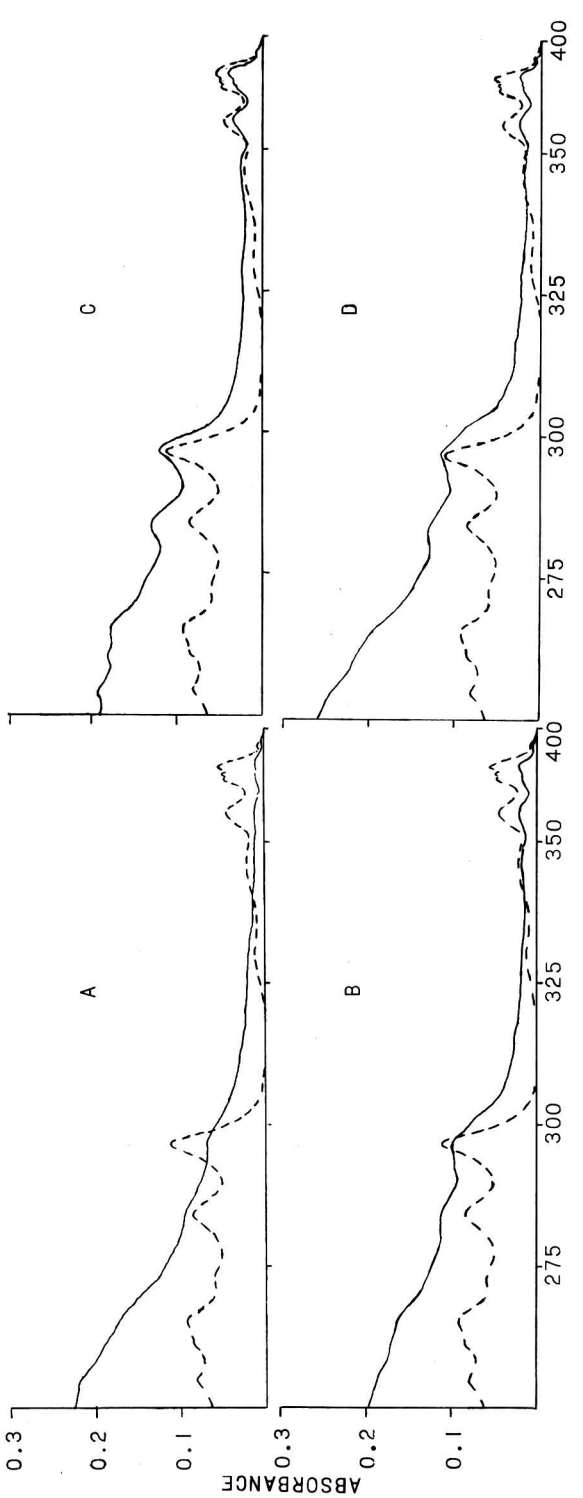

Fig. 1—Ultraviolet absorption spectra of benzo(a)pyrene. Solid line, unknown; broken line, reference. A. 0.5 ppb level recovered from 100 g haddock; B. isolated from 100 g smoked ham; C. 2.0 ppb level recovered from 150 g haddock; D. isolated from 100 g smoked fish.

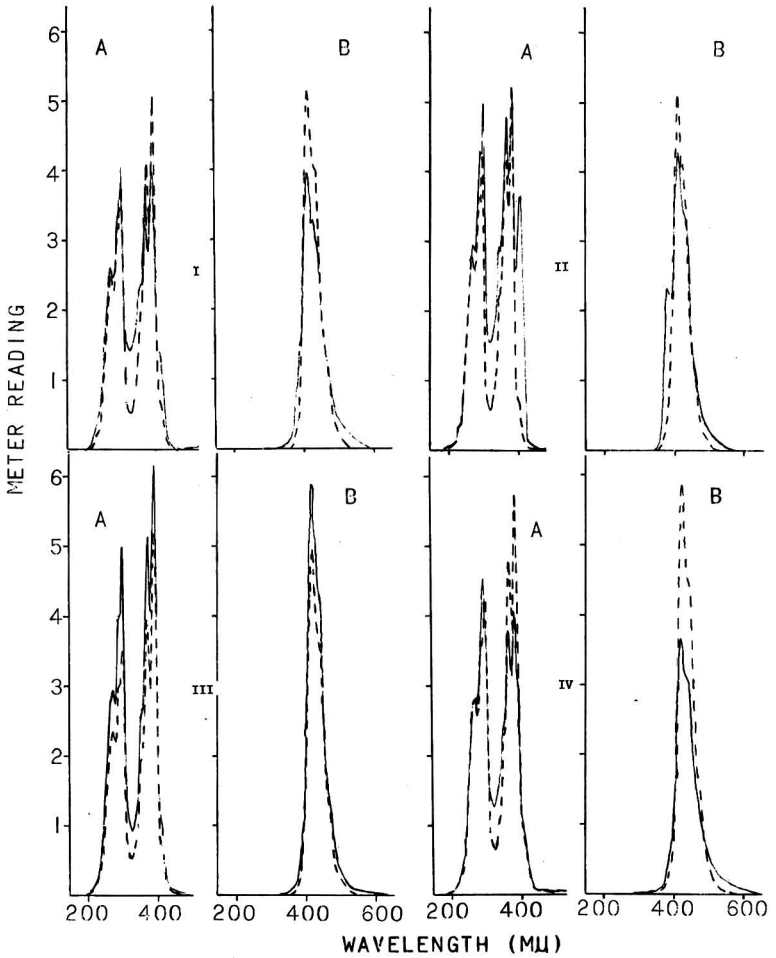

Fig. 2—**Fluorescence spectra of benzo(a)pyrene, reference standard 1.0 mg/L. Solid line, unknown or recovery; broken line, reference. I. Recovery from 100 g haddock at 0.5 ppb level. A. Excitation spectra at emission 415 mμ, sensitivity 50, 0.003 MM; B. Emission spectra at excitation 390 mμ. II. Isolation from 100 g smoked fish; A. Excitation spectra at emission 415 mμ, sensitivity 50, 0.01 MM; B. Emission spectra at excitation 390 mμ. III. Recovery from 150 g haddock at 2 ppb level; A. Excitation spectra at emission 415 mμ, sensitivity 50, 0.01 MM; B. Emission spectra at excitation 390 mμ. IV. Isolation from 100 g smoked ham; A. Excitation spectra at emission 415 mμ, sensitivity 50, 0.01 MM; B. Emission spectra at excitation 390 mμ.**

with a standard solution containing 0.3 μg/ml of benzo(a)pyrene.) With ultraviolet spectroscopy, the lowest detectable level with the procedure as described was 0.5 ppb. As shown in Fig. 1A the maxima of benzo(a)-pyrene (0.05 μg recovered from a 100 g sample) are barely discernible. The fluorescence spectra are given in Fig. 2I.

Work is in progress to extend the sensitivity of the ultraviolet determination by the development of suitable micro-spectrophotometric techniques. Current findings indicate that such techniques may increase the sensitivity at least several-fold.

The procedure has been applied to the analyses of smoked ham and fish products which had been found to contain benzo(a)-pyrene at levels of 3.2 and 1.0 ppb, respectively, in a previous undertaking (18). Although the samples were of the same type and brand as those previously analyzed, they were purchased at a different time and

therefore no direct correlation of the results obtained in the two studies can be made. Benzo(a)pyrene was again isolated from both the ham and fish products at levels of 1.7 and 2.0 ppb, respectively. Ultraviolet and fluorescence spectra confirming its identification are shown in Figs. 1 and 2. The carcinogen was also found in a cooked smoked ham sample at a level of 1.3 ppb, which suggests that cooking does not remove any appreciable amount of this hydrocarbon.

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FRUITS AND FRUIT PRODUCTS

Rapid Flame Photometric Method for Sodium in Fruits

By FREDERICK E. BOLAND (Division of Food Standards and Additives, Food and Drug Administration, Washington, D.C. 20204)

The flame photometric method of Pro and Mathers for sodium in wines and distilled spirits has been adapted to the analysis of sample solutions of fruit and subjected to collaborative testing. A sample solution of fruit is aspirated directly into the flame photometer, which eliminates the need for ashing the sample and thus effects a saving of time. Six Food and Drug Administration District Laboratories, two State laboratories, and the Associate Referee participated in the collaborative study. The results indicate that the method is sufficiently accurate for the determination of sodium in fruit, and the method is recommended for adoption as official, first action.

The increasing use of low sodium foods has created a need for a rapid and accurate method of determining sodium. The natural

sodium content of fruits is seldom more than a few parts per million. However, added sodium finds its way into fruit products such as jams and jellies through the addition of invert sugar (inverted with hydrochloric acid, which is later neutralized with sodium bicarbonate). In canning operations, apples, peaches, quinces, and other fleshy fruits are often immersed in salt solution or lye-peeled. Significant amounts of sodium may be absorbed by the fruits. Determination of sodium by the flame photometer is much more rapid than the current conventional gravimetric procedure.

The flame photometric method of Pro and Mathers (*This Journal,* **37**, 945–960 (1954)) for sodium in wines and distilled spirits was adapted for sample solutions of fruit and studied collaboratively. A sample solution of fruit was prepared by method **20.003** (10th Ed.) and aspirated directly into the flame photometer, so that the need for ashing the sample was eliminated.

Collaborative Study

Chemists from six Food and Drug Administration District Laboratories, two State laboratories, and the Associate Referee participated in the collaborative study. Collaborators were directed to follow the prescribed method.

In addition to the flame photometric method, gravimetric procedures were also used by two collaborators. The Associate Referee used the magnesium uranyl acetate method, **6.023–6.024** (10th Ed.), on solutions D, E, and F; Collaborator 2 used the sodium zinc uranyl acetate method, *This Journal,* **37**, 586–600 (1954), on solutions D and F.

Six samples (A–F) were sent in pint-size polyethylene bottles to each of eight collaborators. Each sample consisted of a sample solution of fruit made up in accordance with method **20.003**. Each solution was preserved with formaldehyde (5 ml/2 L of sample solution). Samples A, B, and C contained only the natural sodium in the fruit. Samples D, E, and F contained sodium added as NaCl; *see* Table 1 for recovery tests. The fruits used to prepare the solutions were as follows:

Table 1. Recovery of sodium from salted fruits by flame photometer (mg Na_2O/100 g fruit)

	Currant Juice	Blackberry Juice	Strawberry Juice
Amount added	70.2	70.2	65.3
Amount recovered	71.6	69.8	66.0
% Recovered	102	99.4	101

Sample A and D, tomato juice;

Sample B and E, apple juice;

Sample C and F, sample solution of apricots (100 ml equivalent to 15 g fruit).

METHOD

Reagents and Apparatus

(a) *Sodium standard solns.*—Dry reagent grade NaCl at 100° overnight and dil. 2.5422 g to 1 L with H_2O. (Soln contains 1000 ppm Na.) Dil. 10 ml to 100 ml, and further dil. 1, 2, 4, 6, 8, and 10 ml dild soln to 100 ml to make std solns contg, resp., 1, 2, 4, 6, 8, and 10 ppm Na. Store in clean, dry polyethylene bottles.

(b) *Flame spectrophotometer.*—See **11.026** (b).

Determination

Prep. sample soln as in **20.003**. Dil., if necessary, to reduce Na concn to range covered by flame photometer (preferably 4–10 ppm Na). Aspirate sample soln (dild or undild) directly into flame.

Det. %T for stds and plot curve of %T against ppm Na. Det. %T for sample and use std curve to det. ppm Na in sample or use procedure specified in instruction manual supplied with flame photometer employed, making check detns as necessary. If internal std instrument is used, add appropriate amount of LiCl to both std and sample solns.

Report as mg Na_2O/100 g sample. Na × 1.3478 = Na_2O.

Results and Recommendation

The results (Table 2) obtained by the flame photometric method agreed very closely with the corresponding values obtained by the magnesium uranyl acetate and the sodium zinc uranyl acetate methods. The Associate Referee, in determining Na_2O by the magnesium uranyl acetate method, also

This report of the Associate Referee was presented at the Seventy-ninth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 11–14, 1965, at Washington, D.C.

Table 2. Analyses of fruit for Na_2O by the flame photometer (mg Na_2O/100 g fruit)

Coll.	A Tomato Juice	B Apple Juice	C Apricots	D Tomato Juice	E Apple Juice	F Apricots	Instrument Used
1 (AR)	3	2	2	285 (296)[a]	75 (77)	482 (473)	Beckman DU
2	3	2	2	300 (285)[b]	77	500 (485)	Beckman DU
3	3	2	2	286	80	485	Beckman DU
4	3	2	2	300	78	493	Beckman DU
5	4	2	3	322	77	505	Beckman DU
6	3	2	3	293	77	510	Baird-Atomic
7	3	2	4	277	77	506	Baird-Atomic
8	3	2	3	303	86	478	Baird-Atomic
9	3	1	4	291	77	490	Baird-Atomic
Av.	3	2	3	295	78	494	
Std Dev.				13	3	11	

[a] Magnesium uranyl acetate method, **6.023–6.024.**
[b] Sodium zinc uranyl acetate method, *This Journal*, **37**, 586–600 (1954).

ran a standard stock solution of Na_2O simultaneously and obtained a recovery of 101%. The average flame photometer value (295 mg Na_2O/100 g fruit) for tomato juice, Sample D, represents 105% recovery of added sodium, whereas the corresponding figure (494 mg Na_2O/100 g fruit) for apricots, Sample F, represents 102% recovery of added sodium. The amount of sodium added to apple juice, Sample E, was not determined. The results obtained by the flame photometric method indicate that equally good results may be obtained by both the Beckman Model DU and the Baird Atomic flame photometers. Results indicate that the method is sufficiently accurate for the rapid determination of sodium in fruits.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee D, and was adopted by the Association. See *This Journal*, **49**, 172–175 (1966).

It is recommended that the flame photometric determination of sodium in fruits be adopted as official, first action.

Acknowledgments

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B. L. Glendening, Public Health Laboratories, Kansas State Department of Health, Topeka, Kan.; H. G. Thigpen, Division of Technical Services, Virginia Department of Agriculture and Immigration, Richmond, Va.; and the following members of the Food and Drug Administration; Walter Holak, New York District; B. M. Brunstad, San Francisco District; John F. Weeks, New Orleans District; Ronald E. Joyce, Buffalo District; Joseph A. McDonnell, Los Angeles District; and Thomas J. Williams, St. Louis District.

Investigations of the Gas-Liquid Chromatography of Fruit Acids

By EARL HAUTALA (Food and Drug Administration, 518 Federal Office Bldg., San Francisco, Calif. 94102)

Present analytical methods for the determination of fruit acids are long, since most require a separate determination for each individual acid. Gas-liquid chromatography offers a method capable of determining many acids by one procedure. Citric and *l*-malic acid esters were successfully prepared and chromatographed. Tartaric acid esterification and chromatography were attempted without satisfactory results. It is recommended that other stationary liquids and derivatives be studied.

The present official methods for the determination of fruit acids utilize many different techniques of analysis. The methods for citric and tartaric acids by Hartmann and Hillig (1) separate the polycarboxylic acids as the lead salts, determine regenerated tartaric acid by titration (after fractional crystallization) from an alcoholic solution, and measure citric acid gravimetrically as pentabromoacetone (after oxidation by $KMnO_4$ in the presence of KBr).

In Ferris' method for malic acid (2), the free acid is regenerated from the lead salt and separated on a standardized silicic acid column. The eluted malic acid is determined by titration. Ferris (3) later extended the use of the standardized silicic acid column to separate citric, isocitric, and tartaric acids by collecting the eluted fractions and titrating them as they came off the column.

Lactic acid is determined by Hillig's method (4), in which the acid is extracted from the other materials present by a continuous extraction apparatus and determined colorimetrically as an acid-$FeCl_3$ complex.

Other approaches have been applied to the problem. Owens, Goodban, and Stark (5, 6) separated the organic acids from products and determined many of them by paper chromatography. Harlow and Morman (7) separated and determined complex mixtures of dilute water-soluble acids by ion exclusion chromatography, using an ion exchange resin and an automatic titrating device.

For regulatory purposes, an ideal analysis would separate and quantitate the major acid constituents of a fruit or fruit product by one procedure, and this result could be used to establish acid profiles characteristic of standard fruit products. Rumsey, *et al.* (8), using gas-liquid chromatography, have come closest to this type of analysis; they have separated and semiquantitatively determined ten acids found in forage and rumen fluid after a simple esterification.

Experimental

A preliminary study of the applicability of gas chromatography to the analysis of fruit acids was carried out in this laboratory. The common fruit acids, citric, malic, and tartaric, were chosen as the basis for the study. Free citric acid was found to chromatograph without treatment, but the resulting peak was very broad and poorly shaped.

METHOD

Apparatus

(a) *Gas chromatograph.*—A Barber-Colman Model 5000 gas chromatograph, equipped with an electron attachment (radium cell), argon ionization (radium cell), and hydrogen flame detector (with Aerograph Model A-650 hydrogen generator).

(b) *Chromatographic columns.* — Three 6 mm. i.d. columns were tested: (*i*) 2′, 5% neopentylglycolsuccinate (NPGS) on Chromosorb W, 100/110 mesh; (*ii*), 2′, 5% Carbowax 20M on Chromosorb W, 100/110 mesh; (*iii*), 6′, 10% DC-200 silicone oil on Chromosorb W, 100/110 mesh.

Procedure

Esterify citric, *l*-malic, and tartaric acid standards by refluxing with MeOH and H_2SO_4. Neutralize mixture with sodium methoxide. Separate precipitated sodium sulfate by centrifugation and analyze liquid portion by gas-liquid chromatography.

Results and Discussion

The literature indicates that both polar and nonpolar columns have been used for the separation of fatty acid esters (9). The best results in these experiments were obtained on the NPGS column with the electron attachment detector and the following conditions: flash heater, 125°; column, 117°; detector, 205°; carrier gas, N_2, 70 ml/min.

The *l*-malic acid ester chromatographed as a sharp, nearly symmetrical peak at 4.2 minutes. The extent of malic acid esterification was not determined. Citric acid was uniformly esterified and chromatographed as a low, nearly symmetrical peak at about 28 minutes.

Tartaric acid does not satisfactorily elute from any of the tested columns, either as the free acid or after the esterification step at

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elevated column temperatures. Other columns will be tested to find a stationary phase suitable for the separation of all the major fruit acids.

If methyl tartrate does not elute from any of the common stationary phases, the trimethylsilyl derivatives will be prepared and tested (10).

Recommendation

It is recommended that research on fruit acids be continued to find a suitable derivative and stationary liquid phase for their gas chromatographic separation and quantitation.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee D, and was accepted by the Association. See *This Journal*, **49**, 172–175 (1966).

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Collaborative Study of the Determination of *l*-Malic Acid in Lemon Juice

By HENRY YOKOYAMA (Fruit and Vegetable Chemistry Laboratory, Western Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture, Pasadena, Calif. 94102)

Additional collaborative studies were conducted on the *l*-malic acid content of lemon juice. Results were accurate and precise; the method is recommended for adoption as official, first action.

Analytical methods for determining the content of total amino acids and total polyphenolics in lemon juice were adopted as official, first action at the 1964 AOAC meeting. At the same time further study of the method for determining the content of *l*-malic acid in lemon juice was recommended (*This Journal*, **48**, 179 (1965)). In accordance with this recommendation, additional collaborative study was carried out this year.

Samples of single-strength lemon juice were submitted to six collaborating laboratories with the request that determinations be made for *l*-malic acid.

As in the previous year, the nine analysts in the six participating laboratories were provided with samples from two lots (A and B) of single-strength lemon juice (80–100 meq. acid/100 ml juice). One subsample of each was sent to each analyst; the subsample from lot A consisted of one 6 oz can of single-strength lemon juice, and the subsample for lot B consisted of one 6 oz can of the same single-strength lemon juice as used for Sample A. Each analyst was requested to run triplicate analyses on the contents of each of the two cans received.

METHOD

Determination

In graduated cylinder mix 15 ml sample with 45 ml alcohol and let stand 10 min. Centrifuge pectin ppt. Evap. alc. juice to thick sirup (ca 1–2 ml) in rotary vac. evaporator (not $> 50°$). Add 13–14 ml H_2O to sirup and mix thoroly. Pipet 2 ml pectin-free sample into

100 ml beaker, and add 25 ml H_2O. Titr. potentiometrically to pH 8.4 with stdzd 0.1*N* NaOH. Acidity (meq./100 ml pectin-free sample) = 5 × (ml alkali).

Pipet 10 ml pectin-free sample into 25 ml vol. flask, add 1 drop phthln, and proceed as in detn of std rotation. *l*-Malic acid concn in dild, neutralized sample, $[MA]_D = R_{std} \times (\alpha_i - \alpha_u)$. Calc. citric acid:malic acid ratio by dividing 0.4 times acidity of pectin-free sample by $[MA]_D$.

Results and Discussion

Results submitted by the participating analysts are shown in Table 1.

The analyses of variance (Table 2) indicate good reproducibility of results by an analyst on a given sample. The mean value for *l*-malic acid is 20.2. The variance of the mean for 9 analysts, 2 samples, and 3 determinations is 0.118. The standard error of the mean is 0.344. The 95% confidence interval is 20.2 ± 0.69.

Data received from three collaborators was so complete that finite values for acidity (calculated as anhydrous citric acid) and for *l*-malic acid could be calculated (Table 3).

On the basis of this study and the one conducted last year, the recommended method is satisfactory for determining *l*-malic acid content in lemon juice.

Table 1. Results for citric acid/*l*-malic acid ratio

Coll.	Citric Acid/*l*-Malic Acid Ratio A	B
1	20.9	21.2
	19.9	19.5
	19.5	19.9
2	20.6	21.5
	20.1	21.0
	20.1	21.6
3	19.1	–
	18.8	17.2
	17.0	24.1
4	16.7	19.6
	17.2	17.1
	18.4	19.7
5	22.7	18.5
	22.5	20.5
	20.0	21.1
6	24.0	21.7
	21.7	21.2
	20.5	20.8
7	19.5	22.4
	20.3	21.3
	21.7	20.5
8	21.9	18.5
	21.8	19.8
	19.0	19.0
9	21.5	19.8
	19.5	19.0
	19.5	20.5

Table 2. Analyses of variance

Source of Variation	Degrees of Freedom	Mean Squares	Expected Value of Mean Squares[a]
Collaborators	8	6.31	$\sigma^2 + 3\sigma_s^2 + 6\sigma_a^2$
Samples	9	2.73	$\sigma + 3\sigma_s^2$
Analytical Errors	35	1.84	σ^2

[a] Where σ^2 = variance among determinations; σ_a^2 = variance due to analysts; and σ_s^2 = variance among sample means; $\sigma^2 = 1.84$; $\sigma_a^2 = 0.60$, and $\sigma_s^2 = 0.30$.

Table 3. Finite values by three collaborators

Coll.	Acidity Calcd as Anhydrous Citric Acid meq/100 ml	g/100 ml	*l*-Malic Acid meq/100 ml	mg/100 ml
	Sample A			
1	98.25	6.29	4.76	319.2
	98.10	6.28	4.88	327.0
	98.30	6.29	4.90	328.6
5	100.00	6.40	4.57	306.4
	100.00	6.40	4.58	307.1
	100.50	6.44	5.34	358.1
9	100.25	6.42	4.66	312.5
	100.25	6.42	5.14	344.7
	100.50	6.44	5.15	345.3
	Sample B			
1	98.60	6.31	4.59	307.8
	98.40	6.30	4.69	314.5
	98.55	6.31	4.56	305.8
5	99.55	6.37	5.38	360.8
	100.15	6.41	5.06	339.0
	100.00	6.40	5.26	352.7
9	99.5	6.37	5.03	337.3
	99.5	6.37	5.23	350.7
	99.5	6.37	4.85	325.2

Recommendation

It is recommended that the analytical method for determining the content of *l*-malic acid in lemon juice be adopted as official, first action.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee D, and was adopted by the Association. See *This Journal*, **49**, 172–175 (1966).

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Examination of Three Concentrated Black Raspberry Juices for Authenticity

By DAVID JORYSCH and S. MARCUS (H. Kohnstamm & Co., Inc., 161 Avenue of the Americas, New York, N.Y. 10013)

Gradient elution, paper, and thin layer chromatography were used to detect adulteration in black raspberry juice concentrates. Results show that a combination of gradient elution chromatography plus thin layer chromatography of the anthocyanin isolates provide definite indications of the purity of concentrated fruit juices.

Gradient elution, paper, and thin layer chromatography were used to detect adulteration of black raspberry juice concentrates.

Three black raspberry juices (A, B, and C) were analyzed by the methods. Sample B was an authentic, 5-fold black raspberry juice concentrate. A concentrated elderberry juice and a red raspberry concentrate were also checked, since these products would be likely adulterants of black raspberry juice.

The raspberry samples were diluted 1 + 4 (v/v) with deionized water, and the elderberry concentrate was diluted 1 + 5 (v/v) with deionized water. Analyses were performed on the diluted juices only.

METHODS

Gradient Elution Chromatography of Organic Acids

The method of Palmer (1), as modified by Sullivan, *et al.* (2) and Prill, *et al.* (3) and later used by Jorysch, *et al.* (4) for fruit juice analysis, was used.

The volume of diluted fruit juice found to contain 4 meq. of free acidity was diluted to 150 ml with deionized water and passed through a prepared (4, 5) Dowex, 1-X10 ion exchange resin column. The organic acids were eluted by a gradually increasing concentration of formic acid into test tubes, the water and excess acid were evaporated, and the residual nonvolatile organic acids were titrated with 0.04*N* NaOH. Curves were prepared for each juice by plotting titrant volume vs. tube number. These data were used to calculate the total GEC acidity and percentage acidity of each peak, as shown in Table 1.

Paper Chromatography of Organic Acids

Organic acids from 10 ml of diluted fruit juices were isolated as their lead salts according to the methods of Way (6), Jorysch, *et al.* (7), and Fitelson (5, 8).

Paper chromatographic analysis was performed by streaking four 3 μl volumes of an organic acid-isolate solution over ¾″ on the paper and drying with cool air between each 3 μl application.

Patterns were developed by an ethyl ether-formic acid-water system (8), and the re-

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solved organic acids were detected by a xylose-aniline reagent (8).

Thin Layer Chromatography of Anthocyanins

Two methods were used to prepare the fruit juice samples for spotting on the thin layer plates:

Mattick Method (9).—Diluted juice (5 ml) was shaken with 5 ml of absolute methanol in a 50 ml glass centrifuge tube and allowed to stand 1 hour. The precipitate was centrifuged, and 4 drops of 37–38% HCl were added to 5 ml of clear solution. Four 1 μl volumes were spotted (capillary tube pipet) on the cellulose-covered plates (10).

Modified Nybom Method (11).—Diluted juice (10 ml) was depectinized by adding 10 ml of normal propanol. The insoluble material was centrifuged; the clear liquid was treated with 10 g of Dowex 50W-X4 or 50W-X8, 100–200 mesh ion exchange resin. The resin with the absorbed anthocyanin pigments and other components was treated in either of two ways:

Centrifugation-Column Isolation (CN-CL).—The resin was centrifuged, washed with water and absolute methanol, and transferred to a 10 × 300 mm sintered glass chromatographic tube containing a cotton plug (5 mm thick) resting on the sintered filter. The resin column was eluted with 150–400 ml of 2% HCl (37%) in absolute methanol (v/v). The eluate was evaporated in a 120°F water bath under vacuum. The residue was taken up into solution in two ways: either (0.98*M* solution) by adding 2 ml of a 2% solution of HCl (37%) in absolute methanol (v/v), or (0.75*M* solution) by adding 1 ml each of a 50% solution of HCl (37%) in absolute methanol (v/v) followed by 1 ml of the 2% solution.

All-Column Isolation (AL-CL).—The resin, in contact with the depectinized and centrifuged juice, was transferred to a 10 × 300 mm chromatographic tube fitted with a cotton plug over its sintered glass filter, and allowed to stay this way overnight or for at least 1 hour. The excess liquor was allowed to partially drain, and then the column was carefully washed with 100 ml of deionized water and 50 ml of absolute methanol. Elution was performed as above. The eluate was evaporated and the residue was dissolved as above.

Thin layer plates, 20 × 20 cm, were used for these studies. The plates contained a 250 μ coating of MN 300 (normal) cellulose powder (10). Plates were heated at 105°C for 5 minutes and cooled in a desiccator before use. Sample volumes of four or six 1 μl portions were deposited on the cellulose layer 1.5″ from the bottom of the plate and separated by distances of 1.25″. A maximum of 6 samples were spotted on a plate. The spotted plate is allowed to equilibrate in the solvent atmosphere 2 hours before development. During development, the solvent was allowed to migrate 10 cm from the spotting line before it was removed. The plate was then dried by a fan, exposed to concentrated HCl vapors, and inspected in daylight and under the influence of longwave ultraviolet light.

Results and Discussion

Gradient Elution Chromatography

Table 1 gives the available or free acidity determined by titration of unchromatographed, diluted fruit juices; it also lists the total GEC acidity and peak percentages of existing acids or acids produced by acidification of their salts present in the juices.

Peak shape may be used for qualitative estimation only. The percentages of the individual peak acidities of the various juices can be compared for a more quantitative determination.

Elderberry has a very large Peak I, a lesser though appreciable Peak II, and a small Peak III.

Red raspberry has small Peaks I and II, especially when compared to Peak III, which is quite high and broad.

Black raspberry A is significantly different from black raspberry B and C. Peak I of A is rather large in comparison to Peak III. Peak II is disproportionately large in comparison to Peaks I and III of B and C.

Black raspberry B and C are similar; they differ only slightly but noticeably in the heights of Peaks I and II. Peak I was higher in B; Peak II, in C.

Adulterating black raspberry juice with elderberry juice could conceivably account for the relative increase in Peaks I and II and decrease in Peak III of A, when compared to B and C.

By gradient elution chromatography, black raspberry C appears similar to B. The slight difference in the height of Peak I for C may be due to differences and extent of depectinization of the samples.

On the other hand, black raspberry A

Table 1. Gradient elution chromatography (GEC) of juices, meq. acid/100 ml juice (meq./100)

Juice Sample	Free Acid, meq./100	Vol. GEC[a]	GEC Acidity						
			Total	Peak I		Peak II		Peak III	
			meq./100	meq./100	%	meq./100	%	meq./100	%
Black raspberry (A) #110	11.80	33.9	13.775	2.718	19.72	2.543	18.46	8.514	61.82
Black raspberry (B) #111	12.46	32.1	15.020	2.343	15.58	0.463	3.08	12.214	81.34
Black raspberry (C) #112	15.46	25.9	18.592	1.780	9.57	1.241	6.68	15.571	83.75
Elderberry, #113	11.30	35.4	13.009	9.695	74.52	2.022	15.55	1.292	9.93
Red raspberry, #115	24.52	16.0	26.094	1.158	4.44	0.936	3.56	24.000	91.97

[a] ml diluted juice containing 4.0 meq./100 ml juice.

definitely appears different from either red raspberry or black raspberry C. When the curves for elderberry juice are used as a basis, it can be concluded that a noticeable quantity of elderberry had been added to a black raspberry base, resulting in definite change in Peaks I and II of black raspberry A.

Paper Chromatography

The organic acids were isolated by the lead salt method (Pb) and detected by a xylose-aniline spray reagent with heating at 105°C for about 5–10 minutes.

Elderberry juice has a very weak spot at R_f 0.92 and a weak spot at R_f 0.53. Red raspberry juice has a very weak spot at R_f 0.65 and a very strong spot at R_f 0.59. Black raspberry A has a very weak spot at R_f 0.65 and a moderately strong spot at R_f 0.55; B has a strong spot at R_f 0.57; and C has a very weak spot at R_f 0.65 and a strong spot at R_f 0.58.

All patterns showed two organic acid spots, except black raspberry B, which showed only one. Red raspberry and black raspberry C have the most similar patterns except for the intensity of spots at R_f 0.58–0.59; the black raspberry spot is not as intense as that for red raspberry.

Black raspberry A has an acid peak at R_f 0.65 like red raspberry and black raspberry C, but the spot at R_f 0.55 is midway between elderberry (R_f 0.53) and black raspberry B (R_f 0.57).

None of the other samples have an acid spot at R_f 0.92 similar to the very weak spot of elderberry.

