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DRUG RESIDUES IN ANIMAL TISSUES

Determination of Melengestrol Acetate in Bovine Tissue: Collaborative Study

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Seven laboratories collaboratively studied a method for the assay of melengestrol acetate at the 0, 10, and 20 ppb levels in bovine fat, liver, muscle, and kidney. The study included fortification of tissue by each laboratory and analysis of fat samples taken from treated heifers which had endogenous levels of 0, 10, and 20 ppb melengestrol acetate. The multistep cleanup procedure used included extraction, solvent partition, column chromatography, and electron capture gas-liquid chromatographic determination. Results of the study for muscle, liver, kidney, and fat showed that the method gave satisfactory recoveries and accuracy. In fat, the most critical tissue, recovery was >93%. A statistical comparison of the results reported for fat tissue from treated heifers demonstrated that 5 of the 7 laboratories obtained similar results. The results produced by the method can be expected to be repeatable within and among laboratories. On the basis of the collaborative results the method has been adopted as official first action.

Melengestrol acetate (17-hydroxy-6-methyl-16-methylenepregna-4,6-diene-3,20-dione acetate) (MGA®, The Upjohn Co.) is an effective oral progestational agent (1, 2) which is incorporated into the diet of feedlot heifers at levels up to 0.5 mg/head/day to increase feed efficiency and rate of gain (3). Numerous methods for the assay of MGA have been developed over the years, each more sensitive and shorter than its predecessor (4–6). We recently published an electron capture gas-liquid chromatographic (GLC) method sensitive to 25 ppb MGA in bovine tissue (7). This method was modified and submitted to 8 laboratories in a collaborative study. Seven laboratories completed the study.

L. F. Krzeminski received the Associate Referee of the Year Award presented at the 1975 AOAC meeting.

Collaborative Study

Preweighed frozen control samples (25 g) of bovine fat, liver, kidney, and muscle from an untreated heifer were prepared. Preweighed samples were provided to avoid the problems that might be encountered in thawing and refreezing samples between assay days. In order to obtain endogenous fat tissue levels of 10 and 20 ppb MGA for use as unknowns, a number of beef heifers were fed 3 mg MGA/head/day (a 6-fold overdosage from the maximum Food and Drug Administration-approved level). Perirenal fat was removed surgically, cooled, ground, frozen, and analyzed for MGA content. Sufficient fat was collected so that all laboratories were supplied with identical preweighed aliquots from one animal with 10 ppb and another with 20 ppb MGA levels. Similar fat samples from an untreated heifer served as the unknown control.

Each laboratory received MGA standard, 20 packets of each control tissue, 12 packets of unknown fat samples (4 each of 0, 10, and 20 ppb content, labeled A–L), Florisil, report forms, and a copy of the method. An assay schedule indicated the day on which each tissue was to be assayed, and the random order in which the 6 samples were to be fortified (0, 10, and 20 ppb) and analyzed on each assay day. Each tissue was assayed on 2 separate days with the unknown fat samples being assayed on the last 2 days of the study.

Each laboratory was asked to run one or more trials of a solvent and an MGA-fortified solvent blank to establish the cleanliness of its equipment and whether the determination of MGA

This report of the Associate Referee was presented at the 89th Annual Meeting of the AOAC, Oct. 13–16, 1975, at Washington, DC.
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in the absence of tissue was acceptable (80–90%). Fortification of the control samples was carried out by the collaborator at the first extraction step by pipetting an aliquot of MGA directly onto the tissue in the Waring Blendor. The assay was carried out with the modified 10 ppb MGA procedure provided. A 10 or 20 ppb MGA standard was injected alternately before and after each 3 samples and the peak heights were calculated by the baseline method.

In addition to the specifications in the method, the collaborators were also instructed to report MGA in the samples as follows:

(*1*) Report as <10 ppb if sample peak height is smaller than peak height of 0.25 ppm standard when equal volumes of sample and standard are injected under identical conditions. (*2*) Report as 10 ppb or some larger value when the sample peak height exceeds the peak height of the 0.25 ppm standard under the same conditions. Generally peak heights for the 0.25 ppm standard will be about the same as that for 20–25 ppb. The peak height is always equivalent to 10 ppb in the 25 g sample.

For the recovery study, they were instructed as follows:

(a) *Fortification of reagent blank (fat extraction).*—For those using this method for the first time, either for recovery study or tissue assay, a solvent blank and solvent fortified with MGA should be processed through the entire procedure. This preliminary operation will establish whether or not the solvents and glassware are free from contamination and demonstrate the level of recovery of standard MGA. Level of recovery for fortified solvent should be in the same range as the fortified samples. Place 150 ml hexane into 250 ml beaker. To another 150 ml hexane in 500 ml beaker, add 0.5 ml of 0.5 ppm standard (10 ppb equivalent in tissue). Assay both samples as described in the method.

(b) *Fortification of the fat samples.*—Weigh 25 g portions of unfortified tissue into 250 ml beakers and set half aside to serve as tissue blanks. Add, to the remaining samples, 0.5 ml of 0.5 ppm standard to serve as fortified samples (10 ppb). Assay both fortified and unfortified tissue as described in method, beginning with heating step.

(c) *Column profile procedure.*—Pipet 1.0 ml of 1.0 ppm standard into clean 50 ml round-bottom flask and roto-evaporate solvent. Chromatograph sample on Florisil column. Express results as ppb MGA recovered in hexane-acetone (8+2) fraction.

METHOD

Melengestrol Acetate (MGA)

Gas Chromatographic Method
Official First Action

41.B01 ***Principle***

MGA is extd from lean tissue with CH_3CN and ext is partitioned with hexane. MGA in fatty tissues is extd with hexane and then transferred into CH_3CN. Residue from either ext, after evapn of solv., is chromatgd on Florisil to remove interfering lipid materials with hexane and hexane-acetone (95+5). MGA is eluted with hexane-acetone (80+20). Residue is dissolved in hexane-acetone and detd by GLC.

For those liver samples where MGA is poorly resolved on chromatogram, hexane-acetone is evapd, partitioned with aq. 70% MeOH-hexane, transferred into $CHCl_3$, and evapd. Dry residue is dissolved in hexane-acetone and reinjected onto GLC column.

41.B02 ***Apparatus***

(a) *Adapters.*—⅋ 24/40, Nos. 5225-10 and 5205 (Ace Glass, Inc., or equiv.).

(b) *High-speed blender.*—Waring Blendor Model 702-B with 1 L glass bowl having polyethylene gaskets (*see* (i)), or equiv.

(c) *Chromatographic tubes.*—Glass, 400 × 19 mm id, fitted with medium porosity fritted glass disks, Teflon stopcocks, and ⅋ 24/40 tops.

(d) *Containers.*—Plastic, Romac No. AP06C with No. LA-16P lids (Romac Container, Inc., 33625 Pen Oak Pkwy, Avon Lake, OH 44012), or equiv.

(e) *Flasks.*—R-b, 50, 500, and 1000 ml.

(f) *Funnels.*—Medium porosity fritted glass funnels, 350 ml (Arthur H. Thomas Co., or equiv.).

(g) *Gas chromatograph.*—F&M Model 402 (available from Hewlett-Packard Co., Rt 41, Avondale, PA 19311), or equiv., with all-glass on-column injection system, ^{63}Ni electron capture detector, and 1 mv strip chart recorder. Operating conditions: temps (°)—column 240–250, injection port 240–250, detector 270–275; flow rates—He carrier gas 60–80 ml/min (40 psi, 3.0–3.5 rotameter setting), Ar-CH_4 purge gas (95+5) 135–150 ml/min (40 psi); attenuation 16× or 32×; pulse interval 150; electrometer sensitivity 1×10^{-12} amp full scale deflection with 1 mv recorder. Approx. retention time of MGA under these conditions is 5–6 min.

(h) *Gas chromatographic column.*—Use borosilicate glass tubing, 0.2362±0.013″ (6.00±0.33 mm) od and 0.118±0.01″ (3.00±0.25 mm) id (Wilkens-

Anderson Co., 4525 W Division St, Chicago, IL 60651, or equiv.). Bend 3′ (0.9 m) piece of tubing into proper design for instrument. Pack column with 1% OV-17 on 100–120 mesh Gas-Chrom Q (max. operating temp., 350°, Applied Science Laboratories, Inc., or equiv.), and plug both ends with 0.5 cm loosely packed silanized glass wool. Pack far enough from ends so that no part of column packing or glass wool is inside injection port or detector inlet fittings. Connect column to injection port and cap detector inlet. Condition column 1 hr at 240° with He carrier gas at 40 ml/min, and then 16 hr at 275° with He carrier gas at 80 ml/min. Remove cap and connect column to detector.

(i) *Gaskets.*—Polyethylene, cut from 1 qt (1 L) freezer containers.

(j) *Nitrogen pressure manifold for columns.*—(Optional). Adapters No. 5205 (Ace Glass, Inc., or equiv.) connected thru manifold regulated at 3 psi, with individual control valve.

(k) *Pipets.*—Transfer pipets, 9″ Dispo-pipettes (Scientific Products, Inc., or equiv.).

(l) *Reservoirs.*—250 ml ⧩ 24/40 r-b flasks with ⧩ 24/40 male joint in bottom, or equiv.

(m) *Rotary evaporator.*—4–6 small size Rinco evaporators (Rinco Instrument Co., 503 S Prairie St, Greenville, IL 62246), or equiv., controlled with 4 mm bore stopcocks connected to manifold that leads to 2 condensation traps connected in series to vac. pump with free air capacity of 140 L/min. Cool traps with solid CO_2-alcohol mixt. Connect each sample in r-b flask with 2 adapters in series to evaporator, and heat in thermostatically controlled H_2O bath at 45°.

(n) *Separators.*—With Teflon stopcocks, 500 and 1000 ml.

(o) *Silanized glass wool.*—Applied Science Laboratories, Inc., or equiv.

(p) *Syringe.*—10 μl, Hamilton No. 701N, or equiv.

41.B03 *Reagents*

(All solvs must show no impurities when processed thru entire detn in absence of tissues.)

(a) *Argon-methane, 95+5.*—Purge gas (Matheson Gas Products, PO Box 85, 932 Paterson Plank Rd, E Rutherford, NJ 07073, or equiv.).

(b) *Diatomaceous earth.*—Celite 545 (Johns-Manville Products Corp., or equiv.).

(c) *Florisil.*—60–100 mesh (available from Floridin Co.). Activated by manufacturer at 1225–1250°F (650°C). Heat in oven at 130° ≥48 hr before use.

(d) *Glassware cleaner.*—Haemo Sol (Scientific Products, Inc., or equiv.).

(e) *Helium.*—99.5% min. purity (Matheson Gas Products, or equiv.).

(f) *Solid carbon dioxide.*

(g) *Solvents.*—Acetone, CH_3CN, benzene, $CHCl_3$, hexane, and MeOH. Distd-in-glass grade (Burdick & Jackson Laboratories, Inc., or equiv.).

(h) *Solvent mixtures.*—(v/v). (*1*) *Hexane-acetone.*—(8+2). (*2*) *Hexane-acetone.*—(95+5). (*3*) *70% Methanol.*

(i) *Anhydrous sodium sulfate.*—Mallinckrodt Chemical Works, or equiv. Wash with $CHCl_3$, dry in 110° oven, and store in g-s bottle until used.

41.B04 *MGA Standard Solutions*

(a) *Stock solns.*—(*1*) *A.*—1 mg/ml; 1000 ppm. Dissolve 100.0 mg melengestrol acetate (99.5% purity, Upjohn Co.) in 100 ml acetone. Soln is stable 2–3 months. (*2*) *B.*—100 ppm. Dil. 10.0 ml soln *A* to 100 ml with MeOH. Prep. soln fresh daily. (*3*) *C.*—10 ppm. Dil. 10.0 ml soln *B* to 100 ml with MeOH. Prep. soln fresh daily.

(b) *Intermediate solns.*—(*1*) *D.*—0.5 ppm. Dil. 5.0 ml soln *C* to 100 ml with MeOH. (*2*) *E.*—1.0 ppm. Dil. 10.0 ml soln *C* to 100 ml with MeOH. (*3*) *F.*—1.5 ppm. Dil. 15.0 ml soln *C* to 100 ml with MeOH.

(c) *Working solns.*—0.25, 0.50, 0.75 ppm. Transfer 5.0 ml solns *D*, *E*, and *F* into sep. 50 ml r-b flasks and evap. on rotary evaporator. Dissolve residues in 10.0 ml portions hexane-acetone (8+2).

41.B05 *Extraction*

(Wash all glassware in detergent and rinse in H_2O to remove traces of cleaning agent. Then rinse with MeOH, acetone, or $CHCl_3$. *Caution: See* **51.004**, **51.043**, **51.046**, and **51.061**. Store samples in freezer.)

(a) *From fat.*—Transfer 25.0 g sample to 250 ml beaker. Add 150 ml hexane and warm on steam bath without boiling. Stir with spatula until fat dissolves. Place 20 g diat. earth (2 heaping tablespoons) in fritted funnel and wash with 100 ml CH_3CN. Discard wash. Filter warmed fat soln thru cake on funnel with vac. into 1 L filter flask. Rinse beaker with <50 ml hexane to remove solids, and transfer to funnel. Remove top 3 mm diat. earth cake and transfer to blender bowl. (Some diat. earth is left in funnel for next filtration.)

Add 150 ml hexane and homogenize 3 min at low speed. Filter soln thru diat. earth cake into filter flask. Rinse blender bowl with enough hexane to remove solids, and transfer to funnel. Adjust combined filtrates to ca 400 ml with hexane in filter flask. Rinse beaker and blender bowl with two 50 ml portions CH_3CN, and transfer to funnel. (Rinse cake thoroly, since MGA may adsorb onto diat. earth from hexane.) Warm filter flask on steam bath in hood and transfer filtrate to 1 L separator. Rinse flask with 5–15 ml CH_3CN, and transfer to separator. Shake vigorously 1 min. Let layers sep. 30 min. Drain lower layer into 1 L r-b flask. Add 100 ml CH_3CN to separator. Repeat extn and sepn twice. Add 50 ml benzene to r-b flask and evap. on rotary evaporator.

(b) *From muscle, liver, and kidney.*—Transfer 25.0

g frozen tissue to blender bowl. Let thaw 5–10 min at room temp. Add 150 ml CH_3CN, 20 g diat. earth (2 heaping tablespoons), and 50 g anhyd. Na_2SO_4 (2 tablespoons). Homogenize at low speed 3 min. Place 20 g diat. earth into fritted funnel and wash with 100 ml CH_3CN. Discard wash. Filter soln thru cake with vac. into 1 L filter flask. Rinse blender bowl with <50 ml CH_3CN to remove remaining solids. Sep. tissue cake from filter pad and transfer to blender. (Do not disturb diat. earth below tissue cake. Household fork is good transfer tool.)

Add 10 g diat. earth, 25 g anhyd. Na_2SO_4, and 150 ml CH_3CN to blender bowl. Homogenize 3 min at low speed, filter, and rinse. Transfer combined filtrate to 1 L r-b flask and add 50 ml benzene. Evap. to dryness in rotary evaporator. (*Caution:* Bumping may occur.) To dry residue, add 200 ml hexane and 100 ml CH_3CN thru adapter. Remove adapter, and transfer solv. mixt. to 1 L separator. Add another 200 ml portion hexane to r-b flask and transfer to separator. Shake vigorously 1 min. Let layers sep. 30 min. Drain lower layer into 1 L r-b flask. Add 100 ml CH_3CN to separator. Repeat extn and sepn twice. Add 50 ml benzene and evap. on rotary evaporator.

41.B06 *Column Chromatography*

Before analysis of samples, confirm, using MGA std soln, that hexane-acetone (8+2) elutes MGA completely, as follows: Pipet 1 ml 1 ppm MGA std soln into 50 ml r-b flask and evap. solv. on rotary evaporator. Chromatograph on Florisil column as indicated below. Det. recovery of MGA. If recovery is <95%, det. new elution vol. or obtain new batch of Florisil.

To 19 mm id glass tube, add cooled Florisil to ht of 10 cm with tapping. Push small wad of glass wool into tube until it touches Florisil. Place reservoir on top of column. Consecutively prewash column with 100 ml hexane, 100 ml acetone, and 100 ml hexane. (N pressure may be used to speed up this washing.) Remove reservoir.

Dissolve sample residue in 20 ml hexane and transfer to top of column. Replace reservoir and consecutively wash flask with 20 ml hexane, 200 ml hexane, and 300 ml hexane-acetone (95+5), and add each washing to column; if N pressure is used, add adapter. When last of solv. has reached top of column, place 500 ml r-b flask under column, wash r-b flask with 150–170 ml hexane-acetone (8+2), and transfer to column for MGA elution. Elute sample until column goes dry, using N pressure to blow out last of solv. Evap. to dryness on rotary evaporator. Quant. transfer dried residue with five 2 ml portions acetone to 50 ml r-b flask and evap. on rotary evaporator. Dil. sample to 1.0 ml with hexane-acetone (8+2).

MGA gives poorly resolved chromatogram with some liver samples. Following addnl cleanup is necessary to remove interferences: Evap. remainder of 1 ml hexane-acetone soln on rotary evaporator. To dried residue, add three 20 ml portions hexane and transfer to 500 ml separator. Add 50 ml 70% MeOH, shake vigorously 1 min, let sep. 15 min, and drain lower layer into second 500 ml separator. Add 50 ml 70% MeOH to first separator. Repeat extn and sepn twice. To MeOH layer in second separator, add 1.0 ml satd Na_2SO_4 soln, 100 ml deionized H_2O, and 50 ml $CHCl_3$. Shake vigorously 1 min. (*Caution:* Vent frequently.) Let layers sep. 15 min and drain lower layer into 500 ml r-b flask. Add 50 ml $CHCl_3$ to separator and repeat extn and sepn twice. Evap. $CHCl_3$ on rotary evaporator. Quant. transfer dried residue with five 2 ml portions acetone to 50 ml r-b flask and evap. on rotary evaporator. Add 1.0 ml hexane-acetone (8+2) and reinject on column.

41.B07 *Gas Chromatography*

Alternately inject 2–4 μl aliquots sample blank and 0.25 ppm MGA std soln until reproducible peak hts are obtained for std. Inject 1–4 μl 0.25 ppm MGA std soln. Adjust gas flow and attenuation until 20–25 mm peak ht is obtained. Use this std soln for measurement and calcn of samples at ca 10 ppb level, 0.5 ppm std soln for samples at ca 20 ppb, and 0.75 ppm std soln for samples at 30 ppb.

Inject same sample vol. as used for std soln to obtain 20–25 mm response. Measure peak ht of std, H', and sample, H, at retention time of MGA by baseline technic.

$$\text{ppb MGA} = (H/H') \times C \times (V/I)/\text{g sample},$$

where C = ng MGA std injected on column; V = total ml soln (sample + solv.) in r-b flask (1.0 ml); and I = ml sample soln injected onto column.

Results and Recommendation

The purpose of this study was to determine the ability of a number of laboratories to use this analytical procedure successfully. The design of the study allowed us to measure systematic and random error, to confirm the sensitivity of the procedure, and to compare laboratory-fortified samples with samples from treated animals.

Preliminary statistical examination of the observed per cent recoveries of MGA from the 4 tissues (Table 1), the MGA residues of the fat from treated animals (Table 2), and the values from control tissues (Table 3) indicated that the method was adequate. Since metabolism studies (7) have indicated that fat is the limit-

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee G and was adopted by the Association. *See* (1976) *JAOAC* **59**, 399.

Table 1. Observed per cent recovery of MGA from fortified kidney and liver tissue, muscle, and fat

Run	Sample	Fortification level, ppb[a]	Laboratory 1	2	3	4	5	6	7
			Kidney						
1	4	10	95	129	104	71	107	84	85
		R	93	131	85	71	103	86	73
	6	10	100	99	100	75	111	84	95
		R	89	115	82	79	107	89	70
	2	20	90	65	85	76	113	90	58
		R	89	105	87	74	115	—	—
	3	20	81	70	89	67	111	92	0
		R	—	—	—	—	—	—	—
5	26	10	100	117	92	85	105	85	82
		R	103	123	94	85	103	85	67
	27	10	89	109	84	79	103	86	83
		R	94	122	96	72	103	86	138
	28	20	88	73	90	67	101	88	95
		R	—	—	—	—	—	—	—
	30	20	100	77	81	79	102	—	—
		R	96	79	95	83	102	—	—
Av.			93.4	101.0	90.3	75.9	106.1	86.8	84.6
Std dev.			6.2	10.3	4.0	6.5	1.9	1.2	25.4
			Liver						
3	15	10	98	97	95	92	129	88	171
		R	106	106	95	76	125	82	228
	17	10	95	109	107	88	125	93	86
		R	90	105	119	95	129	—	167
	13	20	85	79	77	76	119	84	96
		R	82	84	73	83	101	91	—
	14	20	80	92	79	74	123	—	101
		R	—	—	—	—	—	—	—
6	31	10	85	95	80	78	100	82	145
		R	87	116	89	72	119	86	169
	35	10	79	75	67	78	111	95	138
		R	81	80	73	78	126	87	125
	32	20	75	64	75	68	98	93	133
		R	—	—	—	—	—	—	—
	36	20	73	64	77	62	101	—	—
		R	81	98	89	68	100	—	—
Av.			85.5	91.7	85.4	77.7	114.7	88.1	141.7
Std dev.			5.5	14.2	9.8	6.1	4.4	5.5	36.0
			Muscle						
2	10	10	85	68	69	82	94	94	0
		R	86	87	69	79	97	90	0
	11	10	84	45	75	75	97	96	42
		R	79	56	76	84	101	96	47
	7	20	88	56	84	73	95	101	89
		R	101	48	83	75	95	—	—
	8	20	76	42	76	81	97	92	51
		R	—	—	—	—	—	—	—
4	22	10	73	113	72	84	102	89	88
		R	77	109	79	90	102	89	73
	24	10	78	98	74	76	102	77	72
		R	71	102	79	65	98	87	68
	19	20	78	87	64	94	95	92	79
		R	—	—	—	—	—	—	—
	20	20	86	79	64	100	98	—	—
		R	84	85	75	101	100	—	—
Av.			81.9	76.8	74.2	82.8	98.1	91.2	67.7
Std dev.			5.2	13.0	3.6	8.5	2.1	5.2	17.0

Continued

Table 1. (*Continued*)

Run	Sample	Fortification level, ppb[a]	Laboratory 1	2	3	4	5	6	7
			Fat						
7	37	10	89	106	85	93	95	86	53
		R	93	127	96	88	88	—	98
	39	10	93	103	87	114	95	115	80
		R	91	118	83	95	93	107	62
	41	20	94	81	86	92	98	98	99
		R	90	90	90	84	111	—	—
	42	20	93	84	101	102	101	—	74
		R	—	—	—	—	—	—	—
8	45	10	85	109	95	67	106	93	77
		R	87	119	101	67	104	90	61
	47	10	85	118	101	92	102	92	72
		R	89	140	108	92	94	85	76
	44	20	89	98	91	88	109	85	54
		R	86	116	93	74	102	—	—
	48	20	84	104	86	84	105	87	—
		R	—	—	—	—	—	—	—
Av.			89.1	108.1	93.1	88.0	100.4	93.8	73.3
Std dev.			2.0	7.4	6.5	12.4	3.8	10.4	17.0

[a] R = observed per cent recovery resulting from a second injection into the gas chromatograph of the sample immediately above.

Table 2. Observed MGA levels (ppb) in fat from treated animals

Run	Sample	Sample level, ppb[a]	Laboratory 1	2	3	4	5	6	7
9	A	10	5.8	—	10.9	9.0	9.5	10.8	8.8
		R[b]	5.5	—	11.7	10.4	8.8	10.5	9.2
	E	10	5.8	11.0	9.1	12.2	9.1	10.1	6.6
		R	6.5	12.0	9.5	9.4	9.2	—	7.5
	C	20	5.5	21.1	19.5	16.8	19.6	19.4	15.8
		R	5.9	22.4	20.2	16.6	19.2	19.6	12.5
	F	20	6.8	20.0	19.3	20.8	20.9	19.6	15.6
		R	7.6	21.8	21.1	17.2	21.1	—	22.3
10	I	10	4.8	10.9	9.3	10.3	12.3	9.9	5.5
		R	5.3	11.8	9.3	9.9	12.3	10.4	5.9
	K	10	4.4	10.3	9.2	9.0	12.5	9.7	3.7
		R	5.9	11.6	9.2	8.8	13.5	9.6	4.5
	H	20	7.4	19.6	17.9	18.3	25.4	18.4	7.1
		R	8.0	21.2	17.4	17.0	24.3	18.8	7.7
	J	20	7.1	20.5	18.7	19.9	25.2	18.4	5.9
		R	8.0	20.7	19.3	16.8	26.4	17.8	12.1

[a] A number of fat aliquots were assayed to determine the average level of MGA in these 2 samples.
[b] R = observed per cent recovery resulting from a second injection into the gas chromatograph of the sample immediately above.

ing tissue in withdrawal of MGA from the animal, the statistical analysis that follows will be confined to how well the method worked in the 7 laboratories for the analysis of fat tissue. For this purpose the paired sample analysis of Youden (8) was used.

In our analysis, the observed values of paired samples for each day's run are plotted against each other (2 values each for 0, 10, and 20 ppm levels). An arbitrary circle was drawn for each level with the 10 ppb diameter centered at the theoretical coordinates. Any point that fell within its respective circle indicated that the 2 paired assays were within ±5 ppb of the true level. The closer the points are clustered together around the origin, the more accurate and precise are the results. Within each circle, vertical, horizontal, and 45° lines were drawn through the theoretical value. If the pattern made by the points can be seen as lying on the

Table 3. Observed GLC response in control tissue at the retention time of MGA (ppb)[a]

Run	Sample	Laboratory						
		1	2	3	4	5	6	7
				Kidney				
1	1	0.0	4.6	0.5	0.0	0.6	0.0	0.0
	5	1.5	3.0	0.0	0.0	0.0	0.0	0.0
5	25	0.0	2.6	0.4	0.0	0.7	0.0	0.0
	29	0.0	1.7	0.7	0.0	0.3	—	—
				Liver				
3	16	2.1	1.1	0.3	0.0	4.2	0.0	0.0
	18	2.2	0.5	0.0	0.0	2.5	0.0	0.0
6	33	2.4	0.0	0.4	0.0	0.9	0.6	2.9
	34	2.0	0.0	0.0	0.6	0.8	—	—
				Muscle				
2	9	0.0	0.5	0.5	0.0	0.0	0.3	0.0
	12	0.0	0.2	0.0	1.1	0.0	0.5	0.0
4	21	0.0	0.0	0.0	0.6	0.0	0.4	0.0
	23	0.0	0.0	0.0	0.4	0.4	—	—
				Fat				
7	38	0.5	0.0	0.0	0.0	0.0	0.0	0.0
	40	0.7	0.0	1.0	0.0	0.0	0.0	1.2
8	43	0.6	0.0	0.0	0.0	0.0	0.5	0.0
	46	0.5	0.0	0.7	1.7	0.0	—	—
				Fat (Unknown)				
9	B	0.4	0.0	0.8	1.3	0.0	1.0	1.3
	D	0.4	0.0	0.0	2.2	0.0	0.4	0.0
10	G	0.3	0.0	8.2[b]	0.9	0.0	0.0	0.8
	L	0.4	0.0	0.0	0.4	0.0	0.0	0.0

[a] Observed values in many cases were measured from the baseline to the side of an interfering peak and reported as MGA.

[b] Analyst reported gross contamination (other peaks, etc.).

45° line, systematic error was the major source of error in the study. If all 4 quadrants contain about the same number of points, then random errors were responsible for the scatter (8). Graphic presentation (Fig. 1) showed that Laboratory 7 had inordinately large systematic and random errors; therefore, it was excluded from further evaluation.

The average recovery of the method was shown to be 97 and 93% for 10 and 20 ppb fortified fat samples, respectively (Table 4). The residue recovery rates (excluding those of Laboratory 1) were 103 and 99.5% for 10 and 20 ppb treated heifers, respectively. The coefficients of variation due to systematic errors were observed to be <7 and 3% for fortified and treated fat tissues, respectively. These high per cent recoveries and low per cent coefficients of variation indicated that the method was accurate and efficient.

The repeatability or precision of the assay results from one laboratory to another is reflected

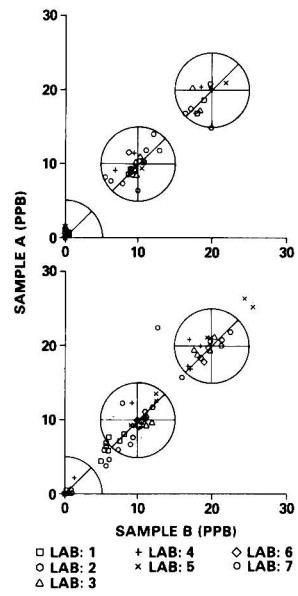

FIG. 1—Paired sample plots for illustration of systematic and random errors. The upper figure is for fortified fat; the lower figure is for fat from treated heifers.

Table 4. Summary of the statistical characteristics of the method

Term	Fortified fat, ppb			Fat from treated heifers, ppb		
	0	10	20	0	10	20
Pooled mean square error (MSE)	0.49	3.96	9.80	0.92	3.03	10.68
total random variance	0.37	3.74	6.10	0.68	2.81	10.66
variance of bias of method	0.06	0.11	1.85	0.12	0.11	0.01
pooled sample variance	0.19	0.82	0.78	0.10	0.65	0.94
pooled sample bias variance	0.15	1.57	4.51	0.41	1.19	4.87
Average residue recovery, ppb	0.24	9.68	18.65	0.35	10.34	19.90
Average recovery, per cent	—	97	93	—	103	99.5
Coefficients of variation, per cent						
due to random error	—	20	13	—	16	16
due to systematic error	—	3	7	—	3	1
Average $\pm 2\sqrt{MSE}$						
lower limit, ppb	—	5.71	12.39	—	6.86	13.36
upper limit, ppb	1.64	13.65	24.91	2.27	13.82	26.44

in the random variations. The coefficients of variation of the total random errors, including variations due to within paired samples, between paired samples, and between laboratories, were estimated to be <20 and 16% for fortified fat and fat from treated heifers, respectively.

As shown in Table 4, the lower limit of the 10 ppb level for both fortified and treated fats did not overlap the upper limits of zero level, which were conservatively estimated, but the intervals between 10 and 20 ppb levels overlapped slightly. It was clear that the method could separate samples with 10 ppb residue from no-residue samples, but the sensitivity was somewhat weaker in differentiating between 10 and 20 ppb residue levels. The same interpretation can be induced by examining the distribution of assays as plotted in Fig. 2. A clear separation between the confidence intervals of zero and 10 ppb levels for all laboratories can be seen, while Laboratory 7 showed difficulty in separation of the 10 and 20 ppb samples.