The paper chromatographic pattern of organic acids for black raspberry A shows the main acid spot to be lighter than that for B, but the former has another spot which seems to be characteristic of red raspberry. It is possible that less red raspberry and more elderberry were added to black raspberry A. If so, the addition of elderberry has reduced the total acidity, although red raspberry has a larger main acid spot than that of black raspberry.

Black raspberry C seems to have a more intense main acid spot and a minor spot at R_f 0.65 characteristic of red raspberry. Thus, only red raspberry could have been added to a black raspberry concentrate.

Thin Layer Chromatography

Thin layer chromatograms were made from anthocyanin isolates of the black raspberry samples, an elderberry, a red raspberry, and a desugared grape skin extract (Enociannina) and compared. Chromatograms were observed in visible light for the red anthocyanin patterns and under long-wave (3660Å) ultraviolet light for fluorescing patterns.

Mattick method.—Very little differentiating fluorescence was found on the chromatograms except for the elderberry sample, which had two light blue fluorescent spots at R_f 0.43 and 0.65, and a bright red spot at R_f 0.80.

Red raspberry had a light blue spot at R_f 0.45, a red spot at R_f 0.65, and a white area at R_f 0.95 in all samples except the elderberry; the spot was weakest in black raspberry B.

Fluorescent patterns of black raspberry A, B, and C were very similar except for relative intensities of the dull red spots; those of A were generally smaller and less intense.

The foreign grape color gave a different fluorescent pattern: the bright red fluorescing material was concentrated at the bottom third of its pattern.

The visible anthocyanin patterns showed the elderberry, red raspberry, and grape color patterns to be different from those of black raspberry A, B, and C. Of the three black raspberries, A had the weakest anthocyanin pattern, and B and C showed different intensities at the same R_f values. Black raspberry C gave a weak red spot at R_f 0.65 not present in A or B but of the same R_f value as the one weak spot found in the red raspberry pattern. The overall pattern of black raspberry B appeared stronger than that of C and noticeably stronger than that of A.

The visible pattern for elderberry is for the most part dissimilar to the black raspberry patterns, except for the red spot at R_f 0.18. Elderberry has two red spots at R_f 0.50 and 0.80, but the two large anthocyanin spots of black raspberry appear at R_f 0.41 and 0.75.

Modified Nybom method (0.75M).—The fluorescent patterns of the chromatographed isolates are relatively more intense and differentiated than in the Mattick method.

Red raspberry had a weak UV pattern; that for elderberry was strong and differentiated. Black raspberry B and C have well-differentiated patterns which are not as intense as that of A. Black raspberry B had the weakest fluorescence among the three black raspberry samples.

Any visible pattern of the anthocyanins was virtually absent for red raspberry and quite weak for elderberry, which just barely showed a spot at R_f 0.49 and a diffuse one at R_f 0.20.

Black raspberry C showed the strongest and most differentiated pattern, and A had the weakest and least differentiated pattern.

Dowex 50W-X8 gave a somewhat stronger anthocyanin pattern for black raspberry A than the 50W-X4 resin; the ultraviolet pattern was brighter and more differentiated.

The all-column (AL-CL) method gave a stronger and more differentiated anthocyanin pattern than the centrifuge-column (CN-CL) method for black raspberry A with the 50W-X4 resin. The fluorescing pattern in the all-column method was slightly more intense than that of the centrifuge-column method because of the greater concentration of red fluorescing anthocyanin pigments.

Modified Nybom method (0.98M).—Thin layer patterns of the black raspberry isolates differed when the residue of the evaporated 2% HCl-MeOH eluate was taken into solution with concentrated HCl in MeOH, either (2 + 98, v/v) or (25 + 75, v/v).

The visible patterns differed between the 0.75*M* and 0.98*M* solutions; the latter gave better and greater resolution of anthocyanin pigments and different R_f values. In both methods, black raspberry A has the weakest visible patterns. However, with the 0.75*M* solution, black raspberry C appeared stronger than B; whereas with the 0.98*M* solution, B was stronger than C.

The two solutions also gave different fluorescing patterns. With the 0.75*M* solution, black raspberry A and C have similar patterns, although A's pattern was brighter. Black raspberry B gave the weakest fluorescence. With the 0.98*M* solution, black raspberry A had the brightest fluorescing pattern, and the patterns of B and C appeared quite similar.

Observations made in visible light by the Nybom method indicate close similarity between black raspberry samples B and C. Sample A appears different in the number of spots present and the intensity of spots. The patterns of elderberry and red raspberry juice showed little definition by this method. Mattick's method shows fairly close similarity among black raspberry samples A, B, and C; however, C shows an extra spot at R_f 0.65 which is also present in the red raspberry pattern. The grape skin color which appears in red raspberry is apparently absent from samples A, B, and C, since they do not show corresponding R_f values in the black raspberry patterns.

Thin layer chromatograms with the Nybom method show bright fluorescence in the elderberry sample but only a slight effect in the red raspberry sample. Ultraviolet patterns of black raspberries A, B, and C are similar, although A has greater fluor-

escent intensity. Sample C has the next brightest fluorescent pattern, and sample B the weakest.

The ultraviolet patterns by the Mattick isolation are much less intense than those by the Nybom method. Samples A, B, and C appear very similar; however, C has a whitish fluorescence at the top of the pattern, as in the red raspberry sample. Sample A appears to have at least one weak light blue fluorescent spot (R_f 0.35) which is present in the elderberry pattern.

Results on visible and ultraviolet thin layer chromatograms confirm previous observations which indicate that elderberry was added to Sample A and that red raspberry is present in Sample C.

Summary and Recommendation

In general, gradient elution chromatography of the organic acids and thin layer chromatography of the anthocyanin isolates show the greatest differences among the three black raspberry samples.

While the organic acid patterns developed by paper chromatography showed differences, they were only slight and were difficult to interpret.

Gradient elution chromatography showed that black raspberry A was definitely different from B and C; there was only a slight difference between B and C.

Thin layer chromatography showed black raspberry A to be weaker than B and C in strength of anthocyanin pigments but of greater ultraviolet fluorescence than either B or C. This could be due to adulteration of the juice with elderberry juice. Paper chromatography of organic acids serves only to confirm these conclusions.

Gradient elution chromatography plus thin layer chromatography of the anthocyanin isolates provide definite indications of the purity of concentrated fruit juices. Standard patterns must be run at the same time for differences to be noted, since fading takes place when samples are compared to older chromatograms.

It is recommended that study be continued on the characterization of fruit juices.

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Rapid Estimation of Recoverable Oil in Citrus Juices by Bromate Titration

By W. CLIFFORD SCOTT and M. K. VELDHUIS (U.S. Fruit and Vegetable Products Laboratory[1], Winter Haven, Fla. 33880)

A new method has been developed for estimation of recoverable oil in citrus juices, based on quantitative combination in acid solution of *d*-limonene, the principal component of distilled citrus oils, with bromine. 2-Propanol is added to citrus juice, the mixture is distilled, and the distillate is acidified with HCl and titrated with potassium bromate-bromide solution. End point is observed by disappearance of color from methyl orange indicator. The distillation requires about 3 min., and complete determination, less than 7 min. Recovery of added *d*-limonene was 100 ± 1%, indicating a high degree of accuracy; replications seldom varied more than 0.001% oil, indicating excellent precision. Recoveries were consistently higher than by the Clevenger method.

Peel oil in citrus juices was considered a contaminant in the early days of the canning industry when mechanical juice extractors were in the process of development, and it was necessary for upper limits to be set by government regulations. Today, the value of peel oil for flavor is recognized and the problem is to maintain its content within optimum limits.

Since peel oil usually contains 90–95% *d*-limonene, measurement of oil recoverable by distillation has been accepted as a satisfactory approximation of peel oil in juice. In 1941, Scott (1) published the first method specifically devised for the estimation of recoverable oil. It consisted of steam distillation and measurement of the distilled oil by volume. By the time this procedure was published and adopted by the U. S. D. A. Agricultural Marketing Administration as the official grading method, Florida citrus juice canners had adapted Clevenger's spice oil technique (2) for the same purpose. After improvement in the design of condenser and oil trap, the Clevenger method was made, and still is, the official grading method.

While the Clevenger method, when used by a careful operator, can give reproducible results directly proportional to the amount of oil present in juice, wide variations between operators occur frequently. Because of the extended time required (up to 90 minutes) the operator is tempted to hurry the warm-up and/or distillation rate, with the result that up to 50% of the oil may be lost. Conversely, at too slow a rate of distillation, oil may reflux in the delivery tube and neck of the flask without ever reaching the condenser. The time involved limits the usefulness of the Clevenger method as a control procedure, since more than 100 tons of fruit could be processed while a single test was being run.

Curl (3) showed that recovery of oil was incomplete with several types of apparatus. He recommended a micro-adaptation of apparatus designed by R. B. McKinnis which permitted use of smaller juice samples (down to 500 ml) and completion of a determination in about 30 min. This apparatus is currently used in some control laboratories, but is not entirely satisfactory.

Burdick and Scott (4) and Burdick and Allen (5) devised a turbidimetric procedure that requires only 7–10 min. to complete. In this method 25 ml acetone was added to 100 ml juice, and 50 ml was distilled. When an aliquot of the clear distillate was diluted, a turbidity, proportional to the oil content, developed and was measured colorimetrically.

The turbidimetric method, as modified by Wolford, Patton, and McNary (6), Kilburn and Petros (7), and others, is currently used in several control laboratories. It is satisfactory but, for reasonable accuracy, the amount of oil distilled in a determination must be kept within narrow limits. In addi-

[1] One of the Laboratories of the Southern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

tion, variables such as distillation rate, distillate temperature, dilution volumes, and mixing techniques must be closely controlled.

The method presented here offers an additional saving in time, but its most valuable features are improved accuracy and precision. The apparatus required is simple; there should be little variation in results between operators.

In this procedure, oil is distilled from a small sample of juice mixed with a completely miscible, volatile solvent to insure solubility and dispersion of oil in the flask and to facilitate its rapid volatilization and carryover. The distillate is acidified and titrated with standard bromate solution. In acid solution, the bromate releases bromine which reacts quantitatively with *d*-limonene through saturation of the double bonds. At the end point, excess bromine completely destroys the color of methyl orange.

METHOD

Reagents

(a) *Standard 0.099N bromide-bromate solution.*—Sec. **42.018–42.019**, (8). For use dilute 1+3 to give 0.0247*N* solution. Calculation of stoichiometric relationships indicates that 1.00 ml of 0.0247*N* bromate supplies bromine to react with 0.00085 g, or 0.0010 ml, of *d*-limonene. The standard solution remains stable for several months, and the dilute solution for 2 weeks or more.

(b) *2-Propanol.*—Reagent grade, ACS.

(c) *Hydrochloric acid.*—Dilute concentrated acid with 2 volumes of water.

(d) *Methyl orange indicator.*—0.1% in water.

Apparatus

(a) *Electric heater.*—With recessed refractory top, 750 watts.

(b) *Still, all glass.*—500 ml round-bottom flask with 24/40 ⅀ -neck; 200 mm Graham condenser with 28/15 receiving socket and drip-tip; connecting trap and adapter as shown in Fig. 1.

(c) *Buret.*—10 or 25 ml, with easily controllable flow to permit dropwise titration. A microburet with spring-loaded Teflon plug has proved most satisfactory, since it can be easily cleaned and quickly refilled with reagent from a polyethylene wash bottle.

(d) *Automatic pipet or dispensing head.*—25 ml.

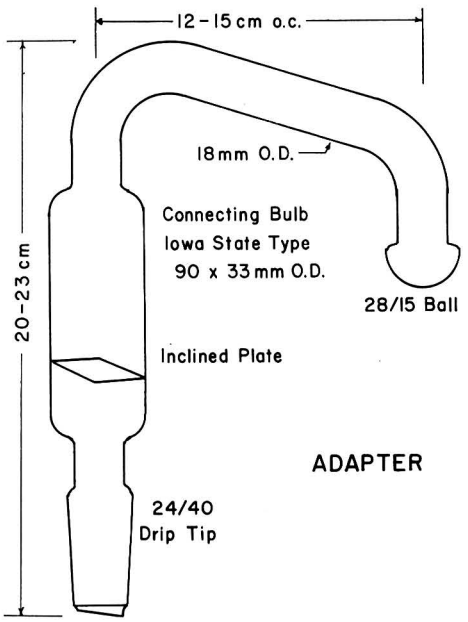

Fig. 1—**Connecting tube adapter for direct distillation.**

(e) *Automatic acid dispensing buret.*—100 ml graduated in tenths, equipped with soda-lime absorption tubes, for dilute HCl.

Procedure

Preheat electric heater. Place 25 ml juice in the distillation flask containing a large carborundum crystal, and add 25 ml 2-propanol. Distill into 150 ml beaker. Boiling will be vigorous as long as solvent is present, will subside and become strong again. Completion of distillation (about 3–3.5 min.) will become apparent by (*1*) the sirupy consistency of the juice because of concentration effect; (*2*) condensation of water in the connecting tube; and (*3*) collection of 30 ml or more distillate.

Stop boiling by removing the flask. Leave the heater on for the next determination.

Place a short magnetic bar in the beaker; add 10 ml dilute HCl and 1 drop of indicator. Titrate with dilute bromate solution while stirring. The major portion of titrant may be added rapidly, but the end point must be approached at about 1 drop/sec. Disappearance of color indicates the end point. Ml titrant × 0.004 = per cent recoverable oil by volume. Blank titrations are usually less than 0.2 ml (0.0008% oil) and may be ignored except at very low oil levels.

Alternative Procedure

Steam distillation may be more convenient than the direct distillation procedure described above when a one-piece microchemical distillation apparatus (9) is available. In this case, pipet 25 ml juice into inner chamber, followed by 15 ml solvent, and distill into a beaker containing another 15 ml portion of solvent. Oil distillation is essentially complete about 30 seconds after beading in the condenser stops (3–4 min.).

Use an automatic titrimeter to eliminate personal bias in reading end points and free the operator from the task of watching a drop-by-drop titration when the approximate oil concentration is not known.

When electrometric titration is used, methanol may be preferred to 2-propanol as solvent, because it affords more pronounced increase in voltage (250–350 mv) at the end point than 2-propanol (150–200 mv). Also, 2-propanol reacts slowly with excess bromine so that the voltage drops, causing the instrument to deliver additional titrant. These shortcomings do not, however, eliminate use of 2-propanol. The smaller change in potential is not important if the original potential of each solution is checked prior to titration and the instrument cut-off is set at about 60 mv higher. If the operator is available to observe the end of the titration, he can turn off the instrument before reaction of solvent with excess bromine calls for additional titrant.

Results and Discussion

Recovery of Added Oil

A reference solution of *d*-limonene freshly distilled from orange oil, 0.1% by volume in 2-propanol, was prepared. Aliquots of 2–15 ml were added to a mixture of 25 ml 2-propanol and 10 ml water (some water is required for the reaction), acidified, and titrated directly to confirm the stoichiometric relationship between titrant and reference solution. To test the adequacy of the distillation procedure, similar aliquots of the reference solution plus enough solvent to make 25 ml were added to 25 ml portions of water and of low-oil orange juice, and distilled. The distillates were acidified and titrated to visual end points.

Results are shown in Table 1. The "blank" titers for water solution and distillate show the amount of bromate solution required to decolorize one drop of methyl orange indicator. "Blank" titers in these and many other tests varied between 0.10 and 0.20 ml, an indication of the limit of precision of the titration. The 0.45 ml blank for orange juice distillate includes both the indicator blank and natural oil distilled from the juice.

After blank values were subtracted from the direct titration volumes, the 0.1% *d*-limonene and 0.0247*N* bromate solutions were equivalent within experimental error. Subtraction of blank values from titers of distillates from water and orange juice samples containing added *d*-limonene indicate that losses in the distillation procedure were insignificant. When volume of bromate solution was converted to equivalent oil percentages, recoveries ranged from −0.0006% to +0.0004% of the oil percentages added; these variations were not affected by the amount of oil present within the range studied. These data strongly indicate that both the accuracy and precision of the method for measuring recoverable oil, as *d*-limonene, are very good.

Comparison with the Clevenger Method

For the Clevenger tests, 4–6 L portions of juice were thoroughly mixed and kept constantly stirred until sampling was completed. The tests were usually made on 1500 ml portions, with 3 L boiling flasks and 1.0 ml oil traps. An antifoam agent was used,

Table 1. Recovery of added oil in aqueous solution and in orange juice

Oil Added, ml 0.1% Solution	Titer, ml 0.025*N* Bromate Solution		
	Direct	Water Distillate	Orange Juice Distillate
0.0	0.14[a]	0.13[a]	0.45[a]
2.0			2.10
3.0		2.93	
4.0	4.01		4.00
5.0		5.07	
6.0	6.01		6.00
7.0		7.02	
8.0	7.96		8.00
9.0		9.04	
10.0	9.96		10.10
11.0		10.97	
12.0	12.06		11.85
13.0		13.02	

[a] Average blank for which titrations in each column were corrected.

and the sample was brought to incipient boil with a Meker burner. This burner was then replaced with a Bunsen burner adjusted to maintain boiling at a rate of about 50 drops/min., which was maintained for 1 hr. The extreme care exercised in these tests was reflected in unusually close agreement among replications.

At the time these particular studies were made, the new procedure consisted of steam distillation with methanol as solvent followed by bromate titration. Test samples were taken from the large samples prepared for the Clevenger runs.

Comparative recoveries of oil from orange and grapefruit juices by the two methods are shown in Table 2. All replications are shown to indicate the degree of precision obtained. The short method was slightly more precise than the Clevenger, and resulted in the recovery of more oil. The difference was fairly consistent at 0.003% oil. The presence of added essence did not change this relationship.

Comparative recoveries from canned tangerine juice are shown in Table 3. Data are also included for comparison of solvents and distillation procedures. Results are reported to an additional decimal place to better illustrate comparative precisions. The first two columns of the table present information similar to that shown in Table 2. The bromate method, in comparison with the Clevenger, was as satisfactory with tangerine juice as with orange and grapefruit juices. By observation of data columns 2 and 3, it is apparent that substitution of 2-propanol for methanol improved both recovery and precision. In the direct distillation procedure, methanol frequently produced excessive foaming, while with 2-propanol, excessive foam did not develop until all solvent had been distilled over and considerable concentration of juice had taken place. Results shown in the last column, together with the 100% recoveries of added oil reported in Table 1, indicate that direct distillation with 2-propanol will serve adequately as a reliable method for oil determination, both in plant control and for quality grading purposes.

Comparative recoveries from lemon and lime products by the Clevenger method and bromate titration following direct distillation with 2-propanol are shown in Table 4. All the concentrates were commercially packed, while the purees were made from fresh fruit by comminution in a Waring Blendor. It is quite interesting that the recoveries from the freshly prepared purees were in almost perfect agreement. Since there is inevitably

Table 2. Comparison of oil recoveries from orange and grapefruit juices: Clevenger method compared to bromate titration after steam distillation with methanol

Type of Sample	Recovered Oil (% by Volume)		
	Clevenger Method	Bromate Titration	Bromate Titration −Clevenger Method
Orange juice			
Reconstituted (A)	0.004	0.011	
	0.006	0.012	+.006
	0.003	0.011	
(A) + added oil	0.026	0.029	
	0.025	0.029	+.003
	0.026	0.029	
Reconstituted (B)	0.005	0.008	
	0.005	0.008	+.003
		0.009	
(B) + added oil	0.011	0.014	
	0.012	0.015	+.003
	0.010	0.014	
Reconstituted (essence added) (C)	0.004	0.007	
	0.004	0.007	+.003
(C) + oil	0.017	0.020	
	0.016	0.020	+.003
Fresh	0.005	0.010	
	0.008	0.011	+.003
	0.008	0.010	
Fresh	0.012	0.016	
	0.015	0.016	+.003
Grapefruit juice			
Reconstituted (Evap. pumpout)	trace	0.0012	
	trace	0.0006	+.001
	trace	0.0014	
Fresh	0.029	0.026	
	0.025	0.024	−.001
Fresh	0.027	0.030	
	0.029	0.030	+.002
		0.030	
Canned (substandard)	0.026	0.028	
	0.028	0.031	+.003
		0.029	

Table 3. Comparison of oil recoveries from canned tangerine juice by the Clevenger and various bromate procedures

Sample		Recovered Oil (% by Volume)			
			Steam Distillation		Direct Distillation
		Clevenger	Methanol	2-Propanol	2-Propanol
A-1		0.0286	0.0306	0.0320	0.0320
		0.0286	0.0320	0.0320	
			0.0306		
			0.0320		
	Av.	0.029	0.031	0.032	0.032
A-2		——	0.0288	0.0310	0.0306
			0.0294	0.0310	0.0308
			0.0266		0.0308
	Av.		0.028	0.031	0.031
B		0.0168	0.0172	0.0198	0.0192
		0.0166	0.0168	0.0198	0.0196
			0.0167	0.0196	0.0190
			0.0170		0.0194
	Av.	0.017	0.017	0.020	0.019

Table 4. Comparison of oil recoveries from lemon and lime products by the Clevenger method and bromate titration after direct distillation with 2-propanol

Samples	Recovered Oil (% by Volume)		
	Clevenger Method	Bromate Titration	Bromate Titration −Clevenger Method
Lemonade, reconstituted	0.006	0.010	+.004
Lemonade, reconstituted	0.004	0.007	+.003
Lemonade, reconstituted	0.006	0.010	+.004
Lemon juice, reconstituted	0.044	0.048	+.004
Lemon (Calif.) puree	0.038	0.039	+.001
Limeade, (Persian) reconstituted	0.005	0.008	+.003
Lime (Persian) puree	0.052	0.052	0
Lemon (Avon) oil, distilled	0.058	0.063	+.005

a slight loss in the Clevenger determination, there must be a component of the fresh oil that does not react with bromine at the same rate as does *d*-limonene, resulting in a lowered titration value.

Florida cold-pressed lemon oil was steam distilled, then subjected to recovery tests from model systems by the two methods. As shown in Table 4, the bromate method gave better recovery. This still amounted to only 94% of the oil added as compared to practically complete recovery of freshly distilled orange oil.

Since some lemon oils contain as much as 4% citral, which is not an important component of orange and grapefruit oils, this compound was tested for its response to the solvent distillation and bromate titration procedure. Citral reacts with only half as much bromine as *d*-limonene, both on direct titration and following solvent distillation. Evidently, only one of the double bonds of citral opened under the conditions of this reaction.

Since lemon oil contains as much as 5% α-pinene, this compound was also tested. Although α-pinene contains only one double bond, it was found to utilize bromine in an amount equal to that of *d*-limonene with two double bonds. This may be due to a secondary reaction following bromination of the double bond, or to molecular rearrangement in acid solution.

Operating Variables

Since a major goal of this investigation was a reduction in time required for a determination, distillations were conducted at maximum rates achievable with the equipment at hand. For both steam and direct distillations, straight condensers occasionally gave erratic results. When water pressure decreased during a series of tests, distillates were as hot as 150°F, which is not conducive to retention of volatiles. This problem was solved by use of spiral condensers. The time required for the distillations reported in Table 1 varied from 2.7 to 3.5 min., as compared to 5–6 min. for steam distillations, and 1.5 hr for Clevenger distillations.

Solvent concentrations were adjusted to give adequate recovery, while avoiding waste, and to maintain complete solubility of oil in the distillate.

Concentration of HCl was varied from 1 ml to 10 ml of concentrated acid in the titrating vessel. Reproducible results with high oil samples were not obtained at the lower level, while at the higher levels, the results were consistently low. The 10 ml por-

tion of acid diluted 1 + 2 was chosen to give 3.3 ml HCl plus enough water to facilitate the bromination reaction. Highly accurate reproduction of the acid component is not critical unless automatic titration is used, and then only to insure replication of initial potentials.

HCl is obnoxious in the laboratory, even when diluted, unless carefully handled. Attempts to replace HCl with H_2SO_4 were not successful. The reaction was so slow that titration end points were overshot by at least 25%. Addition of NaCl with the H_2SO_4, to provide chloride ion in strong acid, did not appreciably affect the reaction rate. Several automatic pipets were tried for dispensing the dilute HCl, but fumes still escaped. A 100 ml dispensing buret, fitted with soda-lime tubes, satisfactorily contained the HCl fumes.

All experimental work reported here was conducted on single strength or reconstituted juices. No special sample preparation would be necessary in plants canning single-strength juices or laboratories grading quality of concentrates where concentrates have to be carefully reconstituted for other purposes. When the oil content of high density concentrates, centrifuge sludges, etc., is needed, the product should be quantitatively diluted so that a 25 ml aliquot would have an oil content within a reasonably specific range. Because of the small amount used, juices and diluted products must be very thoroughly mixed to obtain truly representative sampling. Products containing discrete oil particles should be diluted with solvent.

We believe that these investigations have been broad enough to show that solvent distillation and bromate titration can be used satisfactorily to indicate peel oil levels in all types of citrus juice products now being commercially produced. Many components of citrus oils, other than those specifically studied here, are unsaturated to approximately the same degree as *d*-limonene. Those whose reactions with bromine in acid solution are appreciably different are present in small quantities and do not affect the usefulness of the method.

The procedures described above were presented orally in the form of a progress report to the Florida citrus industry on October 13, 1965. Several quality control chemists tried this method in their laboratories. To date, four have reported excellent results: they compared the new method with those routinely used for oil determinations on a variety of products such as single-strength juices, concentrates, citrus drinks, and emulsions.

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PESTICIDE RESIDUES

Rapid Cleanup Techniques for Chlorinated Pesticide Residues in Milk, Fats, and Oils

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A cleanup procedure suitable for analysis of chlorinated hydrocarbon pesticide residues in milk, fats, and oils at the 0.01 ppm level was developed. Acetone removes the milk solids and water from milk. Pesticides are efficiently separated from milk fat and other fats and oils with a small volume of aqueous acetonitrile. The Mills, Onley, & Gaither Florisil technique is used as the final cleanup. The procedure is rapid, and recoveries from fortified samples ranged from 80 to 106%.

Isolation of pesticide residues from milk and fats or oils for analysis by electron capture gas chromatography usually involves difficult and lengthy cleanup procedures. The classic Mills procedure (1) for chlorinated pesticides with various modifications is probably still the most widely used, but a number of other procedures have been reported. Eidelman (2) used dimethyl sulfoxide to extract chlorinated pesticide residues from acetone and petroleum ether solutions of fats. McCully and McKinley (3) obtained a separation by precipitating the fat from a benzene-acetone solution which was cooled to − 70°C. Ott and Gunther (4) heated butterfat to 190°C and used a stream of nitrogen to sweep the pesticides into a cooled trap. Langlois, Stemp, and Liska (5) ground dairy product samples with Florisil to produce a free-flowing powder which was added to the top of a partially deactivated Florisil column. Pesticides were then eluted in a form said to be suitable for electron capture gas chromatography.

The present procedure actually consists of two parts: (*a*) the isolation of fat from milk and (*b*) the extraction and cleanup of the pesticide residues from fats and oils. The addition of acetone to milk, to extract the fat, results in a coarse precipitate of milk solids which is removed by filtering through glass wool. The filtrate is then extracted with petroleum ether, and the extract is concentrated for the cleanup procedure.

Fats and oils, including the milk fat extract, are distributed on a column of unactivated Florisil, and the pesticides are eluted with 10% water-acetonitrile mixture. The extract is then partitioned into petroleum ether; the Florisil column described by Mills, Onley, and Gaither (6) is used for final cleanup.

METHOD

Reagents

(a) *Acetone.*—Redistilled from glass.[1]

(b) *Acetonitrile.*—Redistilled from glass.[1]

(c) *Petroleum ether.* — Redistilled from glass.[1]

(d) *Florisil.*—Floridin Co., 2 Gateway Center, Pittsburgh, Pa. 15222. 1200°F activation, 60–100 mesh. (1) Unactivated: use as received from the manufacturer. (2) Activated: heat Florisil for at least 5 hours at 130°C.

(e) *10% Water in acetonitrile.*—Add 50 ml water to 500 ml volumetric flask and dilute to volume with redistilled acetonitrile (b).

Apparatus

(a) *Separatory funnels.*—With Teflon stopcocks; 1 L.

(b) *Chromatographic columns.*—25 mm o.d. × 300 mm long, with Teflon stopcocks.

(c) *Kuderna-Danish concentrators with graduated tubes.*

(d) *Disposable glass transfer pipets.*

Sample Preparation

Milk.—Weigh 50 g whole milk into 400 ml beaker containing loose glass wool (about 0.3 g). Add 100 ml acetone and stir gently with a glass rod. Filter the liquid phase through glass

[1] Available from Burdick and Jackson Laboratories, Inc., Muskegon, Mich.

wool in a glass powder funnel into a 1 L separatory funnel, pressing fluid out of glass wool in beaker. Rinse contents of beaker twice with 25 ml portions of acetone, add washings to the separatory funnel, and again press fluid out of glass wool in beaker. Slurry contents of beaker twice with 100 ml petroleum ether and filter into separatory funnel in same manner as before. Shake separatory funnel thoroughly. Add 300 ml water and 10 ml saturated NaCl, mix well, and allow 10 minutes for phases to separate. Discard lower aqueous phase.

Pass the petroleum ether layer through a 3″ column of anhydrous $NaSO_4$ into a Kuderna-Danish concentrator and rinse separatory funnel and column with small amounts of petroleum ether. Concentrate to 5–10 ml. Use this extract for the following cleanup procedure for fats and oils.

Fats and oils.—Weigh 2 g fat or oil in a test tube. Dissolve in 5 ml petroleum ether.

Extraction and Cleanup

Transfer about 2 ml of the petroleum ether solution, using a disposable transfer pipet, to 3″ of dry, unactivated Florisil in a chromatographic column. Let solution sink into the Florisil. Apply vacuum to tip of column to remove solvent. Transfer remainder of sample to the column 2 ml at a time, removing solvent after each transfer. Rinse test tube twice with 2 ml petroleum ether and transfer rinses to column, applying vacuum after each transfer. Remove all solvent from column with vacuum.

Elute column with 70 ml 10% water in acetonitrile and receive into a 1 L separatory funnel containing 100 ml petroleum ether (eluate is about 50 ml). Shake separatory funnel vigorously. Add 300 ml water and 10 ml saturated NaCl to separatory funnel. Mix well and allow 10 minutes for the phases to separate. Discard lower phase. Wash petroleum ether phase twice with 50 ml portions of water. After discarding last water wash, wait 2 minutes, and drain off water that separated.

Complete the cleanup, using an activated Florisil column as described by Mills, Onley, and Gaither (6). Concentrate each eluate from the Florisil column to a definite volume (4–10 ml) using a Kuderna-Danish concentrator. Make determination by gas chromatography, injecting aliquots equivalent to 4 mg fat or oil or 100 mg milk into electron capture instrument. (Use conditions described in (7).)

With the gas chromatographic conditions used (7), the electron capture detector response to 1 ng of aldrin is one-half of full scale with an electrometer setting of 10^{-9} AFS. If the final volume of the cleaned up sample is 4 ml, an 8 μl injection is convenient for testing for pesticide residues at the 0.01 ppm level in 50 g milk and at the 0.25 ppm level in 2 g fat or oil.

Results and Discussion

Whole milk samples were fortified with a mixture of 8 pesticides and DDE at a level of 0.01 ppm for each compound. Other samples were singly fortified with chlordane at 0.01 ppm, methoxychlor at 0.05 ppm, and toxaphene at 0.1 ppm. Recoveries averaged from 80 to 99% for the various pesticides and are listed in Table 1.

Samples of corn oil and butterfat were also fortified with these same pesticides at the 0.75 ppm level for toxaphene and 0.25 ppm levels for the other compounds. Results shown in Tables 2 and 3 indicate average recoveries of 82–106% for the individual pesticides.

In Fig. 1, the lower tracings are the gas chromatographic electron capture response for 1 ng quantities of pesticide standards. Comparable responses in an analysis representing 100 mg milk would indicate pesticides present at an 0.01 ppm level. The sample tracings were obtained in analyses of

Table 1. Recoveries of pesticides from 50 g samples of fortified whole milk

Pesticide Added (0.01 ppm)	Analysis of Unfortified Milk (ppm)	% Recoveries[a] (Corrected for Blank)				
		A	B	C	D, E[b]	Av.
Lindane	0.000	95.7	99.3	103.3		99.1
Heptachlor	0.000	85.3	72.7	82.2		80.1
Aldrin	0.000	84.3	99.2	94.5		92.7
Heptachlor epoxide	0.007	80.3	91.4	95.1		88.9
DDE	0.002	90.8	88.9	86.9		88.9
DDD	0.002	86.8	85.3	83.4		85.2
p,p′-DDT	0.004	78.2	84.8	84.6		82.5
Dieldrin	0.002	89.8	88.8	101.5		93.4
Endrin	0.000	87.7	101.3	94.3		94.4
Toxaphene[b]	0.000				80.5	
Methoxychlor[b]	0.000				88.3	
Chlordane[b]	0.000				92.3	

[a] A, B, and C are replicate samples.

[b] Sample D was fortified with 0.1 ppm toxaphene only; Sample E was fortified with 0.05 ppm methoxychlor and 0.01 ppm chlordane.

Table 2. Recoveries of pesticides from 2 g samples of fortified corn oil

Pesticide Added (0.25 ppm)	Analysis of Unfortified Oil (ppm)	% Recoveries[a] (Corrected for Blank) A	B	C	D, E[b]	Av.
Lindane	0.000	92.5	97.7	91.8		94.3
Heptachlor	0.000	90.3	94.5	90.3		91.7
Aldrin	0.000	90.7	94.3	90.0		91.7
Heptachlor epoxide	0.000	93.7	99.5	95.7		96.3
DDE	0.000	95.6	84.3	95.3		91.7
DDD	0.000	97.4	105.8	101.6		101.6
p,p'-DDT	0.030	103.5	106.8	109.1		106.5
Dieldrin	0.021	95.7	88.3	114.1		99.3
Endrin	0.000	95.5	104.4	98.5		99.5
Toxaphene[b]	0.000				90.0	
Methoxychlor[b]	0.000				91.8	
Chlordane[b]	0.000				87.0	

[a] A, B, and C are replicate samples.
[b] Sample D was fortified with 0.75 ppm toxaphene only; Sample E was fortified with 0.25 ppm each of methoxychlor and chlordane.

Table 3. Recoveries of pesticides from 2 g samples of fortified butterfat

Pesticide Added (0.25 ppm)	Analysis of Unfortified Butterfat (ppm)	% Recoveries[a] (Corrected for Blank) A	B	C	D, E[b]	Av.
Lindane	0.000	99.5	94.4	100.9		98.3
Heptachlor	0.000	97.9	93.0	95.3		96.1
Aldrin	0.000	95.8	91.0	92.5		94.0
Heptachlor epoxide	0.013	98.3	92.2	96.2		95.6
DDE	0.046	97.5	105.8	101.8		101.7
DDD	0.023	107.5	100.0	95.0		100.8
p,p'-DDT	0.065	105.1	96.2	106.2		102.5
Dieldrin	0.066	109.8	112.2	95.4		105.8
Endrin	0.000	108.2	100.0	93.8		100.7
Toxaphene[b]	0.000				90.0	
Methoxychlor[b]	0.000				82.3	
Chlordane[b]	0.000				95.1	

[a] A, B, and C are replicate samples.
[b] Sample D was fortified with 0.75 ppm toxaphene only; Sample E was fortified with 0.25 ppm each of methoxychlor and chlordane.

extracts prepared by the methods described in this paper. The calculated analyses of these samples are found in Table 4. Table 4 also includes analyses for cod liver oil, palm oil, coconut oil, olive oil, and peanut oil.

Emulsions are formed with many of the solvents used to extract fat and pesticides from milk. Special care is required to minimize emulsions, and much time is spent in breaking them. In this method no special precautions are needed to prevent emulsion formation.

All unnecessary apparatus and reagents were eliminated, and manipulations were reduced or simplified.

Glass wool placed in the beaker retains the milk solids and facilitates filtration. The 200 ml of petroleum ether extracts the pesticide residues from the aqueous acetone-milk fluid mixture in one step and minimizes pesticide losses. This large volume of petroleum ether is easily and quickly removed in the next concentration step.

Attempts to develop a one step cleanup for fats and oils proved to be unrealistic. Although results could be obtained, the sample extracts contained extraneous matter which produced undesirable GLC effects such as shortened column life and the need for frequent detector cleaning. Therefore, a preliminary cleanup was combined with the Mills Florisil column cleanup (1) to obtain sample extracts with a minimum of interfering materials for good gas chromatographic analysis. Although some new techniques have been introduced, no new reagents or equipment have been used.

The amount of fat or oil that can be cleaned up by this procedure is limited to 2 g since larger amounts overload the column. Regardless of the amount of starting material (e.g., 0.5–2.0 g), a constant eluting volume will elute the same amount of oil, about 0.1 g under the conditions described. Thus, in this method, the pesticides are separated from fat or oil by solubility rather than chromatography. The Florisil, as used here, serves only as a support for the fat or oil. The degree of activation is not critical, and Florisil may be used as received from the supplier.

The pesticides are completely removed by the first 50 ml of the 10% water-acetonitrile eluting solvent. Of the total 70 ml eluting solvent used, about 20 ml remains on the column and serves merely to force the other 50 ml through. The use of aqueous acetonitrile reduces the amount of oil carried through, without affecting the pesticide

recovery. The same amount of 100% acetonitrile will elute about 0.5 g of oil from the column.

To reduce the carry-through of oil, all solvents used to transfer the oil or fat sample to the column must be removed. Vacuum from ¼″ rubber tubing connected to an aspirator is used for this purpose. The tubing is slipped on to the tip of the column for up to 3 minutes after each sample transfer. Before attaching the rubber tubing, it is often helpful to apply vacuum momentarily to draw solvents down into the Florisil. Then, with a constantly applied vacuum, the solvents will be drawn off as vapors. The application of vacuum in this manner is not a critical step; pesticides have been retained on the column quantitatively when vacuum has been applied for as long as 15 minutes.

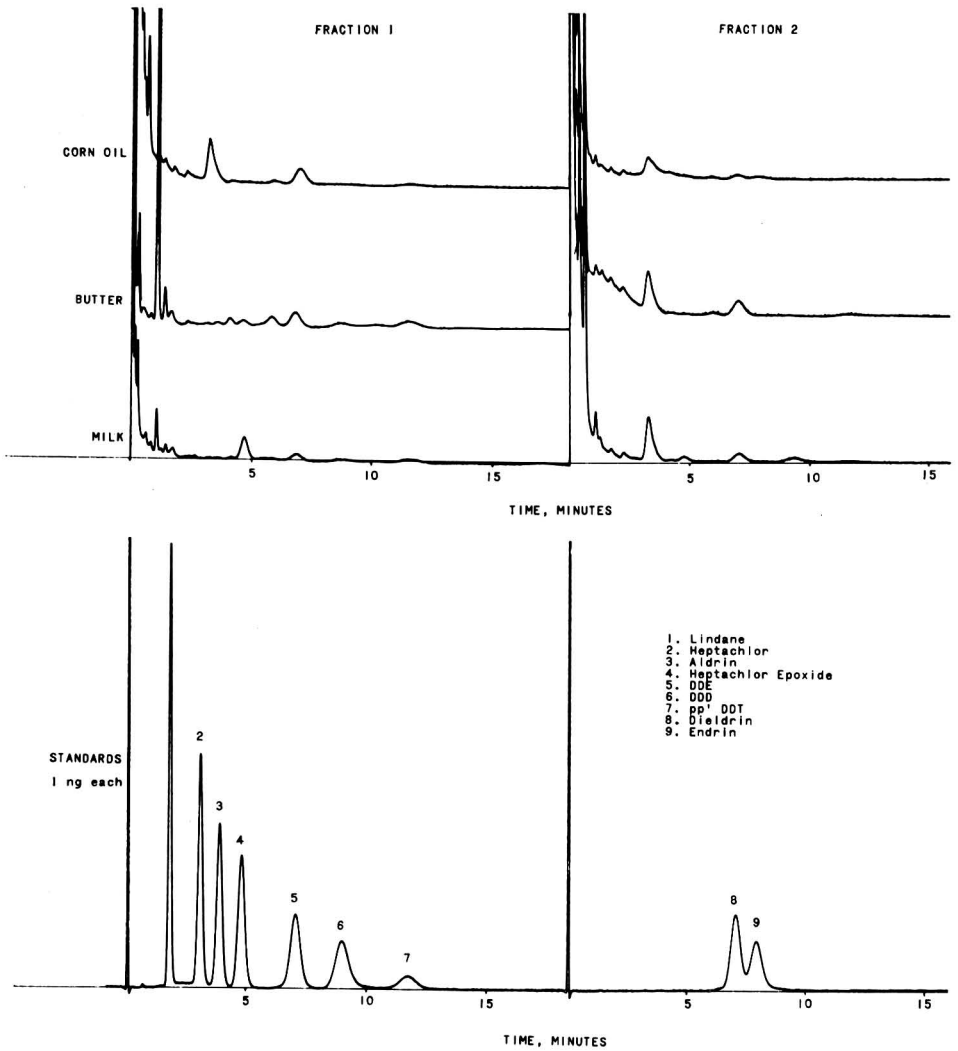

Fig. 1.—**Gas chromatograms of corn oil, butter, and milk samples, and mixture of chlorinated pesticide standards. The upper chromatograms represent unfortified samples obtained by injecting aliquots equivalent to 4 mg corn oil, 4 mg butter, and 100 mg milk; the lower show the response for 1 ng quantities of common pesticides at the same sensitivity setting as the above traces. If present in the above samples, these peaks represent levels of 0.25 ppm in corn oil and butter and 0.01 ppm in the milk.**

Table 4. Analyses of various oil and dairy product samples

Sample[a]	Found (ppm)						
	Aldrin	Heptachlor Epoxide	DDE	DDD	*p,p'*-DDT	Dieldrin	Endrin
Milk	0.000	0.005	0.003	0.002	0.006	0.002	0.000
Butterfat	0.000	0.013	0.046	0.028	0.065	0.066	0.000
Corn oil	0.000	0.000	0.000	0.000	0.030	0.021	0.000
Cod liver oil	0.000	0.038	0.496	0.834	0.598	0.100	0.036
Palm oil	0.000	0.000	0.000	0.012	0.036	0.011	0.013
Coconut oil	0.000	0.000	0.007	0.000	0.062	0.053	0.129
Olive oil	0.000	0.011	0.000	0.025	0.047	0.013	0.000
Peanut oil	0.027	0.018	0.025	0.101	0.033	0.601	0.000

[a] Sample size used was 2 g except for milk which was 50 g.

An hour is required to process four milk samples to a concentrated petroleum ether solution containing pesticides and milk fat. Four fat or oil samples can also be prepared for the final cleanup in an hour.

Conclusions

Procedures have been developed for the preparation of milk, fat, and oil samples suitable for 0.01 ppm level analysis of chlorinated hydrocarbon pesticides by electron capture gas chromatography. Cleanup of these products has been a major problem and troublesome because of the affinity of pesticides for fat and oil. An excellent separation can be achieved easily with a small volume of aqueous acetonitrile as the eluant. In a preliminary treatment of milk, milk solids and water are quickly separated by using acetone. Pesticides are recovered quantitatively, and the extracts are sufficiently cleaned up so that there are no deleterious column or detector effects produced.

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Paper Chromatography in Pesticide Residue Analysis[1]

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Some of the paper chromatographic techniques and detection methods used for pesticide residue analysis are reviewed. Procedures utilized for quantitative estimation pesticide residues separated by paper chromatography, such as measurement of spot size, combusion prior to analysis, and elution before chemical analysis, are also discussed. The advantages and disadvantages of paper chromatographic techniques and the problems inherent in the paper chromatographic separation and detection of minute amounts of pesticide residues are outlined. Additional applications and modifications of paper chromatography to extend its usefulness in pesticide residue analysis are suggested.

[1] This paper was presented as part of the Symposium on Unit Processes in Residue Analysis conducted at the 149th Annual Meeting of the American Chemical Society, April 4–9, 1965, at Detroit, Mich.

Paper chromatography has been defined as the separation of a mixture of substances by the passage of solvent through paper in a definite direction (1). Ideally, each substance in a mixture will move along with the solvent at a unique rate, so it will occupy a distinct position somewhere along the solvent path. The basic technique can be varied in many ways by changing features such as direction of solvent movement, type of paper, modification of the paper by chemical treatment, impregnation of the paper, and type and function of the solvent system. Each variation is particularly useful for certain purposes, but only a few have been used extensively in pesticide residue analysis.

This paper does not attempt to review all the applications of paper chromatography to pesticide residue analyses. Rather, a few applications are discussed and an attempt is made to point out some of the advantages, disadvantages, purposes, and future applications of paper chromatographic techniques to the problems associated with the analysis of pesticide residues.

Purposes of Paper Chromatography in Pesticide Residue Analyses

Paper chromatography has played a major role in the analysis of pesticide residues. Acree, *et al.* (2) used paper chromatography to extract organophosphate residues from insect tissues and Menn, *et al.* (3) used the technique as a cleanup procedure to separate insecticides and their metabolites from lipids in insect tissue extracts. Major and Barry (4) separated pesticides from plant waxes with this method. However, the major uses of paper chromatographic techniques have been the separation, detection, and identification of pesticides in cleaned up extracts. The applications of paper chromatography for these purposes are very numerous and have been thoroughly discussed in the review articles by McKinley (5) and Getz (6). Egan and Evans (7) and San Antonio (8) quantitatively determined pesticide residues by measurement of spot size on the paper chromatogram.

Until the recent appearance of gas chromatography and thin layer chromatography, paper chromatography was the only procedure generally applicable to the separation and identification of many pesticide residues. The greater sensitivity and concurrent quantitative estimation possible with gas chromatography have led to its widespread use, although paper chromatography is still used for confirmation of the relatively nonspecific gas chromatographic results. Thin layer chromatography, because of increased resolution and shorter development time, is rapidly replacing paper chromatography in many areas of pesticide residue analysis.

Paper Chromatographic Techniques

Since paper partition chromatography is not generally applicable to water-insoluble compounds, which include the chlorinated hydrocarbon and organophosphate pesticides of major interest, it has been applied to residue studies only in a limited number of cases. It was used by Acree, *et al.* (2) for extraction and by Major and Barry (4) and Menn, *et al.* (3) for cleanup. Zweig and Archer (9) used paper partition chromatography for the separation and identification of Sevin and 1-naphthol in wine. This technique has also been used for the determination of herbicides, such as monuron by Bleidner (10) and 3-amino-1,2,4-triazole by Mitchell (11), and for the separation of phosphamidon and its metabolites by Anliker and Menzer (12).

Reverse phase chromatography is the technique which has generally been applied to pesticide residue studies. In this technique, the paper, in theory but not always in practice, acts only as a support for an immobile solvent, and the compounds of interest are separated by partitioning between the immobile solvent on the paper and the mobile solvent moving through the paper. Vegetable oils, mineral oils, silicones, propylene glycol, and dimethylformamide are materials commonly used for immobile phases. This technique is especially applicable to the separation of compounds with very low water solubilities, and thus is useful for compounds such as chlorinated hydrocarbon and organophosphorus pesticides.

In general, paper chromatography is used

to separate pesticide residues without chemical modification of the chromatographic paper. However, fiberglass papers have been used for the reverse phase chromatography of organophosphorus compounds (13) and dithiocarbamates (14). In addition, McKinley and co-workers (13, 15) have used acetylated papers for reverse phase chromatographic separation of organophosphorus pesticides.

Selection of Solvent Systems

Several factors should be considered in choosing solvents for the paper chromatographic separation of pesticide residues; e.g., solubility and stability of the pesticide in the solvent, rate of solvent movement through the paper, and effect of the solvent on the subsequent detection and quantitative measurement of the residue. In reverse phase chromatography, the immobile and mobile solvents must not be miscible to any appreciable extent. Thus, the choice of possible solvents for specific applications is limited, but the actual selection of adequate solvent systems has been, and still is, largely a process of trial and error.

Detection Techniques

Generally, the detection technique must be capable of great sensitivity, i.e., detecting a few micrograms of the residue and, in many instances, detecting less than 1 μg of the compound.

One widely-used technique for chlorinated pesticide residues involves spraying the developed chromatogram with a solution of silver nitrate and then exposing the sprayed chromatogram to ultraviolet light (16). The chlorinated compounds appear as black or brown spots on a white background. Papers must be thoroughly washed before chromatography for a satisfactory background.

Other techniques used to a limited extent for the detection of chlorinated pesticides on paper chromatograms involve spraying with potassium hydroxide and ferric sulfate (1), methyl yellow followed by exposure to ultraviolet radiation (17), and indophenol blue with a weak organic acid (18).

Organophosphorus pesticide residues may be detected on paper chromatograms by several techniques. One of these is the enzymatic technique based on cholinesterase- or carboxylesterase-inhibition by pesticide residues. This is extremely sensitive for most organophosphorus pesticide residues if they are converted to active inhibitors prior to the application of the enzymatic detection procedure. This conversion has most often been accomplished by bromination of the developed chromatogram. Although acetylcholinesterase-inhibition has been used by many workers, probably the most familiar of these techniques is that developed by Getz and Friedman (19) from the procedure originally outlined by Cook (20). Human blood plasma is the enzyme source and acetylcholine is the substrate; color is developed on the chromatogram by using an acid-base indicator to detect the acetic acid produced in the chromatogram by the action of the enzyme on acetylcholine. McKinley and Johal (21) described a procedure based on the inhibition of carboxylesterases in which a beef liver homogenate was the enzyme source and 1-naphthyl acetate the substrate. A diazo compound (Azoene Fast Blue RR salt) was used to detect the l-naphthol produced over the background of the paper by the action of carboxylesterases on l-naphthyl acetate. The organophosphorus compounds appeared as white or yellowish spots on a brown background. The enzymatic techniques are extremely sensitive for most organophosphorus compounds; the carboxylesterase procedure can detect less than 1 μg of most of the organophosphates and sometimes as little as 5 ng.

Another technique is based on detection of sulfur in the molecule. Since the majority of organophosphorus pesticides contain sulfur, this procedure is applicable to most but not all of the organophosphorus pesticides. Getz (22) used a silver nitrate-bromophenol blue spray followed by washing in a citric acid solution to produce blue spots on a yellow background. MacRae and McKinley (23) described an iodoplatinate spray reagent which apparently detects only those organophosphorus compounds with two or more atoms of sulfur. Other reagents used to detect the sulfur-containing organophosphate pesticides are 2,6-dibromo-*N*-chloro-

p-quinoneimine (24) and metanil yellow, yellow RFS, or methyl yellow after bromination (25). Cook (26) introduced a technique which involved spraying the chromatogram first with *N*-bromosuccinimide in methyl chloroform, then with a slightly basic, ethanolic solution of fluorescein. This has been used for thiophosphates and some other phosphates.

Other spray reagents used for organophosphates are the Hanes and Isherwood reagent (27) and a ferric chloride-salicylsulfonic acid reagent (15).

In addition, many other techniques have been used to detect individual pesticides or groups of pesticides on paper chromatograms.

Quantitative Estimation

Quantitative determination of pesticide residues on paper chromatograms by measurement of spot size has not been widely applied because all stages of the determination for each compound and by each operator must be carefully standardized. Moreover, it is usually applicable only over a very narrow range and, in many chromatographic separations, the spot size appears to be determined largely by factors other than the quantity of the compound present. Probably the best examples of this technique are to be found in the work of Egan and Evans (7) and San Antonio (8) on the quantitative estimation of chlorinated hydrocarbon pesticide residues in pigeon flesh and soils, respectively.

In the application of this technique, the separated components are often eluted from the paper for quantitative determination by spectrophotometric or other procedures. The pesticide itself may be eluted or the elution may be carried out after reaction of the separated pesticide to produce a measurable compound.

Digestion of the paper chromatographic spot followed by chemical analysis has received considerable attention as a quantitative procedure. For example, Coffin and Savary (28) and Blinn (29) used oxygen flask combustion prior to the determination of orthophosphate as a means of estimating organophosphate pesticide residues.

Disadvantages

One of the major disadvantages of paper chromatographic techniques is the relatively poor spot definition often obtained. Spots on paper chromatograms are usually not as well defined as those on thin layer chromatograms and, therefore, it is more difficult to separate and identify the components of complex mixtures. The characteristics of the paper limit the types of detection techniques. High temperatures or strongly acidic or alkaline solutions cannot be used for paper chromatography because high temperatures often cause severe darkening of the paper and strongly acidic or alkaline solutions usually cause serious degradation of the paper. In reverse phase chromatography, materials suitable for the immobile phase may interfere with the subsequent detection or quantitative measurement of the separated components. Some immobile phases cannot be applied with enzymatic detection techniques because excess bromine is difficult to remove from the chromatogram after oxidation of thionophosphates. Silicones are not suitable for immobile phases on chromatograms to be subjected to oxygen flask combustion prior to quantitative determination of the residues, because the silicones are not digested by this procedure. The application of immobile phases in reverse phase chromatography somewhat limits the use of two-dimensional chromatography for the separation of pesticide residues.

Advantages

The advantages of paper chromatography are the relative simplicity of the operations, the small expense for the required equipment, and the relative ease of varying conditions. Features such as the type of paper, immobile phase, developing solvent, direction and length of development, and detection technique may be readily and rapidly altered in paper chromatography. This factor is particularly valuable in the development of procedures and in the use of specific conditions for specific samples and situations. Paper chromatography has a distinct advantage over thin layer chromatography when the sample is to be digested after chromatographic separation and before

quantitative determination since the paper can be readily digested with the sample, whereas it is often necessary to elute the separated components from thin layer chromatograms before digestion. Since paper chromatography has been employed in pesticide residue studies for many years, it currently has the advantage that it is more familiar to many workers than many of the newer chromatographic techniques. This familiarity often results in greater ease and certainty of interpretation of paper chromatographic results.

New Approaches

The use of paper chromatography in pesticide residue studies has generally been limited to a few of the available techniques. Very few attempts have been made to apply paper partition chromatography to pesticide residues. This technique should be applicable for detection of some of the more water-soluble organophosphate pesticides, generally in the area of plant and animal metabolites of the organophosphates, and for many of the more polar herbicide and fungicide residues. The use of paper partition chromatography would eliminate many of the problems associated with use of immobile phases in reverse phase chromatography.

Special or modified papers should be applicable in many areas of pesticide residues, e.g., ion exchange papers for separating some of the more polar pesticide residues including many herbicides, some fungicides, and possibly some organophosphate metabolites. Acetylated papers and fiberglass papers have been applied in some instances and the results would justify further investigation of their application in the chromatography of pesticide residues.

Low temperatures have seen some limited use in the paper chromatographic separation of pesticide residues. Lowering the temperature reduces the rate of development of the chromatogram and usually improves the resolution largely by decreasing the size of the spots on the chromatogram. Lower temperatures may permit the use of certain volatile solvents for both immobile phases and developing solvents. Conversely, higher temperatures may permit the use of certain solvents or solvent systems which are not applicable at the temperatures normally employed for paper chromatography.

Although reverse phase chromatography has been used extensively, the number of materials employed for immobile phases has been very limited. There are many materials, particularly silicones of widely varying characteristics, which should provide separations which cannot be obtained with the chromatographic systems currently in use.

Development of sensitive detection techniques, both general and specific, for the detection of pesticide residues on paper chromatograms is essential. A detection technique applicable to all organophosphorus pesticide and metabolite residues would be of great value in studies on the metabolism and residues of organophosphate pesticides. Sensitive detection techniques for herbicide, fungicide, and carbamate insecticide residues are also needed.

Although paper chromatography has been used extensively for pesticide residue studies, this technique has always been only one stage of the process of residue determinations, which consists of the integrated operations of extraction, cleanup, separation, identification, and quantitative measurement.

It is apparent that paper chromatographic techniques for pesticide residue studies will be largely replaced by other techniques such as thin layer chromatography and gas chromatography, although paper chromatography will continue to have a valuable function in these studies.

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Study of Extraction Procedures for Chlorinated Organic Pesticides in Fresh Eggs

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The Johnson procedure was modified to provide a quicker and more satisfactory method for cleanup of fresh egg samples for chlorinated pesticide residue analysis. Acetonitrile extraction of the sample was followed by centrifugation of the extract and chromatography on a "layered" Florisil-sodium sulfate column. The use of the "layered" column yielded 15% eluates suitable for direct analysis by microcoulometric gas chromatography. Unfortified egg samples were analyzed for DDD, DDE, DDT, heptachlor epoxide, and dieldrin by both this procedure and the Onley-Mills technique, and the results were compared.

The Mills procedure (1) adapted for the determination of chlorinated pesticides in eggs (2) consists of removal of phospholipids, mixed-ether extraction, acetonitrile partitioning, and Florisil column cleanup. This phospholipid removal also eliminated an interference, toxaphene-like but nontoxic to flies, which was present in most samples.

In practice, we found that this egg procedure was time-consuming and that the Florisil column eluates contained excessive sample extractives. To resolve these problems, other extraction solvents were used to eliminate interferences.

Johnson (3) reported that acetonitrile efficiently recovered several pesticides from some food samples. This procedure, when applied to blended fresh eggs, removed

the toxaphene-like interference; however, the pesticides were not efficiently extracted and the 15% eluate contained excessive extractives. Modification of this extraction procedure and Florisil column led to more efficient extraction of the pesticides usually detected and cleaner 15% eluates.

Other solvents such as dimethylformamide, dimethylsulfoxide, a benzene-alcohol mixture, and tetrahydrofuran were also tested. Only tetrahydorfuran appeared to be suitable; the other solvents or mixtures dissolved excess egg material. The investigation of tetrahydrofuran as an extraction solvent is described later in this paper.

The Johnson method as applied to fresh egg samples is as follows:

METHOD

Reagents

(a) *Acetonitrile.*—Redistilled.[1]

(b) *Petroleum ether.*—30–60°C, redistilled.[1]

(c) *Ethyl ether.*—Reagent grade.

(d) *Florisil.*—See *This Journal*, **45**, 983 (1962). Store 24 g portions at 130°C at least 5 hours.

(e) *Eluting mixtures.*—(*1*) 6% ethyl ether in petroleum ether; (*2*) 15% ethyl ether in petroleum ether.

(f) *Sodium sulfate.*—Reagent grade, anhydrous, granular.

Apparatus and Equipment

(a) *Chromatographic columns.*—25 mm o.d. × 300 mm with coarse fritted disc and Teflon stopcock.

(b) *Gas chromatographs.* — (*1*) Micro-Tek Model 2503R gas chromatograph, with Dohrmann combustion furnace and microcoulometric detection system (4). Columns were aluminum, ¼″ mm o.d. × 6′, packed with 20% DC-200 silicone oil (12,500 cst) on 70/80 mesh Anakrom ABS. The GLC was operated to achieve an aldrin retention time of about 6 min.; usually, 210–220°C column temperature and 100–120 ml/min. nitrogen carrier gas flow rate. (*2*) Barber-Colman Model 10 gas chromatograph, with concentric-type, tritium source, electron capture detection system (5). Glass U-shaped columns, 6 mm o.d. × 4′, packed with 10% DC-200 silicone oil (12,500 cst) on 90/100 mesh Anakrom ABS were used. The system was also operated to obtain an approximate 6 min. retention time for aldrin: about 205°C column temperature and 100–120 ml/min. nitrogen flow rate.

Extraction

Pour 50 g blended fresh eggs into 500 ml centrifuge bottle, add 150 ml acetonitrile, stopper, and shake contents intermittently about 10 min. Centrifuge at 1500 rpm for 3–5 min. and decant liquid into a 2 L separatory funnel. Break up cake of egg material and extract as before. Add 150 ml petroleum ether to the combined extracts, shake vigorously, and vent pressure. Add 1500 ml 2% sodium sulfate solution. Again shake vigorously and vent pressure. After layers separate, discard lower aqueous layer. Wash the petroleum ether solution once, using about 1 L 2% sodium sulfate solution, and discard wash. Filter the petroleum ether solution through a 2″ column of anhydrous sodium sulfate into a Kuderna-Danish concentrator. Rinse separatory funnel and sodium sulfate column with several 10 ml portions of petroleum ether and combine the washings with the extract. Concentrate solution to about 10 ml, and proceed with cleanup.

Cleanup

Place half-inch layer of anhydrous sodium sulfate in a chromatographic column. Add half of 24 g portion of Florisil and tap the column gently to settle contents. Add 1″ sodium sulfate, the rest of the Florisil, and another 1″ sodium sulfate. Again tap the column gently to settle contents. Prewash the column with about 50 ml petroleum ether and discard prewash.

Place a receiver (a 400 ml beaker is satisfactory) under the column. Transfer sample extract to the column with several 5 ml portions of petroleum ether. When transfer is complete, elute at about 2.5 ml/min. with 200 ml 6% eluting mixture. Just before the last of this eluting mixture enters the absorbent phase, change receivers, and elute with 300 ml 15% eluting mixture. After elution, transfer the eluates to 500 ml Kuderna-Danish concentrators and concentrate to a volume suitable for gas chromatography (see *Apparatus and Equipment* (**b**)(*1*) and (*2*)).

Thin Layer and Paper Chromatography

These methods were used to confirm the GLC results, to check the efficiency of cleanup, and to test for the presence of interferences.

[1] Available from Burdick & Jackson Laboratories, Inc., Muskegon, Mich.

For TLC, the method of Kovacs (6) was followed; for paper chromatography, the Mills method (7) was used.

Results and Discussion

The above procedure was tested on District quota samples of fresh eggs by several analysts. When pesticides were present, the Onley-Mills method was used for check analysis. Comparative results by these two methods for 26 samples are presented in Table 1. For the modified procedure (23 samples), average recoveries were 0.190 ppm DDE and 0.217 ppm DDT. The comparable values obtained by the Onley-Mills procedure were 0.202 ppm DDE and 0.178 ppm DDT.

In the modified Johnson procedure, samples larger than 50 g overloaded the cleanup column; at the same time, extraction with smaller volumes of solvent significantly decreased the amount of pesticide removed.

The shaking method is preferred to extraction in Omni-mixer and Waring Blendor cups because the blending procedures were found to be no more efficient for removing pesticides. In addition, the blending procedures require more equipment and time, and the egg material sticks to the blender cups and blades, making them difficult to wash. A shaking time of 5–10 min. was used for extraction. Longer times did not improve efficiency.

During the partition step, the acetonitrile extract must be diluted to about 2 L to obtain good recovery of the pesticide. When diluted to 2 L, the extract would be about 15% acetonitrile, as compared to 30% in a 1 L dilution. Good recoveries were obtained for 14 organo-chlorine pesticides from 30% acetonitrile, but more pesticide residue was detected in actual samples with the 2 L dilution. The egg extract therefore affects the recovery of the residues.

The egg fat, as expected, is almost insoluble in acetonitrile; apparently, only water and the yellow constituents are removed. Because a small extract is obtained, only the Florisil column is needed for adequate cleanup.

The amount of Florisil used by Onley and

Table 1. Comparative results of extraction procedures for chlorinated pesticides in fresh eggs

Sample No.	Procedure[a]	Analyst	Pesticides Detected, (ppm)[b] DDT	DDE
1	MJ	A	0.093	0.107
	OM	A	0.08	0.09
2	MJ	A	tr.	tr.
	OM	A	tr.	tr.
3	MJ	A	tr.	
	OM	A	tr.	
4	MJ	A	0.08	0.08
	OM	A	0.06	0.08
5	MJ	B	1.85	1.79
	OM	B	2.03	1.06
6	MJ	B	0.089	0.572
	OM	B	0.210	0.537
7	MJ	B	0.149	0.222
	OM	B	0.133	0.161
8	MJ	A	0.072	0.053
	OM	C	0.10	0.10
9	MJ	A	0.104	0.053
	OM	C	0.08	0.08
10	MJ	A	0.103	0.052
	OM	C	0.12	0.08
11	MJ	D	0.15	0.21
	OM	D	0.103	0.166
12	MJ	A	0.19	0.22
	OM	A	0.20	0.10
13	MJ	A		
	OM	E		
14	MJ	C	0.25	0.26
	OM	A	0.24	0.24
15	MJ	C	0.08	0.04
	OM	A	0.06	0.04
16	MJ	C	0.058	0.096
	OM	F	0.060	0.064
17	MJ	C	0.07	0.07
	OM	F	0.052	0.086
18	MJ	C	0.16	0.20
	OM	F	0.10	0.14
19	MJ	C	0.05	0.08
	OM	F	0.041	0.073
20	MJ	C	0.05	0.08
	OM	F	0.05	0.11
21	MJ	C	0.06	0.07
	OM	F	0.05	0.06
22	MJ	G	0.239	0.266
	OM	F	0.23	0.28
23	MJ	G	0.094	0.085
	OM	F	0.17	0.084
24	MJ	G	0.116	0.127
	OM	F	0.13	0.10
25	MJ	G	0.136	0.165
	OM	F	0.15	0.26
26	MJ	G	0.123	0.09
	OM	F	0.19	0.11

[a] MJ = modified Johnson procedure; OM = Onley-Mills procedure.