The average per cent recoveries and standard deviations of fortified and treated fat samples for each laboratory were plotted in Fig. 3. Laboratory 1 was excluded from this figure due to the problems[1] with the unknown fat tissues. The means were distributed closely on the direction of the 45° diagonal line. Therefore, a good agreement can be interpolated from the laboratory-fortified results to samples from field conditions. All the averages were seen above 80% residue recovery except those from Laboratory 7 where the average recovery rate fell between 60 and 70%. When studying the figure, one also notes that there is no evidence of the dominancy between the lengths of the vertical and horizontal

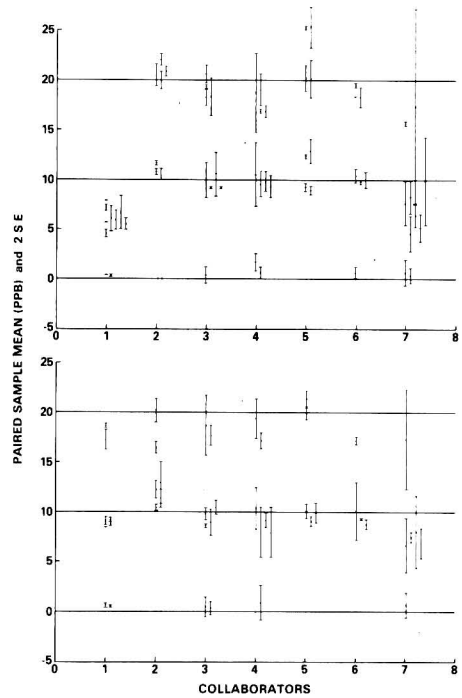

FIG. 2—Paired sample means and their confidence limits. The upper figure is for fat from treated heifers; the lower figure is for fortified fat.

[1] Laboratory 1 encountered solvent problems during the preliminary part of the study which were resolved before the collaborative part began. The records indicated recurrence of problems during the study, which may explain the low recoveries from the unknown fat samples at the end of the study.

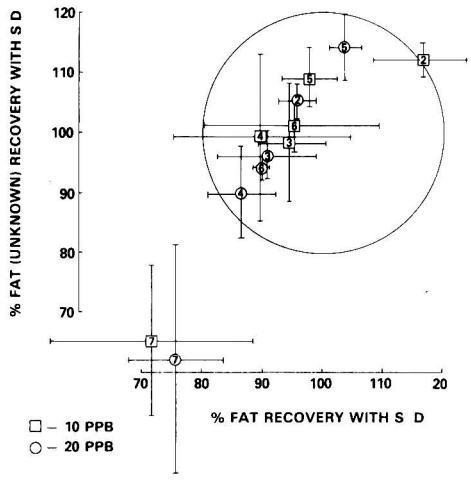

FIG. 3—Sample means and standard deviations for fortified fat and fat from treated heifers. Numbers in the plot represent the laboratories. Numbers without circles are first level (10 ppb) results; those with circles are second level (20 ppb) results. Large circle indicates recoveries from 80 to 120%.

standard deviations. This indicates that the precision of the 2 set assays can be considered as equivalent. When laboratory results were pooled, the total mean square errors and the random components were not significantly different for either the 10 or the 20 ppb level between the 2 fat tissues. The systematic errors of the method were not different at the 10 ppb level, but differences were found at the 20 ppb level where an extremely small bias was found for unknown fat tissues (Table 4). Overall, it can be concluded that the method can be applied to field samples by a number of laboratories with results equivalent to those obtained with laboratory-fortified samples. It is recommended that this method be adopted as official first action.

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TOXICOLOGICAL TESTS

Appraisal of Existing Methodology in Aspiration Toxicity Testing

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New methods or modifications of currently used methods in aspiration toxicology must be capable of accurately assessing the hazard and toxicity potentials of those products which are capable of being aspirated. The methods evaluated in the rat were intratracheal injections, intravenous injections, and modifications of the Gerarde technique. A product containing petroleum distillates and a standard viscosity petroleum distillate sample were administered to Osborne-Mendel rats, rabbits, and guinea pigs in ml/kg doses. The utility of the methods was determined by comparing lung weight increases, lung-body weight ratios, gross pathological lung changes, and mortality. The modified Gerarde technique showed the best potential for predicting aspiration hazard and toxicity based on the severity of the reactions which, using a radiolabeled oil, was shown to be a function of the amount of product which enters and reacts with the lungs. The influence of preventing the swallowing reflex to assure aspiration of the oil was assessed in the rat. The results show that less than 10% of the dose is aspirated when the swallowing reflex occurs. Methods of sacrifice were investigated for an effect on rat lung weight. No tested method except stunning with cervical dislocation produced an effect. Tests were conducted on rats and rabbits to determine differences in lung reactivity to the influence of inhalation anesthetics, pentothal sodium, or no anesthesia in response to petroleum distillate administration and to determine which test species is the better animal model. The results indicate that the rat is the better test model because of degree of lung reaction, size, and economy, and that inhalation anesthesia with ether is preferable over the other choices.

In 1970, the Department of Health, Education and Welfare estimated that there were 20,000 occurrences each year of ingestions involving furniture polishes known to contain petroleum distillates (1). For fiscal years 1973 and 1974, the Consumer Product Safety Commission estimated the number of ingestions of petroleum distillate-containing products (polishes, paint removers, chemicals, lighter fluids, etc.) in children under 5 years of age at 13,000 annually (2). Petroleum distillates are considered as special hazards in terms of the Federal Hazardous Substances Act. This Act requires cautionary labeling on those products which contain $\geqslant$10% w/w of these substances (3) and which have viscosities below 100 Saybolt Universal Seconds (SUS) at 100°F (4).

Although for many years the Gerarde technique (5) has been used in our laboratory, an official test does not exist to predict the potential aspiration hazards of petroleum distillates. Such an aspiration test must be rapid, reliable, and capable of detecting those distillates which have the intrinsic ability to produce even mild degrees of lung irritation. The present paper reports the efforts by which this laboratory has endeavored to improve the predictive quality of the Gerarde test. These modifications include quantitation of the dose on an ml/kg basis; choosing an appropriate general anesthetic; and establishing dose-response relationships based on increases in lung weight, lung-weight-to-body-weight, and mortality ratios, physical appearance of the lungs, and changes in body weight for a 24 hr observation period. In these experiments, 24 hr body weight changes were used as indicators of animal morbidity, stress, and failure to consume normal amounts of food and water.

In order to determine the actual amounts of petroleum distillates aspirated into the lungs by 2 different methods (modified Gerarde and intratracheal (IT)) and under 2 inhalation anesthetics, a radiolabeled oil was administered to

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rats. The information gained from these investigations was then used to select the most appropriate manner in which to conduct aspiration tests on animals. Three species of laboratory animals were then evaluated, using the above modifications of the Gerarde technique, to determine the most appropriate species for use in these tests.

Experimental

Materials

A commercially available patio torch fuel was used for most of the experiments. Gas chromatographic analysis demonstrated that the hydrocarbons of this petroleum distillate ranged from C_9 through C_{16}, corresponding to kerosene. The viscosity of this product was measured with a Wells-Brookfield cone-plate viscometer and was found to be 31.4 SUS. The Brookfield viscosity (centipoise) was converted to SUS at 100°F by the American Society for Testing and Materials conversion tables (ASTM D2161-74). The viscosity value of the second sample, Oil K (National Bureau of Standards Viscosity Sample), was similarly determined to be 104.6 SUS.

The surface tension of both petroleum distillates at 24°C was measured by a Fisher Tensiomat (du Nouy Pt-Ir ring). The patio torch fuel had a surface tension of 25.8 dynes/cm and that for Oil K was 29.9 dynes/cm. While the surface tensions of the torch fuel and Oil K are significantly different it should be noted that for petroleum distillates in general, surface tension variation is small over a wide range of oil viscosities. As a result, viscosity appears to be the more significant parameter for the penetration of these petroleum distillates into the deep structures of the lung (5; R. E. Osterberg, 1974, unpublished observations).

Three species of nonfasted animals were used: Osborne-Mendel albino rats of both sexes weighing 200–300 g, male albino guinea pigs of the Hartley strain weighing 400–700 g, and New Zealand white rabbits of both sexes weighing 1.8–3.0 kg.

Anesthesia was induced in the experimental animals with ethyl ether (anhydrous) or chloroform (ACS) or by the short-acting barbiturate pentothal sodium (Abbott Laboratories) at 20 mg/ml.

Modified Gerarde Technique

Animals were anesthetized with ethyl ether except where otherwise indicated to the point of slow, diaphragmatic breathing which is rapidly followed by apnea. Anesthesia for the rats and guinea pigs was induced in a desiccator jar (approximately 10 L capacity) which contained a desiccator plate to keep the animals above the liquid pool of ether. Rabbits were anesthetized with ether by the open drop technique with a gauze mask.

Following deep anesthesia, the animal is placed in a supine position at ca 120° angle to the table top with the head elevated. The mouth is held open by a hemostat inserted between the jaws and the tongue is pulled forward with forceps to prevent the swallowing reflex (Fig. 1). Petroleum distillate doses of 0.25, 0.5, or 1.0 ml/kg are delivered into the rear portion of the mouth near the tracheal orifice with the aid of a 0.5 ml glass syringe without a hypodermic needle. Then the nostrils are closed with the fingers to force the animal to breathe through its mouth (Fig. 2). The animal is maintained in position until the characteristic sounds of 1 or 2 aspiration cycles (1 or 2 slurps) are heard, the investigator judges that the distillate has entered the trachea, or the rat shows signs of regaining consciousness, whereupon the nostrils and tongue are released and the animal is returned to a holding cage. At this time, if it is believed that the animal has not successfully aspirated the dose as determined by the absence of aspiration cycle sounds or visual confirmation of distillate entrance into the trachea, it is eliminated from further consideration. In those experiments in which animals were not anesthetized, patio torch fuel was administered only by the modified Gerarde technique. Rats were firmly held by hand and received the oil in the above manner. Rabbits were placed in a restraining box and a metal bit was inserted into the mouth between the jaws. The tongue was pulled through a hole in the bit and the rabbit received the oil also in the above manner.

Intratracheal Injection Method

Following the induction of ether anesthesia, the rat is removed from the jar and placed in a supine position on the table top. A midline incision is made through the skin of the neck along the midsagittal plane with a scalpel blade to expose the underlying musculature. Then the muscles overlying the trachea are cut to expose the tracheal rings. Petroleum distillates are injected into the trachea between the rings by a $\frac{5}{8}'' \times 26$ gauge needle attached to a 0.5 ml glass syringe. All incisions are closed with a 7.5 mm Michel wound clip.

Intravenous (IV) Injection Method

The rat is anesthetized with ether and immobilized in a supine position. A small transverse incision is made on either side of the midline just

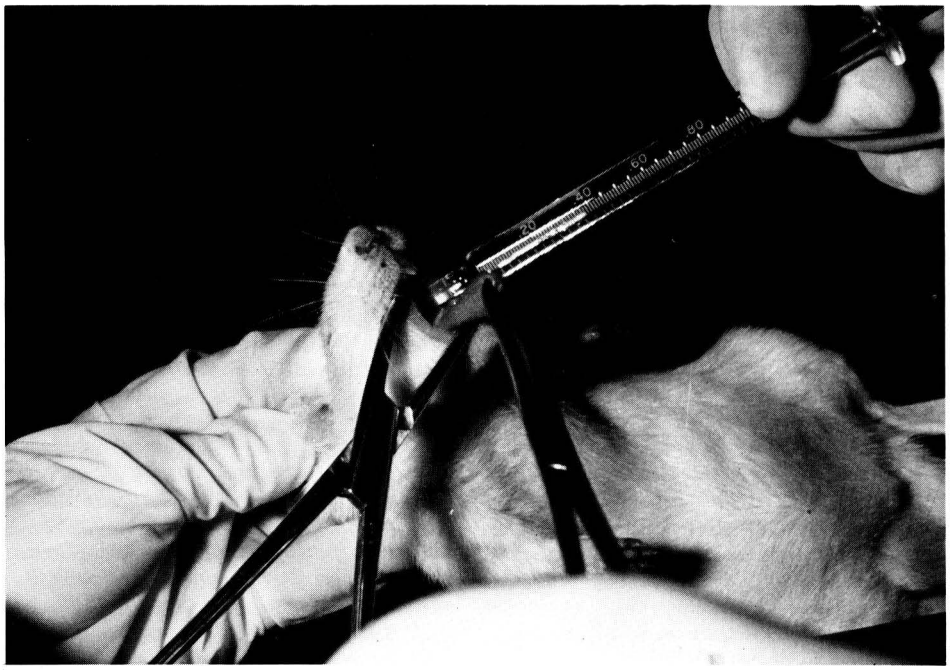

FIG. 1—This picture of the Gerarde technique shows the anesthetized rat with its mouth held open, tongue extended, and with a partially filled syringe in position for oil administration.

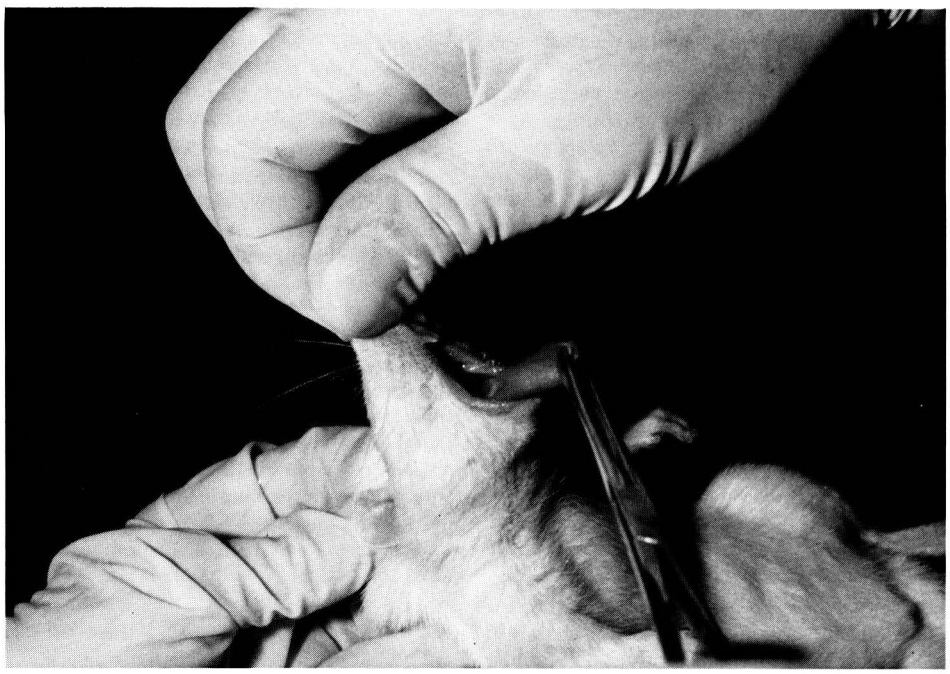

FIG. 2—This picture shows the rat immediately following oil administration. The tongue is still extended to prevent swallowing of the oil and the nose is pinched to force inspiration through the mouth.

above the clavicle and lateral to the midclavicular line by a blunt scissor. This procedure exposes the common jugular vein as it emerges at the supraclavicular fossa. Following the injection of the petroleum distillate, the bleeding is controlled and the incision is closed with a 7.5 mm Michel wound clip.

Anesthetic Trials

The modified Gerarde test was used in the determination of the anesthetic of choice. In these experiments groups of rats received intraperitoneal injections of pentothal sodium at a dose of 40 mg/kg. Groups of rabbits received pentothal sodium by the injection of a bolus at a concentration of 20 mg/kg into a marginal ear vein. Ether or chloroform anesthesia was induced by inhalation.

Observation and Necropsy

Control groups of animals received distilled water in place of an oil for all methods of administration. Following distillate or water administration, all animals were observed for 24 hr and had free access to food and water. Lungs were removed and weighed at the shortest time interval following death but never when stages of rigor mortis were noted. Surviving animals were sacrificed 24 hr following oil administration. Rats were sacrificed by decapitation, rabbits were sacrificed by an IV injection of a lethal dose of pentobarbital sodium, and guinea pigs were sacrificed by ether anesthesia and the lungs were quickly removed from the thoracic cavity. Only those lungs which showed no external signs of murine pneumonia were used. The heart, trachea, and mediastinal structures were then removed and the lungs were gently blotted on disposable tissues or gauze sponges. Lung weights were obtained from a Roller-Smith precision balance with a 25 g capacity.

Animal Sacrifice

In order to determine whether or not different methods of animal sacrifice might affect lung weight, a separate experiment was conducted on 40 untreated rats. Ten animals each were sacrificed by either decapitation, cervical dislocation, terminal ether anesthesia, or stunning (a blow to the head) followed by cervical dislocation. The lungs were removed, and the weights were recorded and compared.

Radioactive Oil Preparation, Administration, and Analysis

Commercially available patio torch fluid was fortified with equal parts of nonane-1-^{14}C (ICN Pharmaceuticals, Inc., Cleveland, OH) and hexadecane-1-^{14}C (ICN Pharmaceuticals, Inc.) to a specific radioactivity of 0.30×10^6 cpm/0.1 ml. Both the nonane-1-^{14}C and the hexadecane-1-^{14}C stock solutions had a specific radioactivity of 200 μCi/ml of the respective hydrocarbon.

Five groups of 4–6 rats each were given 1 ml radioactive patio torch fluid/kg body weight. The groups received the product by the Gerarde technique with ether anesthesia, the Gerarde technique with chloroform anesthesia, the Gerarde technique with the tongue relaxed in the mouth and not pulled forward, or the IT method. All groups except one which received the labeled patio torch fuel by the modified Gararde technique were sacrificed at 60 sec following oil administration. The other group was sacrificed at 5 sec following oil administration to determine if any differences in oil clearance or diffusion to the blood occurred between these times. The purpose of these tests was to ascertain the relative amount of distillate entering the lungs by these procedures. The lungs obtained from rats treated with the radiolabeled oil were immediately placed in closed containers in an ice bath and then the entire lung was combusted as soon as possible in a Packard Model 306 Tri-Carb sample oxidizer. The sample cups held 1–1.5 g tissue so that, in some instances, lungs had to be divided and treated as 2 samples. In this case, the 2-sample results were added for quantitation purposes after scintillation counting. Samples were then counted in the collecting scintillation fluid (equal parts of Carbo-Sorb and Permafluor V, Packard Instrument Co., Downers Grove, IL), using a Beckman scintillation counter until a standard error of 0.2% was achieved. Samples were counted repeatedly to obtain a constant count. The counting efficiency of the oxidizer cocktail was 65% for ^{14}C. A recovery curve for ^{14}C standard added to whole lungs and combusted showed 98% recovery of added activity. Backgrounds on the order of 0.1–0.2% of the previous sample were found. These were subtracted from the following sample.

Statistical analysis of mortality data was computed by the combination of 2×2 tables, using Cochran's test (6). Analyses of lung weight and lung-body weight ratios, based upon animal's body weight at the time of oil administration, were performed either by standard t-tests or analysis of variance techniques. A level of $P < 0.05$ was chosen as the level of significance unless otherwise noted.

This report of the Associate Referee, R. E. Osterberg, was presented at the 89th Annual Meeting of the AOAC, Oct. 13–16, 1975 at Washington, DC.

Results

The dose-response mortality ratios and lung weights by technique, anesthetic, and animal species are presented in Tables 1 and 2. Comparisons discussed refer to these data.

Patio Torch Fuel (31 SUS)

Mortality.—A comparison of mortality with the 3 techniques, using rats and ether, only showed that the Gerarde technique produced the greatest mortality, followed by the IV and IT techniques. Statistically, the Gerarde technique produced significantly higher mortality than both the IV ($P < 0.05$) and IT ($P < 0.0001$) techniques. Although the IV technique produced higher mortality than the IT technique, the difference is not statistically significant ($P > 0.1$), possibly because of the small numbers. For all 3 methods the increase in mortality with increasing dose indicates the suitability of the rat as a model in this type of test. Furthermore, decreased doses markedly increased survival time (mean deaths for the 1 ml/kg groups with ether anesthesia occurred at 43.8 min compared with 172.7 min for 0.5 ml/kg and 270 min for 0.25 ml/kg). The range of mortality from low to high dose for both the Gerarde and IV techniques is also satisfactory.

Further examination of the Gerarde technique data in Table 1 shows approximately equivalent mortality in rats among the 3 anesthetics; these all show greater mortality than the unanesthetized group (only chloroform was not significantly greater, $P = 0.10$). Similar results are seen with the rabbits; the unanesthetized group shows less mortality than the anesthetized groups at the 2 lower doses, with no difference at the 1 ml/kg dose.

The consistency of the Gerarde technique regarding dose-mortality response within the rat, rabbit, and guinea pig is also seen in Table 1. While the guinea pig appears to be more sensitive than either the rat or rabbit, regarding ether anesthesia only, the latter 2 species appear to be equally sensitive to dose and consistent in their reactions with the various anesthetics and with no anesthetic.

Lung Weights.—In regard to ether anesthesia, these 3 methods were also compared for their effects on pulmonary edema as determined by the lung weight responses of the rats at the time of sacrifice or at death if death occurred not sooner than 30 min after oil administration. The results in Table 2 show heavier lung weights with the Gerarde technique compared with the lung weights for either of the other 2 methods. Lung weights with the IV method were not consistently higher than those produced by the IT method. The results of all 3 methods show lung weights which are significantly greater ($P < 0.01$) than those of the 60 control rats for all 3 doses, indicating significant edema formation.

Comparison of the 3 anesthetics and no anesthesia for the Gerarde technique showed that lung weights were related to oil dose. Differences between anesthetic groups were not consistent; the unanesthetized group appeared to have as large a response as the anesthetized groups. With the exception of the low dose of patio torch fuel with pentothal anesthesia, all treated groups showed lung weights which were significantly heavier than those of the controls. The lung weights of the unanesthetized rabbits are somewhat less than those of the anesthetized groups at the 2 lower doses. There is no significant difference in lung weights between the pentothal

Table 1. Patio torch fuel dose-response mortality ratio[a] by technique, anesthetic, and species at 3 doses

		Rat, ml/kg			Rabbit, ml/kg			Guinea pig, ml/kg		
Technique	Anesthetic	0.25	0.5	1.0	0.25	0.5	1.0	0.25	0.5	1.0
Gerarde	ether	2/8	17/21[b]	20/22[b]	1/5	2/5	2/3	4/5	5/5	5/5
	chloroform	1/7	3/8	8/8	—	—	—	—	—	—
	pentothal[c]	0/8	8/10	10/10	1/3	3/3	3/3	—	—	—
	none	0/10	2/10	6/10	0/6	0/4	5/6			
Intratracheal	ether	0/8	1/7	2/8	—	—	—	—	—	—
Intravenous	ether	0/8	4/8	5/7	—	—	—	—	—	—

[a] Number dead at 24 hr/total number.

[b] Additional data from this laboratory on the mortality response at these 2 doses show 60% (24/40) mortality at 0.5 ml/kg and 100% (52/52) at 1 ml/kg. These ratios do not differ significantly from those of the above anesthetics at the specified doses.

[c] Pentothal was given to rats with an intraperitoneal injection at 40 mg/kg. Rabbits received pentothal intravenously (marginal ear vein) at a dose of 20 mg/kg.

Table 2. Patio torch fuel dose-responses of lung weights[a] by technique, anesthetic, and species at 3 doses

		Rat, ml/kg			Rabbit, ml/kg			Guinea pig, ml/kg		
Technique	Anesthetic	0.25	0.5	1.0	0.25	0.5	1.0	0.25	0.5	1.0
Gerarde	ether	1.91±0.33[b] N = 7[c]	3.23±0.14 N = 21	2.92±0.15 N = 22	24.8±5.19 N = 4	25.2±2.6 N = 4	18.4±2.1 N = 3	6.87±0.87 N = 3	10.5±1.84 N = 5	6.18±0.09 N = 4
	chloroform	2.04±0.14 N = 7	2.54±0.18 N = 8	2.81±0.13 N = 7	—	—	—	—	—	—
	pentothal	1.40±0.09 N = 8[d]	2.85±0.32 N = 4	[a]	31.6±2.7 N = 3	32.6±6.5 N = 3	23.2±5.6 N = 3	—	—	—
	none	1.88±0.12 N = 10	2.55±0.28 N = 9	2.85±0.3 N = 10	19.3±0.78 N = 6	20.7±2.4 N = 5	21.2±1.5 N = 2	—	—	—
Intratracheal	ether	1.65±0.21 N = 8	2.91±0.33 N = 7	2.2±0.38 N = 7	—	—	—	—	—	—
Intravenous	ether	1.83±0.08 N = 7	2.3±0.26 N = 6	2.5±0.23 N = 2	—	—	—	—	—	—
Control			1.32±0.02 N = 60			9.8±0.42 N = 42			3.87±0.19 N = 5	

[a] Lungs were not weighed if death time was <30 min following the dose.
[b] Mean lung weights are expressed in g±standard error.
[c] N = number of animals per dose.
[d] Pentothal was given to rats with an intraperitoneal injection at 40 mg/kg. Rabbits received pentothal intravenously (marginal ear vein) at a dose of 20 mg/kg.

and ether rabbits a result possibly related to the small number of rabbits in these groups. All treated groups showed lung weights significantly greater than the controls.

Radiolabeled Patio Torch Fuel.—A comparison of aspiration methods and anesthetic, using radiolabeled patio torch fuel, was also attempted. Table 3 shows the average percentage of petroleum distillate found in the lungs shortly after the aspiration of labeled patio torch fuel both with IT and combinations of the Gerarde technique. Statistical analysis shows no significant difference in the Gerarde technique with either chloroform (53.8%) or ether (65.9%) and there was no additional distillate found in the lungs of the 5 sec sacrifice group (57.7%) as opposed to the 1 min sacrifice group. All of these 3 methods led to significantly ($P < 0.01$) more oil in the lungs than did the IT technique (23.1%).

For the group in which the tongue was not pulled, a comparison shows significantly less oil entering the lungs (7.2%) than with any of the above techniques at either sacrifice time. Furthermore, the results of the former group show large variations in the amount of aspirated oil with one-half of the group failing completely to aspirate any oil.

Oil K (104 SUS)

Oil K was also used to compare the Gerarde and IT techniques. The results presented in Table 4 show that both techniques produced no mortality at the 2 lower doses while the Gerarde technique produced a significantly ($P < 0.001$) larger lung-body weight ratio than both the IT

Table 3. Average percentage of administered ^{14}C-labeled patio torch fuel found in lung by method of administration, time of sacrifice, and anesthetic

Method	No. of animals	Time of sacrifice, sec	Anesthetic	Av. % ±std error
Modified Gerarde	5	60	ether	65.9±4.48
Modified Gerarde	6[a]	5	ether	57.7±6.25
Modified Gerarde	4	60	chloroform	53.8±4.97
Modified Gerarde	6[b]	60	ether	7.2±5.19
Intratracheal	6	60	ether	23.1±4.29

[a] Three additional animals given the oil by this method were found to have lung abcesses upon sacrifice. The average percentage of oil found in their lungs was 38.9±6.8.
[b] Tongue not held.

Table 4. Comparison of average lung weight, lung-body weight ratios, and mortality ratios for Oil K, using both the modified Gerarde and the intratracheal techniques in the rat

	Modified Gerarde test				Intratracheal technique			
Dose, ml/kg	N[a]	Lung wt, g	Lung-body wt ratio × 100	MR[b]	N	Lung wt, g	Lung-body wt ratio × 100	MR
1.0	12	2.48±0.25	0.96±0.10	2/14	7	1.49±0.13	0.65±0.07	0/7
0.5	7	1.65±0.04	0.76±0.01	0/7	6	1.53±0.47	0.59±0.02	0/6
0.25	6	1.44±0.08	0.57±0.01	0/6	6	1.62±0.07	0.56±0.03	0/6
0	60	1.32±0.026	0.56±0.02					

[a] N = number of animals per dose.
[b] MR = mortality ratio.

Table 5. Ratio of rats with more than 75% total lung hemorrhage on gross examination by technique, anesthetic, dose, and oil

		Patio torch fuel, ml/kg			Oil K, ml/kg		
Technique	Anesthetic	0.25	0.5	1.0	0.25	0.5	1.0
Gerarde	ether	1/7	17/21	20/20	0/6	0/7	3/12
	chloroform	0/6	6/8	7/7	—	—	—
	pentothal	0/8	3/3	[a]	—	—	—
	none	0/10	2/9	7/10	—	—	—
Intratracheal	ether	1/8	3/7	2/7	0/6	0/6	1/7
Intravenous	ether	[b]	—	—	—	—	—

[a] All animals died within 30 min.
[b] Well defined areas of hemorrhage were not produced by this method.

group and the controls at the 0.5 ml/kg level.[1] At the 1 ml/kg dose level, the Gerarde technique produced 14% mortality while none of the IT group had died within 24 hr. For both lung weight and lung-body weight ratio, the Gerarde technique produced significantly ($P < 0.05$) larger values when compared with those of the IT method and control. This is an indication of the sensitivity of the Gerarde over the IT technique at the present legal limit of 100 SUS where lung reactions are usually less severe. In addition, these results show that with the Gerarde technique oils of different viscosities can be compared. The 1 ml/kg dose of Oil K produces a mortality ratio much lower than that for the patio torch fuel (2/14 vs. 20/22).

Comparison of Lung Hemorrhage—Rats Only

Table 5 shows the ratio of rats having more than 75% of the total lung surface area involved with hemorrhage and congestion as judged by gross examination. The results show increased areas of hemorrhage with increased dose, equivalence among the 3 anesthetics, and more hemorrhage in the anesthetized groups. Although the patio torch fuel produced significantly more congestion than Oil K in both the Gerarde ($P < 0.000001$) and IT techniques ($P = 0.05$) the differences seen with the Gerarde technique were far greater. It can also be seen that the Gerarde technique produces significantly ($P < 0.001$) more congestion than the IT technique for the patio torch fuel but not for Oil K.

Methods of Sacrifice Using Untreated Rats

Table 6 shows the average lung weights and lung-body weight ratios (%) which were obtained on untreated Osborne-Mendel rats following sacrifice by any of 4 methods. An analysis of variance for either lung weights or lung-body weight ratios shows that stunning plus cervical

Table 6. Rat lung weight and lung-body weight ratio comparisons of 4 methods of sacrifice

Method	N[a]	Mean lung wt, g, ±std error	Lung-body wt ratio × 100
Decapitation	10	1.46±0.08	0.56±0.03
Cervical dislocation	10	1.36±0.06	0.50±0.02
Ether	10	1.29±0.02	0.51±0.01
Stunning and cervical dislocation	10	2.46±0.21	0.90±0.07

[a] N = number of rats per treatment.

[1] The lung-body weight ratios were compared because these 2 dose groups were not of the same body weight initially. The mean weight of the Gerarde test group was 214.4 g while the IT group weighed 256.6 g. This difference is statistically significant ($P < 0.001$). Lung-body weight ratios are discussed below.

dislocation causes significant increases (approximately 80%, $P < 0.00001$) in both measures compared with lung weights and lung-body weight ratios obtained following sacrifice by the other methods. There were no significant differences in lung weight or lung-body weight ratios noted among the other 3 methods of sacrifice. Lung-body weight ratio has been established as a sensitive indicator of lung weight change because it is normally relatively fixed within narrow limits for any particular animal species, independent of its age or size (7).

Changes in Body Weight

As the dose of Oil K increased there was a direct relationship to 24 hr body weight decrease in the rat, using the Gerarde technique. In this instance the largest mean weight loss was 20.3 g at the 1.0 ml/kg dose level. Similar inspection of the patio torch fuel data showed that body weight loss was variable with dose and anesthetic (5.6–20.5 g) but, in all instances, body weight declined. Data from control groups which aspirated water showed a 24 hr average weight gain of 1.23 g.

Discussion

Aspiration hazard may be defined as the ability of a liquid to enter into the trachea and deeper structures of the lung. Thus aspiration toxicity would be a measure of the damage (pulmonary and/or whole body) which resulted from the aspiration of such a substance. With these definitions in mind we studied various modifications of 3 different techniques to determine which technique is the most sensitive predictor of potential aspiration hazard and toxicity. As a quantitative measure of hazard we used radiolabeled oils to determine the quantity of oil entering the lungs by the various methods. As measures of toxicity we used dose-response relationships of mortality, lung weight, lung-body weight ratios, areas of lung hemorrhage, and body weight decline. These results showed that with respect to aspiration hazard the Gerarde technique permits a larger percentage of dose to enter the lungs compared with the IT technique (66 and 23%, respectively), using patio torch fuel. Based on rat response it would appear that the same holds true for Oil K aspiration. The importance of pulling on the tongue and correctly holding it to prevent swallowing was exemplified by the use of radiolabeled patio torch fuel. Table 3 shows that there is a wide variation in amounts of aspirated oil when the tongue is not properly held and that only an average of 7% of the dose may get into the lungs. Such poor technique could produce false toxicity information regarding the petroleum distillate.

With respect to toxicity for both low and high viscosity petroleum distillates, we have seen the greater sensitivity of the Gerarde technique over both the IT and IV techniques. We have also seen the consistency of the Gerarde technique in dose-response, using 3 animal species and 3 anesthetics. Furthermore, the Gerarde technique is satisfactory in its range of sensitivity at dose levels thought comparable to those which might be aspirated by humans (*see* below).

In considering which of the 3 techniques best mimics the human hazard situation, it becomes clear that the Gerarde technique considers the factors of hazard and toxicity. The IT technique can only measure aspiration toxicity, since this route bypasses the oral cavity and does not completely simulate the conditions which prevail during an accidental aspiration in which product viscosity plays a role.

The IV method was also used to consider lung and mortality responses to the distillates. This route was chosen on the supposition that sufficient quantities of a large ingested dose could be absorbed from the gastrointestinal tract and could result in blood levels similar to those produced by the IV injection. While significant results were obtained to assess petroleum distillate toxicity, this method, like the IT method, is unsuitable for determining potential aspiration hazard since the normal influences of the oral cavity and the tracheobronchial tree are removed. In addition, the intravenous injection does not allow differentiation between the uniform lung hemorrhage possibly produced by blockage of lung capillaries by oil droplets and/or irritation of alveolar membranes. Last, both the IT and IV methods require surgical intervention which possibly adds more stress to that experienced by the rats following oil administration.

The information contained in Table 2 suggests a dose-response relationship regarding increases in lung weight above control for the rat

and rabbit. However, the apparent attainment of a plateau at the 1 ml/kg level is probably dependent upon the time to death for members of those groups. Gerarde (5) has shown that those animals which survive the longest following oil aspiration have the heaviest lungs, a fact which has been confirmed in our studies.

It has been reported that the volume of a single swallow in a child in the age group most frequently exposed (1–5 years) is approximately 4.5–5 ml or 1 teaspoonful (8). It has also been reported that the ingestion of as little as ½ teaspoonful (2.5 ml) of kerosene has produced death in young children (9). Furthermore the aspiration of 1 ml directly into the lungs can produce a severe chemical pneumonitis (10). However, large amounts (greater than 30 ml up to 60–90 ml) are rarely ingested by young children (11).

With these reports in mind, if we consider a hypothetical case of a child weighing 10 kg and the reported doses of 5, 2.5, and 1 ml, this child would receive 0.5, 0.25, and 0.1 ml distillate/kg. Thus, the doses used in these tests are probably within the range of those aspirated in actual clinical situations.

Once the method for aspiration testing has been selected, the selection of the appropriate animal species must follow. An analysis of Tables 1 and 2 shows that the rat and the rabbit are consistent in their mortality and lung weight responses to the patio torch fuel. The guinea pig, however, is too sensitive to the lethal effects of the distillate and would be inappropriate for further use. In deciding between the 2 suitable species for potential aspiration toxicity testing, the size, ease of handling, and cost of an animal enter into the decision. It is for these latter reasons that the rat appears to be more suitable and should be considered for routine use in this toxicological test.

On the supposition that choice of anesthetic could affect the results of aspiration testing we investigated ether, chloroform, and pentothal sodium. The results show that there is little, if any, difference in mortality ratio and lung weight responses between the inhalation anesthetics ether and chloroform when rats are forced to aspirate patio torch fuel. This has been confirmed, using radiolabeled patio torch fuel, since there was no difference in amounts of distillate entering the lungs. Surprisingly, the increase in bronchial secretions produced by ether and not by chloroform has no effect on these parameters. However, the onset of anesthesia and apnea when chloroform is used is much quicker than for ether and thus poses less of a margin of safety for the survival of the animal; its use therefore should be placed second to the use of ether. Pentothal sodium was also evaluated as a general anesthetic for use in the rat and rabbit. As seen in Table 2, rat lung weights at the 1 ml/kg dose were not obtained, since all members of this group died within 30 min. This indicated that the amount of central nervous system (CNS) depression produced by pentothal was relatively long-lasting in the rat in contrast to that produced by the inhalation anesthetics. Thus upon patio torch fluid aspiration, deeper CNS depression was produced and the rat could not survive. It should be noted that it is a well established fact that kerosene fractions are strong CNS depressants (12, 13). An inhalation anesthetic is recommended, since it is more humanitarian to the welfare of the animal than not using one, makes animal handling easier, and gives acceptable animal responses which can be used to predict potential aspiration hazard and toxicity.

The method for analyzing total lung hemorrhage is performed by eye estimation of surface damage. Although subjective, it can be used successfully to distinguish highly irritating and slightly irritating oils (Table 5). A comparison of the results in Table 5 with those of Tables 1 and 4 show that the incidence of severe hemorrhage correlates closely with the mortality ratios produced at identical doses. In these respects, amounts of total lung hemorrhage should be considered as important additional information in the total assessment of petroleum distillates as potential aspiration hazards.

Methods of animal sacrifice should also be considered in a test method when a sensitive organ system, in this case the lung, may be adversely affected by the procedure. The results of the 4 sacrifice methods used indicate that stunning and cervical dislocation produce substantial lung trauma as manifested by hemorrhage and edema. It is recognized that pulmonary edema has been a reported complication following head injuries (14). The other 3 methods did not result in any appreciable lung damage. Such data indicate that the method of sacri-

fice can influence the results of aspiration tests and that stunning with cervical dislocation should be avoided.

Conclusions and Recommendation

These experiments have shown that the Gerarde test is capable of giving rapid, reliable, and consistent results when used as a predictor of potential aspiration hazard and toxicity.

The inhalation anesthetic ethyl ether appears to be the anesthetic of choice and gives an adequate margin of safety for the animal.

The rat is the better animal for use based on reliability, size, ease of handling, and cost.

The method of animal sacrifice does appear to influence the experimental results; thus the procedure must be quick and should produce no lung damage by itself.

In view of the above results, it is our opinion that the modified Gerarde test is reliable and sensitive, and that the next phase of testing should be a collaborative study.

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The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee G and was accepted by the Association. *See* (1976) *JAOAC* **59**, 400.

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ANTIBIOTICS

Turbidimetric and Diffusion Assay of Bacitracin in Feeds

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A manual turbidimetric method for determining zinc or methylene disalicylate bacitracin in feeds was developed. When zinc ions ($10^{-4}M$) were added to the medium and the phosphate ion concentration in standard solutions was decreased to 1%, the median response of *Streptococcus faecalis* was about 0.038 unit. Feeds analyzed by the turbidimetric assay should be prewashed with petroleum ether and extracted with pyridine as in 42.204. Zinc bacitracin standards added to swine and broiler rations were recovered at 93.1–102.6% by the turbidimetric method. Excellent agreement between the manual turbidimetric method and the plate assay was also obtained for finished feeds containing the zinc or methylene disalicylate salt of the antibiotic. The turbidimetric method appears to have high accuracy and precision. It is more rapid and less costly than the plate assay.

In 1965, an agar-diffusion method for determining bacitracin in mixed feeds was described by Craig (1). The interfering substances in the feed were removed by acetone washing. Bacitracin was extracted from the feed with acid-pyridine followed by addition of methanol to precipitate undesirable proteins if present. Pyridine was evaporated and, after pH adjustment to 6.5, sample extracts were diluted with 5% phosphate buffer to 0.1 unit bacitracin/ml (reference concentration). This method was adopted by the AOAC as official first action. In 1969 (2), the method was modified for the analysis of low levels (4–10 g/ton) of bacitracin in feeds. The modification involved adding neomycin to the agar medium to increase assay sensitivity (0.04 unit/ml as reference concentration). Samples were cleaned up with petroleum ether in the Goldfisch fat extraction apparatus. In both of these studies, however, only one type of feed (broiler ration) was examined. In 1971, Grynne (3) extracted the feed sample with 2% HCl in methanol without sample cleanup or solvent evaporation. Feeds containing 20–350 ppm (18.16–317.8 g/ton) gave results comparable to those for the pyridine method. The advantages of the method over the pyridine assay are that it is simple and it uses an organic solvent that is less toxic than pyridine. The presence of 25% methanol in bacitracin solutions (v/v) decreased the size of inhibition zone of 0.1 unit/ml by 0.88 mm. This caused an apparent negative bias in antibiotic potency of 28%. However, no recovery studies or relative standard deviation values were reported and the sizes of inhibition zones were rather small, thus degrading assay quality.

Recently, we have successfully determined some antibiotics in feeds by the turbidimetric method. It appeared necessary then to examine the plate diffusion assay by both the pyridine and methanol methods and to attempt to develop a turbidimetric assay for bacitracin in feeds.

Experimental

The experimental objectives were as follows:

(*1*) To investigate the photometric response of *Staphylococcus aureus* and *Streptococcus faecalis* to gradient concentrations of bacitracin and define the optimum conditions (pH, amount of inoculum, incubation period, zinc and phosphate ion concentration, etc.) for high assay sensitivity. Furthermore, the effect of methanol and pyridine on turbidimetric response was defined.

(*2*) To perform antibiotic recovery studies in the presence of feed extracts. Standard solutions of zinc bacitracin were added to 2 rations (swine and poultry) at levels comparable to 50, 100, and 200 g bacitracin activity/ton feed. Analysis by the plate method (pyridine and methanol extraction) was compared with the turbidimetric assay.

(*3*) To compare the plate and turbidimetric

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methods for the 2 rations when fortified in the laboratory by feed-grade zinc bacitracin (label claim, 40 g/lb). The fortification levels of each feed were 50, 100, and 200 g zinc bacitracin/ton. Two collaborators participated in this study.

(*4*) To evaluate methanol as a solvent for extracting bacitracin methylene disalicylate (MD). Commercial swine concentrate samples fortified with bacitracin MD at the levels of 20, 50, and 100 g/ton were used. Four collaborators participated in this evaluation.

Preparation of Fortified Feeds

Two commercial unmedicated rations were used in zinc bacitracin studies. The swine ration was basically (%): crude protein, 40; crude fat, 2.5; crude fiber, 7.0; calcium, 4.0–5.0; phosphorus, 2.0; iodine, 0.001; salt, 2.5–3.5. Ingredients listed on the label were dehydrated alfalfa meal and vitamin and mineral supplements. The poultry ration was prepared commercially by mixing (lb): breeder supplement, 500; ground corn, 1380; oyster shell, 70; ground limestone, 50; and grit. The breeder supplement contained (%): soybean meal, 63.2; meat and bone meal, 10; blood meal, dehydrated alfalfa meal, fish meal, dicalcium phosphate, ground limestone, salt, and vitamin and mineral mix.

For laboratory-fortified feeds zinc bacitracin premix was added to each ration in a double-cone V blender, mixed 0.5 hr, and riffled.

For experiments on bacitracin MD a swine concentrate sample (courtesy of S. B. Penick) fortified with bacitracin MD premix at 100 g/ton was used. It was further diluted with corn to 50 and 20 g bacitracin activity/ton feed. The composition of swine concentrate was (%): soybean meal, 49; cottonseed meal, 17.5; meat scraps, 11.4; dehydrated alfalfa meal, 6.25; corn gluten feed, 4.5; potassium carbonate, 4.75; dicalcium phosphate, 2.41; salt, 1.75; vitamin supplement, 0.125; and mineral supplement, 0.25.

METHODS

Microorganisms

For turbidimetric assay, maintain stock cultures of *S. faecalis* ATCC 8043 on Bacto-Penassay seed agar slants. Use 24 hr culture to inoculate 200 ml Penassay broth (Difco antibiotic medium 3). Incubate 12–18 hr at room temperature. Use only fresh inocula.

For plate assay, maintain *Micrococcus flavus* ATCC 10240 as in 42.195(a) (4).

Reagents

(a) *Phosphate buffer.*—1%, pH 6.5. Dissolve 4.43 K_2HPO_4 and 5.57 g KH_2PO_4 in water and dilute to 1 L. Adjust to pH 6.5 if necessary.

(b) *Methanol-phosphate buffer.*—Mix 50 ml methanol (analytical reagent grade) and 950 ml 1% phosphate buffer. Adjust pH to 6.5.

(c) *Pyridine.*—40% pyridine-buffer prepared as in 42.193(i) (4).

(d) *Polysorbate 80.*—Weigh 10 g of the liquid. Add 80 ml water and dissolve by heating on steam bath. Dilute to 100 ml with water.

(e) *Zinc chloride.*—$10^{-2}M$. Dissolve 1.363 g $ZnCl_2$ in 1 L water.

(f) *Stock solution.*—Weigh ca 100 mg USP Zinc Bacitracin and dissolve in enough 0.01*N* HCl to give exactly 100 units/ml. Prepare fresh daily.

Standard Response Line

Pyridine method (all dilutions with 5% phosphate buffer as in 42.193(d)).—Prepare working solution containing 1.0 unit/ml. Pipet 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, and 10 ml quantities in 100 ml volumetric flasks and dilute to volume. Use bacitracin concentrations of 0.01, 0.02, 0.04 (reference concentration), 0.06, and 0.08 unit/ml for plate assay and 0.0 (phosphate buffer), 0.02, 0.03, 0.04, 0.05, 0.06, 0.08, and 0.1 unit/10 ml tube for turbidimetric assay.

Methanol method.—Prepare same standard concentrations as in pyridine method except use methanol-phosphate buffer as diluent.

Atomic Absorption Analysis of Minerals in Feeds and Media

Follow AOAC method 7.077 (4) as modified by Everson (5).

Sample Extraction and Dilution

On day before assay, weigh 10 g (20 g if labeled <50 g/ton) feed. For plate assay, wash with acetone as in 42.204 (4); for turbidimetric assay, wash with petroleum ether in Goldfisch apparatus (2). Let feed dry overnight.

Pyridine method.—Extract feed with HCl (1+32), 40% pyridine-buffer, and methanol; evaporate pyridine, add 5% phosphate buffer, and adjust pH to 6.5% as in 42.204 (4). For plate assay, dilute with 5% buffer to 0.04 unit bacitracin/ml. For turbidimetric assay, prepare 2 dilutions (with 5% buffer) to contain 0.06 and 0.04 unit/ml.

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Methanol method.—Add 25 ml 1*N* HCl to feed in centrifuge bottle. Adjust pH to <2.0 (if required) by adding concentrated HCl with stirring. Note volume of HCl added. Add 25 ml methanol (do *not* let sample stand in HCl >5 min) and 50 ml methanol-buffer. Stir gently and centrifuge 10 min at 1800 rpm. Carefully decant supernate into funnel containing small amount of glass wool and collect supernate in beaker. Adjust to pH 6.5 and dilute (with methanol-buffer) as follows:

If theoretical potency of sample is 20 g bacitracin/ton, pipet 15.0 ml into beaker. Adjust pH to 6.5 (±0.05), using 10*N* NaOH first and then 1*N* NaOH as pH 6.5 is approached. Transfer quantitatively to 50 ml volumetric flask and dilute to volume. Pour into centrifuge tube and centrifuge 5 min at 1800 rpm. The supernate represents 1/16.7 dilution. From this extract dilute 16.7 ml with 7.0 ml methanol-buffer (1/23.7 dilution). Use both dilutions for plate assay.

If theoretical potency of sample is 50 g/ton, adjust pH of 28.0 ml sample extract to 6.5, dilute to 100 ml, and centrifuge. The centrifugate represents 1/35.7 dilution. Dilute 20 ml with 6 ml methanol-buffer (1/46.4) and 16 ml with 10 ml methanol-buffer (1/58) and use for plate assay.

For bacitracin potencies of 100 and 200 g/ton, volumes for pH adjustment are 14.0 and 7.0 ml, respectively. Follow same dilution scheme as for 50 g/ton to obtain 1–92.9 and 1–116.0 dilutions for 100 g/ton feeds and 1–185.8 and 1–232 for 200 g/ton feeds.

Turbidimetric Assay

Prepare Bacto-Penassay broth (Difco antibiotic medium 3) by dissolving appropriate amount of dehydrated medium in water. Sterilization is not necessary if medium is made on same day of analysis. Add 1 ml $10^{-2}M$ $ZnCl_2$, 0.3 ml Polysorbate 80, and 1.0 ml glucose per 100 ml assay medium. Inoculate with *S. faecalis* at the rate of 1 ml inoculum/100 ml assay medium. Incubate at 37°C (static) until absorbance is ca 0.03–0.04 at 600 nm (flowcell and 10 mm light path), using uninoculated Penassay broth as blank.

Follow same procedure reported earlier (6). Add 1 ml standard or sample solution and 9.0 ml inoculated Bacto-Penassay broth (Difco antibiotic medium 3) in replicates of 4 tubes. Prepare medium blank tubes containing 1.0 ml pH 6.5 phosphate buffer and 9.0 ml inoculated assay broth. Refrigerate blank tubes. Incubate all other assay tubes ca 2 hr at 37°C. When absorbance of cultures in absence of antibiotic is 0.4–0.5, stop growth in all tubes by heating 10 min at 80°C and then cool rapidly to room temperature. Shake by inverting each culture once. Adjust spectrophotometer to 0.0 absorbance, using medium blank tubes. Read turbidity at 600 nm (10 mm light path) using flowcell Model 8495-L10 (Arthur H. Thomas Co., Philadelphia, PA).

Calculations

In turbidimetric assays, plot log *A* vs. *C* on semilogarithmic paper. Draw line of point to point between 0.03 and 0.06 unit of zinc bacitracin. Carefully read unknown concentration from plot and multiply by dilution factor to obtain antibiotic concentration as unit/g feed. To convert results to g/ton, multiply by 21.619.

In plate assay, plot log *C* (concentration in units) vs. *X* (zone diameter) on semilogarithmic paper. Draw line of best fit between 0.01 and 0.08 unit of zinc bacitracin. Read unknown and calculate as in turbidimetric assay.

Results and Discussion

Figure 1 shows the dose-response curves of zinc bacitracin in log probability form for *S. aureus* and *S. faecalis*. When no zinc ions (other than those present in Penassay broth) were added, the median response (MR) values for *S. aureus* and *S. faecalis* were about 0.37 and 0.2 unit of bacitracin, respectively. As the concentration of zinc added to the medium increased to $10^{-4}M$, the assay sensitivity increased (about 0.068 unit for *S. aureus* and 0.038 unit for *S. faecalis*). At $2 \times 10^{-4}M$ zinc, there was no significant increase in sensitivity, indicating no further enhancement of antibiotic activity. These

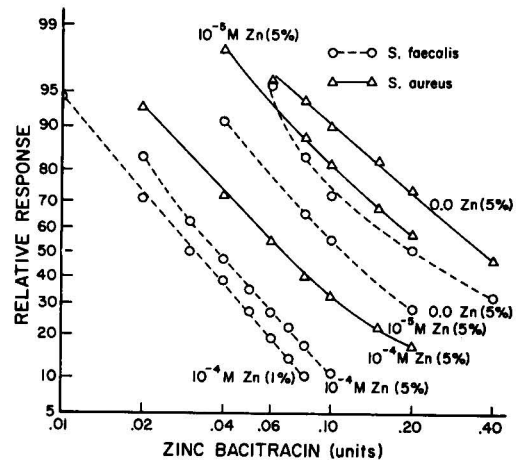

FIG. 1—Dose-response curves of *S. faecalis* and *S. aureus* to zinc bacitracin in phosphate buffer (5%) in log probability form.

concentrations of zinc ions did not inhibit the growth of either organism in absence of the antibiotic. Similar enhancement of bacitracin activity by zinc ions was reported by Weinberg (7). Figure 1 also shows that, for *S. faecalis*, there was further improvement in assay sensitivity as the concentration of phosphate decreased from 5 to 1.0%. Kavanagh (8) also noted that decreasing the phosphate ion concentration limited the growth of *S. faecalis* in the turbidimetric assay of monensin. *S. faecalis* showed greater sensitivity than *S. aureus*, so it was chosen for further turbidimetric studies. It should be noted that these experiments were conducted with zinc bacitracin. At the present time, the methylene disalicylate salt (bacitracin MD) is also incorporated in many feeds. It would be of interest to examine the response of *S. faecalis* to the pure form of this antibiotic. Enhancement by zinc and thermal stability of both salts of bacitracin during incubation in the turbidimetric assay should be compared.

To establish the appropriate incubation period, the log of relative bacterial concentration (N) was plotted vs. time (Fig. 2). As the antibiotic concentration increased from 0.0 to 0.08 unit, the shapes of the growth curves were displaced to the right. Between t_1 and t_2 (hr), the slope of response was the same in presence or absence of antibiotic. This indicates that the action of the antibiotic was killing part of the bacterial population. Some evidence of lysis was observed for 0.1 unit of bacitracin between 3 and 4 hr. Thus in the turbidimetric assay of bacitracin, the incubation period can vary between 1 and 2 hr without significantly influencing the slope response. A smaller inoculum, however, will increase the length of incubation time. At an incubation temperature of 30°C, the growth of *S. faecalis* was slower (incubation period of about 5 hr) as compared to growth at 37°C. Similarly, when the pH of the medium was adjusted to 6.0, assay sensitivity decreased. This was probably due to a decrease in antibiotic activity. At pH 7.0 and 8.0, no significant difference in MR values was observed. The pH of Penassay broth is usually 7.0, so there was no advantage in pH adjustment.

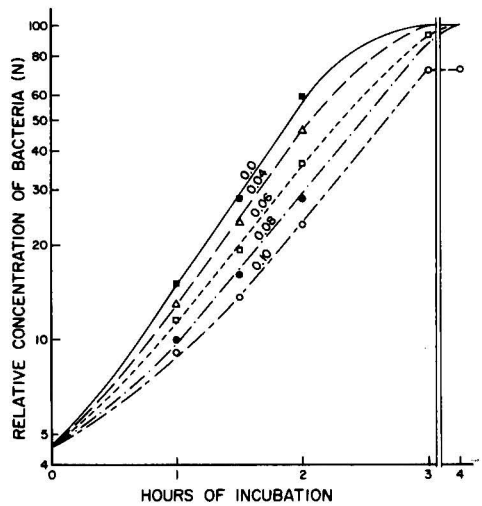

FIG. 2—Effect of incubation time on response of *S. faecalis* to zinc bacitracin.

To compensate for interference by fats present in feed extracts, Kavanagh (8) added Polysorbate 80 to the monensin assay medium. In the bacitracin assay, the addition of Polysorbate 80 (0.3 or 0.6 ml/100 ml medium) did not significantly affect the slope of response. The growth of *S. faecalis* appeared to be rapid and consequently a shorter incubation period (about 1.5 hr) was possible.

In order to minimize long lag period, the assay organism was allowed to preincubate at 37°C to an absorbance of about 0.04 (about 45 min) before exposure to the antibiotic. Under such conditions the inoculum rate varied from 0.5 to 2.0 ml and the slope of the response remained the same. In general the sensitivity of the assay decreased by increasing the amount of inoculum.

The effect of methanol on photometric response of *S. faecalis* showed that above 10% (v/v) concentration of methanol there was an apparent activity. For example at 15 and 40% methanol, biological activity comparable to about 0.01 and 0.04 unit of bacitracin was detected. At a concentration of 5% methanol, no significant activity was detected (absorbance of 0.413 as compared with 0.416 for 0.0 zinc bacitracin).

Pyridine at concentrations of 0.80% (v/v) per 10 ml tube showed negligible activity. Higher pyridine concentrations, however, showed considerable inhibition of the organism. The combined action of zinc bacitracin (0.04 unit) and pyridine (0.8%) showed a positive bias (about 116%). Therefore, in turbidimetric assays of

bacitracin by pyridine extraction, the solvent was removed by evaporation.

The precision of measurement in the turbidimetric assay of zinc bacitracin standard in phosphate buffer or in methanol-buffer solutions was compared by the automated (Autoturb®) and manual methods. Tables 1 and 2 show that variations (underlined) among replicates could be as high as 3.5% T by the automated system and 5.7% T by the manual method. This could result in some error in results. In this case the measurement could contribute errors of −2.5 to +8.8% and −8.3 to +5.5% by the automated and manual systems, respectively. One collaborator, using the automated system and an on-line computer, found that the relative standard deviation values were rather large (about 8%) at both ends of the standard response line. Within the linear portion of the response line, the relative standard deviation values were between 1 and 5% (Table 3). In the monensin assay, Kavanagh (9) reported that a relative standard deviation value of about 1% is quite satisfactory for this assay and is about the size of error caused by an uncertainty of 1 mv in measuring turbidity. Variations in bacitracin assay, however, are within reasonable limits due to the steepness of the response line. For example, an increase in concentration of zinc bacitracin in methanol-buffer of 0.01 unit (from 0.04 to 0.05 unit per 10 ml tube) increased the response from 51.8 to 62.0% T by the manual method. It is interesting to note that by comparison the size of zones of inhibition (measured by a Fisher Lilly zone reader connected to a digital voltmeter) showed similar variation (relative standard deviation about 2–5) by diffusion methods (Table 4). The reason for variability in % T among replicates may be due to some protoplast formation (7). Another possible explanation is that slight variation in the temperature of incubation of each assay tube may affect the antibiotic activity. Preliminary experiments in this laboratory showed some improvement in replication (about 2% T) when each test tube carrier was completely filled with assay tubes. Apparently this allows more uniform temperature distribution during the incubation period.

In general, the precision of the automated measurement was better than that of the manual method. The slope of standard response lines in phosphate buffer or in methanol-buffer was ap-

Table 1. Typical dose-response for manual and automated turbidimetric assay of zinc bacitracin in 5% phosphate buffer

Antibiotic concn, unit	Automated, % T[a,b]			Mean	Absorbance[c]	Antibiotic concn, unit	Manual, % T[b,d]			Mean	Absorbance[c]
0.0	32.5	32.2	30.5 30.6	31.6	0.500	0.00	22.1	22.2	22.2 22.1	22.2	0.654
	32.3	31.0	32.5			0.020	34.6	34.8	35.5 33.8	34.7	0.460
0.010	32.4	31.4	32.8 31.6	32.1	0.494	0.030	41.7	40.5	38.5 40.1	40.2	0.396
0.015	34.5	32.9	34.1 32.9	33.6	0.474	0.040	53.9	53.6	51.9 51.2	52.7	0.278
0.020	39.1	38.5	39.9 38.5	39.0	0.409	0.050	61.6	62.2	63.4 57.7	61.2	0.213
0.030	51.9	49.8	51.9 50.6	51.1	0.292	0.060	71.9	74.1	73.1 71.6	72.7	0.138
0.040	62.5	60.7	64.2 61.6	62.3	0.206	0.070	80.3	82.1	80.6 82.4	81.4	0.089
0.060	78.5	76.7	78.9 77.1	77.8	0.109	0.080	85.4	84.2	84.9 83.9	84.6	0.073
0.080	86.2	85.2	86.3 87.1	86.2	0.065						

[a] Automated results were provided by Collaborator D.
[b] Values underlined represent large variation between replicates observed in the assay.
[c] Averages are reported to nearest 0.001 absorbance.
[d] All assay tubes in one test tube carrier.

Table 2. Typical dose-response for manual and automated turbidimetric assay of zinc bacitracin in methanol-phosphate buffer

Antibiotic concn, unit	Automated, % T[a,b]			Mean	Absorbance[c]	Antibiotic concn, unit	Manual, % T[a,d]			Mean	Absorbance[c]
0.00	33.1	32.8	32.9	33.0	33.1	0.480	0.00	19.4	19.1	19.3	19.7	19.5	0.711
	33.2	33.3	33.2	32.5				19.6	19.6	19.4	19.6		
0.010	33.5	34.0	34.5	34.8	34.2	0.466	0.020	27.9	27.3	27.3	27.4	28.2	0.549
0.015	36.7	37.0	38.9	39.0	37.9	0.421	0.030	36.4	41.1	39.2	38.5	38.8	0.411
0.020	46.6	47.5	46.4	47.4	47.0	0.328	0.040	52.5	51.0	50.1	53.7	51.8	0.285
0.030	60.9	61.2	61.8	62.1	61.5	0.211	0.050	61.5	64.1	61.6	60.7	62.0	0.208
0.040	71.2	71.7	70.3	70.9	71.0	0.149	0.060	72.3	71.4	72.6	70.9	71.8	0.144
0.060	81.8	81.6	81.2	81.4	81.5	0.089	0.070	78.2	78.0	79.7	76.7	78.2	0.107
							0.080	84.4	86.5	87.1	87.1	86.3	0.064

[a–d] See Table 1.

proximately the same by manual and automatic methods. The linear portion of the standard response line was about 0.03–0.06 by manual assay. At concentrations >0.08 unit of zinc bacitracin, a drift was observed in the automated assay.

When zinc bacitracin standards were added to swine and broiler rations, recovery by pyridine extraction showed good agreement between the turbidimetric (93.1–102.6%) and AOAC methods (88.0–112.0%). Since the same feed extracts were processed by both methods on each day of analysis, it appears that either method can be used for analysis of feeds without significant difference in results. Relative standard deviation values (Table 5) were approximately the same for both methods. For example, the values for swine ration were 6.38–10.55 and 7.56–13.78 for the turbidimetric and plate assays, respectively. A similar coefficient of variation (about 11%) was reported by Craig (1) for the AOAC method, indicating the same precision for turbidimetric and plate assays. It should be mentioned that unmedicated feeds showed no antibiotic activity by either method.

Methanol extraction of zinc bacitracin yielded rather poor recovery (60–70%) by the turbidimetric method. Therefore these results were not included in this paper. Before this assay can be successfully developed, it is necessary to investigate the effect of substances that are extracted from feeds by methanol on the photometric response of *S. faecalis*. For the plate assay the methanol method showed good recovery (about 95.5–118.6%) and good precision (relative standard deviation 5.26–9.22) for both feeds (Table 5).

For feeds fortified in the laboratory with commercial zinc bacitracin premix, the plate assay was studied collaboratively by 3 laboratories. Results showed that recovery by the pyridine method was 71.5–75.3% for the swine ration and 87.3–98.6% for the broiler ration (Table 6). The low recovery in swine ration may be due to some loss of bacitracin activity during decantation of acetone in the prewash step. Preliminary results in this laboratory indicate the Goldfisch petroleum ether prewashing to be superior. Methanol extraction showed some positive bias (103–114%) for both feeds. Furthermore, there were some differences between assay results from 2 weights of the same sample and between

Table 3. Precision of automated[a] system in zinc bacitracin assay[b]

Sample[c]	Concn, unit	Sample volume, ml 0.10		0.15		Calcd response				Mean Response	Mean Rec., %	Rel. std dev.
1	0.00	56.00	56.00	57.00	57.00							
		56.09	56.07	57.14	57.15							
2	0.01	56.85	57.12	59.69	59.14							
3	0.02	63.01	63.92	71.63	72.05							
4	0.03	72.05	72.45	81.55	81.82							
5	0.04	79.86	79.16	89.26	88.27							
6	0.06	87.66	87.87	92.93	92.77							
7	0.08	92.25	91.62	93.83	94.49							
8	0.01	56.81	57.27	59.23	58.90	0.010	0.010	0.009	0.009	0.010	100	5.03
9	0.02	62.53	63.80	71.38	71.55	0.019	0.020	0.020	0.020	0.020	100	1.46
10	0.03	73.48	74.53	84.66	84.73	0.032	0.033	0.033	0.034	0.033	110	3.10
11	0.04	80.61	79.49	88.27	89.23	0.042	0.041	0.038	0.044	0.041	102.5	5.24
12	0.06	88.29	88.90	93.01	92.83	0.062	0.065	0.062	0.061	0.062	103.3	2.88

[a] Automated results were provided by Collaborator E.
[b] Zinc bacitracin standard prepared in methanol-buffer.
[c] Samples 1–7 were used for plotting the standard response line; 8–12 are known concentrations of zinc bacitracin which were considered as unknown samples. The reader unit of automated system was connected to a computer to fit the response line and calculate the points.