[b] DDD was also detected in trace amounts in most samples. Two samples also contained small amounts of heptachlor epoxide, and one sample contained a small amount of aldrin.

[c] 1.90 ppm dieldrin was detected by analyst A and 1.93 ppm dieldrin by analyst E.

Mills in their egg method was determined to be 24–26 g; smaller amounts were found to be inadequate for cleanup. The "split-column" technique effectively provided a clean 15% eluate, whereas the eluate from the undivided Florisil column was not suitably cleaned up for microcoulometric GLC. Moffitt (8) states that an additional layer of sodium sulfate stops channeling. In this laboratory, multiple layers of sodium sulfate have been used in the past, but for the sake of uniformity, only the single layer was used.

Different batches of Florisil vary in pesticide elution patterns, and Moddes (9) developed test procedures useful for detecting variations. We found that dieldrin was only partially eluted with 200 ml of the 15% eluant; therefore, 300 ml was used to insure complete elution. Because of possible variations, each batch of Florisil should be standardized before use.

When the Onley-Mills method was used for check analysis, the following limitations, precautions, or modifications were made to obtain eluates satisfactorily free of extractives.

First, sample size was limited to 50 g to prevent overloading of the cleanup column.

Second, analysts were instructed to repeat the boiling step until the egg extract began to precipitate in the boiling acetone. The number of excessively dirty eluates resulting from incomplete removal of the mixed-ether extraction solvent, indicated by solubility of the egg extract in large volumes of cold acetone, was thus decreased.

Last, the "split" Florisil column was used to obtain 15% eluates sufficiently free of extractives.

Tests of tetrahydrofuran (THF) as a pesticide extraction solvent in this laboratory showed that it efficiently extracts most pesticides from some foods[2]. Because it quantitatively extracted the egg fat, it appeared suitable for egg work. Since acetonitrile removed very little fat and mixed ethers removed only a portion, a THF extraction was a means to study the relation of fat removal to extraction of pesticides.

[2] M. J. Matherne, unpublished work, this laboratory, 1962.

Six samples, listed in Table 1, were extracted with THF, the extracts were partitioned into petroleum ether, and the solvent was evaporated. The extract was then subjected to a petroleum ether-acetonitrile partitioning and Florisil column cleanup. The detection results were compared with those of the other two procedures, and these observations were made.

First, for low levels (up to 1 ppm) of pesticide residue, THF was no more efficient than acetonitrile or the mixed ethers. There was some indication, however, that this solvent may be more efficient when higher levels of pesticide residues are present.

Second, the toxaphene-like interference described by Onley and Mills was not eliminated. This interference was most noticeable on thin layer chromatograms. It caused a raised baseline with the microcoulometric detection system, but, strangely, it did not noticeably affect electron capture detection.

Because most samples contained low levels of residues and because the interference was not eliminated, the testing of this solvent was discontinued. No future testing is planned since few samples contain large amounts of pesticide residues.

Conclusion

The modified Johnson procedure is more rapid, is more efficient, and gives cleaner extracts than the Onley-Mills method for levels of DDT usually detected in eggs.

It is again stated that this study concerned the analysis of actual samples, not samples fortified in the laboratory.

Only DDE, DDD, DDT, dieldrin, and heptachlor epoxide were detected. Similar data are needed for such compounds as toxaphene, chlordane, BHC, and endrin (heptachlor and aldrin would be metabolized to heptachlor epoxide and dieldrin, respectively). As these other compounds are encountered as residues, they will be subjected to the same type of study.

Acknowledgments

The author wishes to thank Caesar A. Roy for his assistance with the development of the procedure using tetrahydrofuran. In addition, the author is indebted to ten other

analysts in this laboratory who assisted by analyzing many of the samples.

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Determination of Oxidative Metabolites of Dimethoate and Thimet in Soil by Emission Spectroscopic Gas Chromatography

By C. A. BACHE and D. J. LISK (Pesticide Residue Laboratory, Cornell University, Ithaca, N.Y. 14850)

The conversion of dimethoate to its oxygen analog and the production of oxidative metabolites of Thimet in soil has been shown by using emission spectroscopic gas chromatography for their determination. Preparative thin layer chromatography was used for prior separation of Thimet oxidation products. The sulfoxide and sulfone of Thimet were the major determinable products.

Combination of gas chromatography with the emission spectroscopic detector described by McCormack, *et al.* (1) was very effective for determining organophosphorus insecticide residues (2). In the work reported, this detector system is used for determining oxidative metabolic products of dimethoate (*O,O* - dimethyl *S*-(*N* - methylcarbamoylmethyl)phosphorodithioate) and Thimet (*O,O*-diethyl *S*-ethylthiomethyl phosphorodithioate) in soil following thin layer and gas chromatographic separations.

METHOD

Reagents

(All solvents were redistilled before use and all reagents were reagent grade.)

(a) *Silica gel H.*—Brinkmann Instruments Inc., Westbury, N.Y.

(b) *Developing solution.*—Dissolve 15 ml methanol in benzene and dilute to 500 ml with benzene.

(c) *Chromogenic agent (see Ref. 3).*—Dissolve 5 ml 5% palladium chloride solution and 1 ml concentrated HCl in 95% ethyl alcohol, and dilute to 100 ml.

(d) *Buffer solution, pH 6 (see Ref. 3).*—Dissolve 5.7 ml 0.1*N* NaOH and 50 ml 0.1*M* potassium dihydrogen phosphate solution in water, and dilute to 100 ml.

(e) *Stock solutions of organophosphates.*—1 mg/ml solutions of dimethoate (D), dimethoate oxygen analog (DOA), Thimet (T), Thimet sulfoxide (TSO), Thimet sulfone (TSO_2), Thimet oxygen analog (TOA), Thimet oxygen analog sulfoxide (TOASO), and Thimet oxygen analog sulfone ($TOASO_2$) in acetone.

Apparatus

(a) *Gas chromatograph.*—The gas chromatograph and detector systems were identical to those described earlier (2). All of the columns were borosilicate glass, U-shaped and 3/16″ i.d.

(b) *Thin layer chromatography equipment.*—Research Specialties Co., with 8 × 8″ and 2 × 8″ glass plates.

(c) *Rotating evaporator.*

Soil Preparation

Place 10 g portions of sieved Canfield silt loam (pH 5.8) in each of twenty 5-oz plastic cups. Transfer 1 ml dimethoate stock solution to each of 10 cups, and add 1 ml acetone to each of the 10 remaining cups to serve as controls. Use the same procedure for

Thimet to give 100 ppm in the soil. Evaporate acetone, mix 2 ml water into each soil sample, cover cup with aluminum foil, and store at room temperature. Keep the soil moist by additions of water during the test period. At intervals, take cups of insecticide-treated and control soils for analysis.

Extraction, Isolation, and Determination

Blend total soil sample in cup with 25 ml $CHCl_3$ for 2 min. Add 2 teaspoons anhydrous Na_2SO_4 and blend for another min. Filter supernatent liquid through S & S 595 filter paper into 125 ml round-bottomed flask. Add 25 ml $CHCl_3$ to soil in blender and blend for 1 min. Repeat this step twice, filtering the supernatant liquid into the flask after each extraction. Evaporate combined filtrates just to dryness in a rotating evaporator in a water bath at 30°C.

Dimethoate oxygen analog (DOA).—Dissolve residue in flask in exactly 2 ml $CHCl_3$. Inject 5 µl into gas chromatograph. (A 6 ft column packed with 5% FFAP (Wilkens Instrument and Research Inc.) on 80–100 mesh Gas Chrom Q was used; column temperature was 130°C and argon flow rate was 80 ml/min. The retention time for DOA was 9.8 min.) This was the only column found suitable for chromatographing DOA; however, it was not suitable for chromatographing dimethoate, since the latter merged almost immediately with the solvent.

Dimethoate.—Transfer solution remaining after analysis of DOA to a 50 ml volumetric flask with $CHCl_3$, and dilute to volume. Inject 5 µl for analysis. (A 2 ft column packed with 5% SE 30 on 80–100 mesh acid-washed Chromosorb W was used. With a column temperature of 155°C and gas flow of 110 ml/min., the retention time for dimethoate was 5.5 min.) This same column was used for analysis of Thimet and its metabolites.

Thimet.—Soil samples were extracted for Thimet and its metabolites as described above. For analysis of Thimet, evaporate combined $CHCl_3$ filtrates after extraction, transfer solution to a 50 ml volumetric flask, and dilute to volume. Inject 2–5 µl into the SE 30 column for analysis. (With column temperature of 150°C and flow rate of 80 ml/min., the retention time for Thimet was 4.1 min.)

Separation of Thimet metabolites and determination of TSO, TSO_2 and TOA.—Transfer $CHCl_3$ extract remaining after analysis of Thimet to the round-bottomed flask and evaporate to about 2 ml.

Thin layer chromatography.—Plate preparation was a modification of Blinn's procedure (3). To 40 g silica gel H in a 250 ml glass-stoppered Erlenmeyer flask, add 80 ml pH 6 buffer solution. Shake contents vigorously, transfer the slurry to spreader, and coat a series of 8 × 8″ and 2 × 8″ plates with a 0.044″ thick layer. Let plates dry overnight, then activate for 1 hr at 120°C.

With an eye dropper drawn to a fine tip, apply the entire 2 ml $CHCl_3$ solution in a band 1 cm wide across an 8 × 8″ plate about 1″ from the bottom. Rinse with additional $CHCl_3$ for quantitative transfer. Spot 10 µg standards of each insecticide and metabolite on a separate 8 × 8″ or 2 × 8″ plate. Develop plates containing samples and standards simultaneously in tank with developing solution (reagent (**b**)). Remove plates when solvent has moved 10 cm above point of sample application. Locate the spots with the chromogenic agent as described by Blinn (3).

Scrape off the areas corresponding to TSO_2, TSO, TOA, TOASO, and $TOASO_2$ and transfer solids to separate 8 ml glass-stoppered test tubes. Add exactly 2 ml acetone to each and shake (wrist action shaker) for 1 hr. Centrifuge contents of each at 1000 rpm and inject 2–5 µl of supernatant solution into the SE-30 column.

Results and Discussion

Table 1 lists the retention times and R_f values for Thimet and its metabolites obtained by using the same instrument operating parameters in each case as were used for Thimet. Since a column suitable for chromatography of TOASO and $TOASO_2$ could not be found, these possible metabolites were not determined. Thimet could be determined in the presence of TOA even though their retention times were similar, because the concentration of Thimet was many times greater. The necessary dilution, therefore,

Table 1. Retention time and R_f values for Thimet and its metabolites

Compound	Retention time, min.	R_f
T	4.1	0.68
TSO	15.3	0.10
TSO_2	13.4	0.40
TOA	3.2	0.24
TOASO	—[a]	0.00
$TOASO_2$	—[a]	0.10

[a] Not determined because of poor response on chromatographic column used.

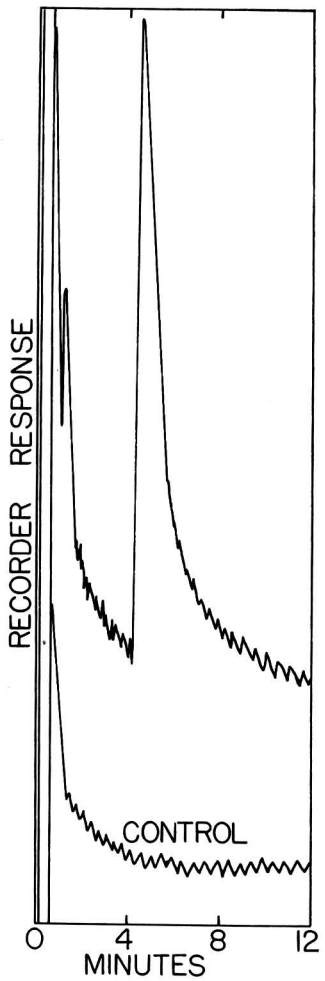

Fig. 1—**Chromatograms of Thimet sulfoxide (4.3 min.) in soil after 11 days and control soil.**

prevented visible response from TOA in the chromatogram. As is shown, TSO_2 was easily separated from the other metabolites on the basis of its R_f value. TSO and TOA were easily separated on the basis of their relative retention times and, although scraped off in the presence of TOASO and $TOASO_2$, the latter two did not interfere since they could not be chromatographed. Although Table 1 lists the retention times for TSO and TSO_2 as 15.3 and 13.4 minutes, respectively, the instrument operating parameters were adjusted to yield retention times within 4–5 minutes. Figure 1 shows chromatograms of TSO in soil after 11 days and in control soil. The instrument was adjusted to allow the appearance of TSO after 4.1 minutes. TOA is also visible at about 1 minute. Figure 2 shows chromatograms of DOA in soil after 20 days and in control soil. Table 2 shows the recovery of the insecticides and their metabolites. For recovery studies, dimethoate and DOA, and Thimet, TSO, TSO_2, and TOA were added to control soil in mixture prior to extraction. The sensitivity of the method for dimethoate, DOA, Thimet, TSO, TSO_2, and TOA, respectively, was about 0.02, 0.02, 0.1, 0.2, 0.2, and 0.1 ppm.

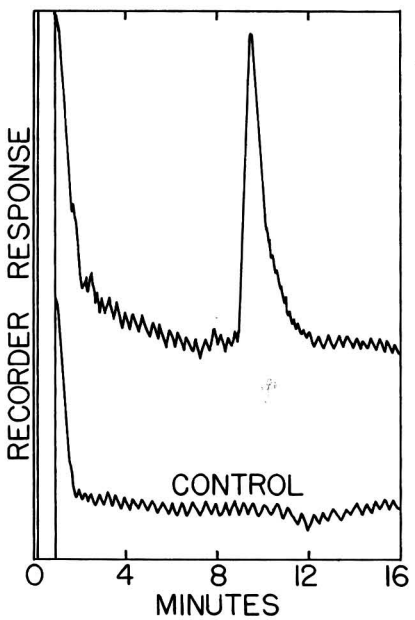

Fig. 2—**Chromatograms of dimethoate oxygen analog (9.8 min.) in soil after 20 days and control soil.**

Table 2. Recovery of insecticides and oxidation products[a]

Compound	Added, ppm	Recovery, %
D	1.0	97.6
DOA	4.9	86.2
T	1.0	89.8
TSO	20.0	94.7
TSO_2	4.0	104.0
TOA	2.4	86.7

[a] Amounts of TOASO and/or $TOASO_2$ are not given because of poor chromatographic response.

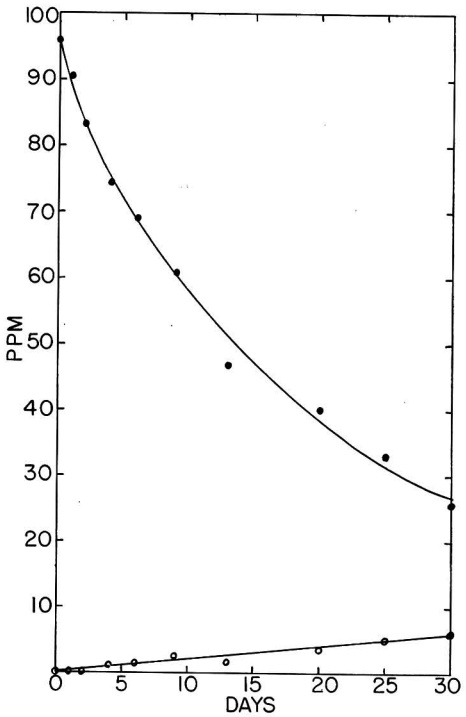

Fig. 3—**Disappearance of dimethoate (●—●) and production of its oxygen analog (○—○) in dimethoate-treated soil.**

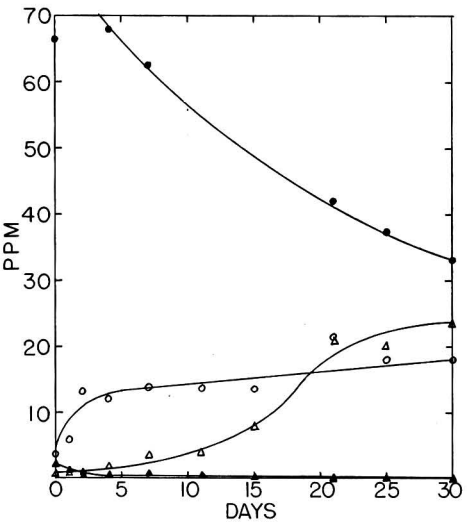

Fig. 4—**Quantities of Thimet (●—●) and its oxidation products, TSO (○—○), TSO_2 (△—△), and TOA (▲—▲) in Thimet-treated soil.**

Figure 3 shows the disappearance of dimethoate and the production of DOA in soil with time. Figure 4 illustrates the disappearance of Thimet and production of metabolites TSO and TSO_2. The concentration of Thimet found in the soil (66 ppm) on day zero immediately after adding 100 ppm may have been due to dilution error, considering the sensitivity of the method. Two additional soil samples were fortified with Thimet at 100 ppm and analyzed, but no improvement in the recovery at this high level resulted. In this connection, several other soil samples, fortified with Thimet at 100 ppm to study its degradation and analyzed by other isolation procedures, showed the following results: 0 time, 45.5 ppm; 1 day, 47.9 and 52.5 ppm; and 2 days, 43.6 and 50.0 ppm. TOA was present as an impurity in Thimet and simply disappeared as shown. This possible metabolite was found to be produced in only minor amounts in the study of metabolites of trithion (*O,O*-diethyl *S*-(*p*-chlorophenylthio) methyl phosphorodithioate) in lettuce (4). The major oxidative metabolites of trithion produced in lettuce were the sulfoxide and sulfone (4). These were also prominent (Fig. 4) as metabolites of Thimet in soil.

Acknowledgment

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Analysis by Microcoulometric Gas Chromatography

By H. P. BURCHFIELD[1] and R. J. WHEELER (Southwest Research Institute, San Antonio, Texas)

Microcoulometry provides an absolute and stoichiometric measure of chlorine, sulfur, and phosphorus in pesticides. The practical limit of detection of most pesticides containing these ions is 0.01–0.1 μg with the Dohrmann Model C-100; with the Model C-200, 0.001 μg chlorine can be detected. When the coulometer is used in the reducing mode, phosphorus is detected as PH_3; when it is used in the oxidizing mode, chlorine is detected as HCl, and sulfur is detected as SO_2. Temperature-compatible polar and nonpolar columns can be housed in the same oven, so that relative retention times, as well as elemental compositions, can be cross-checked by using either the oxidation or reduction modes.

Microcoulometric detection eliminates interfering peaks; reduces enhanced baselines; minimizes tailing from the solvent peak; allows programming without column bleed; and permits use of more volatile and less stable liquid phases than is possible with ionization detectors.

The modern era of pesticide residue analysis had its beginnings in 1960 when Coulson, *et al.* (1) introduced microcoulometric gas chromatography and Goodwin, *et al.* (2) applied the electron capture detector to the analysis of crop extracts for traces of chlorinated pesticides. Prior to this, most analyses were carried out by colorimetry; a specific method was required for each compound. Theoretically, it should have been possible to use gas chromatography for the analysis of pesticides since the introduction of this versatile technique by James and Martin (3) in 1951. However, the requirement for cleanup of tissue extracts was too great to make the method feasible for practical use. Traces of naturally occurring metabolites present in most samples obscured pesticide peaks or rendered the baseline too high to be tolerable. These difficulties were overcome by the invention of detectors which are highly selective for certain elements commonly present in pesticides such as chlorine, sulfur, and phosphorus.

The electron capture detector possesses high relative specificity because it yields very high responses to chlorinated hydrocarbons and very low responses to nonpolar hydrocarbons, which are unable to capture electrons. Thus, carbon tetrachloride yields a signal about 400 million times greater than that obtained with hexane when passed through this instrument. Compounds having intermediate polarity yield intermediate signals. The magnitude of the response is dependent upon the composition and the configuration of each individual molecule. Some nonchlorinated organic compounds yield responses as high as or higher than those reported for pesticides. For instance, the response of the electron capture detector to dimethyl fumarate is of the same order of magnitude as its response to lindane. This sometimes makes the interpretation of results difficult. Bowman, *et al.* (4) observed many small peaks in extracts from soils that had been stored in sealed containers before the advent of DDT. The inspection of a chromatogram obtained by the electron capture method does not reveal whether highly polar compounds such as pesticides are present in trace quantities or if slightly polar compounds present in relatively large amounts are responsible for the observed signals.

These difficulties can be circumvented by the use of the microcoulometric detector, which provides an absolute and stoichiometric method for determining the amount of chlorine, sulfur, or phosphorus passing into the cell. Basically, microcoulometry is an electrochemical method of analysis comparable to classical gravimetric and volumetric procedures with respect to specificity and stoichiometry. Unlike ionization and ther-

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mal detection techniques, the response obtained is not unique for each compound analyzed. Instead, the method gives an absolute measure of the microequivalents of chlorine, sulfur, or phosphorus in each molecule. Therefore, all that is required to compute the amount of residue present is a knowledge of the identity of the compound.

Detection of Halogen and Sulfur in the Oxidation Mode

The apparatus which is used for this purpose is shown schematically in Fig. 1. The sample, dissolved in an organic solvent, is injected into the apparatus through a glass injection port, which is heated externally. The compounds contained in the sample are transported through a gas chromatographic column with nitrogen as the carrier gas. At the end of the column additional nitrogen is introduced as a sweep gas, and oxygen is added to combust the sample. The gas mixture then passes through a combustion tube heated to 800°C. Organic compounds contained in the sample are oxidized to carbon dioxide, water, hydrogen chloride, and/or sulfur dioxide. The effluent from the combustion tube is passed through a titration cell, which consists of four electrodes that function as an anode-cathode generator pair and a sensor-reference pair. For the detection of chloride ion, the sensor and generator anode are silver, the generator cathode is a platinum spiral, and the reference electrode is silver in saturated silver acetate. The electrolyte is 75% acetic acid. When a chloride ion enters the cell, a silver ion is precipitated. The electrical imbalance created by this is sensed by the microcoulometer, and silver equivalent to the amount precipitated is regenerated from the generator anode. The current required to regenerate the silver ion is recorded in the form of a gas chromatographic peak. Thus the cell is sensitive only to compounds that can precipitate silver ion from extremely dilute solutions. Compounds other than hydrogen chloride that can precipitate silver under these conditions include hydrogen sulfide, phosphine, and hydrogen bromide. However, sulfur and phosphorus are oxidized to SO_2 and P_4O_{10}, respectively, during passage through the combustion tube and are not measured. Organic bromides are converted to elemental bromine, which yields one-half the detector response of the hydrogen halide due to its hydrolysis to HOBr and HBr (5); only the latter precipitates silver ion. If required, it would probably be possible to eliminate the signal from bromine entirely by inserting a subtraction tube containing oleic acid coated on an inert solid support between the outlet of the oxidation tube and the inlet of the titration cell.

It is possible to measure sulfur selectively by a modification of this system. The silver cell is replaced by a cell in which all of the electrodes are made of noble metal. The reference electrode is platinum in saturated triiodide, and the electrolyte is 0.04 to 0.05% potassium iodide in 0.4% acetic acid. Sulfur dioxide entering the cell is titrated by iodine to sulfate automatically. The recorder system measures the current required to replace the

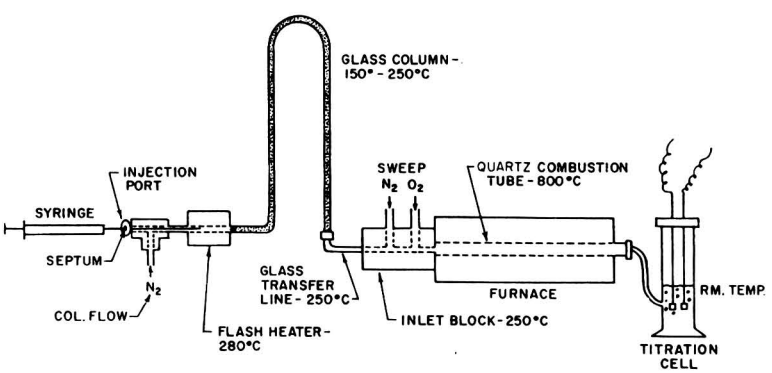

Fig. 1—**Schematic diagram of coulometric detection system.**

electrolyte component and yields a gas chromatographic peak.

When operated as described above, the microcoulometric system can be used to detect and quantitatively determine chlorinated hydrocarbons with the halide cell, as well as many pesticides containing sulfur, such as Tedion, with sulfur cell.

Detection of Phosphorus, Sulfur, and Halogen in the Reduction Mode

The microcoulometric method has been employed successfully to detect many pesticides containing halogen and sulfur. However, it is also necessary to be able to detect organic phosphates stoichiometrically with high specificity. Many of these contain sulfur as well as phosphorus, but the sulfur is often lost through atmospheric oxidation or metabolism.

Organic phosphates cannot be measured readily by the oxidation method. Presumably the phosphorus is converted to P_4O_{10}, which has very low volatility and is probably bound irreversibly by the walls of the combustion tubing. However, organic phosphates can be measured as phosphine by reducing them with molecular hydrogen at an elevated temperature. A schematic diagram of the apparatus used for this process is shown in Fig. 2. The sample is injected into a heated glass port and carried onto the gas chromatographic column by a stream of hydrogen. The organic phosphates are separated on the column in the usual manner. Solvent is vented to the atmosphere through outlet (F) before it enters the reduction zone. The chromatographic column is connected with a quartz tube by a graded seal. This tube passes through a furnace heated to a temperature of approximately 950°C where the phosphates are reduced to phosphine. The reduction is actually carried out within the confines of an empty, narrow bore, quartz

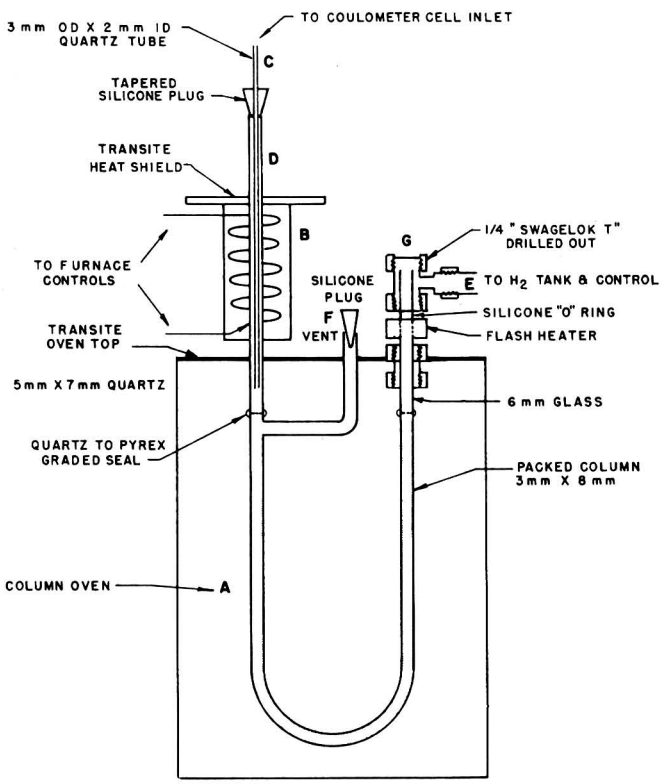

Fig. 2—**Schematic diagram of reduction oven system.**

tube inserted into the larger quartz tube projecting from the reduction furnace and connected to it with a tapered silicone plug. Dimensions of this tube are critical and appear to be optimum at 25 cm × 3 mm o.d. × 2 mm i.d. under the conditions studied. This concentric arrangement is employed so that the tube in which the reduction actually takes place can be replaced conveniently in a matter of seconds. After 5–7 runs the tubes are cleaned by placing them in a muffle furnace at 600–700°C for about one-half hour.

Following reduction, the gas stream is bubbled through a titration cell equipped with silver generating and sensing electrodes. Silver phosphides (equivalent to a stoichiometry of Ag_2PH) are precipitated, and the silver removed from the system is regenerated as in the measurement of halogen. Reduction products of hydrocarbons, oxygenated hydrocarbons, and amines do not yield responses. However, pesticides that contain chlorine or sulfur as well as phosphorus give responses due to the formation of hydrogen chloride and hydrogen sulfide, respectively, since these also precipitate silver ion. Therefore, the detection *per se* is not specific for phosphorus. This situation can be remedied easily by the introduction of a subtraction tube or GSC column which selectively removes or separates the interfering gases. The technique which has been most successful so far is to insert a small tube containing either aluminum oxide or silica gel between the exit of the reduction tube and the titration cell. Figure 3 illustrates a chromatogram obtained on the insecticide Ronnel ($C_8H_8O_3SPCl_3$) in the absence of a subtraction tube or GLC column. The large peak, A, represents the sum of chlorine, sulfur, and phosphorus. However, if a GSC column containing silica gel is placed between the exit of the reduction tube and titration cell, hydrogen chloride is subtracted quantitatively, and the peaks for phosphine and hydrogen sulfide are resolved, B. Although peak profiles of the hydrogen sulfide and phosphine peaks differ markedly, their areas are identical to within experimental error. If it is desired to detect phosphorus only, a subtraction tube containing aluminum oxide

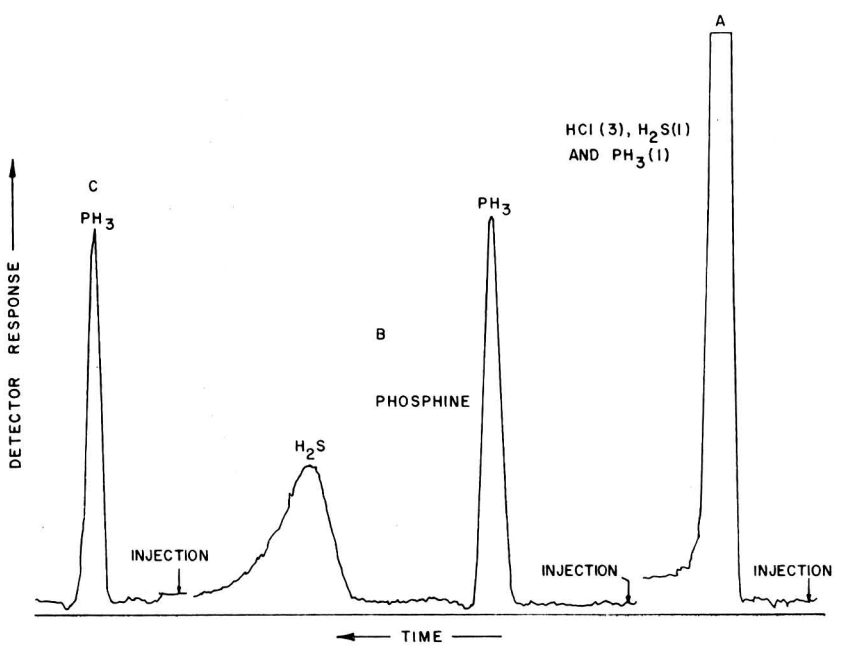

***Fig. 3*—Chromatogram of Ronnel.**

is placed between the exit of the reduction tube and the titration cell. This material binds hydrogen chloride and hydrogen sulfide while permitting free passage of phosphine as seen in the third injection. Thus the system has absolute specificity for phosphorus-containing compounds.

The application of this system to temperature-programmed gas chromatography of organic phosphate insecticides is illustrated by Fig. 4, which shows the separation of eight of these materials from one another. A tube containing aluminum oxide was inserted between the outlet of the reduction tube and the inlet of the titration cell so that the response obtained was due to the presence of phosphorus only.

It is possible by this method to detect phosphorus with absolute specificity, to separate peaks representing sulfur and phosphorus, or to measure the sum of the response due to sulfur, phosphorus, and chlorine. When this method is used to detect total response, sensitivity for organic phosphates and related compounds is enhanced substantially.