Table 4. Precision of measurement of response of zinc bacitracin by the plate assay

	Size of zone of inhibition, mm[a]									
	0.01	0.02	0.04	0.06	0.08	0.01	0.02	0.04	0.08	0.16
Statistic	Std in 5% phosphate buffer					Std in methanol-buffer[b]				
No. of detns	8	8	16	8	8	10	10	10	10	10
Average, mm	13.1	15.3	17.3	18.4	19.2	9.9	12.5	14.8	17.6	20.7
Std dev.	0.588	0.385	0.666	0.736	0.765	0.591	0.422	0.502	0.517	0.553
Rel. std dev.	4.506	2.509	3.843	3.996	3.978	5.946	3.382	3.390	2.930	2.671
Range	11.9–13.8	14.8–16.0	16.0–18.7	17.5–19.4	18.4–20.2	9.0–11.0	12.0–13.1	13.5–15.2	17.0–18.5	20.0–21.8

[a] Zones of inhibition were measured by Fisher Lilly zone reader connected to a voltmeter. Results are reported to the nearest 0.1 mm.
[b] Results reported by Collaborator B.

Table 5. Comparison between the plate assay (AOAC pyridine and methanol) with the turbidimetric assay of zinc bacitracin in feeds

Statistic	Pyridine method Turb[a]	Plate	Turb.	Plate	Turb.	Plate	Methanol method Plate	Plate	Plate
	Swine Ration								
Level of fortification,[b] g/ton	50	50	100	100	200	200	50	100	200
No. of detns	23	18	23	18	27	20	15	16	9
Average, g/ton	49.9	45.8	95.3	88.0	186.2	178.1	56.0	103.3	191.0
Recovery, %	99.8	91.6	95.3	88.0	93.1	89.1	112.0	103.3	95.5
Std dev.	4.3	6.31	6.08	6.65	19.63	18.55	4.92	9.53	17.14
Rel. std dev.	8.607	13.78	6.38	7.56	10.55	10.41	8.69	9.22	8.97
	Broiler Ration								
Level of fortification, g/ton	50	50	100	100	200	200	50	100	200
No. of detns	28	9	30	13	23	11	14	13	10
Average, g/ton	51.3	56.0	99.5	100.1	188.0	188.9	59.3	106.3	206.1
Recovery, %	102.6	112.0	99.5	100.1	94.0	94.5	118.6	106.3	103.0
Std dev.	6.18	2.03	11.87	10.62	22.51	16.81	4.94	8.97	10.85
Rel. std dev.	12.04	3.63	11.93	10.61	11.98	8.90	8.34	8.44	5.26

[a] Turbidimetric assay results represent pyridine extraction of feeds prewashed with petroleum ether.
[b] 10 g portions of unmedicated feed were prewashed with acetone and zinc bacitracin standard solution was added. The samples were extracted according to the pyridine or methanol methods as specified in text.

Table 6. Plate assay comparison of pyridine (AOAC) and methanol extraction of zinc bacitracin in feeds fortified in the laboratory with commercial premix

Laboratory		Level of fortification, g/ton					
		Pyridine method[a]			Methanol method[a]		
		50	100	200	50	100	200
		Swine Ration					
A	Day 1	35.8	75.8	144.1	52.5	97.0	178.4
	2	39.4	81.1	164.1	58.9	105.7	191.8
	3	38.6	88.8	169.0	62.7	117.4	237.7
	Av., g/ton	37.9	81.9	159.3	58.0	106.7	202.6
B	Day 1	—	—	—	58.0	106.7	202.6
	2	—	—	—	63.6	126.6	257.0
	Av., g/ton	—	—	—	59.5	122.4	250.8
C	Day 1	33.6	68.7	137.0	54.1	105.7	186.3
	Grand av., g/ton	35.8	75.3	148.2	57.2	111.6	213.2
	Rec., %	71.5	75.3	74.0	114.4	111.6	106.6
		Broiler Ration					
A	Day 1	48.7	92.8	222.8	51.3	103.1	231.2
	2	48.9	100.6	217.3	55.4	91.9	193.7
	3	50.2	89.2	195.2	55.4	—	218.8
	Av., g/ton	49.3	94.2	211.8	54.0	97.5	214.6
B	Day 1	—	—	—	46.4	120.5	188.5
	2	—	—	—	60.2	99.0	239.0
	Av., g/ton	—	—	—	53.3	110.0	213.8
C	Day 1	49.3	80.4	167.6	62.3	101.4	212.5
	Grand av., g/ton	49.3	87.3	189.7	56.5	103.0	213.6
	Rec., %	98.6	87.3	94.6	113.1	103.0	106.8

[a] Average of 2 dilutions.

dilutions of the same sample extract. The reason for the positive bias in results is difficult to explain. Phosphate buffer (1%, pH 6.5) solutions containing methanol at 5, 10, and 20% (v/v) showed no activity. When zinc bacitracin solutions (0.04 unit/ml) containing 5 and 10% (v/v) methanol were evaluated in terms of standard in 1% buffer, no significant changes in potency were observed. These results agree with those observed by Grynne (3). It appears that methanolic extracts of feeds used in these studies contained certain ingredients which enhanced the antibiotic activity. Atomic absorption analysis of the swine and broiler rations revealed that the swine ration had higher (almost 3×) copper, manganese, and zinc concentrations (Table 7). Since zinc enhances bacitracin activity (7), it would be expected that greater positive bias would be observed in this feed as compared to the broiler ration. Both feeds, however, showed approximately the same magnitude of bias. This may be due to the presence of adequate concentrations of zinc in both feed extracts. Other potential sources of zinc in the assay are the test medium and the antibiotic solutions used. Analysis of Difco antibiotic medium 1 (Lot No. 0263-01) revealed zinc to be the only trace metal detected in considerable quantities (21 ppm). The concentrations of other trace metals were 11 ppm iron and 12 ppm cobalt. Manganese was present in negligible amounts. Sykes (10) reported that different batches of medium may drastically differ in their trace mineral content. Furthermore, highly purified lots of bacitracin standard may have the zinc removed by purification (7). All these factors could account for variations in potency between the same weights and between dilutions of the same sample.

Pyridine extraction of zinc bacitracin showed excellent agreement between the turbidimetric

Table 7. Atomic absorption analysis of metal cations in swine and broiler rations

Metal	Swine ration, ppm	Broiler ration, ppm
Cu	21.5	6.7
Zn	217.5	82.0
Mn	300.5	100.0
Fe	390.0	460.0

Table 8. Manual turbidimetric assay of zinc bacitracin in feeds fortified in the laboratory with commercial premix[a]

Level of fortification, g/ton	Swine ration			Broiler ration		
	50	100	200	50	100	200
Day 1	43.0	80.0	164.3	44.9	84.8	179.7
Day 2	42.0	85.0	171.4	49.4	104.6	168.3
Day 3	36.6	72.8	156.0	52.8	99.0	199.3
Av., g/ton	40.5	79.2	163.9	49.0	96.1	182.4
Rec., %	81.0	79.2	82.0	98.0	96.1	91.2

[a] Ten g feed samples were prewashed with petroleum ether in Goldfisch apparatus. Antibiotic was extracted by pyridine method.

(Table 8) and plate assay (Table 6). The turbidimetric method then appears to be feasible. Commercial samples at different levels of fortification should be tested before the accuracy of each method is established.

For bacitracin MD, similar results were obtained by 4 laboratories on swine concentrate feeds fortified at 20, 50, and 100 g/ton (Table 9). The grand average showed antibiotic recovery of 96.6–104.1% by the pyridine plate assay and 119.1–132.0% by methanol extraction. The turbidimetric assay of these 3 feeds (Table 10) agreed very well with the plate pyridine method. According to these results, methanol extraction of either zinc or methylene disalicylate bacitracin from feeds yields some positive bias in results. Except for 20 g/ton feed, the magnitude of this bias was within the tolerances (<130%) established by the Association of American Feed Control Officials (11). The turbidimetric assay showed high accuracy and the recovery of the antibiotic was comparable to the plate pyridine method. Thus, for the analysis of bacitracin in feeds, laboratories that are equipped for diffusion assays could employ the methanol method as a reasonable substitute for the AOAC pyridine extraction. Laboratories which can adapt to turbidimetric assays and require high accuracy can employ the pyridine extraction and obtain results on the same day of analysis.

Conclusions

Bacitracin standards are complex mixtures and may contain varying amounts of less active forms of bacitracin (12). Test organisms may

Table 9. Plate assay comparison of pyridine (AOAC) and methanol extraction of bacitracin MD in feeds fortified with commercial premix

Laboratory[b]		Level of fortification, g/ton[a]					
		Pyridine method			Methanol method		
		20	50	100	20	50	100
I	Day 1	17.1	45.9	108.1	23.2	56.4	113.7
	2	16.2	42.7	108.1	26.3	56.2	117.4
	3	—	43.2	97.3	27.6	61.2	119.4
	Av., g/ton	16.4	44.0	104.5	25.7	57.9	116.8
II	Day 1	20.1	49.4	103.5	28.5	60.5	115.3
	2	18.3	46.4	92.8	25.5	63.1	134.0
	3	20.3	52.4	97.1	26.7	65.5	136.4
	Av., g/ton	19.6	49.4	97.8	26.9	63.0	128.6
III	Day 1	19.4	50.3	90.2	29.0	69.9	121.0
	2	15.3	43.2	98.4	29.8	79.3	131.0
	3	22.3	42.8	116.0	25.2	59.4	110.0
	Av., g/ton	19.0	45.2	101.5	28.0	69.5	120.7
IV	Day 1	21.1	53.5	110.6	24.8	58.6	104.8
	2	25.2	55.8	118.0	25.3	58.8	106.9
	3	22.5	54.5	109.3	25.0	64.7	118.5
	Av., g/ton	22.9	54.6	112.6	25.0	60.7	110.1
	Grand av., g/ton	19.5	48.3	104.1	26.4	62.8	119.1
	Rec., %	97.4	96.6	104.1	132.0	125.6	119.1

[a] The feed sample was a swine concentrate fortified at 100 g/ton with bacitracin MD premix. Levels of 20 and 50 g/ton were prepared by dilution with corn

[b] One laboratory used large plate assay and one laboratory used stainless steel templates instead of cylinders.

Table 10. Manual turbidimetric assay of bacitracin MD in feeds fortified with commercial premix

Day	Level of fortification, g/ton		
	20	50	100
1	16.9	40.6	90.4
2	20.2	52.3	100.1
3	21.0	52.0	89.0
Av., g/ton	19.4	48.3	93.2
Rec., %	96.8	96.8	93.2

respond differently to these mixtures. Future collaborative studies should involve the use of one standard, perhaps USP Zinc Bacitracin. The use of different types of bacitracin (regular or zinc) in a laboratory may contribute to variation in sample potency.

Further turbidimetric studies are necessary to define the effect of temperature distribution during incubation and the factors influencing the linear portion of the standard dose-response line.

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Modified Assay Medium for the Turbidimetric Assay of Chlortetracycline in Feeds

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In previous experiments, the turbidimetric method for determining chlortetracycline-HCl (CTC-HCl) in feeds showed lower recovery than the AOAC plate method. Although the addition of vitamins to the turbidimetric medium improved results, values by the turbidimetric method remained about 10% lower than by the plate method. A modified (1.7× the weight recommended by the manufacturer) turbidimetric assay medium decreased assay sensitivity but did not significantly change the slope of *S. aureus* response to CTC-HCl. There was no evidence that vitamin fortification of the modified medium had any significant effect on the growth rate of test organism. Examination of about 100 samples of commercial feeds containing CTC-HCl showed excellent agreement in results between the turbidimetric and plate methods.

In a previous report from this laboratory (1) the turbidimetric method for determining chlortetracycline-HCl (CTC-HCl) in feeds gave lower recovery (87.1–93.3%) than the AOAC plate method (99.4–108.3%). Preparation of CTC-HCl standard in sodium hypochlorite-inactivated feed extracts gave results that were essentially the same by both methods (1). The compensating standard curve method, however, is tedious and cannot be easily applied to routine analysis. Preliminary experiments in which vitamins were added to the turbidimetric assay medium without the compensating curve gave improved results (2) but antibiotic recovery still remained lower (about 10%) by the turbidimetric method than by the AOAC plate assay. Weinberg (3) reported that some multivalent ions suppress the tetracycline activity. The suppressing ions vary with the genus of test microorganisms and with the medium used for growth. Therefore, it was necessary to investigate possible modification of the turbidimetric assay medium to eliminate or minimize the negative bias observed in the photometric assay.

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In this paper a slight modification of the turbidimetric medium resulted in excellent agreement between the plate and turbidimetric methods when applied to the analysis of CTC-HCl in commercial feeds.

METHODS

Microorganisms

For turbidimetric method, maintenance of *Staphylococcus aureus* cultures and preparation of inoculum have been described previously (1).

Standard Response Line

For plate assay, dilute CTC-HCl stock standard with pH 4.5 buffer (as in 42.193(g) (4)). For turbidimetric assay, prepare following concentrations: 0, 0.02, 0.04, 0.06, 0.08, and 0.10 μg CTC-HCl/ml, using pH 4.5 buffer.

Turbidimetric Assay

Weigh 1.7× amount recommended by manufacturer of Bacto-Penassay broth (Difco antibiotic medium 3). Follow same procedure reported earlier (1) except do not prepare feed blank tubes. Stop growth of cultures and read absorbance at 600 nm (10 mm light path), using flowcell Model 8495-L10 (Arthur H. Thomas Co., Philadelphia, PA).

Plate Assay

Proceed as in 42.208 (4), incubating *Bacillus cereus* plates at 30°C.

Sample Extraction and Dilution

All samples examined were commercial feeds collected and prepared as suggested in AOAC 7.001 and 7.002 (4). Extraction of the antibiotic and dilution were the same as in 42.209 (4) except no pH adjustment was necessary for the turbidimetric assay. The feed extracts were diluted to 0.04 μg CTC-HCl/ml for the plate assay and 0.08 and 0.06 μg CTC-HCl/ml for the turbidimetric assay. All dilutions were prepared with pH 4.5 phosphate buffer.

Results and Discussion

Figure 1 shows that within the same incubation period the growth rate of *S. aureus* in-

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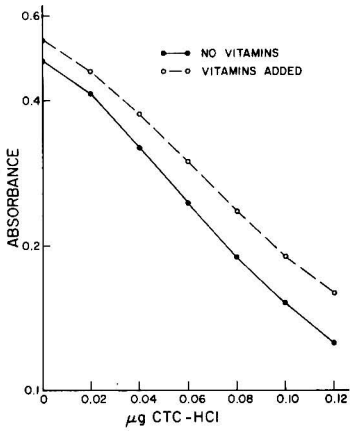

FIG. 1—Effect of vitamin fortification of Bacto-Penassay broth on response of *S. aureus* to CTC-HCl.

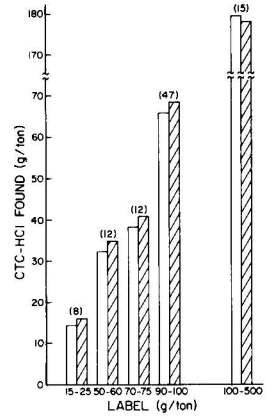

FIG. 2—Comparison between the AOAC plate (shaded columns) and the turbidimetric method (clear columns) in modified medium for the analysis of CTC-HCl in commercial feeds. Numbers on tops of columns represent numbers of samples analyzed.

creased when single strength turbidimetric medium was fortified with a mixture of vitamins. Assay sensitivity slightly decreased (median response increased from about 0.060 to 0.070 μg CTC-HCl), but the slope of response remained approximately the same. Even when the vitamin concentrations were increased 2-fold, there was no further increase in growth rate of test organisms, indicating that these concentrations were probably in excess of the growth requirements.

In all these experiments, culture tubes were placed in one test tube carrier to minimize assay variability due to slight temperature variation during incubation in the water bath. Thus, vitamin fortification of assay medium may in part explain the improved turbidimetric assay results reported earlier (2). However, these results cannot be considered conclusive. Further nutritional studies should be conducted in well-defined simple media. In these experiments Bacto-Penassay broth, which is a complex medium, was used. Furthermore, if certain metal cations present in sample extracts and not in standard solutions affect the antibiotic activity, fortification of the medium by vitamins would not be of much value. The ideal microbial assay would specify preparation of the antibiotic standards in solutions of exactly the same composition (minerals, growth factors, and feed ingredients without the antibiotic) as the sample. Since most feed formulations usually contain considerable amounts of minerals and vitamins, it was thought that an increase in nutrients in the turbidimetric assay medium would be advantageous. This can be accomplished simply by increasing the weight of medium over that recommended by the manufacturer.

When the response of *S. aureus* to CTC-HCl standard in concentrated (1.7× the weight/L) medium was determined, the slope of response did not change significantly compared with the regular medium. In order to determine if the growth factor requirements of the concentrated medium were not limiting to microbial growth, solutions of 0.04 μg CTC-HCl were each fortified with a vitamin (0.4 μg/ml) and compared with CTC-HCl standards in concentrated medium. Potencies of these samples were determined and relative standard deviation values were calculated (Table 1). Results showed excellent antibiotic recovery (about 100%). There was no evidence that vitamin fortification of concentrated medium had any significant effect on the growth rate of the test organism. Had this condition existed, a negative bias in sample potencies would have been observed. Relative standard deviation values (about 1–2%) indicate that the manual assay system (antibiotic, organism, and dilution) was performing reliably. Kavanagh (5) reported that a relative standard deviation of about 1% is satisfactory variation for the Autoturb® system. Thus, the manual assay compares well with the automated system.

The turbidimetric method was then used to determine CTC-HCl in commercial feeds. About 100 samples of various types of feeds

Table 1. Effect of vitamins on response of *S. aureus* to CTC-HCl in concentrated medium[a]

Sample[b]	CTC-HCl, μg	Absorbance, 600 nm (× 1000)	CTC-HCl found, μg (× 1000)	Av.	Rec., %	Rel. std dev.
1	0	475[c]				
2	0.02	405				
3	0.04	326				
4	0.06	259				
5	0.08	206				
6	0.10	166				
7	0.04 + pyridoxine	319, 321, 321, 323, 321, 324	408, 404, 404, 397, 404, 395	402.0	100.5	1.23
8	0.04 + pyridoxal	318, 320, 320, 319, 319, 314	410, 405, 405, 408, 408, 420	409.3	102.3	1.36
9	0.04 + pyridoxamine	313, 315, 315, 318, 317, 317	423, 418, 418, 410, 414, 414	416.2	104.0	1.08
10	0.04 + niacin	317, 321, 321, 321, 321, 327	414, 404, 404, 404, 404, 390	403.3	100.9	1.90
11	0.04 + thiamine	320, 320, 321, 319, 323, 321	405, 405, 404, 408, 400, 404	404.3	101.0	0.64
12	0.04 + pantothenate	320, 321, 320, 318, 318, 323	405, 404, 405, 410, 410, 400	405.7	101.5	0.94
13	0.04 + *p*-amino-benzoic acid	317, 321, 323, 321, 321, 318	414, 404, 400, 404, 404, 410	406.0	101.5	1.25

[a] Medium is Bacto-Penassay broth prepared by weighing 1.7×/L the amount recommended by the manufacturer.
[b] Samples 1–6 represent standard concentrations. Samples 7–13 represent 0.04 μg CTC-HCl/ml and each vitamin at 0.4 μg/ml.
[c] Each absorbance value for standard concentrations represents average of 9 determinations.

were examined by both turbidimetric and plate methods (Fig. 2). Table 2 (for feed supplements) and Table 3 (for complete feeds) show good agreement between the 2 methods. The favorable effect of the concentrated turbidimetric medium may be due to an increase of one or more of the metal contents. Atomic absorption analysis of Bacto-Penassay broth (Lot No. 0243-01) showed it contained iron (28 ppm), zinc (30 ppm), cobalt (13 ppm), and a negligible amount of manganese. It may be that the increased iron concentration suppressed the antibiotic activity. Similar observations (6) were reported for oxytetracycline. Proof can only be convincing if all metal ions are removed from the turbidimetric medium and the effect of individual cations or anions on biological activity is established. However, it has been reported (7) that it is extremely difficult to perform such experiments in a metal-free environment. Furthermore, antibiotic preparations may contain a certain amount of trace metals (8).

In view of the agreement in results between the turbidimetric and AOAC plate methods, it is suggested that both methods be evaluated by a collaborative study.

Table 2. Comparison between turbidimetric and AOAC plate methods for analysis of CTC-HCl in some commercial feed supplements and premixes

Lab. sample	Label claim, g/lb	Found,[a] g/lb			
		AOAC	Rec., %	Turb.	Rec., %
1	0.227	0.296	130.3	0.302	133.0
2	2.00	2.600	130.0	2.600	130.0
3	2.00	1.360	68.0	1.400	70.0
4	2.00	1.530	76.5	1.590	79.5
5	4.00	3.40	85.0	4.00	100.0
6	10.00	6.03	60.3	5.53	55.3
7	20.00	16.80	84.0	17.5	87.5
8	35.00	29.60	84.6	29.1	83.1
9	50.00	32.10	64.2	31.5	63.0
10	50.00	39.60	79.2	43.2	86.4
Overall rec.,%			86.2		88.8

[a] Results represent analyses of 2 sample weighings.

Table 3. Comparison between turbidimetric and AOAC plate methods for analysis of CTC-HCl in commercial finished feeds

Samples analyzed	Label claim, g/ton	Found,[a] g/ton			
		AOAC	Rec., %	Turb.	Rec., %
2	400	252.4	63	253.8	64
4	300	193.1	64	187.6	63
3	200	142.3	71	144.3	72
4	150	116.4	78	108.4	72
45	100	66.6	67	66.2	66
12	70	45.4	65	41.6	60
12	50	34.8	70	33.9	68
3	25	14.9	60	12.7	51
2	15	10.8	72	9.1	61
Overall rec., %			67.8		64.1

[a] Results represent average of 2 determinations.

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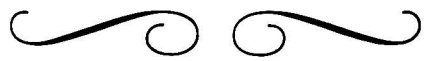

ACS Course—Oct. 16 and 17, 1976
Marriott Motor Hotel, Twin Bridges
Washington, DC

Immediately before the AOAC meeting, The American Chemical Society will hold a course on "Solving Problems with Modern Liquid Chromatography," conducted by Dr. J. J. Kirkland, E. I. Dupont de Nemours Co., and Dr. Lloyd R. Snyder, Technicon Instrument Corp. This new problem-oriented course is designed for chemists with experience in high pressure liquid chromatography, and will be helpful to those who attended the ACS course "Modern Liquid Chromatography." After a brief review of the basics of modern liquid chromatography, topics discussed will include separation control, the latest equipment and column packings, trouble-shooting, mobile and stationary phase selection, preparative separations, gradient elution and related methods, and techniques such as sample preparation and trace analysis. A number of recent applications, collected and organized according to sample type, will be used to illustrate the development of practical liquid chromatography separations.

To register or obtain complete information on the course, convenient lodging, and student discounts, please write or call the Department of Educational Activities, American Chemical Society, 1155—16th Street, NW, Washington, DC 20036 (202/872-4508). The fee is $160 for ACS members, $195 for non-members and includes the textbook, *Introduction to Modern Liquid Chromatography*, by L. R. Snyder and J. J. Kirkland.

FOOD ADDITIVES

Survey of Food Products for Volatile *N*-Nitrosamines

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A variety of food products containing nitrite were analyzed for 14 volatile *N*-nitrosamines by using a method demonstrated to be sensitive to 10 ppb. A total of 121 food samples were screened for volatile *N*-nitrosamine content. *N*-Nitrosopyrrolidine was confirmed in fried bacon at levels up to 139 ppb. *N*-Dimethylnitrosamine, *N*-nitrosopyrrolidine, and *N*-nitrosopiperidine were also confirmed in spice-cure mixtures at levels ranging from 50 to 2000 ppb.

The many publications in the literature during the last 5 years pertaining to the study of volatile *N*-nitrosamines in the environment and in food supplies attest to the concern of the scientific community with these potent carcinogens. It has been shown that nitrosamines are formed by the action of nitrite on secondary and tertiary amines (1, 2). The widespread use of nitrite as a preservative and color fixative, coupled with the natural occurrence of amines in foods, prompted the surveillance of products so preserved to determine the extent of these compounds in our food supply. The food products investigated to date include alcoholic beverages (3, 4), bacon (5–11), fish (12–16), frankfurters (17–19), ham (20), spinach (21, 22), bologna (23), prepared meat products (24–26), and meat spice-cure mixtures (27–29). This report continues the study of volatile *N*-nitrosamines in our food supply. The results of the analysis of 121 samples are given.

METHOD

Apparatus and Reagents

The apparatus and reagents used in this study have been described by Fazio *et al.* (30). The silica gel and the solvents, methylene chloride, pentane, and ethyl ether, were purified and tested prior to use to ensure the absence of interfering peaks.

Procedure

The multidetection method for the analysis of volatile *N*-nitrosamines in foods has been described by Fazio *et al.* (30). Fourteen compounds (*see* ref. 30 for selection criteria) can be determined: *N*-nitroso-dimethylamine, -methylethylamine, -diethylamine, -methylpropylamine, -ethylpropylamine, -dipropylamine, -ethylbutylamine, -propylbutylamine, -methylamylamine, -dibutylamine, -piperidine, -pyrrolidine, -morpholine, and -diamylamine.

Briefly, the comminuted food sample is digested in methanolic potassium hydroxide and the nitrosamines are extracted with methylene chloride in a liquid-liquid extractor, distilled from alkaline solution, further isolated by extraction and column chromatographic techniques, and determined by gas-liquid chromatography (GLC), using a modified thermionic detector.

When a peak was observed at the retention time of one of the 14 nitrosamines, the identity of this compound was confirmed by mass spectrometry (MS). To ensure adequate cleanup for this analysis, the concentrated methylene chloride eluate from the silica gel column was passed through an acid-Celite column. This procedure has been described by Howard *et al.* (13).

The identities of the nitrosamines isolated were confirmed with a combined GLC-MS system. A Varian Model 5600X, 2-stage membrane separator was interfaced to an Atlas CH-4 mass spectrometer. The separator is an integral part of the Varian Model 1700 gas chromatograph. This unit has the facility of venting the solvent to the atmosphere and maintaining the MS vacuum. Accordingly, large quantities of solvent can be injected onto the GLC column, vented, and prevented from entering the mass spectrometer. In this study, 30 µl of the final methylene chloride extract (equivalent to 100 ng nitrosamine) was injected onto the GLC column.

Results and Discussion

Before the recovery studies, the individual food products were analyzed for the 14 volatile *N*-nitrosamines. In instances where a nitrosamine was suspected, recovery values were ad-

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This report of the Associate Referee, T. Fazio, was presented at the 89th Annual Meeting of the AOAC, Oct. 13–16, 1975, at Washington, DC.

Table 1. Recoveries of nitrosamines[a] added at levels of 10 ppb to 25 g food product

Sample	Product	Rec., % Range	Rec., % Av.
1	Bacon, hams, other pork products (liver)	77–95	82
2	Baby foods	76–95	83
3	Cheeses (imported)	71–94	85
4	Fats and oils	67–100	83
5	Total diet products	75–100	86
6	Spice-cure mixtures	74–87	81
7	Icelandic foods	84–100	91

[a] 14 volatile *N*-nitrosamines as described in text.

justed appropriately. As shown in Table 1, recoveries of the 14 volatile *N*-nitrosamines added at 10 ppb (μg/kg) to 25 g of the food products ranged from 67 to 100%, with an overall average of 84%. Representative chromatograms of a spiked sample and the blank for cheese are shown in Fig. 1. A total of 121 food samples were analyzed during this survey, including bacon, lard, baby food, total diet samples, miscellaneous pork products, spice-cure mixtures, imported cheeses, and Icelandic national foods.

All bacon samples were purchased at local retail markets. Bacon was analyzed raw and also after frying at 340°F in an electric frypan (Sunbeam Model T61B, or equivalent) for 3 min on each side. Excess fat was removed from the bacon by blotting with paper toweling. As shown in Table 2, *N*-nitrosopyrrolidine was found in fried bacon at levels ranging from 7 to 139 ppb. Figure 2 shows a typical chromatogram of fried bacon after acid-Celite cleanup.

The fat cooked out of some of the bacon samples was analyzed as described by White *et al.* (31) and was found to contain from 19 to 92 ppb *N*-nitrosopyrrolidine. All values in Table 2 were confirmed by combined GLC-MS, except Sample 16 which had a low concentration. No

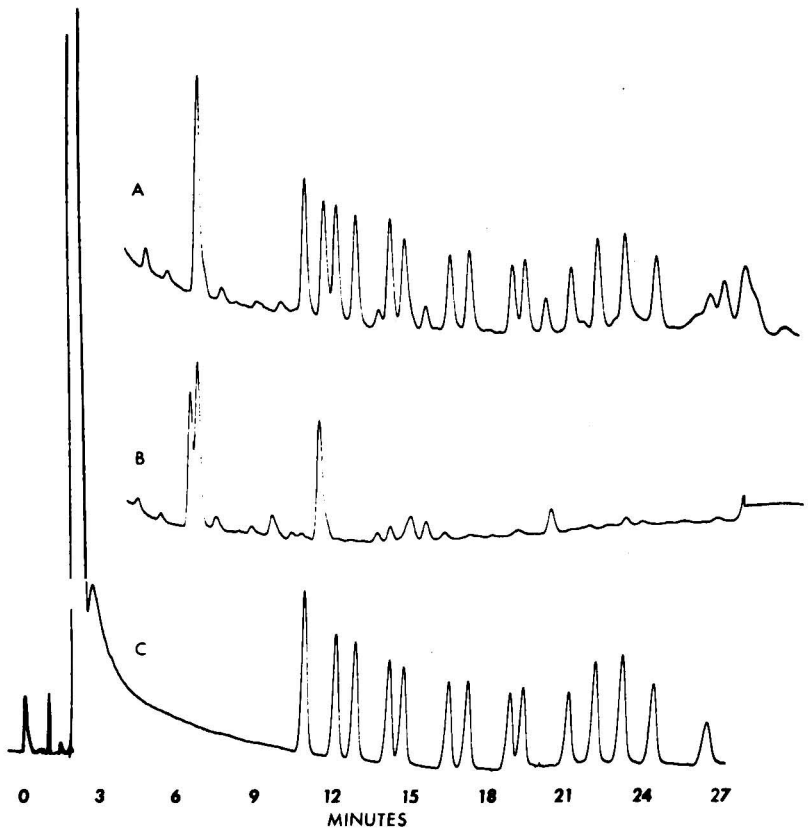

FIG. 1—Gas-liquid chromatograms of A, cheese fortified with 10 ppb nitrosamines; B, cheese extract; C, reference nitrosamine standards (0.5 μg/ml).