Gas Chromatographic Equipment

Gas chromatographic equipment developed in these laboratories for use with the microcoulometric titration system is illustrated in Fig. 5. The unit to the right consists of a Micro-Tek MT-220 gas chromatograph used in the oxidation mode. The unit to the left consists of a modified Micro-Tek 2500 gas chromatograph converted to operate in the reduction mode. Both units feed into the same titration cell. It should be noted that when the instrument is used in the reduction mode, the titration cell inlet must be modified and a surfactant added to the electrolyte to insure good dispersion of the carrier gas (6). This is required because of the low solubility of phosphine in the electrolyte.

For analysis of pesticides, and particularly pesticide metabolites, the injection port of the column and the transfer line should be constructed entirely of glass. This is especially true for the injection port, since this is generally the hottest part of the chromatograph and, therefore, the most conducive to decomposition of unstable compounds. The literature contains a variety of conflicting information on the merits of glass versus metal systems. Also, opinion is divided on the merits of glass versus quartz. While some workers recommended glass, others claimed to have used metal systems successfully. A preponderance of evidence, however, indicates that glass construction is preferable. It may be true that some pesticides and pesticide metabolites do not decompose on contact with hot metal surfaces but, on the other hand, others undoubtedly do. To insure optimum results, particularly in metabolism studies, it is best to prepare for the analysis

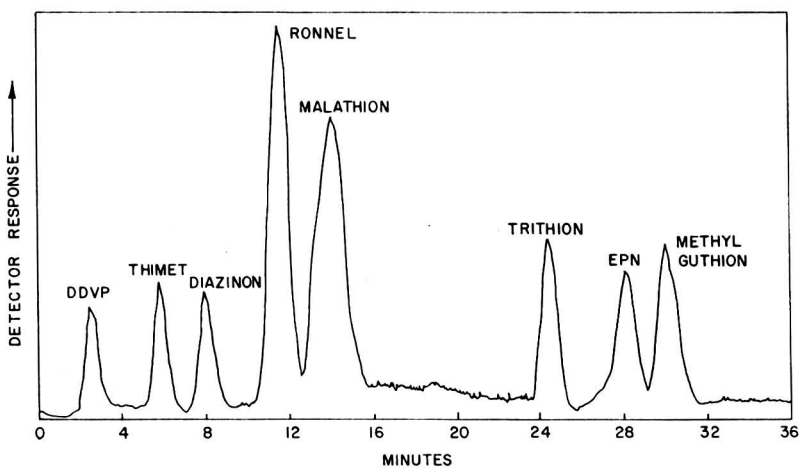

Fig. 4—**Chromatogram of 8 organophosphate pesticides.**

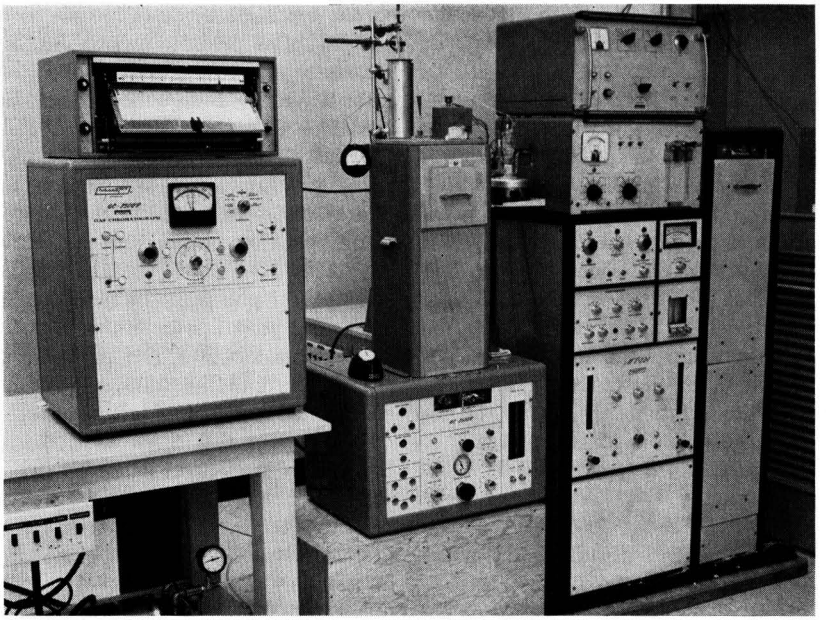

Fig. 5—**Photograph of MT-220 and GC 2500R.**

of the least stable rather than the most stable compound, particularly when the nature of the compound being sought is unknown.

Therefore, glass is recommended for general purpose use. This should not be misconstrued to mean that metal should not be used in cases where compounds of known stability are being analyzed. The requirement for an all-glass system extends not only to the injection port and the column, but also to the transfer line connecting the column with the detector. In addition, the transfer line should be as short as possible and be uniformly heated so that hot or cold spots are not present.

An illustration of the effects produced by a metal transfer line on an unstable compound is shown in Fig. 6. The symbol CP denotes chlorpromazine, a tranquilizing drug, and the symbol CPSO denotes chlorpromazine sulfoxide, one of its metabolites. In obtaining these chromatograms, the conditions were identical with a single exception. In one case the transfer line was made of aluminum; in the other case the transfer line was made of glass. About the same peak area was obtained for chlorpromazine by either system. However, considerable loss of the chlorpromazine sulfoxide was encountered with the aluminum transfer line.

The injection port of the Micro-Tek MT-220 chromatograph can be equipped with a removable glass or quartz tube inserted in the flash heater. This is advantageous, since most tissue extracts contain large quantities of nonvolatile compounds even after extensive cleanup. Therefore, deposits of these materials will build up after repeated injections. This accumulation of nonvolatile materials in the inlet can lead to absorption of sample components and result in low recoveries of pesticides. Also, continued bleed from this source reduces the column life. These problems can be avoided by changing the inserts as required. This system is not required with highly purified extracts or when extremely small samples are injected and the detector is operated at its maximum sensitivity.

The oven of this instrument is equipped to accommodate four 6′ U-shaped columns. The U-shape is not considered critical but is preferred when glass columns are used, since they are easier to pack than coiled columns.

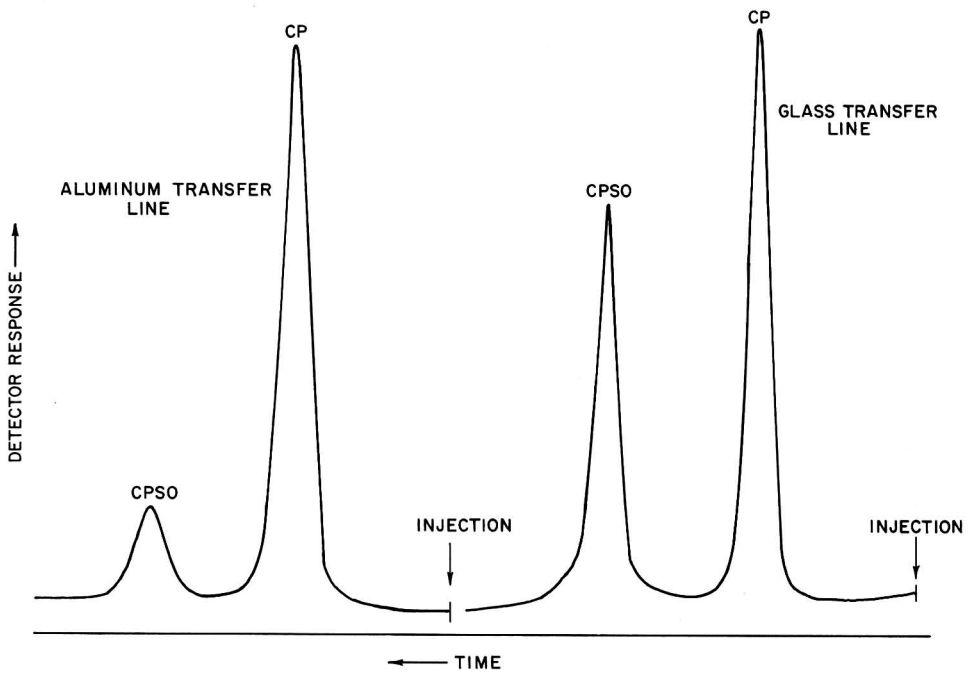

Fig. 6—**Comparison of effects of aluminum and glass transfer lines on chlorpromazine sulfoxide.**

Also, replacement columns can be prepared by individuals with limited glass-working experience. One of the columns used for pesticide residue analysis is nonpolar (for example, silicone oil DC-200), while another is polar (for example, a mixture of QF-1 and SE-30). Having the polar and nonpolar columns positioned on the same chromatograph is distinctly advantageous, since tentative identifications can be confirmed readily by comparing retention times on the two columns. The two columns are temperature-compatible; each is provided with its own injection port. The chromatograph oven is equipped with temperature programming for which several linear program rates are available. Temperature programming is not presently used in routine analysis of pesticide residues, but it is likely that it will be used in the near future, since it is possible to obtain better resolution than when the column is operated at a single temperature.

Operation of the Microcoulometer

By using the Dohrmann Instruments Co. Model C-100 microcoulometer, it is possible to detect 0.01 to 0.1 μg of chloride. Recently a modified version of this instrument (Model C-200), which can detect as little as 0.001 μg of chloride, has become available. Thus, the method now approaches the flame ionization detector in sensitivity and far exceeds it in selectivity.

The Model C-100 microcoulometer requires a well-trained technician for successful operation, but it has proved remarkably stable and trouble-free in experience extending over a period of more than four years. Experience with the C-200 microcoulometer is less extensive. Some difficulties were experienced during early trials with it, but these have largely been resolved.

The circuitry of the Model C-200 differs from the model C-100 in that solid-state components are employed and there is no mechanical servo system. The operation of the titration cell also differs in one important aspect. In the Model T-200 titration cell[2] the sensor electrode surface is located on the op-

[2] C-100 microcoulometer.

posite side of the cell from the carrier gas inlet. The solution is well stirred so that an imbalance in the average silver ion concentration of the entire solution is sensed. In the C-200 system,[3] a 10 to 20-fold gain in sensitivity has been obtained by placing the sensor electrode directly in front of the carrier gas inlet. This results in the gas impinging directly on the sensor rather than being dispersed throughout the entire cell. Consequently, the concentration of reactant at the sensor electrode surface is much higher for a given amount of reactant than with the C-100 system. As a consequence, it is necessary to set the amplifier gain very low to avoid overshoot. Therefore, when the system is first turned on and the bias voltage is set, it requires about 15 to 20 minutes for the T-300-S cell to come to equilibrium and about 5 to 10 minutes for the I_2/I_3^- cell to come to equilibrium. This suggests that the response of the cell might be too slow to follow a gas chromatographic peak. However, these cells are operated as constant concentration cells. Reduction of the silver ion concentration at the surface of the sensor electrode is followed continuously by regeneration of silver ion from the generator electrode. Thus, even though a long time is required to reach equilibrium conditions after changing electrolyte, response is still fast enough so that the emergence of peaks from the column can be followed. This is illustrated by Fig. 7 in which the time is plotted against generator voltage immediately after the change of electrolyte. In the period of time equilibrium conditions were being established, three identical injections of aldrin were chromatographed. The peaks observed are of approximately the same size, even though the concentration of silver ion had not yet reached the value demanded by the bias voltage. Thus, it is evident that the response is fast enough to meet the requirements of a gas chromatographic system.

Because a localized change in silver ion concentration is sensed, this system is quite sensitive to flow rates, since this changes the amount of reactive gas impinging on the

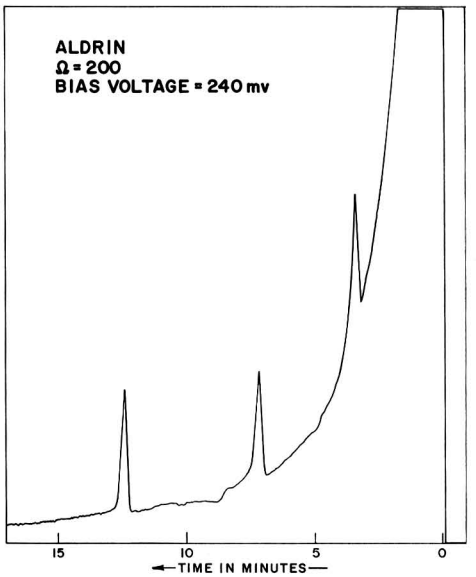

Fig. 7—Cell equilibrium-response time.

electrode per unit time. Changes in stir rate can also change the concentration of reactant within the sensing zone. The generator electrode reacts correspondingly, so that over- or under-generation of silver ion results. Increasing the stir rate over the optimum causes the peaks to appear to tail. Too slow a stir rate leads to overshoot due to over-generation of silver ion. Overshoot is very undesirable, particularly when accompanied by column bleed. Therefore, it is best to adjust the stir rate to avoid overshoot. This adjustment often leads to peaks that appear to tail slightly. However, this is not a serious defect unless the peaks are crowded. It is recommended that an integrator always be used with the system, since areas computed by conventional triangulation methods are often erroneous. To obtain quantitative results with this system, it is necessary to operate under standardized conditions. First, the flow rates should be adjusted to preset values in the neighborhood of 100–150 ml per minute total. The bias voltage should be set at 240 mv, the amplifier gain at 200, and the sensitivity setting at 200 ohms. The stir rate should then be adjusted so as to obtain a nearly symmetrical peak for a standard insecticide such as aldrin. If overshoot oc-

[3] T-300 cell.

curs, the stir rate should be increased so as to decrease the residence time of the sample at the surface of the sensor electrode. By careful adjustment, symmetrical peaks can be obtained. A slight amount of tailing rather than overshoot is preferred, as noted above. Once this adjustment is made it is recommended that the magnetic stirrer be permitted to run continuously.

Due to the critical nature of these settings, it will sometimes be desirable to operate the Model C-200 microcoulometer in the C-100 mode. This can be accomplished by placing the sensor electrode at an angle of 15° from the carrier gas inlet and increasing the amplifier gain. The gain must be increased by a factor of 10 to 15 to compensate for the smaller changes that are detected at the sensor electrode. As a result of increased gain, noise due to the stir bar and turbulence in the electrolyte is increased, resulting in lower effective sensitivity. When used in this manner, the Model C-200 coulometer system is only 2 to 5 times more sensitive than the C-100 system as compared to 10 to 20 times when the carrier gas is allowed to impinge directly on the sensor electrode and low gain is used. However, the stability of the system is improved. Therefore, for measurements at intermediate sensitivity the use of the Model C-200 in the C-100 mode is recommended. However, the direct impingence method is still available when high sensitivities are required. A kit is being prepared by the Dohrmann Instruments Co. that will make it possible to adjust the gain more easily than is the case with the original instrument.

As a result of the higher sensitivity of the Model C-200 microcoulometer, it is much more likely to give erroneous signals due to stray currents. The major source of this difficulty has been found to reside in the harness connecting the microcoulometer to the titration cell. The effect is enhanced when the two are connected through the furnace oven. When the generator wiring and the sensor wiring are in the same shielded cable, the sensor wiring picks up a signal from the generator wiring, which has a considerably higher voltage. This, in effect, gives an increased signal to the sensor, thus calling for additional generator current. As a result of this effect, apparent recoveries of aldrin as high as 150 to 170% have been observed. This effect can be virtually eliminated by connecting the titration cell to the coulometer with four independent cables. Figure 8 shows the results obtained on chromatographing a mixture of two chlorinated hydrocarbon insecticides with the two types of cables. Peaks labeled (A) and (B) were obtained with all of the leads contained in the same shielded cable. The peaks labeled (C) and (D) were obtained when all four leads were separate. The Dohrmann Instruments Co. now supplies all coulometers with separate leads. This capacitance effect has been demonstrated independently by connecting 50 to 100 $\mu\mu$ farad capacitors across the generator and sensing electrodes. The same type of peak exaltation was obtained when all of the leads are contained in the same cable.

Despite these initial difficulties, excellent recoveries can be obtained with the C-200 coulometer when it is operated under optimum conditions. These are achieved by the following means:

(a) adjusting the flow rate to a preset constant value;

(b) adjusting the stir rate to obtain optimum peak profile and letting the stir bar motor run continuously;

(c) keeping at a minimum the capacitances and resistances in the harness connecting the cell to the coulometer;

(d) using the Model C-200 in the Model C-100 mode when the increased sensitivity is unnecessary.

Discussion

The principal advantage of the microcoulometric system is that it is specific in the sense that chemical methods of analysis are specific. Chloride ion is detected through the precipitation of silver chloride, and sulfur dioxide by oxidation to iodine as in classical gravimetric and volumetric procedures. Moreover, ancillary procedures can be in-

This work was supported in part by the Food and Drug Administration under contract numbers Proc 63-105 (Neg.) and FDA 65-13 (Neg.) and by the U.S. Public Health Service under contract numbers PH-86-64-74 and PH-86-65-69.

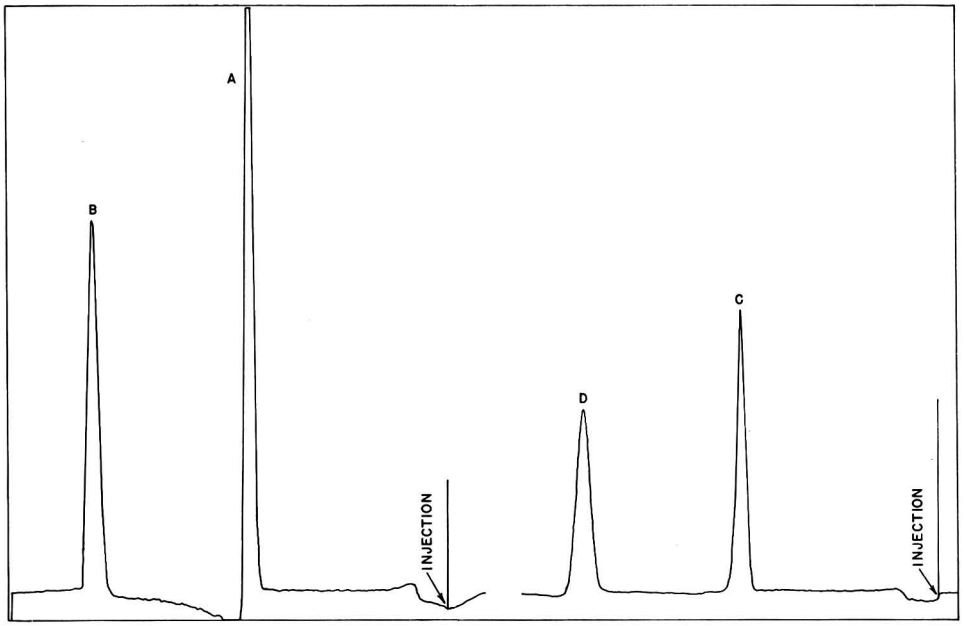

Fig. 8—**Comparison of results of identical samples, changing C-200 cell harness. A and B, all leads contained in same shielded cable; C and D, all four leads contained in separate cables.**

corporated to increase specificity. Phosphine, hydrogen sulfide, and hydrogen chloride all precipitate silver ion. However, phosphine can be measured in the presence of the other two compounds by using alkaline materials such as Ascarite or aluminum oxide, which do not absorb phosphine but do irreversibly bind HCl and H_2S.

By contrast, ionization and thermal detection methods possess relative rather than absolute specificity. Response will vary from compound to compound, usually in an unpredictable manner. Consequently, these instruments require calibration for each material to be analyzed. In a sense this is also true with microcoulometry. Although the detection instrument itself is stoichiometric, losses of compounds on gas chromatographic columns tend to be variable. Even so, the microcoulometer possesses an important advantage in this respect.

The possibility of quantitatively measuring the amount of the element being analyzed for in the carrier gas effluent automatically makes it possible to compute yields, and from these work out optimum gas chromatographic conditions. It is very difficult to do this with ionization detectors, since it cannot be predicted what the detector response should be when there are no losses from the columns or other parts of the system. The microcoulometric method serves as a guide for optimizing column conditions, as well as makes it possible to calculate results directly from peak areas.

These capabilities are very important in residue analysis for several reasons. The ability to analyze a peak for chlorine, sulfur, and phosphorus makes identifications much more certain than those based on relative retentions alone. This can be important in residue analysis for legal considerations as well as identification of new metabolites. The use of the microcoulometric system in both the oxidation and the reduction modes provides considerable additional information. When the reduction mode is used, it is possible to measure the sum of sulfur, chlorine, and phosphorus, measure sulfur and phosphorus in the same compound independently, or measure phosphorus specifically (Table 1). Moreover, by decreasing the oven tem-

Table 1. Theoretical total response of various pesticides with reduction mode

Compound	Moles of Element			Total Equivalents
	Cl	S	P	
Aldrin	6	0	0	6
DDT	5	0	0	5
2,4-D	2	0	0	2
Dieldrin	6	0	0	6
Heptachlor	7	0	0	7
Paraoxon	0	0	1	2
Parathion	0	1	1	4
Phosdrin	0	0	1	2
Ronnel	3	1	1	7
Thimet	0	2	1	6
Trithion	1	3	1	9

perature to 700°C it is possible to measure the sulfur attached to a phosphorus atom without interference from the phosphorus. When used in the oxidation mode, chlorine can be measured as HCl and sulfur as SO_2. The capacity for measuring sulfur as H_2S in the reduction mode and SO_2 in the oxidation mode serves as an excellent confirmation of the presence of this element.

Microcoulometric detection tends to alleviate many practical problems encountered in the chromatography of pesticides. Some of the advantages are as follows: (*1*) interfering peaks from compounds that do not contain chlorine, sulfur, and phosphorus are eliminated from the chromatogram; (*2*) enhanced baselines due to general compound "noise" are greatly reduced; (*3*) tailing from the solvent peak is minimized, which makes it possible to detect the presence of compounds having very short retention times; (*4*) the column temperature can be programmed at will without interference from column bleed; (*5*) because of insensitivity to column bleed, it is practical to use more volatile and less stable liquid phases than is possible with ionization detectors; such columns may give better compound resolution than is possible with conventional high temperature liquids; and (*6*) multiple runs on the same sample make it possible to analyze each gas chromatographic peak for sulfur, chlorine, phosphorus, and potentially nitrogen. (It seems likely that it will be possible to incorporate a modification for the detection of nitrogen into the system at an early date.) From these measurements molar ratios can be computed. This makes identifications much more authoritative than those based on relative retention times alone.

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Rapid Screening for Some Anticholinesterase Insecticide Residues by Automated Analysis[1]

By DANIEL E. OTT and FRANCIS A. GUNTHER (Department of Entomology, University of California, Riverside, Calif. 92502)

Various sample preparation methods with two new modifications of a previously reported automated system are presented for the determination of anticholinesterase insecticides. An analyst may select a total set of conditions which may be used as a rapid screening test for the detection of some mammalian toxic organophosphorus pesticide residues in a wide variety of plant material substrates. Suggestions are included for obtaining some degree of specificity, when desired, for certain inhibitors of cholinesterase. Typical summary data from analysis of fortified substrate are as follows: (1) 0.25 ppm DDVP in both broccoli and cauliflower; (2) 0.04 ppm parathion (99.6%) in canned peaches after a manual bromine-water oxidation procedure; (3) 0.1 ppm technical grade parathion in canned peaches and water plants (from wildlife pond); (4) paraoxon and technical grade parathion at 0.4 and 0.5 ppm each in Valencia orange peel; and (5) phosphamidon at 0.1 ppm in both the peel and the pulp of white potatoes.

Since Winter (1) introduced an automated analysis scheme for anticholinesterase (anti-ChE) compounds in 1960, and the topic was later discussed by Gunther (2) and by Winter and Ferrari (3), this valuable technique has been lying dormant awaiting a practical method for its use as a screening technique for these residues in food substrates. A practical use closely related to insecticide residues is the combined manual and automated method for certain purified organophosphorus insecticides, indirectly described by Lovell (4) who employed, with slight modification, the automated cholinesterase (ChE) determination method of Winter (5).

A polarographic screening procedure for the detection of parathion and other "nitrophosphate" pesticides was described by Gajan (6). Blinn (7) described a total phosphorus technique by Schöniger flask combustion which could be used as a screening test for organophosphorus pesticide residues.

The objective of the present paper is to present sample preparation methods so that the automated continuous-flow system for wet-chemical analysis can be used, with modification as described, for screening for above-tolerance levels of anti-ChE insecticide residues.

In our laboratories, the automated oxidation procedure to convert some poor inhibitors to strong ChE inhibitors, e.g., parathion to paraoxon, with dilute bromine-water (1) was unworkable. Attempts to develop alternative automated oxidative procedures have not yet been successful. However, the manual test tube procedure for the oxidation of parathion by bromine-water described here does work and has been used successfully prior to AutoAnalysis.

METHOD

Apparatus

The several modules of the AutoAnalyzer[2] system are arranged according to the flow diagrams shown in Fig. 1 or 2. The double row sampler plate is a special item with Technicon. Since about 30 min. is required for a given sample to flow through the entire system, it is most convenient to use the greater speed of 2-speed proportioning pumps for washout purposes.

Reagents

Prepare all reagents exactly as described by Winter (1) except for the following:

Add the surfactant Brij-35 to a concentration of 0.5 ml/L in all reagents except the enzyme source solution and the enzyme substrate solution.

Acetylcholine bromide, more readily avail-

[1] Presented in part at the September 1965 Technicon Symposium, "Automation in Analytical Chemistry," New York, N.Y. Paper No. 1677, University of California Citrus Research Center and Agricultural Experiment Station, Riverside, Calif.

[2] Technicon Controls, Inc., Ardsley, N.Y.

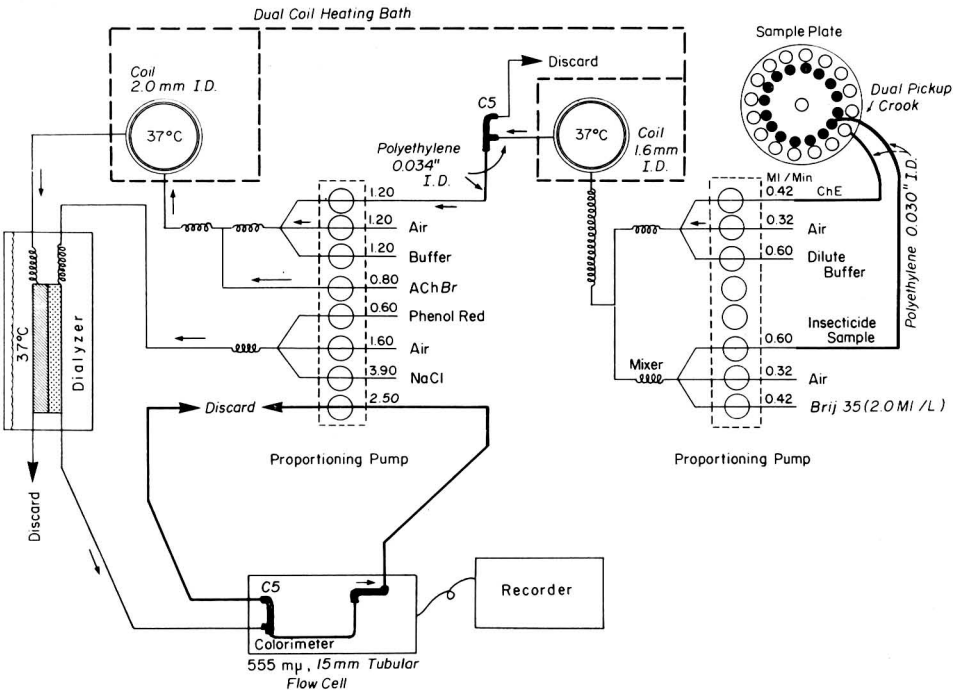

Fig. 1—**Flow diagram of an automated system for determining anti-ChE pesticide residues; redrawn from Winter (1) and, in part, from Ott and Gunther (10). Heavy lines and italic print indicate changes from the Winter paper.**

able at lower cost than acetylcholine iodide, is compatible in this system. Dissolve 16.6 g acetylcholine bromide in 1 L water.

Adjust buffer solution to a predetermined pH (about 8.2) with 0.1*N* NaOH or HCl; when pumped through the analytical system with all the other reagents in the absence of ChE, it will produce a reagent baseline between 10 and 15% T. Add dilute acid or base to the 10% NaCl solution while pumping all reagents and with the colorimeter and recorder operating if it is necessary to adjust reagent baseline up or down.

If dilute bromine-water is required, prepare fresh daily from a known saturated solution of bromine in water. (Droplets of bromine should be visible at bottom of "saturated" solution; if not, add more bromine and mix vigorously.) Prepare dilute bromine-water solution by diluting 0.12 ml saturated solution to 100 ml with water.

Preparation of Sample

Choice of stripping solvent will be left to the individual analyst within the limits to be discussed here. The sample is then prepared for analysis according to one or more of the following procedures:

(a) *Equilibration from n-hexane stripping solution into an aqueous phase.*—Concentrate substrate stripping solution, if necessary for usable sensitivity, down to the 10.0 ml mark of a previously calibrated Kuderna-Danish (K-D) tube by conventional K-D technique (8). Add 5.0 ml water by automatic pipet, stopper with a ground glass stopper, and shake for 2 min. Let phases separate; then remove upper organic phase with an aspirator suction stick. Use aliquot of aqueous phase (lower layer) for AutoAnalysis.

(b) *Extraction from a solvent-free stripping solution residue by boiling in aqueous phase.*—Concentrate stripping solution aliquot to a small volume by K-D technique. Add 2 drops glycerine as a "keeper" and concentrate to dryness under a jet of filtered air from a manifold while immersing the tube in a warm water bath (60°C max.). Add 5.0 ml water by automatic pipet and a fresh boiling chip, and cover tightly with a double layer of aluminum foil. Place in a 110°C oven for 10 min. in a metal test tube rack.

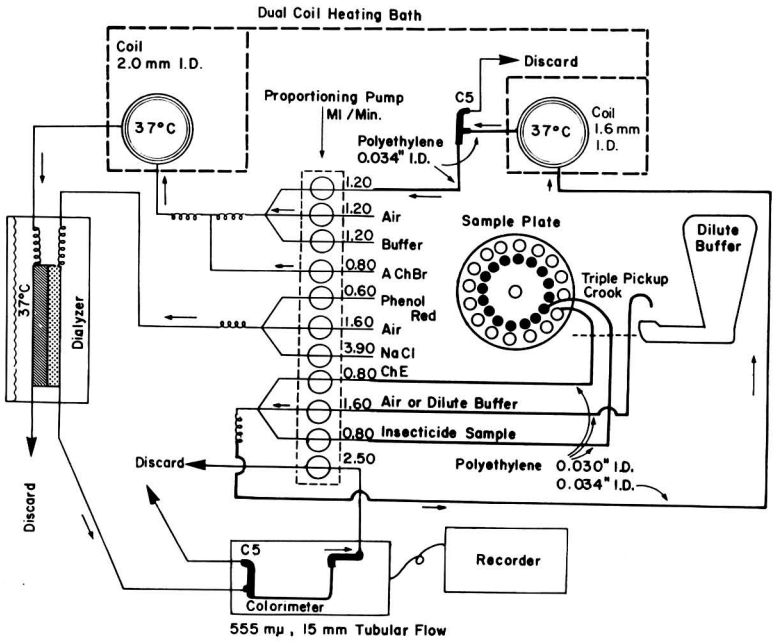

***Fig. 2*—Flow diagram of a single proportioning pump automated system for determining anti-ChE insecticides.**

(c) *Equilibration from chloroform stripping solution into an aqueous phase.*—Concentrate an aliquot of substrate stripping solution, if necessary for usable sensitivity, to 2.0 ml in a previously calibrated K-D tube. Add 4.0 ml *n*-hexane[3] from one automatic pipet and 5.0 ml water from another automatic pipet. Shake and treat as in (a), starting with "Let phases separate . . ." (Although this method may be more specific for certain compounds when chloroform is used as a stripping solvent compared to its use in method (b), we have not used it for crop substrates.)

(d) *Dilute bromine-water oxidation method.* To oxidize the test compound to a more potent ChE inhibitor, e.g., parathion to paraoxon, substitute 0.12% dilute bromine-water solution for water in procedures (a), (b), or (c). Then in (a) and (c), after equilibration and phase separation, discard the organic phase and submit the aqueous phase to the same procedure as in the last part of (b), starting with "a fresh boiling chip. . . ."