Table 2. *N*-Nitrosopyrrolidine (ppb) found in commercial bacon

Sample	Raw	Fried	Fat cooked out
1	0	67	92
2	0	91	
3	0	74,[a] 64[a] 111,[b] 129[b]	
4	0	30	
5	0	119, 139	
6	0	129,[c] 104[c]	
7	0	33, 33, 33	47
8	0	34	48
9	0	10	
10	0	16, 13	24
11	0	10	
12	0	28, 29	
13	0	42, 51	
14	0	28, 30	55, 44
15	0	22, 20	19
16	0	7,[d] 7[d]	
17	0	23,[b] 19[c]	
18	0	13, 12, 13	
19	0	8	
20	0	42, 34	49, 44
21	0	36,[c] 29[c]	
22	0	65, 59, 72, 64	

[a] Held at 72°F for 8 hr.
[b] Held at 72°F for a total of 34 hr.
[c] Held at 72°F for 36 consecutive hr.
[d] Not confirmed.

nitrosamines were found in the raw bacon. One sample of dry-cured bacon (not shown in the table) was analyzed; no nitrosamines were found in either the fried or the raw product. It is evident that pan-frying does induce the formation of *N*-nitrosopyrrolidine in bacon, but the actual mode of formation during the cooking process has not been definitely established. It may be through the formation of nitrosoproline from proline and nitrite and subsequent decarboxylation to nitrosopyrrolidine, or by direct interaction of pyrrolidine, which could arise from proline or putrescine and nitrite (32–34).

Four bacon samples were also analyzed after having been kept at room temperature (72°F) for 34–36 hr, before frying. It has been suggested by Cohen (34) that bacterial action on meat tissue could result in higher concentrations of polyamines. In the presence of nitrite these could, in turn, react to produce larger concentrations of *N*-nitrosamines upon frying. Samples 3, 6, 17, and 21 in Table 2 were held at 72°F $\leqslant$36 hr. The samples preceding them in the table were stored under normal use conditions (40°F) (Samples 2, 5, 16, and 20). Samples 3 and 17 showed considerable increase in *N*-nitrosopyrrolidine concentration after $\geqslant$34 hr above that of Samples 2 and 16, while Samples 6 and 21 showed little or no increase. A reason for these apparent inconsistencies could be the amount of residual nitrite present in the bacon. Producers use different concentrations of nitrite in their cures. Bacon with a low residual nitrite does not show an increase in nitrosamine formation after storage at room temperature, since there is an insufficient quantity of nitrite available to react with the additional amines formed. On the other hand, bacon with a high residual nitrite which can continue to react with the amines as they form at room temperature may show a greater formation of nitrosamines. After frying, the bacon was found to contain increased levels of *N*-nitrosopyrrolidine.

Table 3 shows the results of *N*-nitrosamine analyses on a variety of food products. Two types of baby foods that contained meat cured with nitrite were analyzed, but no nitrosamines were found (Samples 27 and 28). Of the 5 variety meat samples analyzed (Samples 29–34), only the fried canned bacon (Sample 32) showed *N*-nitrosopyrrolidine to be present. A peak at the retention time of *N*-nitrosodimethylamine (DMNA) was also observed but the concentration was too low to confirm its identity.

Six total diet samples were analyzed (Samples 35–40) comprising (*1*) grain and cereal products; (*2*) meat, fish, and poultry; (*3*) leafy vegetables; (*4*) root vegetables; (*5*) oils, fats, and shortenings; and (*6*) dairy products. (These composites represent the typical diet of an American male teenager between 15 and 19 years of age.) Because of the physical nature of the oils, fats, and shortenings sample (Sample 40) and the dairy products sample (Sample 35), they were analyzed by the procedure of White *et al.* (31) for fat cooked out of bacon. None of the 6 total diet samples analyzed was found to contain *N*-nitrosamines.

Since lard is produced from rendered pork fat (in some instances a cured product) and high levels of *N*-nitrosopyrrolidine have been found in fried bacon and fat cooked out of bacon, it was decided to analyze a sample of lard (Samples 41 and 42). No *N*-nitrosamines were found, even after heating at 340°F for 10 min.

Several workers have reported finding DMNA and *N*-nitrosodiethylamine in cheese which had been processed with nitrite. However, none of

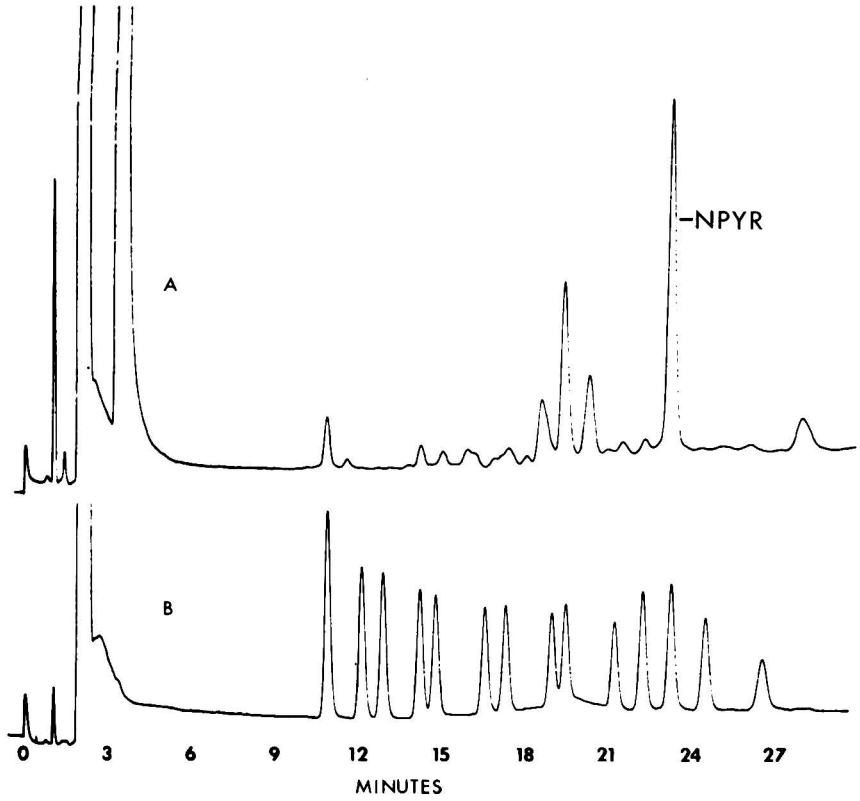

FIG. 2—Gas-liquid chromatograms of A, fried bacon showing N-nitrosopyrrolidine (NPYR); B, reference nitrosamine standards (0.5 μg/ml).

the data had been confirmed (9, 35). In order to verify these findings we surveyed 10 imported cheeses (Samples 43–52). None of the 14 volatile *N*-nitrosamines was found in any of these products.

It was brought to our attention that some Icelandic national dishes were processed under atypical conditions theoretically conducive to nitrosamine formation. For example, shark and skate are cut into pieces, coated with coarse salt, and buried in the ground for weeks until a certain stage of fermentation is reached. The putrified fish is hung for 1–3 months outdoors, and then eaten without further preparation. Other fish, such as trout and salmon, are pickled in a salt solution containing 0.6% nitrite before smoking. Lamb is also prepared by soaking it in a similar salt solution and further treated by smoking for 6–8 hr over sheep manure. The high concentration of amines normally found in fish coupled with the added nitrite and unusual mode of food processing should have provided an ideal environment for nitrosamine formation. As shown in Table 3, 18 different samples were analyzed (Samples 53–70). Despite the apparently ideal environment, no nitrosamines were found in any of the samples.

A problem concerning the formation of nitrosamines in meat spice-cure mixtures has recently been investigated (27–29). Salts, including nitrite, are added to a variety of spices in the formulation of meat spice-cure mixtures. Amines present in spices could react with the added nitrite and form nitrosamines. Both Sen *et al.* (27) and Joe (29) have confirmed DMNA, *N*-nitrosopyrrolidine, and *N*-nitrosopiperidine in meat curing mixtures at levels up to 25 ppm.

Until recently spice-cure mixtures have been marketed in several forms and formulations, such as single packaging of spices and curing salts, "piggy-back" (separate packaging of spices and curing salts), with and without sodium carbonate

buffers, and with and without protective coating of the nitrite crystals (zein corn protein).

A survey of 32 spice-cure mixtures was undertaken to determine the effectiveness of the 3 marketing processes and also to determine the effect of storage time and temperature on nitrosamine formation. The 9 samples found to contain nitrosamines are shown in Table 4. All data in the table were confirmed by MS.

Three samples were part of a time-temperature stability study. Each was buffered with sodium carbonate. The spice-cure mixtures were analyzed at room temperature and 100°F at 0, 30, and 60 days storage. Samples 1 and 2 in Table 4 were nitrosamine-free when first analyzed, but after heat and storage, both contained *N*-nitrosopiperidine from 150 to 420 ppb and *N*-nitrosopyrrolidine from 50 to 95 ppb. The third sample (a control not shown in Table 4) did not contain nitrite and, as expected, no nitrosamines were found.

Sample 3, found to contain 343 ppb DMNA, 730 ppb *N*-nitrosopyrrolidine, and 2000 ppb *N*-nitrosopiperidine, was a spice-cure mix being used at the time in a cured meat product. From the data shown in the table it is evident that buffering with sodium carbonate does not block nitrosamine formation. Samples 4, 7, and 9 were buffered and still DMNA, *N*-nitrosopiperidine, and *N*-nitrosopyrrolidine were found. Ascorbate was also found to be ineffective in blocking nitrosamine formation as shown by Samples 7 and 8 which contained this compound and yet had levels of *N*-nitrosopiperidine and *N*-nitrosopyrrolidine up to 232 ppb. Sen *et al.* (27) obtained similar results with ascorbate. Two samples of spice-cure mixtures utilizing the piggy-back method of packaging were analyzed. No nitrosamines were found. A limited study of 4 samples of spice-cure mixtures containing nitrite crystals coated with zein were analyzed and all 4 were found to be free of nitrosamines. Our studies have indicated that piggy-back pack-

Table 3. Food products analyzed: no nitrosamines detected

Sample	No. of analyses	Product
		Pork Products
23	2	pork liver (raw)
24	1	pork liver (fried)
25	1	Polish style sausage (raw)
26	1	Polish style sausage (boiled)
		Baby Foods
27	5	beans, potatoes, and ham casserole
28	6	creamed potatoes and ham
		Variety Meats
29	2	crumbled bacon
30	2	crumbled ham
31	1	bacon (imported canned; raw)
32[a]	1	bacon (imported canned; fried)
33	1	beer sticks
34	1	smoked pepperoni
		Total Diet Samples
35	1	dairy products
36	1	meat, fish, and poultry
37	1	grain and cereal
38	1	leafy vegetables
39	1	root vegetables
40	1	fats, oils, and shortenings
		Fats and Oils
41	1	lard (uncooked)
42	1	lard (heated)[b]
		Cheese
43	2	Edam
44	2	Gouda
45	2	Samsoe
46	2	Danbo
47	2	Gouda (baby)
48	2	Blue
49	2	Tilsit Sq. Havarti
50	1	Port Salut
51	1	Tybo
52	1	Tilsit Havarti
		Icelandic National Foods
53	1	smoked lamb's leg
54	1	salted lamb
55	2	smoked mutton sausage
56	2	singed lamb's head
57	1	smoked salmon
58	1	smoked lamb's leg
59	1	singed lamb's leg
60	3	smoked mutton sausage
61	1	smoked trout
62	2	putrescent skate
63	2	singed lamb's head (oil flame)
64	1	smoked lamb (birchwood)
65	1	smoked lamb (beechwood)
66	1	smoked salmon
67	1	putrescent shark (gler hakarl) (raw)
68	2	putrescent shark (skyr hakarl) (dried)
69	1	salted lamb
70	1	smoked mutton sausage

[a] Contained 6 ppb *N*-nitrosopyrrolidine, confirmed by GLC-MS.
[b] Heated at 340°F 10 min.

Table 4. Analysis of spice-cure mixtures for nitrosamines (ppb)[a]

Sample	Buffer	0 days	30 days		60 days	
		72°F	72°F	100°F	72°F	100°F
1	Na_2CO_3	0	0	0	0	50 NPYR
2	Na_2CO_3	0	0	150 NPIP	0	420 NPIP 95 NPYR
3	Na_2CO_3	343 DMNA 730 NPYR 2000 NPIP				
4	$Na_4P_2O_7$	72 DMNA				
5	none	106 DMNA 314 NPIP 28 NPYR				
6	none	768 NPIP 144 NPYR				
7[b]	Na_2CO_3	232 NPIP				
8[b]	none	160 NPIP 24 NPYR				
9	Na_2CO_3	29 DMNA 91 NPIP 49 NPYR				

[a] DMNA, *N*-nitrosodimethylamine; NPYR, *N*-nitrosopyrrolidine; NPIP, *N*-nitrosopiperidine.
[b] With ascorbate added.

aging is the most effective method of preventing nitrosamine formation, since the reactants do not come in contact until they are mixed with the comminuted meat. Federal laws in the United States and Canada now prohibit the marketing of meat spice-cure mixtures in any way other than the piggy-back method (36).

Our investigations are presently being extended to fin fish and shellfish and the results will be reported in a subsequent paper.

Recommendation

It is recommended that study be continued on this topic.

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COSMETICS

Determination of Bergapten in Fragrance Preparations by Thin Layer Chromatography and Spectrophotofluorometry

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A method has been developed for the determination of bergapten (5-methoxypsoralen), a known phototoxin, in perfumes, colognes, and toilet waters. The bergapten and other lactonic compounds were first isolated from the sample by a series of extractions. The extract containing the bergapten was diluted to a known volume and an aliquot was spotted on a thin layer chromatographic (TLC) plate coated with silica gel G. After 2-dimensional development with hexane - carbon tetrachloride - *tert* - butylamine (180+12+9) and hexane-toluene-ethyl acetate-acetic acid (100+10+15+0.5), the TLC plate was dried and the emitted fluorescence of bergapten was measured, using a spectrophotofluorometer equipped with a TLC accessory and coupled to an automatic digital integrator. The amount of bergapten was determined by comparing its peak area to those of bergapten standards. The average recovery for levels of 0.001, 0.005, and 0.01% bergapten was 88%.

Bergapten (5-methoxypsoralen) is one of a group of naturally occurring furanocoumarins found in various essential oils such as expressed bergamot and lime, angelica seed, and root oils (1). Its presence is also suspected in some other oils of the citrus family. Many of these oils are used in the formulation of fragrances. Bergapten has been implicated as the agent responsible for the phototoxic reaction resulting from the exposure of skin in contact with expressed bergamot oil to sunlight or ultraviolet (UV) radiation. Marzulli and Maibach (2) concluded from their studies that the minimum amount of bergapten in a fragrance which could elicit an adverse reaction would be 0.001–0.002% of the total product weight. Opdyke (3) reviewed a portion of the extensive literature dealing with the phototoxic effects of expressed bergamot oil, one of the principal sources of bergapten in fragrance products.

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Based on the findings of Marzulli and Maibach (2), there appeared to be a need for the development of an analytical method sensitive enough to detect the presence of bergapten down to a level of 0.001% (10 ppm) in fragrance products such as perfumes, colognes, or toilet waters. Several researchers have quantitatively measured the bergapten content as a constituent of various individual essential oils or admixed with similar previously isolated isomeric compounds. Steck and Bailey (4) separated it from these admixed coumarins and psoralens by gas-liquid chromatography (GLC). Quantitative GLC of some of these compounds was investigated by Furuya and Kojima (5). Recently, high-pressure liquid chromatography has been investigated for the identification and determination of bergapten in treated and untreated bergamot oils down to levels of 10 ppm (6).

Several thin layer chromatographic (TLC) techniques have also been employed in the determination of bergapten. Its fluorescence when exposed to shortwave UV radiation has been useful for both its identification and determination. Karlsen *et al.* (7) separated various admixed furanocoumarins, including bergapten, with a 2-dimensional technique. Cieri (1) separated the nonvolatile constituents of expressed bergamot oil, including limettin (citropten or 5,7-dimethoxycoumarin), from bergapten by TLC. He then scraped the bergapten from the plate, eluted it, and measured the UV absorbance of its solution. Similarly, Chambon *et al.* (8), employing paper chromatography, eluted and determined bergapten in expressed bergamot oil with UV and fluorometric spectrometry.

Madsen and Latz (9) determined various substituted coumarins in lemon, lime, and bergamot oils through the use of *in situ* fluorometry on thin layer chromatograms. This technique was applied to the development of a method for the determination of bergapten in perfumes, colognes, or toilet waters at levels as low as 0.001%

(10 ppm) after the suitable isolation, TLC, and fluorometric parameters were determined. Amounts of bergapten as low as 0.2 μg were readily detectable on the TLC plate.

METHOD

Apparatus

(a) *Air-drying tube.*—Connect rubber tubing from air source to wide end of glass dropping pipet which is, in turn, inserted into one hole rubber stopper secured and supported with Bunsen clamp attached to ringstand.

(b) *TLC developing tank.*—For 20 × 20 cm plates (3 required, Analtech, Newark, DE 19711, No. SJ-1003, or equivalent).

(c) *Template.*—Analtech, No. 5J-1010, or equivalent.

(d) *Pipets.*—2 μl Microcaps (Analtech, No. D-1002, or equivalent).

(e) *Pre-coated TLC plates.*—20 × 20 cm, 250 μm silica gel G (Analtech, No. 1011, or equivalent). Pretreat all plates prior to use to remove impurities contributing to background fluorescence (10) by eluting with methanol in developing tank. Remove after solvent front has reached top edge of plates and dry at room temperature overnight. They may be stored several months before procedure must be repeated.

(f) *Chromatographic tube.*—2.4 × 60 cm (Glenco Scientific Inc., Houston, TX 77007, No. 3060-246) equipped with porous support disk and "chroma" valve with ¼" stem, No. 3070, or equivalent.

(g) *Fluorescence spectrophotometer.*—Perkin-Elmer Corp., Norwalk, CT 06852, No. MPF-3, equipped with TLC accessory, No. 018-0058, as supplied by Perkin-Elmer Corp. and manufactured by Hitachi Ltd., Tokyo, Japan. Operating parameters: direct mode, reference sensitivity ×30; excitation wavelength 340 nm; excitation slit 6.5 nm; emission wavelength 452 nm; emission slit 12.0 nm; filter 430 nm placed before emission beam.

(h) *Automatic digital integrator with printer.*—Infotronics, Columbia Scientific Industries, Austin, TX 78702, No. CRS-204, or equivalent.

(i) *UV viewing cabinet.*—Brinkmann Instruments, Inc., Westbury, NY 11590, No. 0410920-1, or equivalent.

Reagents

All solvents are ACS grade, or equivalent.

(a) *tert-Butylamine.*—Eastman Kodak, Rochester, NY 14650, No. 6772.

(b) *Polyamide powder.*—For TLC, polyamide 6 (Brinkmann Instruments, Inc., No. 660066).

(c) *Celite 545.*—Johns-Manville Products Corp., Baltimore, MD 21202.

(d) *Oil bergamot extra.*—Fritzsche, Dodge & Olcott, Inc., New York, NY 10011.

(e) *Developing solvents.*—(*1*) Hexane-carbon tetrachloride-*tert*-butylamine (180+12+9). (*2*) Hexane-toluene-ethyl acetate-acetic acid (100+10+15+0.5).

(f) *Standard bergapten solutions.*—(*1*) *Stock solution.*—0.5 mg/ml. Accurately weigh ca 25 mg bergapten (see *Preparation of Bergapten Standard*) and add to 50 ml volumetric flask. Dissolve with methylene chloride and dilute to volume. Wrap flask with aluminum foil and store in dark. (*2*) *Working solution.*—0.1 mg/ml. Add 2.0 ml stock solution to 10 ml volumetric flask and dilute to volume with methylene chloride.

Preparation of Bergapten Standard

Remove volatile portion of 500 ml expressed bergamot oil by vacuum distillation at 1–2 mm Hg. Isolate lactonic fraction from distillation residue according to method of Sethna and Shah (10) and dilute lactonic fraction with 2 volumes of ether. Place in refrigerator overnight. Collect crystals (bergapten and limettin (5,7-dimethoxycoumarin)) on Büchner funnel. Recrystallize 4 times with minimum of hot methanol.

Place plug of glass wool at bottom of 60 × 2.4 cm id glass column equipped with porous support disk and add 45.0 g polyamide powder and Celite 545 (1+1, v/v) as slurry in hexane. Adjust level of hexane so that it remains just above settled adsorbent. Add slurry of 0.5–1.0 g Celite-polyamide mixture and methylene chloride solution containing 0.1–0.5 g crude bergapten (*see* ref. 4) to 50 ml beaker. Stir mixture continuously at room temperature under gentle air jet until dry. Add dry mixture to column and rinse all traces down to bed of adsorbent with hexane. Add 200 ml hexane in portions to column. Apply 5 psig to column head to facilitate adequate flow rate. Discard hexane eluate; then elute with 200 ml benzene. After reducing volume of benzene eluate on steam bath, transfer to 50 ml beaker and concentrate further. Eliminate final traces of benzene by placing beaker under gentle air jet at room temperature. Elutriate solid residue several times with small amounts of ether. Decant ether wash solution each time. White crystalline residue of bergapten melts at 189–190°C (11); at this mp the limettin contamination is negligible.

Preparation of Samples

Accurately weigh ca 10 ml sample containing 2–30% fragrance oil in tared 100 ml beaker. Add 3.0 ml NH_4OH and stir; then add 15.0 ml 1.0*N*

NaOH and enough ethanol (95%) to obtain homogeneity (usually 30–40 ml), in that order. Stir 1–2 min and transfer to 500 ml separatory funnel. Rinse beaker with 100 ml water and add rinse to funnel with 10–20 ml methanol. Extract 3 times with 50 ml portions of ethyl ether. If emulsion does not break, add few Na_2SO_4 crystals. Let layers separate and retain both layers. Combine ether extracts and wash with three 25 ml portions of water. Combine all aqueous extracts, including wash solutions. Discard ether extracts. Acidify aqueous solution with HCl until strongly acid to Alkacid test ribbon and extract with three 50 ml portions of ether. Wash combined ether extracts with two 50 ml portions of water, followed with three 10 ml portions of dilute ammonia (1+50). If emulsion forms at any time, add 10–25 ml water and/or methanol while swirling gently.

Discard all aqueous extracts, transfer ether extract to 250 ml Erlenmeyer flask, and dry 1–2 hr over anhydrous Na_2SO_4. Concentrate solution to 25–30 ml in 100 ml beaker on steam bath (rinse Na_2SO_4 with small portions of ether and add rinses to beaker). Transfer to 50 ml centrifuge tube containing small boiling chip. Rinse beaker several times with small portions of ether and add rinses to tube. Evaporate on steam bath to ca 100–200 μl. If water is present in concentrate, add 20–30 ml anhydrous ethyl ether and remove aqueous layer (lower) with 500 μl syringe; redry with anhydrous Na_2SO_4. Evaporate on steam bath to ca 100–200 μl.

Determination

(a) *Initial screening and evaluation of the chromatogram.*—Dilute sample extract to volume in 1 ml volumetric flask with methylene chloride. Fit 2 developing tanks with filter paper liner and add developing solvent 1 to one tank and developing solvent 2 to other. Let equilibrate 30 min before use.

Spot 6.0 μl sample along with 6.0, 4.0, and 2.0 μl bergapten working solution on 20 × 20 cm TLC plate. Develop plate twice in solvent 1, drying several min under gentle air jet between developments. View under shortwave UV light and note whether yellow spot is visible at R_f similar to bergapten standard. If spot is absent but may be obscured by other fluorescent spots at this R_f, rotate plate 90° counterclockwise and develop twice in solution 2. If bergapten is present at either step but at greater intensity than that of standards, dilute sample to volume in larger volumetric flask and repeat procedure. Examine TLC plate under UV light and estimate amount of bergapten in sample spot by comparing its intensity with those of standards.

(b) *Quantitative procedure.*—With aid of TLC template, place 4 very slight indentations onto silica coating of TLC plate with dull pencil point 2.5 cm above and parallel to bottom edge of plate every 4.0 cm, starting 3.0 cm from left edge of plate. Bottom edge should be that which had been immersed in methanol solution for pretreatment. Use 2.0 μl delivery pipet for spotting solutions. Place 6.0 μl appropriate sample dilution on extreme left indentation and amount of standard to most closely match this on spot immediately to its right, followed by standards containing 0.2 and 0.4 μg less than the first standard in the remaining 2 positions. Develop TLC plate twice in both solvents 1 and 2 as before and examine under shortwave UV light in UV viewing cabinet. After plate has dried (9) bracket each bergapten spot (standards and unknown) by scoring on each side with sharp pencil point through to underlying glass surface, taking care not to disturb the integrity of the compound.

Place scored plate silica side down in TLC accessory attached to spectrophotofluorometer. Connect automatic digital integrator with printer to instrument to obtain accurate area readouts. Follow directions in instruction manual for all preliminary adjustments. Do not use slit plate supplied with TLC accessory to allow largest possible area of excitation beam to scan bergapten spots. Open lid of TLC attachment in darkened room and manually align excitation beam along X axis so that it scans between scored lines of first bergapten spot. After closing lid, choose optimum X setting which registers greatest emission intensity by slightly varying position of knob to both left and right of original setting and then observe that position corresponding to maximum meter and/or recorder pen deflection. When found, leave setting undisturbed and adjust Y knob so that beam strikes plate below the spot. Close lid; then turn on automatic Y scan on TLC attachment. Follow instructions for determination of *in situ* emitted relative fluorescent intensities as outlined in manual provided for TLC accessory. Scan each bergapten spot ≥3 times and calculate its average emission intensity (9). Note that there is a time lag of ca 0.5–1.0 min after lid of TLC accessory is closed before noise to integrator disappears.

Calculation

$$\% \text{ Bergapten} = (A_x \times W_s \times V_x)/(A_s \times V_{xp} \times W_x \times 10)$$

where A_x and A_s = areas of bergapten peak of sample and standard, respectively, in arbitrary units; W_s = weight of bergapten standard on plate

in μg; W_x = total weight of sample in g; V_{xp} = volume of sample solution spotted on plate in μl; and V_x = total volume of sample solution in ml.

Results and Discussion

Preliminary investigations into the use of GLC for the determination of bergapten at a 0.001% (10 ppm) level in perfumes were unsuccessful. Although bergapten was adequately separated from other coumarins and psoralens present in expressed bergamot oil, quantitative recovery of known added amounts corresponding to 0.001% from commercial perfumes proved to be more difficult because of the apparent presence of many other lactonic compounds with similar retention times.

The *in situ* spectrophotofluorometric procedure for the determination of fluorescent compounds on TLC plates is based on that developed by Madsen and Latz (9) for 5-geranoxy-7-methoxycoumarin and limettin. Two-dimensional TLC coupled with the use of different solvent systems was successfully employed to effectively maximize separation of bergapten from other psoralens and coumarins present in expressed bergamot oil. This method was then applied to the determination of known amounts of bergapten added to bergapten-free perfumes at levels corresponding to 0.001, 0.005, and 0.01%. The TLC solvent systems previously reported were not practical for *in situ* spectrophotofluorometric measurements. The silica gel G plate was initially developed in hexane-carbon tetrachloride - *tert* - butylamine (180+12+9), dried, and then developed in a direction at a right angle to the first in hexane-toluene-ethyl acetate-acetic acid (100+10+15+0.5).

Concentration of the bergapten fraction of the fragrance product was a major prerequisite for its determination, especially at lower levels. This procedure was based on the formation of water-soluble furanocoumarin salts derived from the reaction of a strong base with furanocoumarins. Initially, isolation of bergapten from bergamot oil or fragrances led, in all cases, to decomposition of the small amounts of bergapten under consideration as either controls or spikes in samples. This decomposition, which occurs when 1.0*N* sodium hydroxide is used for its separation and isolation, was effectively reduced to minimal levels by adding concentrated ammonium hydroxide to the product prior to addition of 1.0*N* sodium hydroxide and 95% ethanol. All of the basic and neutral compounds were initially separated from the acidic fraction by ethyl ether extraction of the alkaline solution. After regeneration of the acidic fraction, stronger acids were removed by using a weakly basic wash (dilute ammonium hydroxide). Remaining are weakly acidic materials such as bergapten, other psoralens, phenols, and coumarins. After this fraction is spotted along with

Table 1. Recoveries of added bergapten from commercial perfumes

Sample (10.0 g)[a]		Added %	Added mg	Rec., %
1		0.001	0.1	74
				85
				84
	Av.			81
		0.005	0.5	101
				105
				81
	Av.			96
		0.01	1.0	101
				98
				85
	Av.			95
2		0.001	0.1	97
				97
				90
	Av.			95
		0.005	0.5	84
				77
				64
	Av.			75
		0.01	1.0	75
				78
				85
	Av.			79
3		0.001	0.1	80
				83
				82
	Av.			82
		0.005	0.5	80
				88
				86
	Av.			85
		0.01	1.0	103
				108
				100
	Av.			104

[a] 10.0 g samples, each containing 10–20% fragrance oil in alcohol.

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the bergapten standard, the TLC plate is developed and viewed under UV light. The presence of bergapten in the sample was detected by comparison of R_f values and fluorescent colors of spots derived from sample and standard. By overspotting extract with standard bergapten, further verification was obtained.

Bergapten corresponding to 0.001% (0.1 mg), 0.005% (0.5 mg), and 0.01% (1.0 mg) was added to 10 g samples of bergapten-free perfume (10–20% fragrance oil). Each level was repeated in triplicate. This was repeated for 2 other bergapten-free perfumes for a total of 27 determinations. Recoveries are presented in Table 1. The value of each recovery is the average result of $\geqslant 3$ scans of each spot. The integrated intensity of the emitted light (452 nm) is proportional to the amount of material present. A linear relationship was found to occur in the range of interest, i.e., 0.2–1.0 μg bergapten.

Recommendation

It is recommended that study on the topic of Essential Oil and Fragrance Materials, Components of, be continued.

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INDUSTRIAL CHEMICALS

Determination of Hexachloro-1,3-butadiene in Spinach, Eggs, Fish, and Milk by Electron Capture Gas-Liquid Chromatography

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Hexachloro-1,3-butadiene (HCBD), a waste product formed in the manufacture of perchloroethylene and trichloroethylene, has been found in fish from the lower Mississippi River basin. The AOAC official method for organochlorine pesticide residues in fatty and nonfatty foods has been modified for the determination of HCBD residues in selected food commodities. Acetonitrile extracts of nonfatty foods, or the combined acetonitrile extracts obtained in acetonitrile-petroleum ether partitioning of fat isolated from fatty foods, are diluted with water and extracted with petroleum ether. The petroleum ether extracts are chromatographed on Florisil and HCBD is eluted with petroleum ether. The eluate is analyzed by gas-liquid chromatography with an electron capture detector. Average recoveries of HCBD from fortified samples of fatty and nonfatty foods were greater than 90% in the interlaboratory trials of the method.

The current interest in hexachloro-1,3-butadiene (HCBD) as a food contaminant can be traced to the discovery by U.S. Department of Agriculture workers in 1973 of hexachlorobenzene (HCB) residues in a large number of cattle in the lower Mississippi River basin. Investigations by several Federal and State agencies revealed that the residues were the result of pollution of the cattle grazing area with by-product HCB from nearby chemical plants manufacturing perchloroethylene.

Information obtained from industry sources indicates that the manufacture of perchloroethylene leads to the formation of a by-product tarry residue, called hex waste, in which HCB and HCBD are the principal components. Approximately 10 million lb HCBD and 5 million lb HCB are generated annually as hex waste in the United States. Although some hex waste is recycled to recover HCB for commercial use, there are no major commercial applications for HCBD in this country and most of the hex waste is either incinerated or disposed of in deep wells, land-locked lagoons, or landfills. The cattle incident has established that the disposal or release of hex waste from perchlorination plants may contaminate the environment and, ultimately, the food supply with HCB. Since HCBD is the major component of hex waste, it is possible that it would also be the major component of the residue resulting in foods from such contamination.

Shortly after the cattle incident, the Food and Drug Administration's New Orleans laboratory found HCB in several fish samples from the lower Mississippi River basin. Our laboratory obtained portions of the HCB-contaminated fish samples to determine whether they also contained HCBD. Electron capture gas chromatography showed evidence of HCBD as well as HCB in extracts of the fish samples. The presence of HCBD and HCB was confirmed by gas-liquid chromatography-mass spectrometry (GLC-MS). HCBD is known to be toxic to mammals (1–4), so it is desirable to determine the extent to which HCBD residues are present in fish and other foods. This necessitated the development of suitable analytical methods.

METHOD

Principle

Residues of HCBD in selected food commodities are determined by using methods similar to those normally employed for organochlorine pesticide residues (5). Spinach, whole eggs, and fish samples are extracted according to the method for nonfatty foods (**29.011**). Fat is isolated from milk as described in **29.012(c)**, with minor changes to avoid volatilization of HCBD residues, and then subjected to acetonitrile-petroleum ether partitioning (**29.014**) with additional acetonitrile ex-

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tractions to improve the recovery of HCBD. Since petroleum ether quantitatively elutes HCBD from Florisil and yields a very clean sample eluate, the Florisil column cleanup (29.015) is modified to use petroleum ether, instead of ethyl ether-petroleum ether (6+94), as the eluting solvent. The eluate is analyzed by electron capture GLC on a 6% OV-101 column and/or a 10% Carbowax 20M column.

Reagents

(a) *General reagents.*—*See* 29.002. Check reagents for interferences, using GLC parameters in this method.

(b) *Hexachlorobutadiene.*—Eastman Kodak Co. No. P5517. Prepare 1 mg/ml stock standard solution in isooctane. Dilute stock solution as necessary for GLC working standards.

Apparatus

(a) *General apparatus.*—As described in 29.005 with addition of 25 ml graduated receiving flasks (Kontes Glass Co., No. K570050, size 2525) for the Kuderna-Danish concentrators described in 29.005 (e).

(b) *Gas chromatograph with electron capture detector.*—As described in 29.008; with one or both of the following GLC columns: (*1*) 6′ × 4 mm id glass column packed with 6% OV-101 on 80–100 mesh Chromosorb W (HP); (*2*) 6′ × 4 mm id glass column packed with 10% Carbowax 20M on 80–100 mesh Chromosorb W (HP). Prepare columns and column packings by techniques described in 29.008(b). Condition OV-101 column at 250–260°C and Carbowax column at 230–240°C with nitrogen carrier gas flow ca 60 ml/min ≥16 hr before connecting column to detector. Operating conditions: nitrogen flow 60 ml/min for 6% OV-101 column, 50 ml/min for Carbowax 20M column; temperatures (°C)—column 105, injector 150, detector 200; recorder 5 mv span, chart speed 0.5″/min; electrometer sensitivity 3×10^{-9} amp for full-scale deflection. Operate electron capture detector (pin cup) at dc voltage which produces one-half full-scale deflection for 0.5 ng HCBD. At these conditions, retention time of HCBD is 6 min on OV-101 column and 2 min on Carbowax column.

Sample Preparation and Extraction

(a) *Leafy vegetables.*—Proceed as described in 29.011. After transferring residues to petroleum ether, 29.011(e), concentrate extract to 5–10 ml in Kuderna-Danish concentrator for transfer to Florisil column.

(b) *Fish.*—Remove scales, head, and viscera. Grind sample in food chopper or meat grinder and mix thoroughly. Weigh 50 g portion into high-speed blender and determine approximate fat content of sample as described in 29.012(e). For analysis, weigh portion of ground fish sample containing ≤2 g fat (maximum sample size, 50 g) and extract as for leafy vegetables.

(c) *Whole eggs.*—Discard shells and thoroughly mix egg yolks and whites. Weigh 25 g into high-speed blender and extract as in (a). Use *T* value of 215 ml for calculation in 29.011(f).

(d) *Milk.*—Isolate fat as directed in 29.012(c), with the following modification: After water-washing combined ethyl ether-petroleum ether extracts, pass extracts through 50 × 25 mm od column of anhydrous Na_2SO_4 and collect ether solution in 500 ml Kuderna-Danish concentrator fitted with 25 ml graduated receiving flask. Wash Na_2SO_4 column with small portions of petroleum ether and evaporate combined extracts and rinses to ca 15 ml in concentrator equipped with Snyder column. Adjust volume of solution in receiving flask to 25 ml with petroleum ether and mix thoroughly. To determine fat content, pipet 5 ml portion of the fat solution into tared 100 ml beaker and evaporate solvent under current of dry air at steam bath temperature. When solvent is completely removed, weigh and record weight of fat. For analysis, pipet 15 ml aliquot of the fat solution (containing 3 times weight of fat recorded above) into 125 ml separatory funnel and proceed with acetonitrile partitioning (29.014) with the following modification: Extract fat solution in 125 ml separatory funnel 6 times, instead of 4 times, with 30 ml portions of acetonitrile saturated with petroleum ether, vigorously shaking separatory funnel 1 min each time.

Florisil Cleanup

Prepare Florisil column as directed in 29.015 and prewet column with 40–50 ml petroleum ether. Place 100 ml volumetric flask under column to receive eluate. Quantitatively transfer concentrated sample extract, maximum volume 10 ml, to column, using 5 ml petroleum ether as rinse. Elute column with petroleum ether until 100 ml eluate has been collected. Transfer eluate to Kuderna-Danish concentrator equipped with Snyder distilling column and concentrate to 5–10 ml on steam bath. Adjust volume of concentrate with petroleum ether so that 3–8 μl aliquot is equivalent to 20 mg nonfatty sample or 3 mg fatty sample (fat basis) and mix carefully.

Caution: Solvent evaporations must be conducted in Kuderna-Danish concentrators equipped with Snyder distilling columns to avoid loss of HCBD. Remove concentrator from steam bath as soon as possible so that approximately 5–10 ml

solvent remains when concentrator has cooled. If further concentration is required, as for GLC-MS confirmatory analysis, carefully evaporate in Kuderna-Danish receiver tube fitted with micro-Snyder column. Do *not* evaporate solvent under jet of air or nitrogen because a significant amount of HCBD residue is lost by using this technique.

Gas Chromatography

Inject 3–8 μl aliquot of concentrated Florisil column eluate containing amount of HCBD within linear range of electron capture detector into gas chromatograph. Determine quantity of HCBD in injected aliquot by comparing peak heights of sample and appropriate HCBD standard. For accurate work, chromatographic responses for sample and standard injections should be similar (±10%). Using recommended injection aliquots (equivalent to 20 mg nonfatty sample or 3 mg fat) and given GLC parameters, the method provides an HCBD quantitation limit (peak height, ca 10% full-scale deflection) of 0.005 ppm for nonfatty foods and 0.04 ppm for fatty foods (fat basis).

Results and Discussion

Chromatograms obtained in the usual analysis for pesticide residues (29.001–29.028 (5)) frequently exhibit large peaks near the solvent front. Since these early eluting peaks have shorter retention times than the pesticide residues of interest, they are often ignored or attributed to coextracted natural constituents of the sample or impurities in the analytical reagents. Our experience indicates that many of these early eluting peaks are caused by residues of halogenated industrial chemicals and their by-products, which, like HCBD, are more volatile than the pesticide residues determined by the AOAC multiresidue method.

The presence of HCBD in a sample may easily be overlooked by the residue analyst because it elutes with or near the chromatogram solvent front at the AOAC-recommended GLC conditions. Figure 1 shows GLC curves obtained at AOAC conditions for (A) a standard solution of HCBD and 4 common organochlorine pesticide residues, and (B) the ethyl ether-petroleum ether (6+94) Florisil column eluate of a Mississippi River fish sample. Although the chromatogram of the fish sample contains a peak at the retention time of HCBD (as marked by the arrow) it is apparent that the identification and

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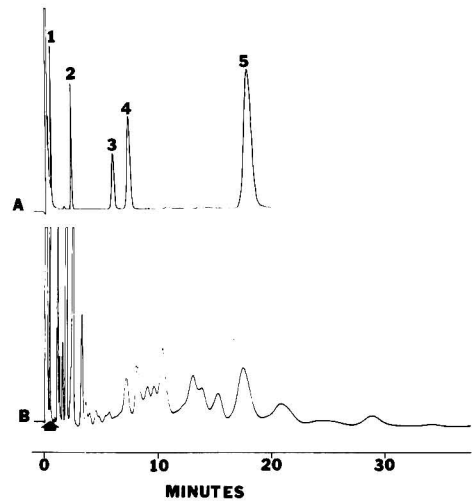

FIG. 1—Electron capture gas chromatograms of A, mixture of reference compounds: (1) 0.14 ng HCBD, (2) 0.33 ng α-BHC, (3) 0.5 ng aldrin, (4) 1.0 ng heptachlor epoxide, (5) 5.0 ng p,p'-DDT; B, ethyl ether-petroleum ether (6+94) Florisil eluate of fish sample (ca 4 mg sample equivalent injected). 10% OV-101 column at GLC parameters in 29.018.

determination of HCBD residues require different GLC conditions than those used to obtain this chromatogram.

HCBD can be adequately gas chromatographed on a variety of GLC liquid phases such as OV-101, OV-210, and Carbowax 20M. The 6% OV-101 and 10% Carbowax 20M columns used in the method provide excellent chromatography for HCBD and differ sufficiently in retention characteristics to be useful for confirmatory purposes. Operating parameters for the GLC analysis were chosen to resolve HCBD from trichlorobenzenes which have been detected in our analyses of some freshwater fish samples. Figure 2 illustrates the GLC separation afforded by each column for a mixture of HCBD and trichlorobenzenes.

The electron capture detector response of our chromatographic system with either the OV-101 column or the Carbowax column was linear for quantities of HCBD in the range from 0.1 to 0.8 ng. Beyond this range, which produced HCBD peak heights from 10 to 80% full-scale deflection, the response veered from linearity. Thus, for quantitation, sample eluates giving an HCBD response greater than 80% full-scale deflection for a 3 μl injection were diluted with petroleum ether so that the HCBD response for

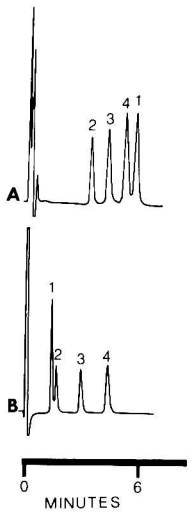

FIG. 2—Electron capture gas chromatograms of HCBD and trichlorobenzene isomers at recommended GLC parameters (see text) for A, 6% OV-101 column; B, 10% Carbowax 20M column. Quantities injected: (1) 0.5 ng HCBD; (2) 5.0 ng 1,3,5-trichlorobenzene; (3) 8.5 ng 1,2,4-trichlorobenzene; (4) 5.0 ng 1,2,3-trichlorobenzene.

a 3–8 μl aliquot of the diluted solution was between 10 and 80% full-scale.

In our studies of the behavior of HCBD in the AOAC multiresidue method, it was found that HCBD is quantitatively recovered from the Florisil column in the ethyl ether-petroleum ether (6+94) eluate. Further investigation showed that HCBD is also quantitatively recovered from the Florisil column with 100 ml petroleum ether. Since many compounds eluted from the Florisil column with 200 ml ethyl ether-petroleum ether (6+94) are not eluted with 100 ml petroleum ether, the latter provides a comparatively clean sample eluate for GLC analysis. This simplifies the identification and determination of HCBD in samples bearing complex mixtures of industrial chemical residues and is especially advantageous in GLC-MS confirmatory analysis. In examining food samples for organochlorine residues, our laboratory now routinely collects 100 ml petroleum ether as the first Florisil eluate before collecting the usual 6+94, 15+85, and 50+50 mixed ether eluates. Although this increases the number of eluates for GLC analysis, it provides a very useful separation into 2 groups of the many compounds normally eluted with 6+94 mixed ethers. Since **29.015** allows transfer of the petroleum ether extract (about 100 ml) from **29.011(e)** to the Florisil column, either directly or after concentration to 5–10 ml, 100 ml petroleum ether would not be expected to change the elution patterns of compounds normally eluted with 15+85 and 50+50 mixed ethers and no such changes have been observed in our laboratory. However, it may be noted that some compounds, such as HCB, have shown a tendency to split in an inconsistent manner between the petroleum ether and 6+94 mixed ether eluates.

The fish and egg extraction procedures described in the method are similar to the AOAC extraction procedure for nonfatty foods and have been in use for several years in Food and Drug Administration laboratories (6). Since they require less analytical time than the fatty food extraction procedures, they were combined with the modified Florisil cleanup for the determination of HCBD. To test the method, samples of spinach, fish, and eggs were fortified with HCBD at levels ranging from 0.1 to 6.0 ppm and analyzed. Recoveries presented in Table 1 ranged from 90 to 103%, using electron capture GLC, and from 91 to 113%, using halogen-specific microcoulometric GLC.

Butterfat was used as a test substrate for development of the fatty food method. HCBD recoveries by a method combining the AOAC acetonitrile partitioning cleanup (**29.014**) with the modified Florisil cleanup described in this paper were 70–82%. It was found that the HCBD losses were occurring in the acetonitrile-petroleum ether partitioning step. Table 2 shows that by increasing the number of partition extractions from 4 to 6, HCBD recoveries from

Table 1. Recoveries of hexachlorobutadiene from spinach, eggs, and fish

Product	Wt, g	HCBD added, ppm	Rec., % EC-GLC	Rec., % MC-GLC
Spinach	100	0.10	90	95
Spinach	100	0.10	98	105
Spinach	100	0.10	103	101
Spinach	100	6.00	99	[a]
Eggs	25	0.10	100	107
Eggs	25	0.10	101	106
Eggs	25	0.10	90	91
Eggs	25	2.0	94	96
Ocean perch	50	0.10	95	113
Ocean perch	50	0.10	93	103
Ocean perch	50	0.10	97	100

[a] Not analyzed by microcoulometric GLC.

Table 2. Recoveries of hexachlorobutadiene from butterfat and corn oil

Product	Wt, g	HCBD added, ppm	No. of CH_3CN partitions[a]	Rec., %
Butterfat	3.0	0.267	4	82
Butterfat	3.0	0.267	4	81
Butterfat	2.6	0.308	4	70
Butterfat	3.0	0.33	6	98
Butterfat	3.0	0.33	6	92
Corn oil	3.0	0.33	6	94
Corn oil	3.0	1.70	6	98
Method blank	[b]	0.33	6	95
Method blank	[b]	1.70	6	96

[a] In petroleum ether to acetonitrile partitioning step.
[b] No sample matrix, fortification assumed a 3.0 g fat sample.

fortified butterfat increased to 92 and 98%. Recoveries from fortified corn oil, using 6 partitionings, were 94 and 98%.

In the AOAC extraction procedure for milk (**29.012(c)**) the fat extracted from the sample with mixed ethers is evaporated in a beaker to completely remove the solvent. Because HCBD is highly volatile, much of the HCBD residue is lost during this evaporation step. To avoid these losses, the mixed ether extracts are evaporated to ~ 15 ml in a Kuderna-Danish concentrator equipped with a Snyder distilling column. After the volume of concentrate is adjusted to 25 ml, a 15 ml aliquot is taken for acetonitrile partitioning and a 5 ml aliquot is evaporated to determine the quantity of fat on which the analysis is based. Table 3 shows that this procedure avoids the large HCBD losses. The modified procedure also reduces by about 0.5 hr the time needed to complete the residue analysis, since the cleanup of the 15 ml aliquot and the determination of fat in the 5 ml aliquot can proceed simultaneously.

Table 3. Recoveries of hexachlorobutadiene from milk

Fat in sample,[a] %	HCBD added,[b] ppm (fat basis)	Solv. evapn technique[c]	Rec., %
3.5	0.86	1	32
3.2	0.94	1	31
3.6	0.83	2	87
3.5	0.86	2	91

[a] 100 g whole milk.
[b] 3 μg HCBD added to each sample.
[c] 1, solvent evaporated as described in **29.012(c)**; 2, modified solvent evaporation technique described in this paper (*see* text).

Sixteen fish samples obtained from Food and Drug Administration District Offices in New Orleans, Buffalo, Minneapolis, and New York City were analyzed. HCBD was found in 8 of the 16 samples, with 7 of the positive samples from the lower Mississippi River basin and 1 from Lake Erie. The levels of HCBD in the positive samples ranged from a trace (less than 0.005 ppm) to 4.6 ppm as listed in Table 4. All HCBD residues found by electron capture GLC were confirmed by halogen-specific microcoulometric GLC. In addition, 2 HCBD residues were confirmed by GLC-MS. Figure 3 shows portions of the mass spectra obtained by GLC-MS at the retention time of HCBD for (A) HCBD and (B) the petroleum ether Florisil column eluate of a Mississippi River fish sample. The spectra are virtually identical, except for the presence of the fragment clusters at m/e 145, 147, 149 and 180, 182, 184 which are attributable to trichlorobenzene residues not completely resolved from HCBD at the operating conditions used for the GLC-MS analysis.

Figure 4 shows chromatograms obtained using a 6% OV-101 column at the GLC parameters given under *Method* to analyze (A) a standard HCBD solution, and (B) the petroleum ether

Table 4. Environmentally incurred hexachlorobutadiene residues in fish

Species	Origin	Wt, g	Fat, %	HCBD found, ppm[a]	
				6% OV-101	10% Carbowax
Catfish[b]	La Place, LA	10.0	17.2	4.65	4.59
Catfish[b]	Arkansas fish farm	24.0	8.2	0.79	0.77
Gaspergoo	La Place, LA	25.0	2.1	0.20	0.20
Buffalo	La Place, LA	25.0	5.2	0.10	0.10
Mullet	Buras, LA	50.0	1.3	trace	trace
Sea trout	Houma, LA	30.0	5.7	[c]	trace
Catfish	Morgan City, LA	20.0	5.5	trace	trace
Sheepshead	Lake Erie	38.0	5.2	trace	trace

[a] Residue in edible flesh.
[b] Identification of HCBD confirmed by GLC-MS.
[c] Not analyzed on this column.

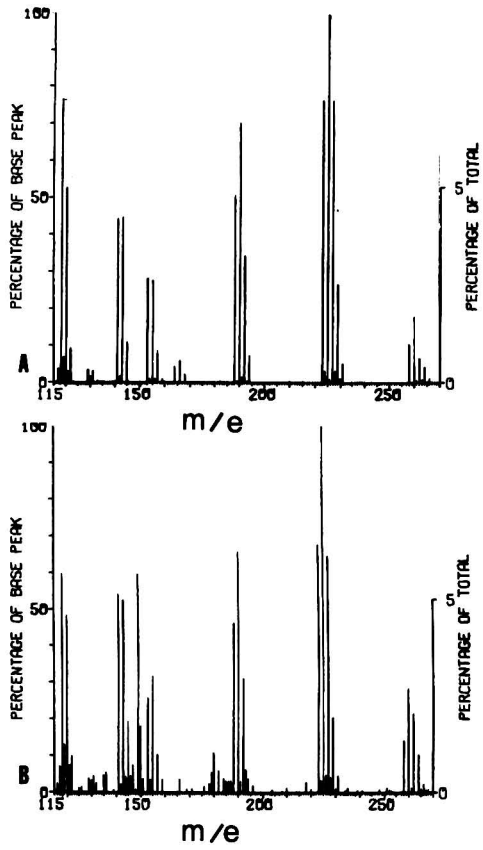

FIG. 3—Mass spectra obtained at GLC retention time of HCBD of A, HCBD standard; B, petroleum ether Florisil eluate of fish sample.

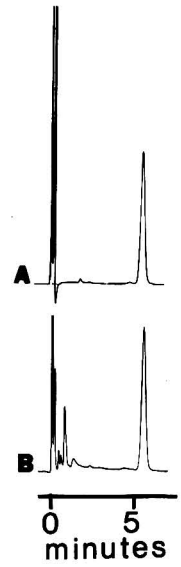

FIG. 4—Electron capture gas chromatograms of A, 0.5 ng HCBD; B, petroleum ether Florisil eluate of fish sample (ca 4 mg sample equivalent injected). 6% OV-101 column at 105°C.

Florisil eluate of a Mississippi River fish sample containing HCBD. Analysis of the 6+94 mixed ether eluate of the same fish sample at the GLC conditions in **29.018** produced the chromatogram in Fig. 1. The differences between the 2 chromatograms of the same fish sample are the result of the differences in GLC parameters and the improved cleanup obtained by using petroleum ether rather than 6+94 mixed ethers as the eluting solvent for the Florisil column.

The residue findings reported in this paper indicate that our environment and portions of our food supply have been contaminated with HCBD. In preparation for a Food and Drug Administration survey to determine the extent of such contamination in foods originating in areas near perchlorination plants, the method has been tested on 4 commodities at laboratories in Dallas and San Francisco. The results of the interlaboratory study indicate that the method is adequate for the determination of HCBD residues. No HCBD was found in blank determinations on the 4 commodities. The sample types, fortification levels, and recoveries are reported in Table 5.

Table 5. Results of interlaboratory trial of HCBD method

Product	Wt, g	HCBD added, ppm	Rec., % EC-GLC	Rec., % MC-GLC[a]
Spinach	100	0.046	102	98
Spinach	100	0.046	100	[b]
Spinach	100	0.094	103	97
Spinach	100	0.094	104	[b]
Eggs	25	0.10	98	92
Eggs	25	0.10	97	95
Eggs	25	2.5	92	94
Eggs	25	2.5	94	90
Sole[c]	50	0.185	108	106
Sole[c]	50	0.185	103	[b]
Sole[c]	50	4.62	107	110
Sole[c]	50	4.62	105	[b]
Milkfat[d]	3	0.10	94	91
Milkfat[d]	3	0.10	94	91
Milkfat[d]	3	2.5	92	94
Milkfat[d]	3	2.5	96	94

[a] Halogen-specific microcoulometric and Coulson conductivity detectors were used interchangeably.
[b] Not analyzed with halogen-specific detector.
[c] 0.88% fat in sample.
[d] Extracted fat was fortified and results are reported on a fat basis.

Acknowledgments

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Survey for Hexachloro-1,3-butadiene in Fish, Eggs, Milk, and Vegetables

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Eighty-four samples (fish, milk, eggs, vegetables) were collected from within a 25 mile radius of 10 perchloroethylene or trichloroethylene manufacturing plant sites and analyzed for hexachloro-1,3-butadiene (HCBD) by a gas chromatographic method. Residues of HCBD were found mainly in freshwater fish caught in the lower Mississippi River. The levels ranged from 0.01 to 1.2 ppm. Two of the 20 saltwater fish contained residues of 0.01 and 0.02 ppm. No HCBD residues were found in eggs and vegetables. One of 20 milk samples contained 1.32 ppm HCBD.

Hexachloro-1,3-butadiene (HCBD) is a waste product formed in the manufacture of chlorinated solvents such as perchloroethylene. It is found in the tarry wastes along with hexachlorobenzene (HCB), hexachloroethane, and other chlorinated by-products. Depending on the method of waste disposal, contamination of the environment by HCBD is possible.

In a 1973 survey of fish for pesticides and phthalate ester residues, an unidentified gas-liquid chromatographic (GLC) peak was observed in the chromatograms of several fish from the lower Mississippi River. This peak was eventually identified as being due to HCBD (1). These positive HCBD findings and the knowledge that the HCBD may be coming from perchloroethylene plants were among the factors which led to the need for more information and for this survey. The basic objective was to determine whether foods grown or produced near perchloroethylene plants were being contaminated with HCBD.

Ten manufacturing plant sites were selected for sampling: Lake Charles, LA; Deer Park, TX; Freeport, TX; Corpus Christi, TX; Wichita, KS; Geismar, LA; Baton Rouge, LA; Plaquemine, LA; Louisville, KY; and Pittsburg, CA. Five Food and Drug Administration Districts were asked to collect 10 samples from farms and streams located as close to each plant as possible, but no farther than 25 miles (40 km) from each plant site and preferably downwind and/or downstream. These samples were to be analyzed for HCBD.

The method used in analyzing for HCBD was essentially the same as the general method for chlorinated and phosphated pesticides (29.001–29.028 (2)). This standard method was modified slightly to make it more suitable for HCBD (1). GLC conditions were changed because of the volatility of HCBD.

Experimental

Extraction

(a) *Vegetables.—See* 29.011.

(b) *Fish.*—Determine fat content. Prepare and extract sample according to 29.012(e), adjusting sample size to obtain ≤2.0 g fat. Maximum sample size is 50 g.

(c) *Milk.—See* 29.012(c). Collect butterfat extract in Kuderna-Danish concentrator and concentrate to 25 ml. Use 5 ml aliquot for fat determination. Use another 15 ml aliquot for rest of procedure.

(d) *Eggs.—See* 29.011(a); use ≤25 g sample.

Cleanup

Milk.—See 29.014, but increase number of partitionings with acetonitrile from 4 to 6.

Florisil Cleanup

Prepare column as in 29.015. Concentrate sample to ≤10 ml before transferring to Florisil column. Complete sample transfer with additional 3–5 ml petroleum ether. Place 100 ml receiver under column and elute with petroleum ether until 100 ml has been collected. Analyze petroleum ether eluate for HCBD by injecting 5–10 μl into gas chromatograph. If no HCBD is detected, transfer eluate to Kuderna-Danish evaporator and concentrate to 10 ml. Inject up to 20 mg equivalent eggs, vegetable, or fish and 3.0 mg milkfat for GLC analysis.

Gas-Liquid Chromatography

Use the following instrumental conditions: 6′ × 4 mm id coiled glass column containing 6% OV-101 or 10% Carbowax 20M on 80–100 mesh Chromosorb W (HP); nitrogen 60 ml/min; col-

umn 105°C, injection port 150°C, electron capture detector 200°C. Adjust voltage so that 0.5 ng HCBD gives ½ full-scale recorder deflection at electrometer setting of 1 or 3×10^{-9} amp full scale. HCBD elutes from OV-101 column in 6 min and from Carbowax column in 2 min. HCBD concentrations can be adequately measured as low as 0.005 ppm in nonfatty foods and 0.04 ppm in fatty foods.

HCBD is quite volatile and analysis for this compound must be done carefully. Use Kuderna-Danish concentrator to prevent losses during solvent reduction.

Results and Discussion

Of the requested 100 samples, 84 were collected and analyzed. Fish were not available at several of the sampling sites. At 2 Texas sites, only fish were available. No samples were available from the Deer Park, TX site and only 4 samples could be collected from the Lake Charles, LA site. The survey results were grouped according to the individual commodity.

Fish

Twenty-eight fish samples from 5 sites were analyzed; *see* Table 1. Nine freshwater fish came from 3 Mississippi River sites in Louisiana and 19 saltwater fish came from 2 sites in Texas (Corpus Christi Bay and Gulf of Mexico). One sample of shrimp was collected from the Corpus Christi site. No fish was available at the other 5 sites in the survey. HCBD residues were reported in 10 fish samples with concentrations ranging from 0.01 to 1.2 ppm. Separating the samples into fresh- and saltwater varieties, 8 of 9 (89%) of the freshwater fish contained HCBD residues compared with only 2 of 20 (10%) of the saltwater fish (including shrimp). Although it appears that most of the contamination is confined to the lower Mississippi River, fish from additional freshwater sites should be examined before such a conclusion may be reached.

Table 1. HCBD residues found in fish

Sample	Site	HCBD,[a] ppm
Catfish	Baton Rouge, LA	0.010
Carp	Baton Rouge, LA	0.062
Gaspergoo	Baton Rouge, LA	0.012
Catfish	Plaquemine, LA	0.010
Gaspergoo	Plaquemine, LA	0.030
Catfish	Plaquemine, LA	0.010
Catfish	Geismar, LA	1.20
Buffalo fish	Geismar, LA	0.12
Whiting	Freeport, TX	0.02
Drum	Freeport, TX	0.01

[a] Additional fish samples from Geismar, LA; Freeport, TX; and Corpus Christi, TX did not contain detectable residues. The limit of detection of the method is 0.001 ppm.

Eggs

Fifteen egg samples were collected from 7 sites. No sample was available at any of the 3 sites in Texas. No HCBD residues were found in any of the samples.

Milk

Twenty samples were collected from 6 sites. No sample was available at the 3 sites in Texas or at Louisville, KY. Only one sample contained HCBD residues: 1.32 ppm (fat basis) in one of the 5 samples collected at the Wichita, KS site. A followup sample from the same area was analyzed and no HCBD residue was found.

Vegetables

Twenty samples were collected from 6 sites. No sample was available at the 3 Texas sites or the Wichita, KS site. Vegetables sampled were cucumber, okra, mustard green, snap bean, cabbage, kale, and collard. No HCBD was found in any sample.

Conclusions

The survey results indicate that eggs and vegetables produced near perchloroethylene plants are not likely to be contaminated with HCBD. The one positive milk sample appears to be an isolated incident but additional sampling in the same area may be necessary to justify a firm conclusion. HCBD residues were found mainly in freshwater fish and apparently the contamination is confined to the lower Mississippi River.

Acknowledgments

The help of the following Food and Drug Administration Districts in obtaining the samples and analytical results is deeply appreciated: Atlanta, Dallas, Kansas City, New Orleans, and San Francisco. The assistance of L. R. Kamps, M. P. Yurawecz, and L. J. Miller, Food and Drug Administration, Washington, DC, is also appreciated. Special thanks are given to P.

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Lewis, Food and Drug Administration, Washington, DC, for gathering the background information on HCBD.

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(1) Yurawecz, M. P., Dreifuss, P. A., & Kamps, L. R. (1976) *JAOAC* 59, 552–558

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ACS Course—Oct. 16 and 17, 1976
Marriott Motor Hotel, Twin Bridges
Washington, DC

Immediately before the AOAC meeting, The American Chemical Society will hold a course on "Solving Problems with Modern Liquid Chromatography," conducted by Dr. J. J. Kirkland, E. I. Dupont de Nemours Co., and Dr. Lloyd R. Snyder, Technicon Instrument Corp. This new problem-oriented course is designed for chemists with experience in high pressure liquid chromatography, and will be helpful to those who attended the ACS course "Modern Liquid Chromatography." After a brief review of the basics of modern liquid chromatography, topics discussed will include separation control, the latest equipment and column packings, trouble-shooting, mobile and stationary phase selection, preparative separations, gradient elution and related methods, and techniques such as sample preparation and trace analysis. A number of recent applications, collected and organized according to sample type, will be used to illustrate the development of practical liquid chromatography separations.

To register or obtain complete information on the course, convenient lodging, and student discounts, please write or call the Department of Educational Activities, American Chemical Society, 1155—16th Street, NW, Washington, DC 20036 (202/872-4508). The fee is $160 for ACS members, $195 for non-members and includes the textbook, *Introduction to Modern Liquid Chromatography*, by L. R. Snyder and J. J. Kirkland.

Determination of Polychlorinated Dibenzo-*p*-dioxins and Dibenzofurans in Commercial Pentachlorophenols by Combined Gas Chromatography-Mass Spectrometry

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Samples of commercial pentachlorophenol (PCP) and its sodium salt (PCP-Na) were examined for the presence of polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs), using a rapid, highly specific method of analysis. Phenolic compounds are removed by alkaline extraction, and the neutral components are fractionated on an alumina minicolumn. After gas chromatographic separation, individual PCDDs and PCDFs are detected by mass fragmentography and their presence is confirmed by complete mass spectral analysis. While some samples had only low amounts of PCDDs and PCDFs, others contained much higher amounts of these components. PCP-Na samples showed the unexpected presence of a tetrachlorodibenzo-*p*-dioxin. Re-analysis of PCP and PCP-Na samples with high PCDD contents on a high-resolution glass capillary column showed the presence of 3 hexa- and the 2 heptachlorodibenzo-*p*-dioxins with nearly constant isomeric ratios.