A practical alternative procedure for the bromine-water oxidation method, when the sample will be analyzed for anti-ChE agents both before and after oxidation, is to use one of the three preliminary methods. Then, take an aliquot of the aqueous phase directly for analysis, add an equal volume of fresh 0.12% dilute bromine-water solution for another aliquot, and follow the same procedure as before. *Note:* In analyzing these aqueous solutions, remember that a two-fold dilution has taken place by the addition of the dilute bromine-water solution.

Analysis

The AutoAnalyzer may be used as described by Winter (1) without modification except for substitution of an aqueous solution of Brij-35 (2.0 ml/L) for dilute bromine-water. The Brij-35 solution improves the bubble pattern as well as the wash characteristics between samples.

To reduce instrumentation cost, the single pump modification shown in Fig. 2 may be used for the analyses described.

In each instrumental arrangement, the reagents are the same and the choice of enzyme source is open. However, for low cost, convenience, and greater sensitivity for some inhibitors, outdated plasma from a blood bank may be used. Plasma may be stored frozen for several months with little loss of activity.

[3] *n*-Hexane is added to aid partitioning of organophosphorus compounds into the aqueous phase.

A working supply is centrifuged to remove particulate matter and is used undiluted in the two-pump method; it is diluted to 20% plasma with dilute buffer for the single pump method. Both undiluted and diluted plasma can be stored for several days at 4°C with little loss of activity.

A wash of 30% ethanol is used between samples to reduce carry-over from one sample to the next. Fill the small special cups with this wash solution, using a large syringe and needle.

Use glass transmission or small diameter polyethylene[4] tubing wherever possible, especially in the parts exposed to the organophosphorus compounds. Butt joints between glass fittings and tubings held together by polyvinyl chloride sleeving pose less serious wash-desorption problems than polyvinyl chloride transmission tubing alone.

An important advantage in an automatic system is the closely controlled reproducibility of volumes and timings. Therefore, when either system is first set up, the insecticide sample and enzyme sample must come in contact at exactly the same time. Shorten the polyethylene tubing of the later stream to achieve this. To make this timing procedure easier, substitute a blue solution of water-soluble food coloring for the insecticide sample and a yellow solution for the enzyme sample. Then put the system into operation with all involved reagents and observe the timing. The system is set correctly when the color from the first mixing coil (for the two separate colors) is the best visibly uniform green obtainable by adjusting the time of arrival of the separate colors.

The sampler is operated at "40 tests per hour," although the actual maximum number is 20 samples/hr because of the alternate wash cups between sample cups.

In system 2 (Fig. 2), a third pickup crook dips into a constant liquid level dispenser after each sample pickup to maintain a relatively constant liquid flow rate through the first part of the system and, at the same time, to provide an extra volume of dilute buffer as wash into the system through the normal air introduction pump tubing. When the other two sample crooks dip down to pick up samples, air is drawn through this third crook to segment the combined sample streams. The insecticide samples are alternated with 30% ethanol; the enzyme samples are alternated with dilute buffer in the dual row sampler plate to wash the respective lines between samples.[5]

In each system an appropriate series of fortified controls should be run in the same analytical schedule as the "unknown" samples. To calculate recovery rates from the fortified controls, an appropriate serial dilution group from a stock standard solution must be included. Controls must also be analyzed to compensate for any background contamination.

For greatest accuracy, an aliquot of all samples, including each series of fortified controls, controls, and standards separated by at least two uninhibited enzyme checks (enzyme solution samples vs. water), should be analyzed completely after a reagent baseline check and at least three uninhibited enzyme checks. This procedure should be repeated with the other aliquots in reverse order after at least three more uninhibited enzyme checks. An average of the two respective % T readout values for a given sample is then taken.

For a more rapid but less precise operation, fortified controls and standards should be arranged and analyzed once in the order of increasing concentration of inhibitor followed (separated by enzyme checks) by the "unknown" samples. A low concentration sample immediately after a high concentration sample should be repeated at the end of the series; all these samples then are arranged in apparent order of increasing concentration of inhibitor.

Treatment of Data

With the above operation, a universal chart reader (Technicon) may be conveniently used. With this, the standard curves are quickly plotted on the chart reader in terms of % T of peak maxima, while the "unknowns" are compared to these standard curves quickly and directly to read out ppm in the sample when comparing it with the fortified control standard curve. Comparison with the standard curve of the organophosphorus compound yields the concentration of the compound in the analytical solution in terms of μg/ml.

To determine the percentage of enzyme in-

[4] Polyethylene is advantageous due to nonwetting properties.

[5] The newer Sampler II (Technicon) with its added features should fulfill the requirements in this paragraph more efficiently than the original model we used, after suitable modification. Included in such a modification would be milling out a second row of inner holes adjacent to the standard holes of the Sampler II sample tray after the manner of Strandjord and Clayson (9).

hibition, the average value for uninhibited peaks (enzyme vs. water) is plotted in terms of % T on the logarithmic scale of semi-logarithmic paper as 0% inhibition, and the % T of the reagent baseline is taken at 100% inhibition. A line drawn to intercept these two points is then used to determine % inhibition of the enzyme by any sample from the intercept of the % T readout of that sample on the line.

Per cent inhibition values of standards or fortified controls plotted on probability × logarithmic paper vs. concentration in μg/ml or ppm on the logarithmic scale yields an essentially straight-line standard curve.

Results and Discussion

Purified bovine erythrocyte ChE was used in all the analyses discussed below under sample preparation (**a**), while human blood plasma was used in the analyses related to the other sample preparation methods; the dual pump analytical arrangement (Fig. 1) was used except where indicated.

Sample preparation (**a**) was used successfully for fortified controls of Valencia orange peel (25 g/test aliquot) in *n*-hexane solution fortified with either paraoxon or technical grade parathion. The lower limits readily detectable were 0.4 ppm paraoxon and 0.5 ppm technical grade parathion. The colorimeter was operated with 15 mm tubular flow cell and without connection to a range expander.

Similarly 0.25 ppm DDVP in fortified *n*-hexane stripping solution of either broccoli or cauliflower was quantitatively detectable. Dibrom field-treated samples of these vegetable crops generally exhibited slightly more inhibition than the pretreatment samples or controls; since stripping solutions of these samples had been in cold storage several months, the results do not represent residues from current field practices. We assumed that no parent Dibrom was left in any of the samples after this storage period; thus DDVP (a decomposition product of Dibrom) was chosen as the standard. Analyses of these samples demonstrated the feasibility of using these procedures for rapid residue evaluations.

Sample preparation (**b**) was used to determine technical grade parathion in fortified *n*-hexane stripping solutions of market canned peaches (25 g). The standard curve from a sample fortified at various levels (Fig. 3A) is shown in Fig. 3B as it would be seen on a chart reader (Technicon). The recorded trace of "unknown" samples would then be substituted under the chart reader, with standard curve drawn for the fortified controls shown. Each peak from an "unknown" sample could be read off directly in ppm of technical grade parathion at the intercept of the line with the peak maximum. Significant inhibition was observed from a fortified sample at the 0.1 ppm level. No inhibition was observed in the unfortified market samples.

Method (**b**) was also used to determine phosphamidon separately in fortified methylene chloride stripping solutions of both the peel and the pulp of white potatoes (25 g each). Significant inhibition of human plasma ChE (phosphamidon is a very poor inhibitor of purified bovine erythrocyte ChE) was observed in each fortified substrate down to 0.1 ppm. Greater inhibition and, therefore, presumably higher recovery of phosphamidon were obtained from fortified potato pulp than from potato peel, as indicated in Fig. 4.

Method (**b**) was performed as written, except that 25 ml *n*-hexane was added prior to concentration of 50 ml benzene stripping solution of waterplants for the determination of technical grade parathion in many waterplant samples from a wildlife study (10, 11). Technical grade parathion at 0.1 ppm in fortified pretreatment samples was the lower limit of detection.

A single pump instrumentation arrangement similar to that shown in Fig. 2, except for the addition of a special valve to meter in appropriately timed portions of dilute buffer and ChE solutions from flasks, was used along with the sample preparation method (**d**, first paragraph, starting with method **b**) to determine parathion (99.6% pure) in fortified *n*-hexane stripping solutions of market canned peaches (10 g) down to 0.04 ppm. The 0.12% bromine-water oxidation procedure produced the same amount of inhibition by a fortified sample at 0.18 ppm as a standard parathion solution at

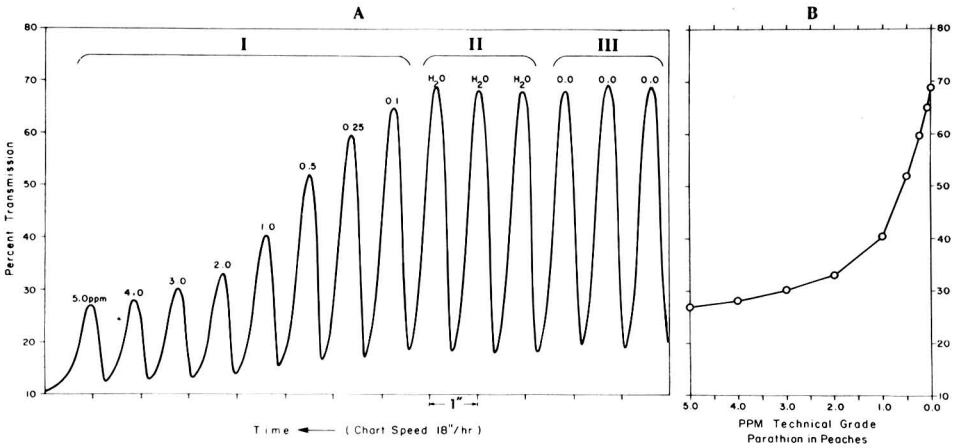

***Fig. 3*—*A.* Representative recording of market canned peaches, unfortified and fortified with technical grade parathion: (I) aliquots from *n*-hexane stripping solution of peaches fortified in terms of ppm; (II) replicate checks of ChE activity (human plasma) when water alone was sampled; and (III) aliquots from unfortified stripping solution of canned peaches. Each aliquot was equivalent to 25 g peaches, and after final preparation the analytical solution represented 5 g/ml. *B.* Standard curve of technical grade parathion-fortified peaches drawn on chart reader (Technicon) from chart record shown in *A*.**

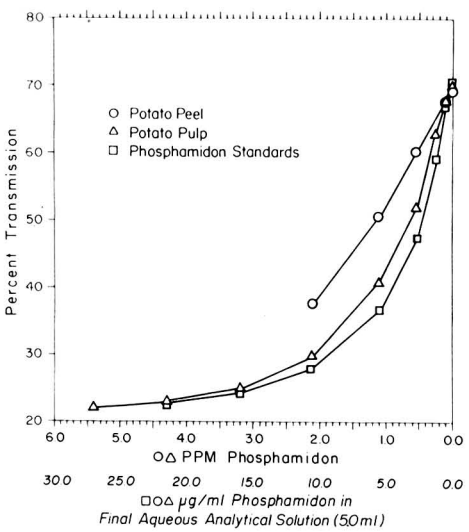

***Fig. 4*—Comparison of the standard phosphamidon curve with phosphamidon-fortified standard curves of potato pulp and potato peel drawn on a chart reader (Technicon). (*Note:* The unfortified substrate exhibited no significant difference from water alone.)**

equivalent concentration did when similarly oxidized, indicating no loss in efficiency of the oxidation in the presence of the peach extractives. However, no significant inhibition was observed in a 0.18 ppm-fortified sample similarly treated with 0.04% bromine-water solutions, thus indicating that all of the bromine was consumed by the plant materials with no excess to perform the oxidation of the parathion present. Therefore, the concentration of bromine-water solution must be determined for each new set of conditions and, most importantly, for the amount and nature of the substrate present. A 0.12% bromine-water solution was suggested in method (**d**) because that level was satisfactory for *n*-hexane stripping solutions of canned peaches representing 10 g/test aliquot. However, for other conditions the concentration might need to be increased.

The following are compounds which, in standard solutions in chloroform (25–100 μg/ml), were equilibrated into an aqueous phase according to method (**c**) for analysis by the single pump automated scheme with special valve: DDVP, Dibrom, paraoxon, parathion (99.6%), parathion (technical grade), and phosphamidon.

Without *n*-hexane added, as called for in method (**c**), DDVP was the only inhibitor in the list above which was measurably equilibrated into an aqueous phase from a chloroform solution. This information is potentially useful for a relatively specific anti-ChE determination of DDVP.

The system in Fig. 2 was used in an extensive study of the interaction and/or simple additive effect of two or more inhibitors when in admixture. Without some useful answers, the general methodology of anti-ChE determinations can never yield definitive quantitative data from "unknown" samples. If the spray history of the crop substrate is known, the quantitative aspects of the method improve markedly.

A certain amount of specificity, even in substrates with an unknown spray history, can be achieved by judicious choice of stripping solvents (e.g., *see* Beroza and Bowman (12)), method of sample preparation prior to AutoAnalysis, and ChE enzyme source. Winter (1) cited three references in which suggestions were offered on choice of enzyme system to increase specificity. Further specificity can be derived from data from analyses of the same sample solution both before and after an oxidative procedure to convert some poor inhibitors to strong ChE inhibitors, e.g., parathion to paraoxon. After making these choices, one can at present only relate the inhibition values obtained from "unknowns" to both the most potent inhibitor and the least potent inhibitor probably present in the actual analytical test solution and thereby obtain a range of semiquantitative data. The information is useful because it shows that a mammalian toxic agent is present, a valuable screening criterion for many organophosphorus pesticide residues; it provides more universal detection than polarography; and it is more specific than total phosphorus methods. Probably the greatest single advantage of this new method is its ease and rapidity in terms of number of samples analyzed per day.

Development of rapid separation techniques will also enhance the value of this method; e.g., combination of thin layer chromatography and a semi-automated procedure to elute compounds from the adsorbent with the automated system (Fig. 2) for determination of anti-ChE agents (13).

Each system, if used separately as discussed, has its merit as a rapid screening procedure. However, the arrangement of Fig. 1 is useless if the automated bromine oxidation step is not successful; even at its best, the efficiency of this reaction does not compare with the efficiency produced in sample preparation (**d**), which requires essentially no more time or effort on the part of the technician than the other sample preparation methods.

Any of the systems potentially permit nonchemists to rapidly turn out useful data which previously could not be obtained so rapidly and which would require years of experience by a skilled technician on a similar manual method to obtain the precision offered by the automated systems.

The potential of coupling this type of system to one or more other continuous automated wet-chemical analysis systems to provide more than one piece of information from a given sample has not been explored. A logical approach would be to split a cleaned up insecticide sample stream at the end of the first pump tubing and pass this second sample stream into a flow system, using a Digestor module (Technicon) adapted for total phosphorus determinations by Winter and Ferrari (3), as modified by Getz (14) and Weinstein, *et al.* (15), and made feasible for organophosphorus insecticides in our laboratories (16). These systems analyze at best no more than 10 samples per hour, thus reducing the overall speed of analysis. However, the data produced are more meaningful than anti-ChE measurements alone. The potential for other combinations is great.

A rapid method to determine the parent phosphate insecticide residue and also its metabolites, both qualitatively and quantitatively, is still needed. At the sacrifice of rapidity, an automated system for anti-ChE determinations could be used following the total method, except the analytical procedure, of Coffin and Savary (17), which is a paper chromatographic separation procedure following a general extraction and cleanup procedure of plant materials prior to determination of organophosphorus residues. The sample obtained after elution from the paper chromatogram would adapt itself readily to stream splitting and simultaneous automated total organophosphorus determinations as discussed above.

Acknowledgment

Technical assistance of Carol A. Lazzaro is gratefully asknowledged.

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Automated Elution-Filtration Analysis of Anticholinesterase Organophosphorus Compounds on Thin Layer Chromatographic Scrapings[1]

By DANIEL E. OTT and FRANCIS A. GUNTHER (Department of Entomology, University of California, Riverside, Calif. 92502)

An automated analysis system for anticholinesterase organophosphorus compounds has been modified to include an automated elution-filtration technique for analysis of these compounds after thin layer chromatography. The entire method offers specificity previously unobtainable by automated anticholinesterase methods and is rapid when combined with a multiple spotter apparatus for thin layer chromatography. The present method has been used to determine parathion residues in fortified canned peaches down to a level of 0.1 ppm.

Recent developments and applications (1–4) using thin layer chromatography (TLC) for the separation, isolation, and semiqualitative and quantitative estimation of organophosphorus pesticides have been most promising.

The general technique of TLC as related to pesticide residue analysis has been reviewed by Conkin (5) and by Abbott and Thomson (6).

Less tedious and more rapid methods combining the advantages of TLC with reasonable accuracy and precision are needed. The present paper describes an approach to help meet these requirements.

METHODS

Apparatus and Reagents

(a) *Thin layer apparatus.*—Applicator and equipment, plus fluorescent silica gel GF 254 (Brinkmann Instruments, Inc., Westbury, N.Y.).

(b) *Sample applicator (Kensco).* — With

[1] Paper No. 1678, University of California Citrus Research Center and Agricultural Experiment Station, Riverside, Calif.

twelve 0.005″ i.d. stainless steel capillary U-tubes (Kensington Scientific Corp., Oakland, Calif.).

(c) *AutoAnalyzer.*—Use system for anticholinesterase determinations described by Winter (7) and modified by Ott and Gunther (8), with a Sample Mixer unit and a Continuous Filter module with the finer type T-3402 filter paper roll (Technicon Controls, Inc., Chauncey, N.Y.). Prepare necessary reagents as described in (8).

(d) *Developing solvent system.*—*n*-Hexane-chloroform-methanol (7:2:1). Redistill all solvents.

(e) *Phosphate buffer solution.*—pH 6.

(f) *Eluting solutions.*—30% ethanol and 0.08% (v/v) dilute bromine-water solution. Prepare fresh daily from a completely saturated bromine-water solution.

Thin Layer Chromatography

Slurry silica gel-buffer solution (1:2) and prepare plates 250 μ thick as described by Blinn (1). (*Note:* Plates are buffered to eliminate hydrolysis of organophosphate compounds.) Air-dry plates overnight at room temperature before use. Score into twelve channels 15 mm wide and mark for 10 cm development distance.

Use Kensco Sample Applicators for mass production spotting. To speed application and minimize spot size, elevate each applicator unit with suitable supports (e.g., Lab Jacks) at each end to permit placement of a hot-air dryer under the applicator with plate. The plate can thus be warmed by conduction. Immerse the stainless steel capillary tubes of the applicator completely in *n*-hexane to fill them. Measure sample solutions into the small applicator vials with a microsyringe and quickly position a capillary tube into each so that the tip barely touches the plate. This will insure that flow is started by capillary action if not by gravity. After the contents of a vial have been applied to the plate, rinse the vial with 0.2 ml or less of solvent, using a clean syringe and needle. After application of rinse solvent, rinse tip of delivery capillary tube with a fresh drop of the same solvent. After spotting is completed, develop plate with solvent system (d) in a closed tank, lined with filter or blotter paper, to a line scored 10 cm above origin. Air-dry developed plate in a fume hood and mark the migrated spots of interest under UV light (2537 and/or 3660Å). Scrape off marked spots into AutoAnalyzer polystyrene sample cups or into small test tubes if the bromine-water oxidative procedure is to be used on the sample. (*Note:* Restrict area scraped off to 15 mm^2.) If first proved safe, scrapings may be stored dry in their stoppered containers in a refrigerator.

Bromine-Water Oxidation-Elution

To oxidize compounds to more potent ChE inhibitors, e.g., parathion to paraoxon, use the following procedure: Add 5.0 ml 0.08% fresh bromine-water solution to each test tube containing scrapings from one appropriate TLC spot. Add two small carborundum boiling chips, cover tube tightly with a double layer of aluminum foil, and heat at 110°C for 10 min. To analyze later, store in a refrigerator.

Automated Analysis

Apparatus.—Anti-ChE determinations are performed with an AutoAnalyzer system essentially as described by Ott and Gunther (8). A single pump is used and an extra wash is provided by a constant volume device containing dilute buffer solution.

The significant changes and additions needed for the present procedure are seen by comparing Fig. 2 (*see* p. 664, ref. (8)) with Fig. 1.

Figure 2 (p. 664) represents the basic flow diagram for anti-ChE determinations, while Fig. 1 is the flow diagram for anti-ChE determinations on TLC scrapings.

Attach Sampler Mixer as diagrammed in Fig. 1 to the pickup crook mechanism of Sampler module and position so that a single cup delay period exists between the second mixing prong and the sampling pickup crook. This permits most of the silica gel particles to settle on the bottom of the sample cup; thus they are not picked up when the Sampler pickup tube is adjusted to dip only about ¾ of the way down into the cup on the pickup stroke.

When the Sampler Mixer is used, place the samples in the outer concentric row of holes of the Sampler plate, and position the cups of enzyme source (diluted outdated human plasma) in the inner concentric row of holes opposite the samples. Alternate samples with cups of 30% ethanol wash, and alternate enzyme source cups with dilute buffer wash in the special small glass tubes provided for wash purposes.

The Continuous Filter module is the main change in this method. The extra pump tube and connecting tubings required for this module delay sample introduction into the system.

Therefore, the new setup must be timed initially so that sample and enzyme will mix downstream from the Filter module at exactly the same time. This timing procedure may be done as described by Ott and Gunther (8): an extra length of polyethylene tubing (0.030" i.d.) on the enzyme line will delay the enzyme source while an extra length of transmission tubing will delay entry of the dilute buffer wash solution from the constant level device. Once this timing procedure is established, these extra lengths of tubing (properly labeled) can be inserted into the positions and the Filter module added to readily change from ordinary anti-ChE analysis to anti-ChE analysis with filtration.

To operate, set the filter paper speed control at midpoint and start both mixer motors (one on the Filter module and one on the Sampler pickup mechanism).

Procedure.—Add oxidized thin layer scrapings directly to standard Sampler cups for analysis. To each cup containing unoxidized samples, add 2.0 ml 30% ethanol[2] from an automatic refilling syringe.

Operate the Sampler module at the 40 tests/hr setting. However, the actual sampling will be reduced to 20/hr because of alternating the sample and enzyme cups with wash solutions.

Results and Discussion

Technical grade parathion in *n*-hexane solution was applied, in varying concentrations, to a fluorescent thin layer plate. After development and examination under UV light, three or four components were observed. A single spot occurred at about 0.2 R_f, an incompletely separated doublet spot appeared at about 0.4 R_f, and the third spot, corresponding in mobility to a parathion (99.6%) standard, had about 0.7 R_f. Only those spots with the R_f of purified parathion were scraped from the plate (15 mm² area) and subsequently oxidized. Results from the automated analysis of these spots are shown graphically in Fig. 2. The compound or compounds from the 0.4 R_f scrapings directly inhibited ChE. However, no significant inhibition (2% or less) was observed from the TLC scrapings of the low R_f-value spots. We repeated this experiment twice with results corresponding very closely to those shown in Fig. 2. On one TLC plate, the two components in the intermediate R_f area were almost completely separated in all channels. These spots were analyzed separately. Surprisingly, the plot of these points was a single line with essentially the same slope as that resulting from analysis of the doublet spot in its entirety (Fig. 2). On the same TLC plate on which the doublet was best separated, paraoxon had also been spotted in a separate channel. Based solely upon comparative R_f data, the lower spot (about 0.3 R_f) in the doublet could have been paraoxon. Both components of the doublet are direct ChE inhibitors, however, and the second component may, therefore, be the S-ethyl isomer of parathion, a known ChE inhibitor and constituent of technical grade parathion (9).

Based on a comparison of R_f data and non-ChE inhibition (Fig. 2), the least mobile spot on the chromatogram may be *p*-nitrophenol.

Since this method is capable of detecting both parent compounds and toxic metabolites or impurities responding to a ChE-type assay, it should be useful to regulatory agencies which are interested in the detection of all pesticide residues in foodstuff resulting from insecticide sprays.

Parathion (99.6%) was analyzed by this method, both before and after TLC, and the recoveries were compared. The samples were oxidized prior to analysis to yield the results plotted in Fig. 3. Essentially quantitative recovery of parathion from the TLC plate was achieved. A similarly prepared inhibition curve of unchromatographed, pure paraoxon[3] overlaps that of unchromagraphed oxidized parathion, also indicating quantitative oxidation to paraoxon.

This method was applied to a simulated residue problem. A methylene chloride stripping solution of market canned peaches, after concentration in a Kuderna-Danish concentrator, was divided so that 11 of the 12 Sample Applicator vials each received the equivalent of 10 g peaches. Parathion (99.6%) in *n*-hexane was added to the first

[2] Concentrations of ethanol up to 30% have no effect on ChE activity.

[3] Generously supplied by R. C. Blinn of American Cyanamid Co., Princeton, N.J.

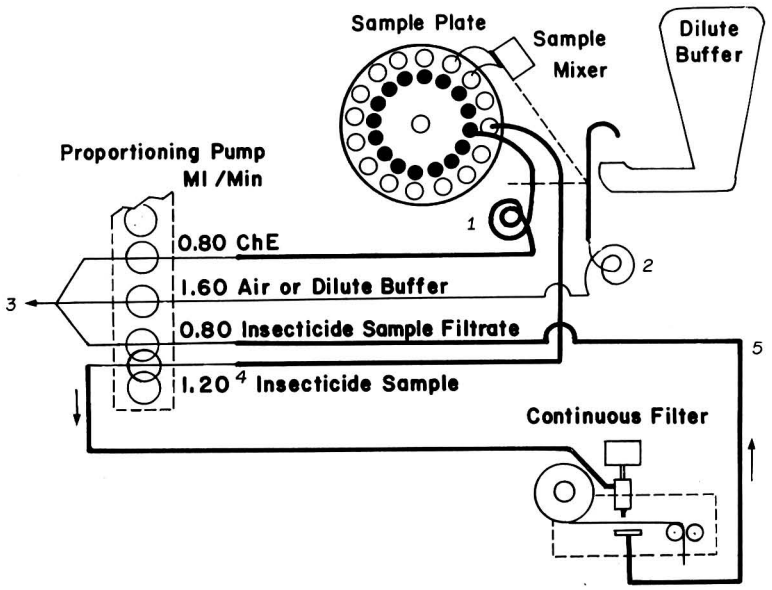

Fig. 1—**Flow diagram of additions and changes to Fig. 2 (p. 664), for an automated filtration-analysis system for determining anti-ChE organophosphorus compounds absorbed on TLC adsorbents. (1) Polyethylene tubing added to delay and time properly addition of ChE solution; (2) transmission tubing added to delay and time properly introduction of dilute buffer solution; (3) connection to rest of automated system from this point is the same as that in Fig. 2; (4) pump tubing with ends cut as short as possible and connected at both ends with lengths of 0.030″ i.d. polyethylene tubings, also as short as possible; (5) same polyethylene tubing used for regular sample pickup line in Fig 2.**

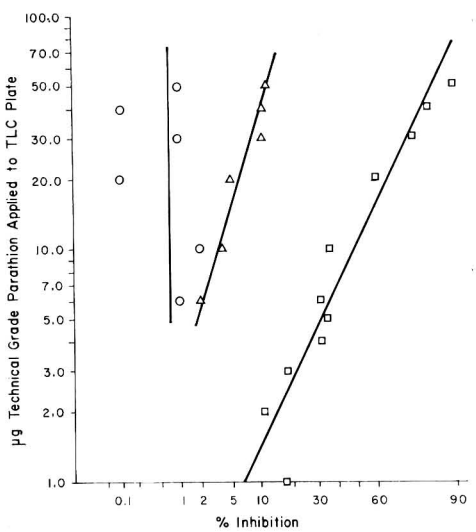

Fig. 2—**Results of automated analysis of TLC scrapings spotted with technical grade parathion. Circles: low R_f component. Triangles: intermediate R_f component(s). Squares: high R_f component after bromine-water oxidation.**

ten vials to fortify the samples over a range of 0.03 to 0.44 ppm; the eleventh vial served as "control" with no added parathion, and the last vial contained only parathion (4.4 µg) solution to serve as the TLC standard. After simultaneous spotting followed by development, the plate under UV light demonstrated that peach extractives obscured the parathion. An area 25 mm long and 15 mm wide at a uniform R_f height range across the plate was removed by scraping. The R_f area ranged from about 5 mm above the marked TLC companion parathion standard to 10 mm below. After oxidation-elution, the samples were analyzed in series with equivalent amounts of oxidized parathion samples as reference standards. Results are shown in Fig. 4, in which the displacement of the two lines indicates about 25% recovery of parathion from fortified peach extractives on a TLC plate. It is possible that higher concentrations of dilute bromine-water might increase

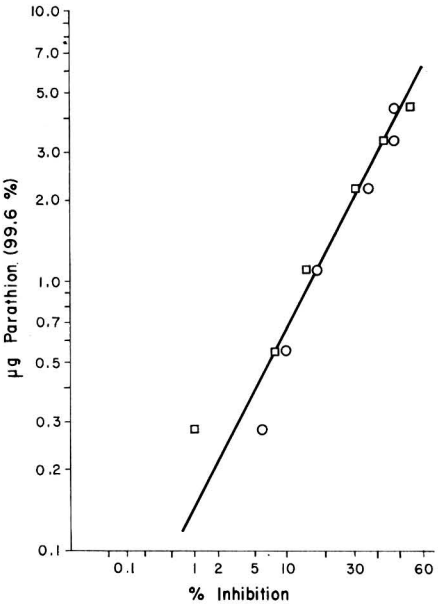

Fig. 3—**Results of automated analysis to check the efficiency of removal of parathion adsorbed on TLC scrapings. Each circle (µg parathion spotted on TLC plate) plotted represents the average of analysis of two TLC samples (same plate) at the same level of applied parathion by comparing with reference standards (squares: µg parathion, unchromatographed). All samples and standards were oxidized by bromine-water prior to analysis.**

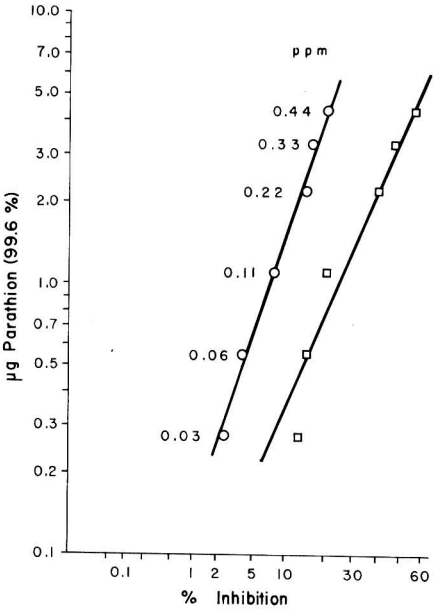

Fig. 4—**Results of automated analysis of TLC scrapings with adsorbed parathion from fortified canned peaches. Each circle (µg added to 10 g, expressed as ppm) plotted represents the average of two analytical replicates of each of 2 TLC samples (same plate) at the same level of fortification as compared with reference standards (squares: µg parathion, unchromatographed). All samples and standards were oxidized by bromine-water prior to analysis.**

recoveries from crop extractives. Even though recoveries are low, the linearity of fortified samples indicates that this method is practical if suitable fortified "controls" for quantitation purposes are analyzed along with the test samples. Almost any automated system can tolerate lower recoveries than those required in comparable manual methods because of the generally greater precision of the former. No inhibition was present in the TLC scrapings from the "control" channel in the R_f range of parathion. Significant inhibition above background levels was seen only in samples fortified at 0.1 ppm and greater.

For a more elegant and time-saving approach, mylar-backed, ready-prepared sheets[4] can be used for TLC. These sheets were developed in redistilled chloroform to achieve separation of the components in technical grade parathion comparable to that discussed previously. After the spots were marked under UV light, they were quickly and conveniently cut out with sharp scissors in a uniform size area. The individual TLC areas were bent carefully and inserted into a test tube or AutoAnalyzer sample cup, and then were treated for analysis as regular TLC scrapings. Evaluation of recovery of parathion (99.6%) from this technique, as in Fig. 3 for regular TLC, produced a set of linear points displaced to the left of those for unchromatographed parathion standards. Recoveries, although less than quantitative, were estimated to be at least 50%.

For supplementary information it would be useful to see the compound of interest on the TLC plate, but at these low levels of fortification in the presence of crop extractives it was impossible. The TLC procedure is used here only as a partial

[4] Eastman Chromagram Sheets (Type K301R), available from Eastman Kodak Co.

cleanup and separative technique which is rapid and convenient with the multiple spotter. This technique is even more rapid and convenient when prepared TLC sheets are used. The total method combining TLC and automated analysis offers greater specificity than previously reported automated anti-ChE methods (7, 8).