Pentachlorophenol (PCP) and other chlorinated phenols are widely used as herbicides, insecticides, wood preservatives, and starting materials for a series of agricultural and industrial chemicals. PCP is produced technically either by chlorination of phenol and chlorophenols or by alkaline hydrolysis of hexachlorobenzene (1). PCP and other chlorinated phenols contain a variety of contaminants (2–4), including the hazardous polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs; *see* Fig. 1 for structures). PCDDs and possibly PCDFs are toxic, teratogenic, and mutagenic at low levels (5, 6) and their presence in these products gave rise to concern (7). In order to prevent environmental contamination with these dangerous compounds, highly sensitive analytical techniques are required to ensure that acceptable quality standards by the manufacturers are maintained. The analysis is complicated by the large number of structurally similar compounds usually present in comparable amounts, requiring extensive sample cleanup and the most specific detection systems (8). The present study was initiated to determine the concentrations of PCDDs and PCDFs in samples of PCP and its sodium salt (PCP-Na) received during 1973 from commercial sources in Switzerland.

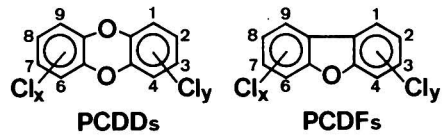

FIG. 1—Structures and numbering (Chemical Abstracts system) of polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs).

Experimental

Apparatus

Gas chromatograph-mass spectrometer.—A Finnigan Model 1015D quadrupole mass spectrometer with electron impact ion source, equipped with venting system and glass jet separator to interface with gas chromatographic (GC) column, was used. *GC conditions.*—Glass column, 2 m × 2 mm id, packed with 3% silicone OV-1 on Chromosorb W (acid-washed, dimethylchlorosilane-treated); column 260°C; injection port 300°C; separator 260°C; helium carrier gas flow 25 ml/min. Mass spectrometer was operated in combination with glass capillary column (silicone OV-61, 18 m × 0.3 mm id) and platinum capillary interface under conditions described previously (9).

Mass spectrometric conditions.—Electron energy 70 ev; ion energy 8 v; Channeltron particle multiplier voltage 1.8 kv. A programmable multiple ion monitoring attachment (Promim®) was used for simultaneous recording of 5 selected m/e values (mass fragmentography). Instrument was periodically calibrated with perfluorotributylamine (FC-43) bleeding from leak valve into ion source, using peaks at m/e 326, 376, 414, 426, and 464.

Preamplifier, recorder sensitivities, and filtering of individual channels were set as required.

Reference Compounds

(a) *2,3,7,8-Tetrachlorodibenzo-p-dioxin (tetra-CDD).*—Obtained from Stickstoffwerke Linz (Linz, Austria).

(b) *Hexachlorodibenzo-p-dioxin (hexa-CDD).*—Prepared by pyrolysis of potassium 2,3,4,6-tetrachlorophenate at 300°C and recrystallization of sublimate from toluene. Purity at least 90% as indicated by GC, mass spectrometry (MS), and microelemental analysis. Major component present, 1,2,3,6,8,9-hexa-CDD, with minor amounts of the 1,2,4,6,7,9- and 1,2,3,7,8,9-substituted isomers (5% each).

(c) *Octachlorodibenzo-p-dioxin (octa-CDD).*—Prepared by pyrolysis of sodium pentachlorophenate at 380°C and recrystallization from xylene; purity >95%. The pyrolyses were carried out on a 1 g scale under conditions previously described (9).

(d) *Standard solutions.*—(*1*) *Solution I (low level samples).*—0.1, 0.2, and 2.5 μg/tetra-, hexa- and octa-CDD/ml *n*-hexane, respectively. (*2*) *Solution II (high level samples).*—0.1, 2, and 50 μg/ml *n*-hexane, respectively, except use benzene for standard solutions of high octa-CDD content.

(e) *Alumina.*—Aluminum oxide (basic, cationotropic), used as received from Woelm (Eschwege, W. Germany).

(f) *Chromatographic solvents.*—(*1*) *Eluant A.*—Methylene chloride-*n*-hexane (1+49). (*2*) *Eluant B.*—Methylene chloride-*n*-hexane (1+1). Prepare eluants immediately before use.

Safety Precautions

Use extreme caution when handling highly toxic PCDD standards, especially during synthesis and purification of the compounds and during preparation of standard solutions.

Preparation of Sample

Add 4.0 g sample (PCP or PCP-Na) to 30 ml methanol in 250 ml separatory funnel. Add 10 ml 2.5*N* LiOH solution and 100 ml water. Extract with 40 ml petroleum ether by vigorous shaking. Use proportionally larger amounts of sample and reagents for extraction of nonhomogeneous samples. Drain lower phase after checking for alkalinity (pH >12). If separation is difficult or incomplete, add 2–5 g anhydrous Na_2SO_4 to break emulsion. Wash organic phase left in separatory funnel with 100 ml water containing 2 ml 2.5*N* LiOH solution. Repeat washing with 50 ml water. Check last washing for neutral pH. Dry organic phase over anhydrous Na_2SO_4 and carefully concentrate 20 ml aliquot (corresponding to 2 g sample) to ca 2 ml in stream of nitrogen.

Prepare alumina minicolumn by adding 1.0 g dry alumina to disposable Pasteur pipet (15 cm × 5 mm id) containing small plug of glass wool. Pack column by gentle tapping. Quantitatively pipet concentrate of sample onto column. Rinse container with 10 ml eluant A and add rinse to column; discard this fraction. Rinse container with 10 ml eluant B, and add rinse to column to elute PCDDs and PCDFs; keep this fraction for analysis. If required, concentrate sample solution to 2–5 ml in stream of nitrogen before analysis.

Determine recovery by adding different amounts of tetra-, hexa-, and octa-CDD to samples before extraction step.

Determination

Adjust individual channels of Promim attachment to m/e values of 320, 358, 388, 422, and 456, respectively, to monitor tetra-, penta-, hexa-, hepta-, and octa-CDD in first series of runs. Inject different amounts of standard solutions (1–10 μl) and prepare calibration curves for tetra-, hexa-, and octa-CDD, using peak height measurements. Ensure linearity of response in desired range. Sensitivity used on different channels must be individually adjusted. Determine specific responses (peak height in mm × sensitivity in mv/ng standard component) for tetra-, hexa-, and octa-CDD from the calibration curves and plot values on semilogarithmic graph paper vs. chlorine number (4, 6, and 8). From this graph, interpolate specific responses for penta- and hepta-CDD.

Inject 10 μl aliquots of sample (corresponding to 2–10 mg of original PCP) and quantitate by measuring peak heights and comparing with standard curves. Use only peaks eluting in required retention time range of 1–3, 2–4, 3–6, 6–9, and 10–12 min, respectively, for tetra-, penta-, hexa-, hepta-, and octachloro compounds (4). If more than one peak is observed in retention time range, add peak heights and report total. Check response factors and m/e settings periodically.

In second series of runs, change m/e values to 304, 338, 372, 406, and 440 to monitor corresponding members of PCDF series. Use same response factors and retention time ranges as for PCDD series.

If desired, re-inject samples onto glass capillary column for analysis of PCDD isomers. *See* ref. 9 for standards and isomer assignment.

Confirmation by Combined Gas Chromatography-Mass Spectrometry

Carry out complete MS analysis with samples requiring confirmation of presence of PCDDs and PCDFs. Concentrate aliquot of sample in order to inject 10–50 ng of desired component. Run mass spectra (m/e 50–650 in 2 sec) before, during, and after elution of suspected component from column. Compare spectra with those of standards.

Results and Discussion

Scheme of Analysis

The sample preparation scheme employed in this study is outlined in Fig. 2 and has been previously described (4). Phenolic compounds are removed by alkaline extraction and the neutral components are further fractionated on an alumina minicolumn. This rapid sample preparation quantitatively removes polychlorinated phenoxyphenols (PCPPs, predioxins) and polychlorinated diphenyl ethers (PCDPEs) that would interfere in the determination of PCDDs and in the mass fragmentographic detection of PCDFs, respectively. The cleanup further removes polychlorinated biphenyls (PCBs) and benzenes (PCBzs). No components other than PCDDs and PCDFs were observed in the final sample extracts. The same analytical scheme was used for the analysis of PCP and PCP-Na, although, in the latter case, lithium hydroxide may not be required. Larger amounts of higher PCDDs and PCDFs may form a precipitate after concentration of the initial extract and care must be taken to ensure a quantitative transfer onto the alumina minicolumn.

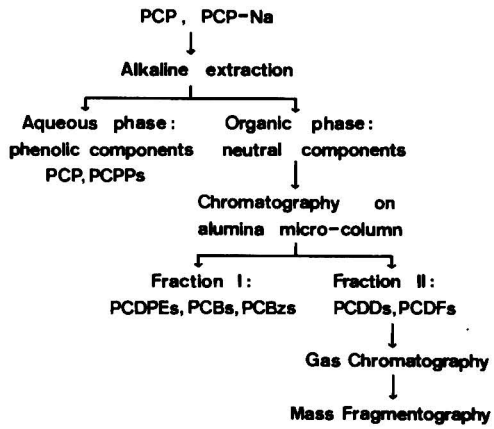

FIG. 2—Scheme of analysis.

Mass Fragmentography

Silicone OV-1 was chosen for GC because this stationary phase is expected to have little selectivity toward isomers, but it still allows the separation of PCDDs and PCDFs according to the number of chlorine atoms in the molecules. After this group separation, individual PCDDs and PCDFs are detected and measured by mass fragmentography, which is one of the most specific means for detecting traces of compounds in a matrix of structurally similar components. Other detectors, such as the electron capture detector, are not sufficiently specific for this application.

PCDDs and PCDFs were specifically detected by mass fragmentography using the molecular ions (M^+) except for penta-CDD (M^+ at m/e 354), where the $[M + 4]^+$ ion of similar intensity was used. The presence of interferences at m/e 354–357 from column bleed and diffusion pump oil background required this change. The molecular ions of PCDDs and PCDFs belong to the most intense ions in their electron impact mass spectra. The expected clustering due to the chlorine isotopes is observed. Although the ^{35}Cl isotope peaks of the molecular ions (M^+) for compounds containing 4 or more chlorine atoms are not the most intense of the corresponding ion clusters, they were chosen for convenience and, in case of some PCDDs, because of possible interference from PCBs at the more intense m/e values ($[M + 2]^+$, $[M + 4]^+$, etc.). More details on mass spectra and fragmentation modes of PCDDs and PCDFs are given in ref. 4.

The multiple ion monitoring attachment (Promim) allowed the simultaneous registration of 5 different m/e values, requiring 2 injections of a sample to determine all tetra- and higher chlorinated species of the PCDD and PCDF series. The sensitivities achieved under these conditions were better than 50, 100, and 200 pg, respectively, for tetra-, hexa-, and octa-CDD. The resulting detection limits of 0.01–0.04 ppm were considered adequate for the present study. If required, the sensitivity can be increased by concentrating the sample solutions. The method is less sensitive to octa-CDD because of its long retention time and the relatively smaller contribution of the ^{35}Cl isotope peak in the molecular ion cluster. The mass fragmentographic responses were sufficiently stable to allow the use of the external standard method. The detection system

is linear for PCDDs from the low picogram to the high nanogram range.

A logarithmic plot of the specific responses of tetra-, hexa-, and octa-CDD was indirectly proportional to the chlorine numbers of these compounds. Response factors for penta- and heptachloro compounds, for which we have no standards available, were determined by interpolation from these graphs. The same response was assumed for positional isomers and the response for PCDFs was assumed to be similar to that of corresponding members within the PCDD series. When multiple peaks were observed (hexa-CDD and hepta-CDD), the peaks were summed and the total was reported, irrespective of isomers. Higher PCDDs and PCDFs may give responses at lower m/e values because of the presence of fragment ions (e.g., $[M - Cl_2]^+$). Because these responses are out of the proper retention time ranges of the lower PCDDs and PCDFs, no confusion occurs.

Results obtained by the external standard method and an internal standard method compared favorably, when known quantities of PCDDs were added to samples before injection. The high specificity of the mass fragmentographic response was established by analyzing standard mixtures of PCDDs and PCDFs and by analyzing PCDDs in the presence of large amounts of added PCBs (H.-R. Buser, 1975, unpublished results).

Recovery

Recovery efficiency was determined by adding tetra-, hexa-, and octa-CDD to PCP and PCP-Na before extraction. Recoveries for tetra-CDD ranged from 80 to 95% at 0.03 to 0.3 ppm (0.12–1.2 μg) in PCP and PCP-Na samples containing low and high amounts of higher (6–8 Cl) PCDDs. Hexa-CDD was recovered from these samples with 80–95% efficiency (0.1–30 ppm) and octa-CDD with 95% efficiency (10–30 ppm). No differences were observed for PCP and PCP-Na samples and the recovery of small amounts of tetra-CDD was not influenced by the presence of significantly larger quantities of higher PCDDs.

Recoveries of PCDFs, with the exception of octa-CDF, were not checked, because standards were not available. Earlier work established complete elution of the PCDFs in the PCDD fraction from the alumina minicolumn (4).

Polychlorinated Dibenzo-p-dioxin and Dibenzofuran Content

In Table 1 the contents of PCDDs and PCDFs in different PCP and PCP-Na samples are reported. The results of some duplicate determinations (Samples 1, 2, 9, 10, and 11) are included to show the repeatability of these results (±10–15%). In Figs. 3 and 4 mass fragmentograms are shown to illustrate the determination of PCDDs and PCDFs in 2 samples, one (Sample 6) with a low, the other (Sample 13) with a high concentration of these components.

From Table 1 the grouping of samples into 2 series can be observed: a first series (Samples 1–8) with low levels and a second series (Samples 9–19) with much higher levels of PCDDs and PCDFs. The ranges of the contents of the higher (6–8 Cl) PCDDs and PCDFs for both sample series are given in Table 2.

The contents of hexa-CDF in all samples were larger than those of hexa-CDD. This was not the case for all samples concerning the contents of other corresponding PCDDs and PCDFs. For most samples the contents of the PCDD and PCDF components were in the order tetra ~ penta < hexa < hepta < octa, and tetra ~ penta < hexa < hepta ~ octa, respectively.

PCP-Na samples showed the presence of unexpected tetra-CDD at 0.05–0.25 ppm. These samples were analyzed for the presence of 2,4,5- and other trichlorophenols by liberation of the free phenols followed by methylation with diazomethane and GC analysis. No trichlorophenol could be detected in either sample (<0.03% relative to PCP). At present we have no well-founded explanation for the presence of tetra-CDD in these samples.

Complete MS analysis confirmed the presence of individual PCDDs and PCDFs in all samples containing tetra- and hexa-CDD above 0.1 and 1 ppm, respectively. The confirmation of tetra-CDD at 0.12 ppm is illustrated in Fig. 5 (Sample 3) and of hexa-CDD at 8–9 ppm in Fig. 6 (Sample 9). The presence of the higher (6–8 Cl) PCDDs and PCDFs in all samples of the second series was confirmed by complete MS analysis (Table 1, Samples 9–19).

Identification of Polychlorinated Dibenzo-*p*-dioxin Isomers

Different isomers of the same PCDD vary significantly in their toxicological properties (5,

Table 1. PCDDs and PCDFs in commercial PCP samples

Sample		Mfgr.	Appearance	PCDD, ppm					PCDF, ppm				
				Tetra-	Penta-	Hexa-	Hepta-	Octa-	Tetra-	Penta-	Hexa-	Hepta-	Octa-
1	PCP	[a]	powder, white	<0.01	<0.03	<0.03	1.0	3.2	<0.02	<0.03	0.15	1.7	1.3
				<0.01	<0.03	<0.03	1.1	2.8	<0.02	<0.03	0.10	1.4	1.2
2	PCP-Na	A	powder, cream-colored	0.16	0.03	<0.03	0.3	1.2	<0.02	<0.03	0.20	1.2	3.0
				0.23	0.03	<0.03	0.3	1.2	<0.02	<0.03	0.10	1.0	2.5
3	PCP-Na	A	powder, cream-colored	0.12	0.03	<0.03	0.3	1.5	<0.02	0.03	0.70	1.3	2.1
4	PCP-Na	A	pellets, cream-colored	0.08	0.03	0.25	2.8	5.1	0.02	0.13	4.1	13	8.6
5	PCP	B	granules, yellow	<0.01	<0.03	0.15	1.1	5.5	0.45	0.03	0.30	0.5	0.2
6	PCP	B	powder, off-white	<0.01	<0.03	0.03	0.6	8.0	<0.02	<0.03	<0.03	<0.1	<0.1
7	PCP-Na	C	granules, light brown	0.06	0.03	0.40	4.2	11	0.02	0.08	1.2	3.6	3.9
8	PCP-Na	[b]	pellets, light brown	0.25	0.08	0.03	0.4	1.5	<0.02	0.03	0.75	2.3	4.1
9	PCP	[a]	flakes, greyish	<0.02	<0.03	9.5	125	160	<0.02	0.05	15	95	105
				<0.02	<0.03	7.2	150	200	<0.02	0.05	19	110	120
10	PCP	[a]	flakes, grey	<0.02	<0.03	9.1	180	280	0.05	0.25	36	320	210
				<0.02	<0.03	9.0	240	250	0.05	0.20	39	280	230
11	PCP-Na	[a]	granules, light brown	0.05	<0.03	3.4	40	115	<0.02	0.05	11	50	24
				0.05	<0.03	3.4	36	105	<0.02	0.03	11	44	29
12	PCP	B	flakes, greyish brown	<0.02	<0.03	10.0	130	210	0.20	0.20	13	70	55
13	PCP	B	flakes, greyish brown	<0.02	<0.03	5.4	130	370	0.07	0.20	9.1	60	65
14	PCP	C	flakes, light brown	<0.02	<0.03	5.2	95	280	0.02	0.40	28	200	230
15	PCP	C	flakes, light brown	<0.02	<0.03	3.3	27	90	<0.02	0.25	12	65	75
16	PCP	D	flakes, light brown	<0.02	<0.03	3.1	50	135	0.04	0.65	23	140	150
17	PCP	D	flakes, light brown	<0.02	<0.03	4.2	54	210	<0.02	0.10	23	160	140
18	PCP	[b]	flakes, light brown	<0.02	<0.03	3.1	54	170	<0.02	0.05	23	180	250
19	PCP	[b]	flakes, light brown	<0.02	<0.03	3.8	90	290	0.02	0.35	30	200	300

[a] Samples from laboratory chemical supplier: 1 = analytical quality, 9–11 = technical quality.
[b] Manufacturer unknown.

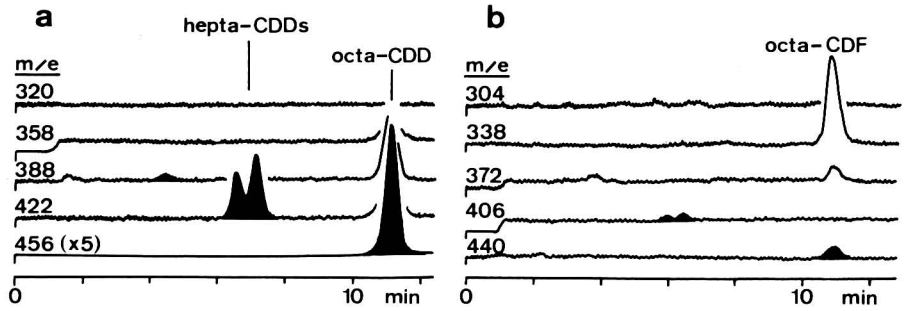

FIG. 3—Mass fragmentograms of PCP Sample 6 illustrating determination of (a) PCDDs and (b) PCDFs. Sensitivity 100 mv or as indicated; 4 mg aliquot injected; results, see Table 1.

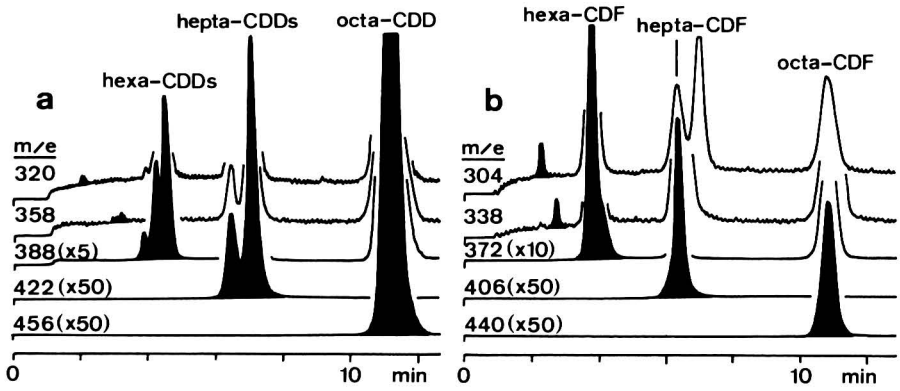

FIG. 4—Mass fragmentograms of PCP Sample 13 illustrating determination of (a) PCDDs and (b) PCDFs. Sensitivity 100 mv or as indicated; 2 mg aliquot injected; results, see Table 1.

Table 2. Range of content of higher (6–8 Cl) PCDDs and PCDFs in commercial PCP samples

	PCDD, ppm			PCDF, ppm		
Series	Hexa-	Hepta-	Octa-	Hexa-	Hepta-	Octa-
Samples 1–8	0.02–0.4	0.3–4.2	1.2–11	0.03–4.1	0.1–13	0.1–8.6
Samples 9–19	3.1–10	27–240	90–370	9.1–39	44–320	24–300

10) and therefore it is important to identify them specifically. For that purpose, all samples containing greater than 0.1 and 1 ppm tetra- and hexa-CDD, respectively, were re-analyzed, using a high-resolution glass capillary column and compared with a reference mixture in which the isomers had been previously assigned (9).

PCP-Na samples with greater than 0.1 ppm tetra-CDD (Samples 2, 3, and 8) showed the elution of one tetra-CDD isomer from the OV-61 glass capillary column. Although these peaks did coincide on OV-61 with the elution of the 2,3,7,8-substituted isomer, re-analysis of these samples on a Carbowax 20M column showed slightly different retention times. The exact chlorine substitution of the tetra-CDD in these samples therefore remains unknown.

In Fig. 7, chromatograms of the reference mixture and of a sample extract (Table 1, Sample 12) show the elution of hexa- and hepta-CDDs (theoretical number of isomers = 10 and 2, respectively). The chromatogram of the sample extract (Fig. 7b) shows the presence of 3 hexa-CDD isomers, tentatively identified as the 1,2,4,6,7,9-, 1,2,3,6,8,9-, and 1,2,3,7,8,9-substituted compounds (ratio = 10:40:50). The chromatogram further shows the presence of both hepta-CDD isomers (ratio = 15:85) in the sample

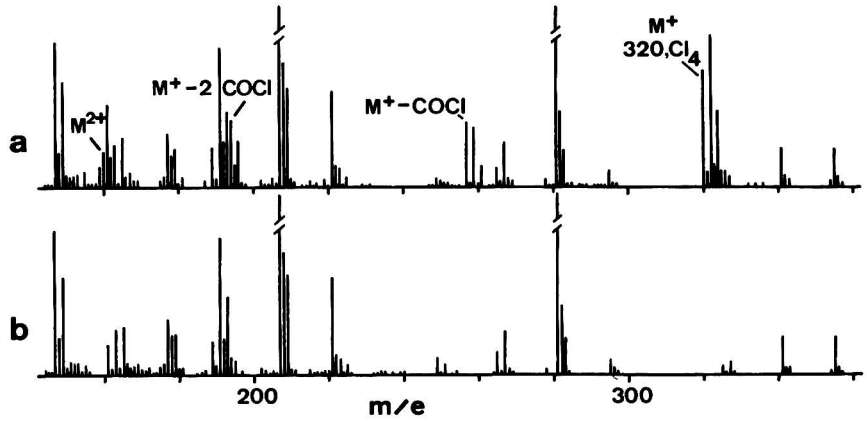

FIG. 5—Mass spectra showing confirmation of tetra-CDD in PCP-Na Sample 3 (Table 1): (a) spectrum at retention time of tetra-CDD (2 min), (b) spectrum of background 10 sec earlier. Differential mass spectrum (a-b) identical to that of standard tetra-CDD.

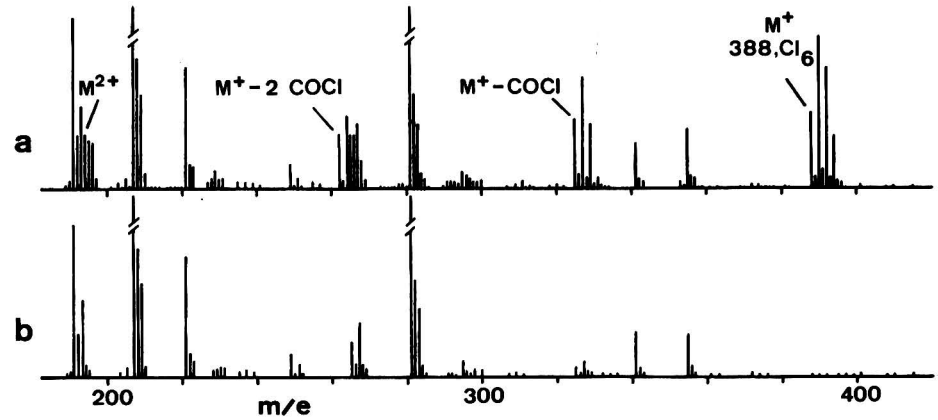

FIG. 6—Mass spectra showing confirmation of hexa-CDD in PCP Sample 9 (Table 1): (a) spectrum at retention time of hexa-CDD (4.5 min), (b) spectrum of background 30 sec later. Differential mass spectrum (a-b) identical to that of standard hexa-CDD.

extract with the 1,2,3,4,6,7,8-substituted compound as the major component. Similar chromatograms with almost identical isomeric ratios of these compounds were found for all samples within this series (Table 1, Samples 9–19). These hexa-CDD isomer identifications are based on pyrolysis experiments of different polychlorophenates (9). The recent observation (11) that Smiles rearrangement reactions may occur during pyrolysis would cause questioning of some of the above identifications of hexa-CDD isomers.

Proposed Specifications

The results of the survey showed, with respect to the levels of toxic PCDDs and PCDFs, large differences in the quality of commercial PCP and PCP-Na. Specifications and legislation are required to prevent environmental contamination with these hazardous compounds through the use of impure products. The specifications proposed by the Swiss Federal Office of Public Health for tetra- and hexa-CDD of 0.1 and 1 ppm, respectively, in chlorinated phenols, which

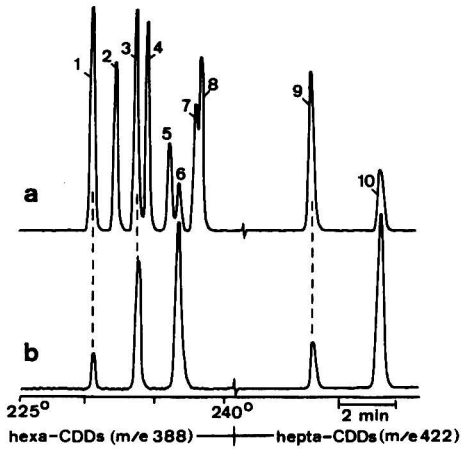

FIG. 7—Partial mass fragmentograms (OV-61 glass capillary column, m/e 388 and 422) of (a) reference mixture and (b) PCP Sample 13 (Table 1) showing elution of hexa- and hepta-CDD isomers. Peak assignments: 1 = 1,2,4,6,7,9-; 2 = 1,2,3,4,6,8-; 3 = 1,2,3,6,8,9-; 4 = 1,2,3,4,6,9-; 5 = 1,2,3,4,7,8-; 6 = 1,2,3,7,8,9-; 7 = 1,2,3,6,7,8-; 8 = 1,2,3,4,6,7-hexa-CDD; 9 = 1,2,3,4,6,7,9-; and 10 = 1,2,3,4,6,7,8-hepta-CDD; assignment for isomers 6 and 7 may be reversed, see text and ref. 11. For experimental conditions see ref. 9.

were based on considerations of toxicity, environmental behavior, and industrial feasibility, could be enforced because a sensitive and specific analytical technique is available now.

Acknowledgments

The authors acknowledge U. Weber for technical assistance in the preparation and analysis of samples, Finnigan Instruments Corp. for the loan of equipment, and S. Allisson, Cantonal Laboratory, Zürich, for the collection of samples.

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MEAT AND MEAT PRODUCTS

Qualitative Tests for Added Coloring Matter in Meat Products

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The rapid qualitative detection of coal tar dyes, chemicals added to color meat products, and some natural coloring materials was investigated through extraction and subsequent identification by spectrophotometric, paper chromatographic, and specific reaction techniques. These techniques will detect the presence of coal tar dyes, imidazole, histamine, histidine, cochineal, beet powder, annatto, alkanet, carotene, paprika, saffron, turmeric, and materials causing discoloration in meats.

Meat Inspection Regulations (1) state: "Coloring matter and dyes other than those specified in the chart in subparagraph 4 of this paragraph, may be applied to products, mixed with rendered fat, applied to natural and artificial casings, and applied to such casings enclosing products, if approved by the Administrator in specific cases. When any coloring matter or dye is applied to casings, there shall be no penetration of coloring into the product. . . ."

Subparagraph 4 indicates that natural and artificial coloring agents may be added "sufficient for purpose" to color casings or rendered fats, and to mark or brand products.

Regulatory laboratories frequently receive samples for identification of coloring materials which have penetrated into meat products, which have been added to them directly, or which are a source of discoloration. These materials may be classified into 3 categories: coal tar or artificial, synthetic dyes; chemicals causing color changes in heme pigments; and natural coloring materials. Quick, inexpensive, qualitative tests for these identifications are of extreme value to the regulatory chemist. The purpose of this paper is to present a compilation of such tests for those materials which have been used in meat products.

This report of the Associate Referee was presented at the 89th Annual Meeting of the AOAC, Oct. 13–16, 1975, at Washington, DC.

Experimental

Samples are often contaminated in small areas or on surfaces. Select a portion of the sample which appears to be the most contaminated and extract with a minimum of solvent.

Coal Tar Dyes (2, 3)

Principle

Dyes are extracted from meat and the visible spectra are compared to standards at various pH levels, or the R_f values are compared to standards by paper chromatography.

Apparatus and Reagents

(a) *Spectrophotometer.*—Recording, capable of operating in range of 700–350 nm.

(b) *Chromatographic developing tank.* — For $8 \times 8''$ sheets.

(c) *Chromatographic paper.*—Whatman No. 1, $8 \times 8''$.

(d) *Hydrochloric acid.*—About 0.2*N*. Dilute 18 ml HCl to 1 L.

(e) *Sodium hydroxide.*—About 0.2*N*. Dilute 11 ml 50% (w/w) NaOH or 8 g pellets to 1 L.

(f) *Inorganic solvent system.*—25% NH_4OH-2.5% sodium citrate-water (45+10+45).

(g) *Organic solvent system.*—*n*-Propanol-ethyl acetate-water (6+1+3).

(h) *Standard dye solutions.*—About 0.1 g/L water for spectrophotometry and saturated solutions for paper chromatography.

Analysis

(a) *Spectrophotometry.*—Slurry meat with minimum amount of warm water or 80% ethanol, let stand 5 min, and filter. Divide filtrate into 3 equal portions. Evaporate each portion just to dryness on steam bath; do *not* boil. Dissolve residue from one portion in water, the second in 0.2*N* HCl, and the third in 0.2*N* NaOH. Filter if necessary. Scan each resultant solution with recording spectrophotometer from 700 to 350 nm. Compare spectra with those of known dyes for bathochromic and hypsochromic effects of pH on absorbance peaks.