Acknowledgment

Technical assistance of Carol A. Lazzaro is gratefully acknowledged.

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COLOR ADDITIVES

Synthetic Organic Colors in Oils

By F. B. JONES (Food and Drug Administration, 850 Third Ave., Brooklyn, N.Y. 11232)

A fast and simple method was developed for the separation and identification of micro amounts of 11 oil-soluble synthetic organic colors. The colors under study were D&C Red Nos. 17, 18, and 35; D&C Green No. 6; D&C Violet No. 2; D&C Yellow No. 11; Ext. D&C Blue No. 5; Ext. D&C Orange No. 4; Ext. D&C Red No. 14; and Ext. D&C Yellow Nos. 9 and 10. Results of the collaborative study were good, and the method is recommended for adoption as official, first action.

Modern marketing techniques require standardization and uniformity in all products. One way of achieving this goal is the addition of color, natural or synthetic. Synthetic organic colors are added in minute amounts to foods, drugs, and cosmetics. Present methods can detect either a limited number of colors in small amounts or all those present only if the amounts are relatively large. Therefore, a procedure is needed to detect and differentiate these colors at the levels normally used. A relatively fast and simple method for the separation and identification of 11 oil-soluble synthetic organic colors, when present in small amounts, has been developed.

The official method (1) uses strong acids in a complicated extraction procedure and determines only four colors. Weiss (2) separated pure oil-soluble coal-tar colors at the 0.5 mg level but gave no directions for separation of the colors from the oil. Silk (3) separated and determined concentrated solutions of 14 oil-soluble colors in food by column and paper chromatography. However, traces of oil remained with the color eluted from the column and obscured the spots in the paper chromatographic identification of the colors. Other difficulties encountered in the procedure are as follows: D&C Violet No. 2 forms a lake in the columns and is difficult to remove; and during the evaporation of the dilute acetone solutions, Ext. D&C Yellow Nos. 9 and 10 may decompose.

The procedures and techniques of Weiss (2) and Silk (3) were combined and adapted for this study. The procedure developed

separates most combinations of oil-soluble colors by combinations of columns and solvents and eliminates interferences from the oily medium. In this method, four columns are used for separation, and the colors are identified spectrophotometrically. When proper care is taken, the procedure is semiquantitative.

Collaborative Study

The colors used in this study were D&C Red Nos. 17, 18, and 35; D&C Green No. 6; D&C Violet No. 2; D&C Yellow No. 11; Ext. D&C Blue No. 5; Ext. D&C Orange No. 4; Ext. D&C Red No. 14; and Ext. D&C Yellow Nos. 9 and 10.

A collaborative study was made of 10 colors divided among three samples. Each sample contained four colors (6 mg/liter) dissolved in cottonseed oil. The portion taken for analysis thus contained 60 μg of each color. The samples contained the following colors:

Sample 1: D&C Yellow No. 11, D&C Green No. 6, Ext. D&C Red No. 14, and Ext. D&C Yellow No. 10.

Sample 2: Ext. D&C Blue No. 5, D&C Red No. 17, Ext. D&C Orange No. 4, and Ext. D&C Yellow No. 9.

Sample 3: D&C Violet No. 2, D&C Red No. 35, D&C Green No. 6, and Ext. D&C Yellow No. 9.

The collaborators were requested to analyze each sample and identify the colors present by the following method (only one determination was required):

METHOD

Principles

Eleven oil-sol. color additives are sepd chromatographically. Colors in fractions are identified spectrophotometrically. Steps are given in Table 1.

Apparatus

Chromatographic tubes.—20 mm i.d. × 300 mm, with stopcock and fritted glass plate (or glass fiber disk over glass wool plug). With device to deliver air pressure at top.

Adsorbents

(a) *Florisil.*—60–100 mesh. Activated at 650° (1200°F) by manufacturer (Floridin Co., 2 Gateway Ctr., Pittsburgh, Pa. 15222). Store at

Table 1. Steps for chromatographic separation of 11 oil-soluble color additives

A Florisil column	Petr. ether	Colorless (discard)
	Ether	Colored, Colored } To column **B**
	Alcohol-ether	Yellow (Natural color, discard)
		D&C Violet No. 2 (81481)
		D&C Yellow No. 11 (83089)
	Acetonitrile	Traces of D&C Yellow No. 11
		D&C Red No. 35 (2425856)
B Alumina column	Petr. ether	Discard
	$CHCl_3$	Colorless (Discard)
	Alcohol-$CHCl_3$	Colored, Colored } To column **C**
C Magnesia column	Petr. ether	Discard
	$CHCl_3$	D&C Green No. 6 (128803), Ext. D&C Blue No. 5 (2646153) } To column **D**
		Ext. D&C Orange No. 4 (2646175)
		Ext. D&C Red No. 14 (1281385)
	Alcohol-$CHCl_3$	D&C Red No. 18 (1281396)
		Ext. D&C Yellow No. 9 (85847) and 10 (131793)
		D&C Red No. 17 (85869)
D Silicic acid column	*n*-Hexane-benzene	D&C Green No. 6
	Benzene	Ext. D&C Blue No. 5

130° in g-s. bottle. For use, add 1.5 ml H_2O to 100 g Florisil in g-s. bottle, shake to break up lumps, and mix thoroly. Let stand overnight before use.

(b) *Alumina.*—80–200 mesh. Adsorption, for chromatographic analysis. Heat 100–200 g 1 hr at 400°. Store in tightly stoppered bottle in desiccator.

(c) *Sea Sorb-43.*—*See* 39.003(j) for source.

(d) *Celite 545.*—Johns-Manville.

(e) *Silicic acid.*—100-mesh, for chromatography (Mallinckrodt Chemical Co. No. 2847, or equiv.).

(f) *Solvents.*—Reagent grade. Petr. ether, ether, alcohol, $CHCl_3$, *n*-hexane, benzene, and acetonitrile. Redistill acetonitrile from H_3PO_4 and P_2O_5, if necessary.

Preparation of Columns

(Place plug of glass wool or piece of Teflon-coated nylon on top of each column.)

(a) *Florisil column.*—Fill tube to height of 4″, tapping to pack and remove air. Wash with petr. ether and drain to top level of column.

(b) *Alumina column.*—Add 50 ml petr. ether to closed tube, add 18 g alumina, and work plunger to break lumps and remove air. Drain to top level.

(c) *Magnesia column.*—Mix equal wts of magnesia (c), and Celite 545. Prep. as for (b), using 9 g of mixt. Compress column with slight air pressure.

(d) *Silicic acid column.*—Add ca 4″ of mixt. of equal wts silicic acid, (e), and Celite 545, (d), to column, using suction. Tamp and smooth upper surface, and wash with *n*-hexane, using pressure.

Determination

Dil. 10 ml oil-based sample with 10 ml petr. ether and place on Florisil column, (a). Elute petr. ether. Discard colorless portion and begin collection when color appears. Continue elution until eluate (No. 1) is colorless. Set eluate aside, change receivers, and elute with ether until eluate (No. 2) is colorless. Set eluate No. 2 aside, start elution with alcohol-ether (1 + 3), and watch eluting colors. Change receivers when eluate color changes. (Identify receivers by position in scheme. Usually first eluate is yellow from natural color of base oil and has no distinctive spectrophotometric curve. Discard this eluate.) Next eluate is *D&C Violet No. 2* (if present); then *D&C Yellow No. 11.*

When alcohol-ether eluate is colorless, begin elution with acetonitrile. (This will elute last trace of *D&C Yellow No. 11;* also *D&C Red No. 35.*)

Evap. individual alcohol-ether and acetonitrile eluates to dryness, dissolve residues in $CHCl_3$, and dil. to vols suitable for spectrophotometer. Scan between 350–700 mμ, and compare against curves of known colors. (Sepn of *D&C Violet No. 2* and *D&C Yellow No. 11* may not be complete but colors can be identified because max. absorption points are widely sepd. These colors may be sepd by extg *D&C Yellow No. 11* with 70% alcohol from soln of their mixt. in petr. ether.) Evap. original ether eluate (No. 2) to remove all ether, add petr. ether eluate (No. 1), and evap. to ca 15 ml.

Transfer carefully to alumina column, (b). When all soln enters column, wash with 50 ml petr. ether and discard eluate. Add two 10 ml portions $CHCl_3$. If $CHCl_3$ eluate is green or blue, add it to following alcohol-$CHCl_3$ eluate; if $CHCl_3$ eluate is colorless, discard it. Continue elution with alcohol-$CHCl_3$ (1 + 3) until eluate is colorless. Evap. solvent completely and dissolve residue in petr. ether.

Add soln carefully to magnesia column, (c), dropwise at side of tube, with pipet. Apply slight pressure until soln just passes into adsorbent; then wash column with 25 ml petr. ether, and discard petr. ether wash. Elute with $CHCl_3$ and watch for colors, collecting individual fractions. (First fraction may contain *D&C Green No. 6* and *Ext. D&C Blue No. 5.* Second may contain *Ext. D&C Orange No. 4* and *Ext. D&C Red No. 14.*) Continue to colorless eluate and change receivers; then elute with alcohol-$CHCl_3$ (1 + 3), changing receivers as different colors appear *(D&C Red No. 18, Ext. D&C Yellow Nos. 9 and 10, and D&C Red No. 17).* Evap. individual solns, dissolve each in $CHCl_3$, and scan from 350 to 700 mμ. Compare curves with those from known colors. If curve for blue-green portion does not conform to known color, use following sepn:

Evap. $CHCl_3$ and dissolve residue in *n*-hexane. Put on silicic acid column, (d), and elute with *n*-hexane-benzene (1 + 1). Collect eluate until colorless and continue elution with benzene until eluate is colorless.

(If curve for *D&C Red No. 17* has min. at 385 mμ, *Ext. D&C Yellow Nos. 9 and 10* may be present. Sep. yellows from *Red No. 17* as follows: Evap. $CHCl_3$. Dissolve residue in min. vol. petr. ether. Put on magnesia column, and elute with alcohol-$CHCl_3$ (1 + 3). Collect individual fractions as color changes.)

Det. color present by evapg solvent in frac-

tion, dilg to vol. with $CHCl_3$, and scanning from 350 to 700 mμ. Compare curves with known color on same chart.

Results and Discussion

The method described will separate and identify many mixtures of oil-soluble synthetic organic colors in dilute oil solutions. The Associate Referee found the recoveries to be from 60 to 100%. Collaborative results (Table 2) did not include quantitative data. The oils used in the study contained four colors each; usually not more than two or three colors are used in a given product.

The pairs of colors, Ext. D&C Yellow Nos. 9 and 10, and Ext. D&C Red No. 14 and Ext. D&C Orange No. 4, which are not separated by this procedure can be determined on a partition column (2). A modification of this procedure is necessary when analyzing for the yellows. As evaporation of the acetone solution may cause decomposition, the acetone-water eluates should first be extracted with petroleum ether. The petroleum ether can then be evaporated with no risk of decomposition.

The maxima of Ext. D&C Red No. 14 and Ext. D&C Orange No. 4 differ by only 5 mμ; these are easily distinguishable when found separately. In the study, some of the collaborators were undecided about the exact wavelength of the maximum of the curve. This is a difficulty inherent in the spectrophotometric identification of colors in general. Scanning the known and unknown on the same chart may eliminate the difficulty of decision.

Collaborators 3, 4, and 6 identified Ext. D&C Red No. 14 as Ext. D&C Orange No. 4 in Sample 1. The Associate Referee examined these curves, however, and thought that they indicated Ext. D&C Red No. 14. Collaborator 6 identified Ext. D&C Orange No. 4 as Ext. D&C Red No. 14 in Sample 2, and Collaborator 4 identified D&C Red No. 17 as D&C Red No. 18 in Sample 2. In each case, the curve looked like that for the correct colors.

Collaborator No. 5 was uncertain about the choice between Ext. D&C Red No. 14 and Ext. D&C Orange No. 4 in Samples 1 and 2. In both cases, the correct color was reported as "more probably present." Collaborators usually reported Ext. D&C Yellow No. 9 and/or No. 10. This was expected, since the curves of the two are identical. The collaborators were not asked to use the Silk procedure (3) to separate these colors.

In Sample 2, Collaborator No. 6 reported D&C Red No. 18 instead of Ext. D&C Yellow No. 9. Examination of flow sheets submitted with the sample indicates incomplete elution with chloroform from the magnesia column; this could have caused confusion in the identification of the colors.

No collaborators indicated any particular difficulty with the procedure. The surface of the columns can be kept smooth, a problem some collaborators mentioned, by placing a plug of glass wool on a piece of Teflon-coated nylon on top of the column. This direction was added to the method, even

Table 2 Collaborative results for identification of oil-soluble colors[a]

Color	Coll. 1	Coll. 2	Coll. 3	Coll. 4	Coll. 5	Coll. 6
Sample 1						
D&C Yellow No. 11	+	+	+	+	+	+
D&C Green No. 6	+	+	+	+	+	+
Ext. D&C Red No. 14	+	+	[b]	[b]	+	[b]
Ext. D&C Yellow No. 10	+	+	+	+	+	+
Sample 2						
Ext. D&C Blue No. 5	+	+	+	+	+	+
D&C Red No. 17	+	+	+	[c]	+	+
Ext. D&C Orange No. 4	+	+	+	+	+	[d]
Ext. D&C Yellow No. 9	+	+	+	+	+	[c]
Sample 3						
D&C Violet No. 2	+	+	+	+	+	+
D&C Red No. 35	+	+	+	+	+	+
D&C Green No. 6	+	+	+	+	+	+
Ext. D&C Yellow No. 9	+	+	+	+	+	+

[a] + Indicates correct color reported.
[b] Identified as Ext. D&C Orange No. 4.
[c] Identified as D&C Red No. 18.
[d] Identified as Ext. D&C Red No. 14.

This report of the Associate Referee was presented at the Seventy-ninth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 11–14, 1965, at Washington, D.C.

though it was not included in the original directions.

A few collaborators indicated a desire for a scheme of elution, which was incorporated in the method.

Conclusions and Recommendation

Most collaborators were able to separate and correctly identify the colors. It is recommended that the method be adopted as official, first action.

Acknowledgments

The Associate Referee wishes to express his appreciation to the following collaborators, all of the Food and Drug Administration: Joseph E. Sperco, Boston; Jerry E. Froberg, Los Angeles; Jane Szpylman, Buffalo; Carolyn N. Andres, Denver; John L. Allen, Minneapolis; and Brenda M. Roediger, Atlanta.

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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 *This Journal,* **49**, 165–167 (1966).

MICROBIOLOGICAL METHODS

Coliform and Total Bacterial Counts in Spices, Seasonings, and Condiments

By FRED WARMBROD and LINDA FRY (Tennessee Department of Agriculture, Food and Drug Division, Nashville, Tenn. 37204)

The regulatory program of the Tennessee Food and Drug Division has been extended to include microbiological contaminants in spices, seasonings, and condiments. Samples were examined for aerobic plate count, coliform count, and acid tolerant bacteria and mold counts. Some samples gave high coliform counts which may possibly indicate contamination. A regulatory program is more meaningful if microbacteriological examinations are made on food products.

A program of testing spices, seasonings, and condiments for bacterial content was started in an effort to determine if these ingredients were responsible for extremely high bacterial contents found in sausages and other prepared meats. Aerobic plate count, acid tolerant bacteria and mold counts, and coliform counts were made on spices, seasonings, and condiments to uncover indications that these products were shipped in an adulterated condition or had become contaminated because of improper handling or storage in the packing plants.

Experimental

Samples of spices, seasonings, and condiments used in various packing plants in Tennessee were collected by the State food and drug inspectors.

The methods for examining spices described by Jensen (1), Tanner (2), and the AFDOUS (3), were used as guidelines for the procedure. Ground spices (1 g) and bulky spices (2 g) were weighed into sterile dilution bottles and were diluted to 100 ml with sterile phosphate buffer. The samples were shaken vigorously for 5 minutes, the coarse materials were allowed to settle, and the samples were plated on the various media.

Aerobic plate counts (APC) were made on nutrient agar (Difco) at dilutions of 1:100, 1:1000, 1:10,000, and 1:100,000 and were incubated at 37°C for 48 hours. Acid tolerant bacteria (ATB) and mold counts were made by plating a 1:100 and 1:1000 dilution on wort agar (Difco) and were incubated at room temperature for 5 days. Coliform counts were made by plating

1:100 and 1:1000 dilutions on violet red bile agar (Difco) and incubating for 24 hours at 37°C. The samples were also analyzed for chlorinated hydrocarbon pesticide residues and extraneous materials.

Results and Discussion

Samples were divided into five groups for reporting results (*see* Table 1). Half of the samples were measured for ATB and mold, and the other half for coliform counts; limited facilities and manpower made it impossible to make both determinations on all samples.

Group I: *Peppers.*—This group had the highest APC of the five groups, with a range of 28,000,000/g to less than 3,000/g. The ATB and mold counts ranged from less than 3,000 to greater than 300,000/g. Of the 19 samples for which coliform counts were made, 12 did not have any colonies at the 1:100 dilution and were reported in Table 1 as less than 100/g.

Group II: *Prepared Sausage Seasonings.*—This group of 22 samples had a wide APC range, i.e., 6,600,000/g to less than 3,000/g; only one sample was under 3,000/g. The average APC for this group (700,000/g) was much lower than that for Group I.

The ATB and mold counts ranged from 50,000/g to less than 3,000. These counts were also less than those in Group I.

Only two of the 14 samples tested for coliform had colonies at the 1:100 dilution, and these counts were only 2,000/g and 330/g.

Group III: *Weiner, Bologna, and Prepared Meat Seasonings.*—This group of 25 samples had an APC range of 2,300,000/g to less than 3,000/g; 12 samples measured less than 3,000/g.

The ATB and mold counts were also low in this group; the majority were less than 3,000/g. Only one of the 12 samples run for coliform counts had colonies at the 1:100 dilution.

Group IV: *Sage.*—The 10 samples in this group had a low APC range of 24,000/g to less than 3,000/g; 3 samples measured under 3,000. Only 4 samples were counted for ATB and mold, and all had low counts. This group had the highest average coliform count of any of the groups (15,000/g). Only one sample was less than 100/g.

Group V: *Miscellaneous.* — This group

Table 1. Aerobic plate count (APC), acid tolerant bacteria (ATB) and mold, and coliform counts on spices, seasonings, and condiments

Sample	APC	ATB and Mold	Coliform[a]
Group I: Peppers			
1	400,000	16,000	—
2	7,500,000	>300,000	—
3	17,000,000	>300,000	—
4	170,000	3,000	—
5	2,800,000	>300,000	—
6	6,000	<3,000	—
7	10,000,000	150,000	—
8	28,000,000	110,000	—
9	88,000	<3,000	—
10	1,100,000	<3,000	—
11	<3,000	—	<100
12	1,400,000	—	<100
13	17,000	—	<100
14	3,000,000	—	34,000
15	20,000	—	<100
16	350,000	—	500
17	<3,000	—	<100
18	<3,000	—	<100
19	21,000,000	—	600
20	<3,000	—	<100
21	<3,000	—	<100
22	870,000	—	<100
23	15,000,000	—	<100
24	8,000,000	—	500
25	650,000	—	<100
26	600,000	—	1,400
27	21,000,000	—	7,500
28	<3,000	—	<100
29	1,400,000	—	250
97	12,000,000	—	<100
Group II: Prepared Sausage Seasonings			
30	90,000	<3,000	—
31	240,000	4,300	—
32	650,000	<3,000	—
33	6,600,000	13,000	—
34	1,300,000	50,000	—
35	110,000	10,000	—
36	1,400,000	43,000	—
37	680,000	<3,000	—
38	520,000	—	2,000
39	<3,000	—	<100
40	140,000	—	<100
41	27,000	—	<100
42	210,000	—	<100
43	83,000	—	<100
44	23,000	—	<100
45	2,500,000	—	330
46	235,000	—	<100
47	11,000	—	<100
48	36,000	—	<100
49	48,000	—	<100
50	87,000	—	<100
51	150,000	—	<100
98	4,000	—	<100
99	<3,000	—	<100

(*Continued*)

Table 1. (*Continued*)

Sample	APC	ATB and Mold	Coliform[a]
Group III: Weiner, Bologna, and Prepared Meat Seasonings			
52	360,000	26,000	—
53	19,000	5,600	—
54	16,000	<3,000	—
55	1,900,000	180,000	—
56	30,000	6,400	—
57	<3,000	<3,000	—
58	<3,000	<3,000	—
59	<3,000	<3,000	—
60	6,000	<3,000	—
61	<3,000	<3,000	—
62	3,000	<3,000	—
63	<3,000	<3,000	—
64	29,000	21,000	—
65	<3,000	——	<100
66	100,000	——	<100
67	4,400	——	<100
68	2,300,000	——	<100
69	<3,000	——	<100
70	60,000	——	<100
71	<3,000	——	<100
72	<3,000	——	<100
73	<3,000	——	<100
74	<3,000	——	<100
75	81,000	——	19,000
76	<3,000	——	<100
100	<3,000	——	<100
101	10,000	——	<100
102	66,000	——	100
103	<3,000	——	<100
104	3,000,000	——	<100
105	<3,000	——	<100
Group IV: Sage			
77	38,000	<3,000	—
78	<3,000	4,000	—
79	4,800	<3,000	—
80	<3,000	<3,000	—
81	7,500	——	75,000
82	9,100	——	10,000
83	10,000	——	<100
84	8,600	——	900
85	24,000	——	6,200
86	<300	——	100
106	<3,000	——	<100
Group V: Miscellaneous			
87	<3,000	<3,000	—
88	<3,000	<3,000	—
89	<3,000	<3,000	—
90	<3,000	3,300	—
91	140,000	26,000	—
92	47,000	5,700	—
93	<3,000	<3,000	—
94	<3,000	——	<100
95	400,000	——	<100
96	<3,000	——	<100

[a] All samples that did not have colonies at the 1:100 dilution were reported as <100/g.

contains all samples that did not fit in Groups I–IV, e.g., meat improver, soy protein, edible flour binder, and prague powder. Of the 10 samples in this group, 7 had an APC of less than 3,000/g and the other 3 ranged from 400,000/g to 47,000/g.

The ATB and mold and coliform counts were also usually low in this group.

Conclusions

The average results in the various groups tended to follow the trend which we usually encountered. The extremely high counts obtained from individual samples indicated that these samples had not been sterilized or had become contaminated by improper handling or storage in the packing plants.

The ATB and mold counts were made because mold in dry sausage and molding of spiced hams are greatly affected by the contamination of spices by mold. Results show that only a few samples measured higher than those usually encountered.

The high coliform counts may possibly be evidence of contamination and would indicate that more care should be exercised in the handling and storage of these products. Many samples that had low counts were packaged in individual packages containing only enough of the seasoning for a certain amount of the product to be made. Some further study should be made to compare spices, seasonings, and condiments that are shipped in bulk with those that are in small batch-size packages.

The testing program will be extended to include frozen foods. Routine examination of food products by microbiological methods is the most effective means for conducting a comprehensive regulatory program.

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NOTES

Note on Chemical Determination of Vitamin D in Evaporated Milk

By S. W. JONES and D. A. LIBBY (Division of Nutrition, Food and Drug Administration, Washington, D.C. 20204)

Since the publication of a chemical method for determining Vitamin D in evaporated milk (1), we have encountered some difficulty with this method in certain unusual brands of evaporated milk. This difficulty is caused by an insufficient amount of interference being removed by column chromatography. Consequently, the 15 second absorbance reading gives a deceptively high value because of the interference on background material.

However, by using a third column of aluminum oxide (2), we can eliminate the excessive interference. With the use of this third column, our chemical values on these difficult samples then agree very well with the rat bioassays.

Since there is no way to predict which evaporated milk samples may have this excessive interference, consideration should be given to the routine use of the third column.

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A New Carotenoid Pigment in Shrimp

By DAMON LARRY and HAROLD SALWIN (Division of Food Chemistry, Food and Drug Administration, Washington, D.C. 20204)

In an effort to develop an objective test of the quality of shrimp based on alteration in the natural pigments during storage, a yellow pigment not previously reported in shrimp was isolated and characterized.

The investigation was prompted by our own observation of the color changes which accompany spoilage and by the importance which other workers have attached to changes in the carotenoid pigments.

Natural pigments of shrimp undergo a variety of changes during processing or storage. Astaxanthin (3,3′-dihydroxy-4,4′-diketo-β-carotene), the principal pigment, fades when exposed to atmospheric oxygen, and lipid peroxide may contribute to this effect (1, 2). Faulkner and Watts (3) used the extent of fading for a quantitative test for the degree of deterioration of shrimp during freezer storage. Dassow and Stansby (4) also measured the fading of astaxanthin in frozen salmon. Lusk, Karel, and Goldblith (5) related changes of freeze-dried shrimp during processing and storage to astaxanthin behavior. Tappel (6) demonstrated the sensitivity of the carotenoid pigments of shrimp and salmon to ionizing radiation.

During storage in ice, the flesh of white shrimp acquires a pink color which becomes progressively more intense. Exudation of an orange slime accompanies spoilage. We investigated these changes as a possible basis for an objective test of spoilage during ice storage. It was thought that the intensification of color might be due to cleavage of a colorless astaxanthin-protein complex and release of the red pigment. Such protein complexes are present in lobster and in crab, and release of the free pigment accounts for the changes in color observed when these shellfish are immersed in boiling water (7, 8).

Attempts were made to measure the amount of free pigment soluble in organic solvents as a proportion of the total pigments. Changes in this ratio during storage were noted, but there was no consistent trend with spoilage. Therefore, efforts to develop an objective test of spoilage based on this principle were dis-

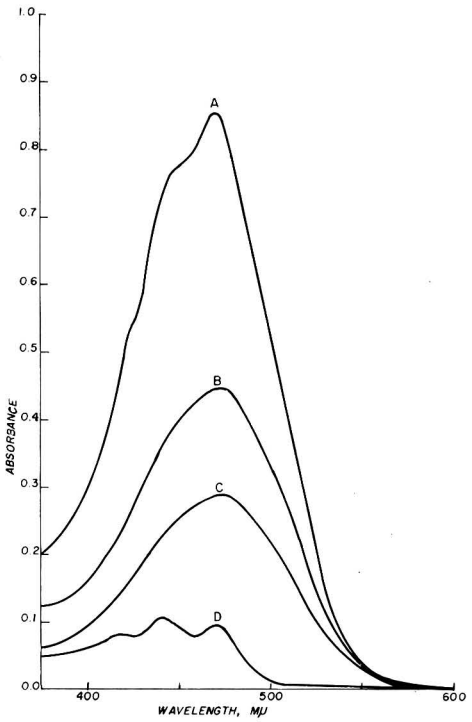

Fig. 1—**Absorption spectra of ether solutions of pigments extracted from shrimp. A. total pigment; B. astaxanthin; C. astacin; D. cryptoxanthin-like yellow pigment.**

continued. However, the extracted pigments were separated and examined because spectral analysis indicated the presence of a pigment in addition to astaxanthin.

Experimental

Pigments were extracted from headless white shrimp (*P. setiferus*) with ethyl ether or with methanol-chloroform (9). The absorption spectrum of the pigments in ether solution had a principal maximum at 472 mμ which corresponds to the maximum of astaxanthin and its oxidation product astacin (3,4,3′,4′-tetraketo-β-carotene). In addition, there were two secondary maxima which appeared as shoulders at 450 and 422 mμ (Fig. 1, curve A). The pigments in hexane solution were then chromatographed on a magnesium oxide-Super-Cel column (10) in order to separate the pigment responsible for the absorption at the shorter wavelengths. Three fractions were isolated: a fast-moving yellow band eluted with hexane-acetone (9:1); a reddish-pink astaxanthin band eluted with hexane-acetone (9:5); and an orange-red astacin band extracted with acetone. Each fraction was examined spectrophotometrically.

The partition test described by Petracek and Zechmeister (11) was used to characterize the fast-moving yellow band separated by chromatography. After evaporation of the eluting solvent, the pigment was partitioned between hexane and 95% methanol. The partition test was also applied to the pigment after hydrolysis according to the method of Brush and Reisman (12). For this purpose, a second extract of the pigment in hexane solution was refluxed with 5% KOH in 95% methanol. The hydrolyzed mixture was just acidified with glacial acetic acid, transferred to hexane, and washed twice with water. The absorbance of the hexane solution was measured at 471 mμ before and after partitioning with 95% methanol to determine the partition coefficient.

The absorption spectrum of the methanol fraction from the partition test on the hydrolyzed pigment was determined before and after adding hydrochloric acid in order to test for the presence of 5,6-epoxides (13).

Results

The partition coefficient of the hydrolyzed yellow pigment in the hexane-methanol system was 80:20. This value suggested a monohydroxy xanthophyll structure (11). Before hydrolysis, the pigment was completely epiphasic, indicating that it was an ester.

The absorption spectra of the ester and of the free pigment were identical. Absorption maxima of the pigment in hexane at 471, 443, and 418 mμ indicated that the pigment may be cryptoxanthin (3-hydroxy-β-carotene) (13). Absorption maxima of the pigment in methanol did not shift to shorter wavelength when hydrochloric acid was added, indicating the absence of a 5,6-epoxide structure (13, 14). Figure 1 shows the absorption spectrum of an ether solution of the total pigment extracted from shrimp that had been held in ice for 11 days (curve A). It also shows the absorption spectra of ether solutions of the fractions separated by column chromatography: astaxanthin (Curve B), astacin (Curve C), and the yellow pigment (Curve D).

The occurrence in shrimp of a cryptoxanthin-like yellow pigment may partly explain the orange coloration and changes in absorption spectrum which accompany spoilage.

This paper was presented at the Seventy-ninth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 11–14, 1965, at Washington, D.C.

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Note on Improved Extraction for Chlorinated Pesticide Residues in Oysters

By ERIC A. ROBERTSON and ROBERT M. TYO[1] (Gulf Coast Shellfish Sanitation Research Center, U.S. Public Health Service, Dauphin Island, Ala. 36528)

The extraction of the pesticide residue from the sample is a critical step in residue analysis. Since there is a direct relationship between the amount of solvent recovery and pesticide residual recovery, improvement of the solvent recovery in the extraction step should improve accuracy of the method.

We use several methods or adaptations of methods (1, 2) in which the residue is extracted by blending the sample with a suitable solvent. The solvent is separated from the homogenate in a separatory or Büchner funnel, or in a "batch" centrifuge, and evaporated at a low temperature.

Our latest modification for separating the residue-containing solvent from the oyster meat homogenate is a "continuous" perforated basket centrifuge head fitted into an explosion-proof centrifuge.

Extracts from oyster meats must be cleaned up in the usual analytical procedure. However, in this study, the cleanup step was omitted and the final concentration of extract from each 50 g sample was diluted to 250 ml. (Note: The usual working volume of an extract from a 50 g oyster sample is 2.5 ml. This step reduced background interference to a minimum and resolved the peaks more clearly on the chromatogram. Omission of cleanup and use of the larger volume of working material provided a means of comparing and evaluating recovery technique more precisely.)

For recovery studies, approximately 500 g of shucked, drained oysters were blended in a Waring Blendor for 5 min. and 50 g portions of the blended oysters were weighed in triplicate for each analysis. The oyster homogenate was fortified with 0.08 mg each of heptachlor, heptachlor epoxide, DDE, and *p,p'*-DDT. A similar amount of each pesticide was added to 250 ml petroleum ether to be used as the gas chromatographic standard for recovery calculations. We consider 5 μl of the pesticide standard (0.0016 μg) to be the optimum amount for good resolution on the instrument[2] used for this study.

Three series of 50 g samples were analyzed

[1] Present address: Federal Water Pollution Control Administration, Pacific Northwest Water Laboratory, Corvallis, Ore.

[2] Gas chromatograph—electron affinity detector; 10^{-9} sensitivity.

Table 1. Chlorinated pesticide recoveries from oyster meat; comparison of "continuous" and batch centrifugation techniques

Pesticide	Av. Recovery[a] (%) "Continuous" Centrifugation	Batch Centrifugation
Heptachlor	99	95
Heptachlor epoxide	97	86
DDE	99	87
p,p'-DDT	115	110

[a] Average based on 3 sample runs.

by the "continuous" perforated basket centrifuge technique as follows: Blend sample with 200 ml acetonitrile for 5 min., then transfer slurry directly into basket centrifuge lined with two layers of Whatman No. 1 filter paper. Spin at about 500 rpm and slowly increase to 1500 rpm. While solvent from first slurry collects in a 2 L separatory funnel containing 1 L aqueous Na_2SO_4 and 100–125 ml petroleum ether, add 100 ml acetonitrile to Blendor jar and reblend 1–2 min. at high speed. Decant the 100 ml wash into the basket centrifuge and collect in the same separatory funnel. Shake funnel vigorously for 1 min., venting as necessary. Add distilled water to bring total volume to about 1850 ml. Again, shake vigorously for 5 min., venting as required.

Let separatory funnel stand until phase separation is complete (usually 5 min.). Discard bottom layer (acetonitrile-water-Na_2SO_4 layer). Rewash top layer (petroleum ether) with 400–500 ml water, let phases separate, and discard the water layer. Drip petroleum ether from separatory funnel through a powder funnel plugged with glass wool and topped with 1.5–2″ of anhydrous Na_2SO_4 into a 250 ml volumetric flask. Wash separatory funnel and Na_2SO_4 several times with small amounts of petroleum ether for complete recovery and dilute to volume with petroleum ether. Then inject a 5 µl aliquot into a gas chromatograph for analysis.

For comparison of recovery technique, samples of oyster meat were extracted according to Johnson (2). Fifty g samples were blended for 5 min., first with 200 ml, then 100 ml acetonitrile. The liquids were subjected to batch centrifugation and decanted after each blending into a 2 L separatory funnel. The solvent was then extracted as described under the previous technique.

Use of the "continuous" perforated basket centrifuge technique shows a 4–12% improvement in extraction over the batch technique, as shown in Table 1. These results were obtained by replicate analysis.

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This work was carried out under GC Project FY 65:2.14, *Chlorinated Pesticides and Other Chemical Pollutants in Shellfish from the Gulf of Mexico and South Atlantic States,* at the Gulf Coast Shellfish Sanitation Research Center, Dauphin Island, Ala. (U.S. Department of Health, Education, and Welfare, Public Health Service, Bureau of State Services, Division of Environmental Engineering and Food Protection, Shellfish Sanitation Branch).

This paper was presented at the Seventy-Ninth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 11–14, 1965, at Washington, D.C.

BOOK REVIEWS

Official Publication, Association of American Pesticide Control Officials (AAPCO), 1965–66. Copies may be ordered from the Treasurer, Robert H. Guntert, State Department of Agriculture, State Office Building, Topeka, Kansas 66612. 94 pp. Price $2.00.

This publication in the form of a Handbook furnishes basic information about the AAPCO and its activities, as well as the proceedings of its last annual meeting, and a directory of key personnel.

Part I, "Association Reference Material", lists current officers, committees and investigators; past officers; By-Laws; statement of incorporation under D. C. Non-Profit Corporation Act; resolutions, policy, and interpretations; Uniform State Insecticide, Fungicide, and Rodenticide Act; Official Regulations under the Model State Insecticide, Fungicide, and Rodenticide Act;

Model Custom Application of Pesticides Act; table of common names for 51 pest control chemicals approved by the American Standards Association; summary of State Pesticide Laws and Registration Requirements; and the AAPCO official sampling procedure (this is also the Association of Official Analytical Chemists' procedure).

Part II is devoted to the proceedings of the 19th annual meeting of AAPCO held in Clemson, South Carolina, August 3–4, 1965. In addition to officer and committee reports, addresses by four invited guests are included.

Parke C. Brinkley, President of the National Agricultural Chemicals Association, discussed the current situation with regard to research on pesticide contamination. George W. Fiero, Chemical Specialties Manufacturers' Association, spoke on the safety of petroleum products used in pesticides. Robert J. Anderson, U.S. Public Health Service, described the teamwork involved among industry and government specialists who deal with pesticides. Justus C. Ward, U.S. Department of Agriculture, discussed problems in implementing the Interdepartmental Agreement on pesticide registration.

Part II also includes a digest of the latest changes in State and Federal pesticide laws and a report on answers by 49 States to a questionnaire on pesticide-fertilizer mixtures.

Part III is a directory of pesticide control officials in the United States and Canada.

PETER CHICHILO
Bureau of Scientific Research
Food and Drug Administration

Organic Acids. By G. C. Whiting. Published by J. Cramer, Weinheim, Germany, 1964. Index + 194 pp. Paperback.

This paperback is a primer of organic acids found in the plant kingdom. The author discusses in a few short pages the classification of organic acids and their extraction, separation, and identification by paper chromatography. The properties and occurrence of the various families of acids, aliphatic, alicyclic, aromatic, heterocyclic, and amino acids, are next discussed. The author further subdivides each of the above groups and presents their characteristics. In many excellent tables, he gives information as to chemical name, formula, common name, physical properties, solubilities, etc. Other tables list the plants and their acid contents.

The second half of the book covers the technology of the acids. The author gives minimum details for obtaining the acids from the plant sources and describes characteristic uses of the acids. There are 24 pages of references, an index of plant names, and an index of organic acids.

In the short time the book has been on the reviewer's shelf, it has resolved several problems concerning organic acids. However, its use as a reference is limited because of its brevity.

GEORGE SCHWARTZMAN
New York District
Food and Drug Administration

Farm Chemicals Handbook. 52nd Edition. Edited by Gordon L. Berg. Meister Publishing Co., 37841 Euclid Ave., Willoughby, Ohio 44094. 1966. 522 pp. Price $15.00.

This Handbook, although in its 52nd year of publication, has several noteworthy new additions: a list of aerial applicators by state for states requiring registration, and a list of over 900 new firms in the Directory of Companies and Plants, which gives extensive coverage of fertilizer and pesticide producers.

In the area of plant foods, consumption statistics have been added for various materials, and a new section lists trace nutrient fertilizers and their sources. The Pesticide Dictionary has increased usefulness with cross-indexing by brand and common names. This Dictionary also includes, for the first time, consumption statistics and LD_{50} data for each product.

The Handbook has four major parts: Section A lists and classifies by type all farm chemical manufacturers by state and location, making it possible to identify all producers available to farm areas; Section B

provides a convenient list of associations and State control officials concerned with agricultural chemicals; Sections C and D include, respectively, dictionaries for plant foods and pesticides which are brought up-to-date. The Dictionary on Plant Foods gives, where possible, standard Association of American Fertilizer Control Officials definitions. The Pesticide Dictionary gives technical information, scientific and common names, and producers for all commercially available pesticides in the United States.

Sections E, F, and G provide the Buyers' Guide for the farm chemicals industry.

This very complete Farm Chemicals Handbook is especially oriented to fit the needs of producers, distributors, sellers, and ultimate buyers of fertilizers and pesticides. However, it also offers a very convenient reference for State and Federal regulatory officials and other officials and groups interested in these chemicals.

LUTHER G. ENSMINGER
Bureau of Scientific Research
Food and Drug Administration

The Analysis of Pesticides, Herbicides, and Related Compounds Using the Electron Affinity Detector. By Benjamin J. Gudzinowicz. Jarrell-Ash Company, Waltham, Mass., 1965. 306 pp. Price $10.00.

This paperback monograph contains 306 pages, including 22 tables and 192 figures. The bibliography with 222 references adequately covers theory and application of electron affinity gas chromatographic detection through early 1965.

Some areas discussed in the text are theory and operating principles of electron affinity and flame ionization detectors; GLC column efficiency; gas chromatographic separation of pesticides, herbicides, and related materials; sensitivity data; and qualitative methods for molecular structure characterization based on retention time-physical property data.

Retention times in minutes are given for 49 compounds on as many as six columns. Sensitivity data in coulombs/nanogram are given for the same pesticides. Sensitivity differences and column effects with different columns are discussed.

Chromatograms obtained by flame ionization detection are shown for 49 pesticides. Chromatograms and response versus quantity plots from electron affinity detection are given for about 40 pesticide chemicals. Chromatograms obtained from different columns are shown for several compounds.

Most of the last 23 pages are devoted to brief abstracts of extraction-cleanup procedures "suitable" for electron affinity GLC analysis of various sample types. Tables are presented matching pesticide, sample type, method reference, and recovery data reported by each investigator. This reviewer finds it difficult to accept all 46 procedures mentioned as "suitable" for electron affinity GLC analysis.

A large portion of the material in the book is of a review nature; 195 of the 222 references are cited in the text.

From the title, this reviewer expected a book written mainly for the applications chemist and specifically the pesticide residue analyst. On this basis, the text is somewhat lacking in information directly applicable in the analytical laboratory. Important aspects of electron affinity GLC detection not covered by the text include a discussion of differently designed electron affinity detectors and their relative merits; quantitative analysis in terms of accuracy and precision to be expected with the electron affinity detector; and a discussion of the detector voltage-response relationship in terms of detector linearity and stability. A thorough discussion of the radioactive source, in terms of standing current, detector sensitivity and linearity, duration of usefulness, need and methods of cleaning, and hazard to health, is lacking. Some chromatograms shown in the text raise questions for the reader; they do not represent the highest quality attainable and should not be considered the standard of achievement from electron affinity GLC detection. The important area of solvent quality necessary for use with electron affinity GLC detection in pesticide residue analysis is not covered. A critical

review of some referenced approaches to sample preparation would have been more helpful than the abstracts of published procedures.

The material presented is of interest to the user of the electron affinity GLC detection technique but fails to satisfy many needs of the investigator who must analyze for pesticides, herbicides, and related compounds.

JERRY A. BURKE
Division of Food Chemistry
Food and Drug Administration

Man Adapting. By René Dubos. Yale University Press, New Haven, Conn., 1965. xvii + 527 pp.; illus., index, bibliog. Price $10.00.

A book on environmental biology dealing with health and disease in man as he responds to environmental factors could be undertaken by scientists from a number of distinct disciplines. From the efforts of the microbiologist Dubos, the reader is treated to a searching, unique, and thought-provoking view of the problems in this area confronting mankind.

The subject of this volume is presented in 16 essays (chapters), each easily read as a self-contained topic on human ecology. Yet all blend with the dominant theme of the book, which is, in the author's own words, "that the states of health or disease are the expression of the success or failure experienced by the organism in its efforts to respond adaptively to environmental challenges." Each chapter discusses a distinct set of environmental factors facing individual man, beginning with prenatal and early postnatal influences in Chapter I (Man's Nature), continuing with topics such as "Nutrition and Infection", "The Evolution of Microbial Diseases", "Environmental Pollution", "The Population Avalanche", "The Control of Disease", and concluding with "Medicine Adapting".

From the complexity of the scientific evidence marshaled by the author from researches in these various fields, this reviewer almost envisioned the individual on a continuous treadmill coping with his changing environment. We live as we can, not as we wish. The author cautions against this view, however, by citing man's creative response to the environment, which he utilizes for self-actualization. Thus, he advises the developing countries to "adapt rather than adopt the techniques and products of Western medicine".

Despite the tremendous vista of unanswered questions in human ecology, the author expresses a hopeful and constructive attitude towards man's response to the challenges of his physical, biological, and social environment. Calculated risks are involved in manipulating environmental factors that present a danger to public health; but he urges that "effectiveness of action must never be sacrificed at the altar of complete intellectual understanding".

In the chapter dealing with the control of disease, he introduces the term "prospective epidemiology" whose aim would be the early containment of new threats to health rather than the Utopian goal of complete prevention or eradication of disease. In his discussion of safety regulations in this chapter and in the chapter on "Environmental Pollution", Professor Dubos discusses briefly Rachel Carson's *Silent Spring* and *Our Synthetic Environment* by Lewis Herber, two books which in recent years have cried out against the threat of new chemicals in our daily life. Unlike the many answers to these books by other scientists, the author does not engage in polemics. *Man Adapting* responds to the threat of chemical pollution, as it does to the various other environmental challenges; it offers a wide vista of the problem that balances the numerous factors and choices in man's response to his total environment.

This book cannot help but raise as many questions as it attempts to answer. Do our scientific methods measure up to modern society? How is the public interest best served in the competition between health and economic welfare? Is man losing control of his environment in his *ex post facto* response to dangers of technological innovation? These are but a few occurring to this

reviewer. Undoubtedly many others will occur to the readers of this volume. A statement in the chapter on "Evolution of Microbial Diseases" is worth quoting because of the hope it expresses for the future of human ecology. "The real problem is not how to apply more effectively the control procedures we already possess, or how to improve them, but rather to search for a qualitatively different kind of knowledge".

This book, concerned with the public health and welfare and in the last analysis with scientific methods and safety regulations, is highly recommended to the readers of *This Journal.*

WILLIAM V. EISENBERG
Division of Microbiology
Food and Drug Administration

The United States Pharmacopeia. Seventeenth Revision. Published by the United States Pharmacopeial Convention, Inc., Mack Publishing Co., Easton, Pa. lxvi + 1156 pages. Price $12.50.

The *United States Pharmacopeia* is in a continuous process of revision, reflecting the progress in the evolution of new therapeutic agents, in improvements of technology which permit better control of the quality of drugs, and in changes in the legal requirements for their control. Progress in these fields is now so rapid that the entire Pharmacopeia is revised quinquennially, and frequent interim revisions must be issued.

Radical changes are not to be expected in such a long established compendium (the U.S.P. dates back to 1820), but the 17th Revision does introduce a number of significant changes. Most readily apparent is the change of Official Titles of many of the drugs; this resulted from new Federal legislation which requires that a drug be labeled by its common name and that it be known *only* by its official name. Thus, Acetylsalicylic Acid becomes Aspirin; Glyceryl Trinitrate becomes Nitroglycerin; and Amobarbital Sodium becomes Sodium Amobarbital. In U.S.P. XVI, the subtitles Eserine Salicylate and Physostigminium Salicylate appeared under the main title Physostigmine Salicylate. These subtitles are deleted in U.S.P. XVII; the first by legal requirement, and the second because all use of Latin names has been discontinued. In the light of these changes, a new chapter on nomenclature has been added which lists other designations of U.S.P. articles, including both the former U.S.P. names and those used in the British and International Pharmacopeias.

A newly introduced provision, applicable to a limited number of articles, requires the assay of individual tablets to establish uniformity of content.

Recognizing the variability among individual spectrophotometers, the new Revision requires that in all spectrophotometric measurements a reference standard be measured concomitantly with the sample, rather than relying upon the relationship of the absorbance of the latter with an absorbance value derived in another instrument under possibly different conditions.

Assays for antibiotics and for biologicals have been removed from the Pharmacopeia; these must instead conform to the requirements of the Federal Food and Drug Administration and the U.S. Public Health Service, respectively.

In keeping up with advances in technology, new sections have been added describing gas chromatography, thin layer chromatography, and polarography, and the sections on radioactivity, spectrophotometry, and design and analysis of biological assays have been expanded.

The changes in official nomenclature have caused the rearrangement of many monographs. Sodium Amobarbital which was, in U.S.P. XVI, contiguous with Amobarbital is now listed alphabetically under S; and the various penicillins, previously grouped together under P, are now widely scattered. The alphabetical rearrangement was not wholly unavoidable; the same reasoning which permits Sterile Sodium Amobarbital to follow Sodium Amobarbital would permit the latter to follow Amobarbital.

The index could have been much more helpful in finding the newly relocated monographs. The subheading Sodium under Amobarbital names the page upon which the

Sodium Amobarbital monograph appears, but the corresponding entry under Phenobarbital fails to do so. It refers simply to the list of Changes in Official Titles, thus necessitating a back-and-forth search to locate the entry. Rigid restriction to official nomenclature further limits the utility of the Index. It gives no clue that sulfadiazine is mentioned in the Trisulfapyrimidines monograph. One searching for the requirements governing variations among individual tablets might look under Variations rather than Uniformity and thus find the requirements regarding uniformity in weight rather than variations in content.

The 17th Revision includes 162 new admissions and has dropped 201 entries which were official in the 16th Revision. For comparison, the 16th Revision had admitted 225 new entries and dropped 159 from the 15th Revision. It is of interest to note that eleven of the latter are included in the new admissions to the latest Revision.

JOSEPH LEVINE
Division of Pharmaceutical Chemistry
Food and Drug Administration

NEW PUBLICATIONS

Bulletin of Environmental Contamination and Toxicology. Vol. 1, No. 1. Jan.-Feb., 1966. Springer-Verlag, Inc., 175 Fifth Ave., New York, N.Y. 10010. Subscription price: $15 per year for institution, $7.50 per year for individuals.

Inaugural issue of a new journal in the field of pesticide research. Five papers as well as the aims and scope of the bulletin are carried.

Examination of Foods for Enteropathogenic and Indicator Bacteria. Edited by K. H. Lewis and R. Angelotti. Public Health Service Bulletin No. 1142, U.S. Department of Health, Education and Welfare. U.S. Government Printing Office, Washington, D.C., 1964. vii + 123 pages. $0.50.

A review of methodology and manual of selected procedures.

Infestation Control: Report of the Infestation Control Laboratory for 1962–64. Ministry of Agriculture, Fisheries, and Food. vi + 100 pages. Her Majesty's Stationery Office, 49 High Holburn, London, W.C. 1, England, 1965, $1.80.

Report of the work on the control of insect pests of stored products and the control of harmful vertebrate pests.

Nomenklatur, geographische Verbreitung und Wirtsbereich des Gelbrostes, Puccinia striiformis West. K. Hasselbrauk. 75 pages. Kommissionsverlag Paul Parey, Lindenstrasse 44-47, 1 West Berlin 66, Germany, 1965. 17.50 Deutsche Mark.

A review about probable origin, occurrence, and economic importance of yellow rust throughout the world.

Requirements for Biological Substances, Report of a WHO Expert Group. World Health Organization: Technical Report Series, 1965, No. 323. Available from Columbia University Press, 2960 Broadway, New York, N.Y. 10027. 71 pages. $1.25.

This report contains revised general requirements for manufacturing establishments and control laboratories as well as revised requirements for poliomyelitis vaccine (inactivated), poliomyelitis vaccine (oral), and smallpox vaccine.

The 1965 Annual Report of the Food Protection and Toxicology Center, University of California (Davis). George F. Stewart, Director. University of California, Davis, California, 1966. Paperbound, 71 pages, no cost.

This report covers the planning, establishment, and first year's operation of the center.

ANNOUNCEMENTS

Standing Committees: Committee on Safety

C. L. Ogg has been appointed to replace T. W. Quigley, Jr., on the Committee. A. B. Heagy and B. L. Samuel have recently been appointed as Committee members.

Disinfectants:

A. F. Petrocci, Onyx Chemical Division, Millmaster Corp., 190 Warren St., Jersey City, N.J. 07302, has been appointed as Associate Referee on Antimicrobial Agents Used by Laundries on Fabrics and Materials.

Pesticides:

M. F. Kovacs, Jr., Pesticides Regulation Division, U.S. Department of Agriculture, Beltsville, Md. 20705, has been appointed as Associate Referee on Carbaryl (Sevin).

Joseph Haus, S. B. Penick & Co., 999 West Side Ave., Jersey City, N.J. 07304, has been appointed as Associate Referee on Sulfoxide to replace Robert W. Price of the same address.

Vitamins and Other Nutrients:

Kenneth R. Griffith, Food and Drug Administration, 1009 Cherry St., Kansas City, Mo. 64106, has been appointed as Associate Referee on Nicotinamide in Vitamin Products.

Antibiotics:

A. W. Neff, Veterinary Research Department, The Upjohn Co., Kalamazoo, Mich. 49002, has been appointed as Associate Referee on Neomycin Sulfate in Feeds and Lincomycin in Feeds.

Color Additives:

William B. Link, Food and Drug Administration, Washington, D.C. 20204, has been appointed as Associate Referee on Subsidiary Colors in Water-Soluble Azo Color Additives.

Robert K. Johnson, Hilton-Davis Chemical Co., 2235 Langdon Farm Road, Cincinnati, Ohio 45237, has been appointed as Associate Referee on Uncombined Intermediates in Triphenylmethane Colors.

Daniel M. Marmion, National Aniline Division, Allied Chemical Corp., 1051 S. Park Ave., Buffalo, N.Y. 14210, has been appointed as Associate Referee on Subsidiary Colors in Other Organic Colors.

Rachel S. Silk, Food and Drug Administration, Washington, D.C. 20204, has been appointed as Associate Referee on Color Additives in Drugs.

Drugs, Acidic and Neutral Nitrogenous Organics:

John L. Allen, Food and Drug Administration, 240 Hennepin Ave., Minneapolis, Minn. 55401, has been appointed as Associate Referee on Barbiturates (Chromatographic) to replace Anthony Romano, Jr.

Fish and Other Marine Products:

John C. Werren, Food and Drug Administration, Washington, D.C. 20204, has been appointed as Associate Referee on Drained Weight in Frozen Glazed Seafoods.

George K. Knoble, Jr., Fish and Wildlife Service, Bureau of Commercial Fisheries, College Park, Md. 20740, has been appointed as Associate Referee on Fat in Fish Meal.

Food Additives:

Richard A. Pimental, Food and Drug Administration, 1521 West Pico Blvd., Los Angeles, Calif. 90015, has been appointed as Associate Referee on Silicate Anticaking Agents in Salt.

Fruits and Fruit Products:

Leo Levi, Food and Drug Directorate, Tunney's Pasture, Ottawa, Ontario, Canada, has resigned as Associate Referee on Instrumental Methods of Analysis of Essential Oils.

J. Fitelson, Fitelson Laboratories, Inc., 254 W. 31st St., New York, N.Y. 10013, has been appointed as Associate Referee on Identification and Characterization of Fruit Juices to replace D. Jorysch, H. Kohnstamm & Co., Inc., 161 Avenue of the Americas, New York, N.Y. 10013.

Preservatives and Artificial Sweeteners:

F. S. Nury, Western Utilization Research and Development Division, U.S. Department of Agriculture, Albany, Calif. 94710, has

resigned as Associate Referee on Sulfur Dioxide (Monier-Williams Method).

Sugar and Sugar Products:

J. W. White, Jr., Eastern Utilization Research and Development Division, U.S. Department of Agriculture, Philadelphia, Pa. 19118, has resigned as Associate Referee on Honey.

Radioactivity:

A. S. Goldin, Northeastern Radiological Health Laboratory, Public Health Service, P. O. Box 232, Winchester, Mass. 01890, has been appointed as General Referee on Radioactivity.

J. C. Drobinaki, Jr., Northeastern Radiological Health Laboratory, Public Health Service, P. O. Box 232, Winchester, Mass. 01890, has been appointed as Associate Referee on Carbon-14 to replace A. S. Goldin of the same address.

Chlorinated Insecticdes and Miticides:

Milton Wilderman, Food and Drug Administration, 1204 U.S. Customhouse, 2nd & Chestnut Sts., Philadelphia, Pa. 19106, has been appointed as Associate Referee on Extraction Procedures for DDT, Captan, and Sulphenone.

Herbicides and Plant Growth Regulators:

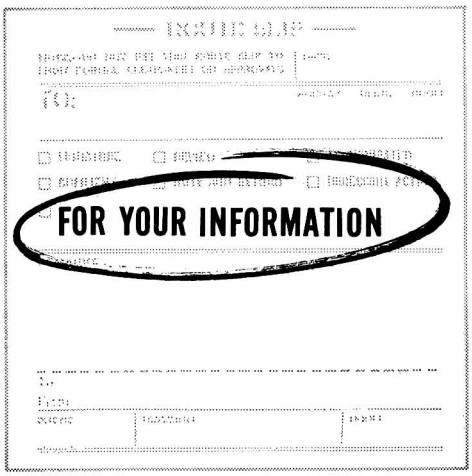
R. C. Rundall, Food and Drug Administration, 240 Hennepin Ave., Minneapolis, Minn. 55401, has been appointed as Associate Referee on Naphthalene Acetic Acid to replace C. W. Cooper of the same address.

Phosphated Insecticides and Miticides:

Robert J. Martin, Food and Drug Administration, 1521 West Pico Blvd., Los Angeles, Calif. 94105, has been appointed as Associate Referee on Demeton (Systox), Di-Syston, and Meta-Syston to replace J. R. Weatherwax of the same address.

CORRECTIONS

This Journal, **48**, 1118 (1965), "Thin Layer Chromatographic Determination of Ethyl *O,O*-Dimethyldithiophosphoryl-1-phenyl Acetate in Both Technical Cidial® and Its 50% Liquid Formulation", by B. Bazzi, *et al.,* p. 1118, 2nd col., 2nd para., line 22	Change "5% metanil yellow" to "0.5% metanil yellow".
Ibid., **49**, 79 (1966), "Report on Preservatives and Artificial Sweeteners," by George Schwartzman, p. 81, line 3	Change "action method for melting point, 26.001" to "action method, 27.001."
Ibid., **49**, 374 (1966), "A Gas Chromatographic Column for Pesticide Residue Analysis: Retention Times and Response Data," by J. A. Burke and W. Holswade, p. 384, caption for *Fig. 3*	Change caption to read as follows, starting with *K*: "*K*, dieldrin; *L*, endrin; *M*, chlorobenzilate; *N*, *p,p'*-DDT; *O*, endrin aldehyde; *P*, *p,p'*-methoxychlor; *Q*, Delta Keto 153; *R*, Tedion."



Reorganization of Subcommittee E

A meeting was held on March 17, 1966 to discuss the reorganization of Subcommittee E. Dr. S. Randle, AOAC President, described the broad purposes of Subcommittee E and expressed the need for realignment in view of the expanding responsibilities covering pesticides.

The following suggestions were considered: whether studies of multi-detection systems should be given emphasis over specific methods; whether General Referees of the Food and Drug Administration should work through District Research Coordinators rather than directly with Associate Referees; whether semi-annual meetings of General Referees should be held; and whether a "higher review system" than the General Referee-Associate Referee system was desirable.

A tentative program will be drawn up for realignment of all General Referee and Associate Referee assignments to become effective after the 1966 meeting.

AOAC President to Attend Society Meetings

Stacey Randle, AOAC President, will speak at the Canadian Food Technologists meeting, June 15–17, 1966, at Toronto, Canada. Dr. Randle will also attend the meeting of the Association of Food and Drug Officials of the U.S. (AFDOUS) in Kansas City as the AOAC representative.

Wiley Award Fund

Due to the generous bequest of the late Mrs. Harvey W. Wiley, the Wiley Award Fund now totals $14,457.39. The AOAC will contribute an additional amount so that interest from the fund will support the Wiley award.

Foreign Scientists to Attend 80th Annual Meeting

Five foreign scientists have accepted the invitation to speak at the 80th Annual Meeting of the AOAC, Oct. 10–13, 1966.

D. A. A. Mossel, Laboratory of Bacteriology, Central Institute for Nutrition and Food Research, TNO, Zeist, The Netherlands, will speak on "Ecological Principles and Some Methodological Aspects of the Enumeration of Food and Feeds."

I. Uritani, Faculty of Agriculture, Nagoya University, Anjo, Aichiprefecture, Japan, will speak on "Abnormal Substances in Foodstuffs Polluted by Fungal Growth".

G. Jacini, Stazione Sperimentale per le Industrie degli Olli e del Grassi, Milano, Italy, will discuss edible oils, specifically olive oils.

H. Egan, Laboratory of the Government Chemist, Cornwall House, Stamford St., London S. E. 1, will describe pesticide residue analysis and regulatory problems.

V. Jans, Association Internationale d'Expertise Chimique, 77, Avenue de Marinville, Saint-Maur, France, will speak on food research in France.

Their attendance will be made possible through Public Health Service Grant IR 13 EF00521-01, authorized by the Committee on Environmental Health.

40th Fall AOCS Meeting

The 40th fall meeting of the American Oil Chemists' Society will be held Oct. 3–5, 1966, at the Bellvue-Stratford Hotel, Philadelphia, Pa. Further information can be obtained from the publicity chairman, Thomas H. Smouse, Campbell Soup Company, 100 Market Street, Camden, New Jersey.

Papers for the 1966 AOAC Meeting—New Requirements

At a recent meeting, the AOAC Editorial Board discussed the difficulties involved in reviewing all the contributed papers submitted for presentation at the Annual Meeting. Last year, for example, 70 manuscripts arrived at the AOAC office the first few days in September; all 70 had to be read and evaluated within less than a week so that the meeting program could be composed and sent to the printer by the beginning of the second week in September. We have set ourselves an all but impossible goal.

The Board agreed that we must find a more practical system. The AOAC cannot accept every paper offered for presentation at the meeting, with no restrictions, because of the limited time available on the program. First place, of course, must be reserved for the referee reports and the transactions of the AOAC's yearly business of adopting new methods.

After much discussion, the Board decided to accept papers on the basis of a submitted abstract which must meet the specifications outlined below. The chairmen of the various meeting sessions will take the responsibility for reviewing the abstracts of the proposed papers in their subject areas and accepting or rejecting them.

The abstracts will be compiled in a booklet for distribution at the meeting, as in past years, and will also be published in a group in the December issue of the *Journal*. This publication should establish priority of the work, in the event that publication of the full manuscript is delayed.

Specifications for Abstracts of Papers to be Presented at the Annual AOAC Meeting

Form of Submission.—Abstracts should be typed double-spaced on one side only of white bond paper. The original and two copies should be submitted *not later than August 26, 1966,* to the Association of Official Analytical Chemists, Box 540, Benjamin Franklin Station, Washington, D.C. 20044.

Length.—The length depends partly on the nature and scope of the full paper. As a general rule, however, the abstract should not be longer than 200 words nor shorter than 75 words.

Nature.—The abstract should be informative; it should contain the significant content of the paper in specific terms, not generalized statements. The author should avoid such statements as "Data are given," "A method is described," etc., with no indication of the nature of the data nor the principles of the method. He should assume that his audience (and the session chairman) has some knowledge of the subject.

The abstract should be completely self-explanatory and intelligible in itself. It should contain no information that is not a part of the full paper; conversely, it should not refer to tables, figures, or bibliographic references within the full paper, since the reader of the abstract will not have access to such information.

Significant Content.—To insure that the abstract will represent the significant content of the paper, the author should follow this outline:

(a) Indicate the objectives of the study and the topics covered by the paper, but do not repeat information given in the title.

(b) State what methods were used. When reporting a new method, give the basic principle, the range of operation, and the degree of accuracy.

(c) Call attention to new reactions, compounds, materials, apparatus, data, concepts, and theories.

(d) Include a brief summary of results and conclusions. Give actual averages, percentage recoveries, standard deviations, etc.

Journal of the Association of Public Analysts

Contents of Vol. 5, No. 1, were as follows: "New Food and Drugs Legislation and Proposals for Regulations, etc., during 1965", by G. H. Walker; "The Determination of Cyclamate in Citrus Fruit Squashes", by A. M. C. Davies; "The Determination of the Composition or Purity of Animal Feeding Stuffs by Microscopy", by J. Hubert Hamence; and "Photography and the Public Analyst", by Miss A. Cook and A. J. Harrison.

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 × 10½ or 8½ × 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. Each article should be accompanied by an abstract of not more than 200 words. Titles of articles should be fully descriptive. Six to ten keyword terms suitable for good indexing should be furnished. The address of the institution from which the paper is submitted should be given and should be in a form (including zip code) 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. Examples: Presented at a scientific meeting; adapted from a thesis; sponsored by an organizational grant; published by permission; change of author's address.

Methods, Results and/or Discussion, Acknowledgments, and Recommendations (applicable to reports of Referees 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: Every table must be cited in 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 rules may be used freely to set off columns. Horizontal rules are used to bound the table at top and bottom and to divide the heads from the columns; otherwise they should be used only sparingly. 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.

Illustrations: Illustrations, or figures, may be submitted as drawings or photographs. All figures must be cited in the text, consecutively. Figures are numbered by arabic numbers, and every figure must be accompanied by a descriptive caption, typed on a separate sheet, *not* on the figure itself. The figure should be identified by number on the back by a soft pencil or (preferably) a gummed label.

Drawings should be submitted either as the original drawing or a glossy photograph of it; photocopies, multiliths, verifax copies, thermofax copies, etc., are not acceptable. Drawings should be done in black india ink (ordinarly blue or blue-black ink is not acceptable) or with drafting tape, such as Zip-A-Line, on white tracing paper or tracing cloth or on "fade-out" graph paper (ordinary graph paper ruled with green or dark blue ink is not acceptable). Lettering should be done with a Leroy lettering set or a similar device; freehand or typewritten lettering is not acceptable.