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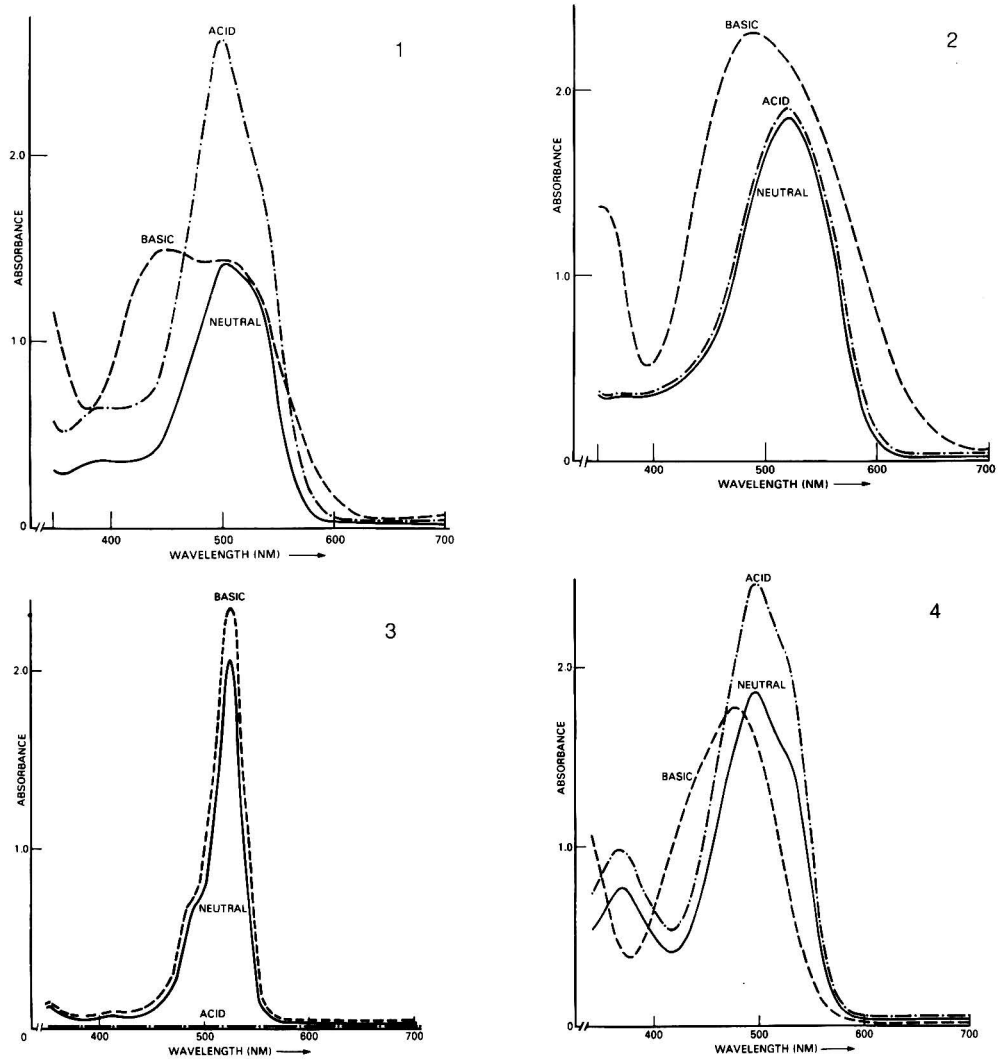
(*See* Figs. 1–19 for typical spectra in acidic, basic, and neutral solutions.)

(b) *Paper chromatography* (*4*).—Prepare filtrate as above; then evaporate to concentrate dye. Using proper spotting techniques, spot unknown dye and standards suspected to be present. Develop chromatogram in saturated development tank until solvent front is 1–2″ from top, utilizing either inorganic or organic solvent system. Approximate R_f values are listed in Table 1. These values may differ slightly, depending on degree of tank saturation; however, this can be controlled by developing paper chromatograms for a specified time. Standard or suspected dyes may be added to samples to allow for co-extractives or detection of mixtures.

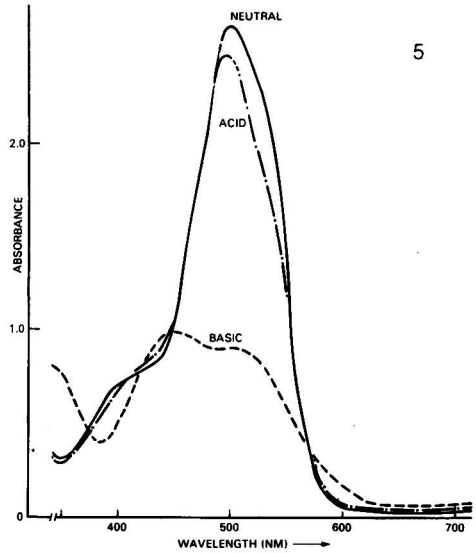
Chemicals Affecting Heme Pigment Coloration (5)

Principle

Chemicals affecting heme pigment coloration, imidazole, histamine, and histidine, are extracted from meat samples. The extract is subjected to paper chromatography and chromatograms are sprayed with chromogenic reagents to produce visible spots. Imidazole reacts with a silver nitrate spray to produce a silver salt that becomes visible



FIGS. 1–19—UV spectra of selected color additives. See Table 1 for identification. Solutions: acidic = 0.2*N* HCl, basic = 0.2*N* NaOH; neutral = distilled water.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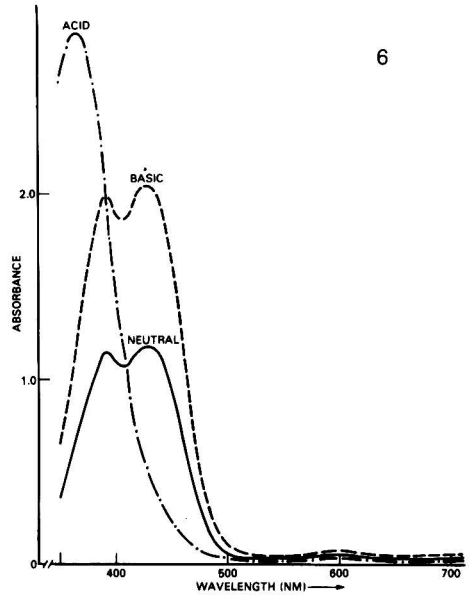
WAVELENGTH (NM)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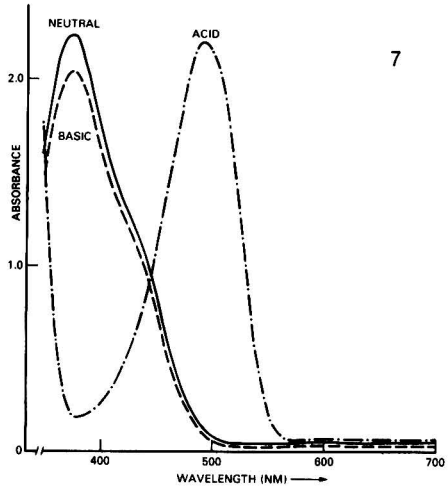
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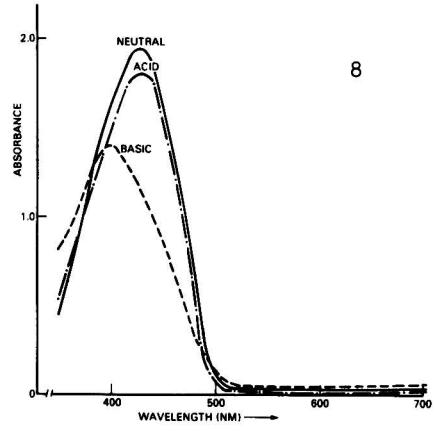
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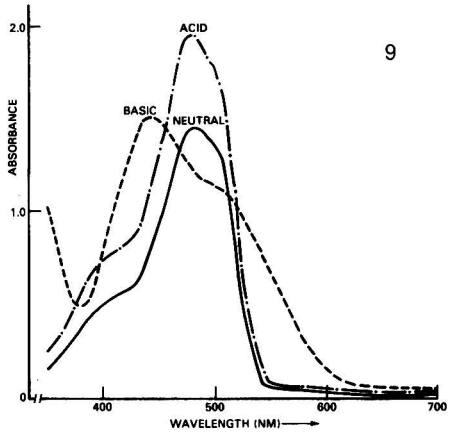
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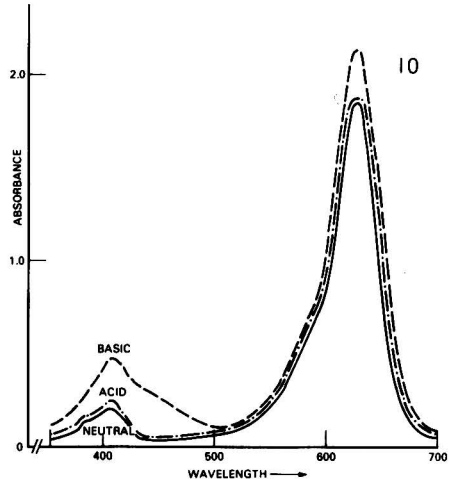
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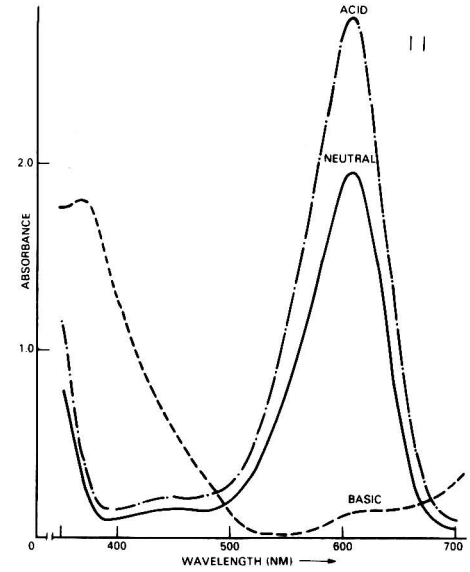
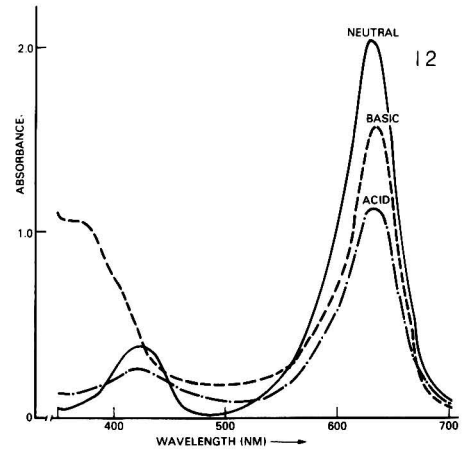
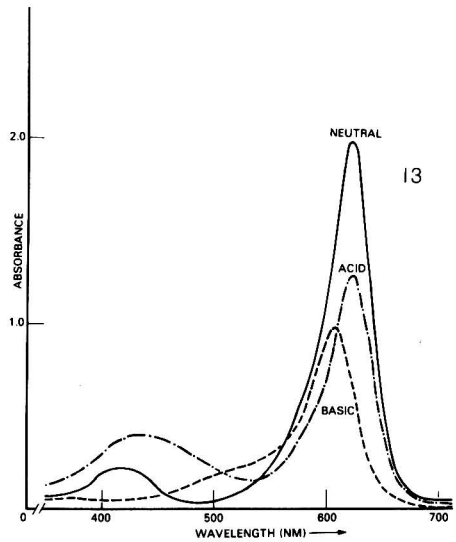
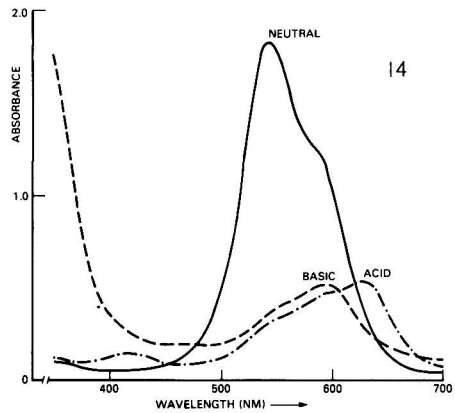








when exposed to ultraviolet (UV) light. Imidazole, histamine, and histidine all couple with diazotized aromatic amines as chromogenic reagents to form C-azo dyes which become orange when resprayed with sodium carbonate solution.

Apparatus and Reagents

(a) *UV light source.*—Longwave.

(b) *Standard solutions.*—Separately dilute 0.1 g imidazole, histamine, and histadine to 100 ml with water.

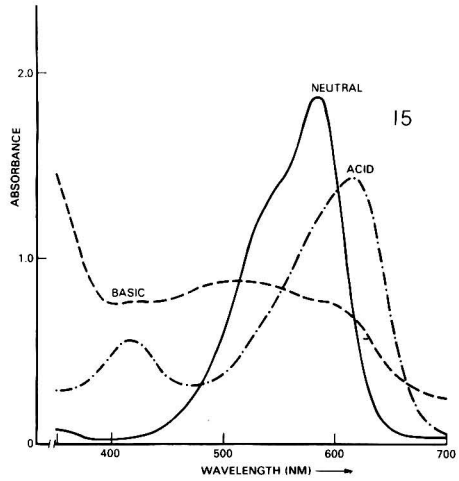
(c) *Developing solution A.* — *n*-Butanol-acetic acid-water (80+20+20).

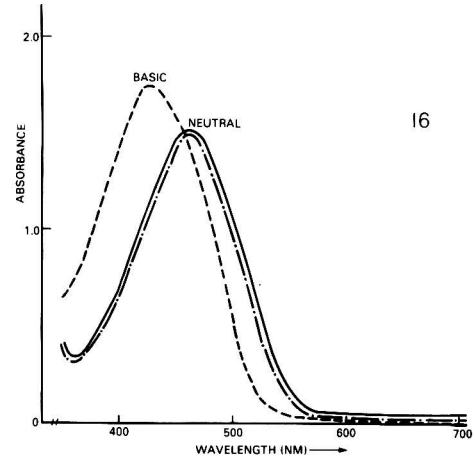
(d) *Developing solution B.* — *n*-Propanol-0.2*N* NH_4OH (3+1).

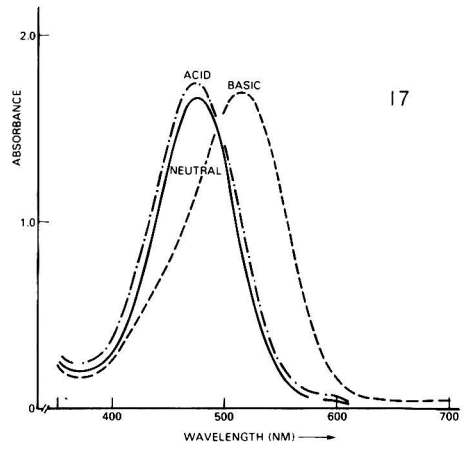
(e) *Silver chromogenic reagent.*—Dissolve 0.1 g $AgNO_3$ in 1 ml water, add 20 ml 2-phenoxyethanol, and dilute to 200 ml with acetone. Add 1 drop 30% H_2O_2, and mix.

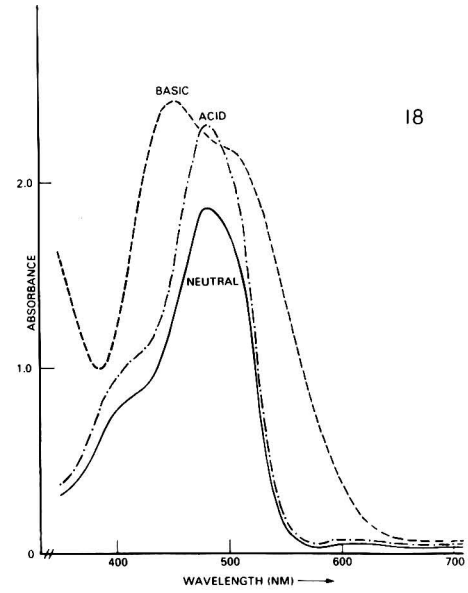
(f) *Diazotized p-bromoaniline solution.* — Dissolve 0.1 g *p*-bromoaniline in 50 ml cold water (use cold beaker). In another cold beaker, dissolve 0.035 g sodium nitrite in 25 ml cold 0.05*N* HCl. Combine the 2 solutions and filter. Store in refrigerator.

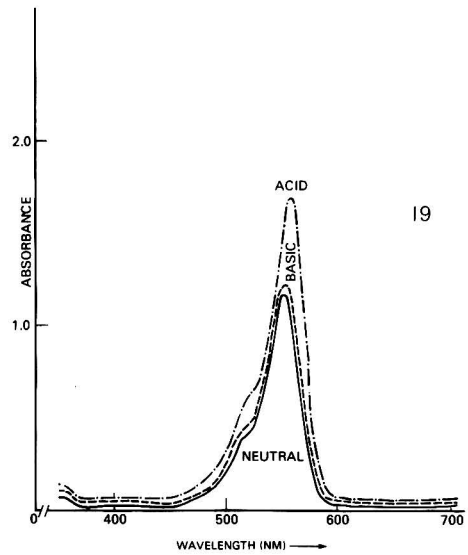
(g) *Diazotized sulfanilic acid solution.*—Dissolve











0.1 g sulfanilic acid in 50 ml cold water (use cold beaker). In another cold beaker, dissolve 0.035 g sodium nitrite in 25 ml cold 0.05*N* HCl. Combine the 2 solutions and filter. Store in refrigerator.

(h) *Sodium carbonate solution.*—1%. Dissolve 1 g Na_2CO_3 in water and dilute to 100 ml with water.

Analysis

(a) *Imidazole.*—Weigh 10 g sample. Dilute to 100 ml with water, mix, and filter. Spot 10 µl filtrate and 10 µl standard imidazole solution on 8 × 8″ Whatman No. 1 chromatographic paper. Develop 2–3 hr in saturated tank with developing solution A. Let air-dry. Spray evenly with silver chromo-

Table 1. R_f values for coal tar dyes

Dye	Fig.	Color Index No.	Inorg. solv.[a]	Org. solv.[b]
FD&C Red No. 1[c]	1	16155	0.15	0.32
FD&C Red No. 2[c]	2	16185	0.55	0.20
FD&C Red No. 3	3	45430	0.05	0.70
FD&C Red No. 4	4	14700	0.42	0.50
FD&C Red No. 40	5	16035	0.35	0.45
FD&C Yellow No. 1[c]	6	10316	0.70	0.50
FD&C Yellow No. 4[c]	7	11390	0.20	0.96
FD&C Yellow No. 5	8	19140	0.85	0.21
FD&C Yellow No. 6	9	15985	0.77	0.35
FD&C Blue No. 1	10	42090	0.95	0.46
FD&C Blue No. 2	11	73015	0.18	0.21
FD&C Green No. 2[c]	12	42095	1.00	0.39
FD&C Green No. 3	13	42053	1.00	0.46
FD&C Violet No. 1[c]	14	42640	0.80	0.65
Methyl violet	15	42535	0.03	1.00
Orange B	16	19235	0.57	0.45
Orange No. 1	17	14600	0.36	0.61
Orange No. 2	18	15510	0.36	0.64
Rhodamine B	19	45170	—	—

[a] NH_4OH-2.5% sodium citrate-water (45+10+45).
[b] *n*-Propanol-ethyl acetate-water (6+1+3).
[c] These colors are no longer permitted for use in foods, drugs, and cosmetics.

genic reagent and let air-dry. Expose to UV light 15–30 min until purple spot at R_f 0.5 for the standard becomes visible. Compare standard and sample chromatogram for purple spot at same R_f indicating the possible presence of imidazole. Confirm presence of imidazole as described in (b).

(b) *Histamine and imidazole.*—Prepare sample and develop chromatogram spotted with sample and standards in (a), except use developing solution B. Spray evenly with diazotized *p*-bromoaniline and let air-dry. Spray with 1% sodium carbonate. Imidazole is bright orange spot at R_f 0.9, and histamine is red spot at R_f 0.6.

(c) *Histidine.*—Prepare sample and develop chromatogram spotted with sample and standards in developing solution B as in (b). Spray evenly with diazotized sulfanilic acid and let air-dry. Spray with 1% sodium carbonate. Histidine is red spot at R_f 0.2.

Natural Coloring Agents and Discoloration

The following tests are all based on specific reactions and must be run in parallel with standards and blanks.

Apparatus and Reagents

(a) *Chromatographic column.*—200–300 cm × 10 mm id with coarse fritted plate and Teflon stopcock (Pyrex 7282, or equivalent).

(b) *Ashing crucibles.*—30 ml, Vycor.

(c) *Borax solution.*—5%. Dilute 5 g sodium borate to 100 ml with water.

(d) *Sulfuric acid.*—1*N*. Dilute 29 ml 94% H_2SO_4 to 1 L with water.

(e) *Stannous chloride.*—40%. Dilute 40 g $SnCl_2$ to 100 ml with water.

(f) *Sodium hydroxide.*—2%. Dilute 2 g NaOH to 100 ml with water.

(g) *Florisil.*—Available from Fisher Scientific Co., No. 100, or equivalent. (No activation is necessary.)

(h) *Alizarin S solution.*—Dissolve 0.1 g alizarin sulfonic acid (or its sodium salt) in ca 100 ml water.

(i) *Sulfanilic acid.*—1%. Dilute 1 g sulfanilic acid to 100 ml with water.

(j) *Sodium nitrite.*—1%. Dilute 1 g $NaNO_2$ to 100 ml with water.

(k) *Methanolic potassium hydroxide.*—Dissolve 10 g KOH in 90 ml methanol.

(l) *Potassium nitrite.*—2%. Dilute 2 g KNO_2 to 100 ml with water.

(m) *Salicylic acid solution.*—Dissolve 0.5 g hydroxybenzoic acid in 10 ml ethanol and dilute to 100 ml with water.

(n) *Potassium thiocyanate solution.*—Dissolve 1 ml KCNS in ca 100 ml water.

(o) *Sodium carbonate solution.*—Dilute saturated Na_2CO_3 solution with water (1+1).

(p) *Folin-Denis reagent.*—Heat 2.5 g sodium molybdate, 9.3 g phosphotungstic acid, and 5 ml 85% phosphoric acid with 75 ml water for 2 hr under reflux condenser. Cool and dilute to 100 ml with water.

Analysis

Cochineal (carminic acid, carmine red) (6).—Weigh ca 25 g meat into beaker. Add 100 ml hot (80°C) 5% borax solution. Mix on steam bath 30 min and filter. Purple filtrate indicates presence of cochineal; yellow is negative. If > 0.1% compound is present in sample, addition of borax solution will give positive test.

Beet powder (7).—Slurry suspected sample with 1*N* H_2SO_4. Purple color indicates presence of beet powder. For confirmation, filter and divide into 3 portions. Adjust portions to pH 2, 5, and 9, respectively, with dilute H_2SO_4 and NaOH. With scanning spectrophotometer, scan each solution from 700 to 400 nm. Maximum absorbance peaks for beet powder will be at 535, 537, and 544 nm at pH 2, 5, and 9, respectively.

Annatto and saffron (8).—Mix 25–50 g sample with 200 ml ethyl ether and 2 ml concentrated HCl and filter through anhydrous Na_2SO_4 in funnel with glass wool pledget. Extract 10 ml dried ether extract with ca 3 ml 2% NaOH. Absorb any color present on strip of filter paper and air-dry. Dip dried paper in concentrated H_2SO_4. Blue color on

paper is positive test for annatto or saffron. To differentiate, add drop of 40% $SnCl_2$ to another strip on which color has been absorbed and let air-dry. If annatto is present, paper will be pink to purple at spot of $SnCl_2$. If annatto is absent, or if previous test was positive due to saffron only, there is no change in color.

Paprika and turmeric.—Prepare chromatographic column to contain 10 cm unactivated Florisil topped with ca 2 cm anhydrous Na_2SO_4. Prewet with ethyl ether. Mix 50 g sample with 200 ml ethyl ether and 2 ml concentrated HCl. Let set 5 min. Filter through funnel containing 10 g anhydrous Na_2SO_4 placed on top of prepared column. Let extract pass through column at 3 ml/min. When extract has sunk into column, remove funnel, and wash column with 50 ml petroleum ether flowing at same rate.

If paprika is present, red band will appear at interface of Na_2SO_4 and Florisil. It will turn yellow and elute from column with ethyl ether.

If turmeric is present yellow band appears at interface. (This is not conclusive evidence, however.) Elute band with 150 ml acetone. Dilute acetone eluant with 300 ml water and mix. Add 3–4 drops of concentrated HCl and few crystals of boric acid. If resultant solution turns red, turmeric is definitely present.

If both coloring agents are suspected, prepare 2 columns and run each of the above procedures separately.

Alkanet.—Extract 25–50 g sample with 100 ml ethanol and filter. Add 10 ml 10% NaOH solution. Blue color indicates presence of alkanet.

Carotene.—Blend 30 g sample 4 min with 40 ml water, 40 ml methanol, and 80 ml $CHCl_3$. Let set 5 min. Filter through glass wool. Dilute 5 ml aliquot of lower ($CHCl_3$) layer to 100 ml with $CHCl_3$. Compare spectrum obtained on recording spectrophotometer with that of solution containing 10 mg β- carotene/100 ml $CHCl_3$. Major peak of absorbance is at 462 nm. (It should be noted that carotenes are natural components in meat and seasonings, and care must be taken in determining if resultant color in product is due to *addition* of carotene.)

Chemicals Causing Discoloration in Meat

Iron, copper, and aluminum.—Ash portion of discolored product in crucible, fuse ash with potassium bisulfate, and dissolve residue in few drops of concentrated HCl. Mix with few drops of KCNS solution and small amount of ethyl ether. Red color in ether layer indicates presence of iron. For aluminum, add 2 drops of alizarin S solution to fused and acidified ash. Add dilute NaOH until mixture is purple. Acidify with concentrated acetic acid and note the formation of a red lake. (Iron interferes by also forming red lake.) For copper, neutralize fused and acidified ash with 10% NaOH and filter. Add 5 drops of 2% KNO_2 and mix. Add 5 drops of 10% acetic acid and mix. Add 3 ml salicylic acid solution, mix, and heat 30–45 min on steam bath. Red color indicates presence of copper.

Biliverdin.—Extract greenish discolored product with water or ethanol and filter. Add filtrate to sulfanilic acid-$NaNO_2$ mixture (5+2). Violet color, intensified upon addition of concentrated HCl, indicates presence of bile pigments.

Chlorophyll.—Extract discolored sample with ethyl ether and filter. Add small amount of methanolic KOH. If chlorophyll is present, mixture changes to brown and then back to green.

Phenolic compounds.—Boil some discolored sample in Na_2CO_3 solution and filter. Add 5 ml Folin-Denis reagent. Blue color indicates presence of phenolic compounds.

Results and Discussion

Spectrophotometric procedures can detect smaller concentrations of dye than paper chromatography, providing the scanned solutions are not turbid. The 0.2*N* solvents were chosen because most analytical meat laboratories already have them prepared for other determinations. Other concentrations of acid and base may be used if desired, but the resultant spectra must be compared with standards having the same concentrations, as shifts in absorbance peaks are affected by stronger solutions. The spectrophotometric method is limited because of its inability to distinguish certain mixtures where absorbance peaks are near the same wavelength.

Paper chromatograms may be affected by coextractives from meat products as well as the degree of tank saturation and the type of tank used. The R_f values given in Table 1 are approximate. Standards must always be spotted with unknowns, and here again, certain mixtures cannot be identified because of similar R_f values. The organic solvent system is preferred; however, it may be necessary to respot and develop with the inorganic solvent system when dyes with similar R_f values can be more readily separated by it. Although the color itself will sometimes indicate which dye is present, this could lead to gross error in some cases. A drop of 0.2*N* acid or base placed on developed dry spots sometimes can differentiate between some dyes. For example, Orange No. 1 turns red with alkali, whereas FD&C Green No. 3 turns purple, and

FD&C Blue No. 2 disappears as does FD&C Red No. 3 in acid.

It is imperative that cold glassware and water be used for diazotization. The *p*-bromoaniline will not completely dissolve in 50 ml cold water, necessitating the filtration step. The diazotized solutions should be colorless. If the solutions are yellow, indicating breakdown products, fresh reagents should be prepared.

There are many chemicals which react with heme pigments to give colors (9–11), some of which are not desirable. Imidazole, histamine, and histidine produce red shades and have been found added to commercial products. Niacin, sulfites, benzoates, and sorbates also produce red colors. Analytical procedures for these already appear in *Official Methods of Analysis* (12). Spectrophotometric identification for the heme pigments is described in ref. 13. When a meat extract is scanned for possible addition of coloring materials, the spectra should always be compared with those of the heme pigments (13, 14).

Many combinations of harmless substances can cause the formation of local or general discoloration in meat. On elimination of bacterial causes, gray or black discoloration is often found to be a combination of a phenolic compound with iron. The most common phenolic compounds are tannin from product contact with wood and pyroligneous acid from natural or artificial smoke. Contact with aluminum often results in gray-to-black "stains." Under magnification, the product often shows chunks or smears of heavy grease from processing equipment, or pieces of charred meat from improperly cleaned kettles or poorly regulated retorts. Green, especially on fatty areas, is due to biliverdin, chlorophyll, or copper. Often it is only an optical illusion caused by a thin film of fatty material formed while cutting a product. Red spots may result from small blood vessels bursting during slaughter due to the animal's stress.

The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

Recommendation

The tests presented in this paper are by no means the only ones available. They have, however, been used successfully in regulatory practice. New compounds are being continually introduced into the meat industry. The Associate Referee recommends further study on this topic with more emphasis on quantitative measurement.

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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* (1976) *JAOAC* **59**, 386.

COLOR ADDITIVES

Quantitative Determination of 4,4′-(Diazoamino)-bis(5-methoxy-2-methylbenzenesulfonic Acid) in FD&C Red No. 40 by Ion Exchange Chromatography

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An ion exchange chromatographic procedure is presented which separates 4,4′-(diazoamino)-bis(5-methoxy-2-methylbenzenesulfonic acid) (DMMA) from FD&C Red No. 40. The DMMA is determined spectrophotometrically. Recoveries of DMMA added to FD&C Red No. 40 at levels of 0.05 to 0.20% ranged from 90 to 110%. DMMA was found in commercial samples of FD&C Red No. 40 in amounts ranging from <0.02 to 0.11%.

4,4′ - (Diazoamino) - bis(5 - methoxy - 2 - methylbenzenesulfonic acid) (DMMA) has been identified as a possible component of FD&C Red No. 40 (1). A high-pressure liquid chromatographic (HPLC) method has been described for its determination (M. Singh, 1975, Food and Drug Administration, Washington, DC, unpublished report). Another HPLC method has been developed and collaboratively studied by Marmion (2). An alternative method of analysis is desirable because many laboratories do not have a high-pressure liquid chromatograph. The method presented here uses a strong basic anion exchange cellulose packing. The buffer and eluant are similar to the primary and secondary solvents of the Singh HPLC method. The fraction containing DMMA is collected and the DMMA is determined spectrophotometrically.

METHOD

Apparatus

(a) *Spectrophotometer.*—Cary Model 118, or equivalent.

(b) *Chromatographic tube.*—22 mm id × 10 cm glass, with ca 200 ml reservoir top and 22 mm fritted disk.

Reagents

(a) *Column material.*—Cellex D, hydroxide form, standard capacity (DEAE cellulose) (Bio-Rad Laboratories, 32nd and Griffin Aves, Richmond, CA 94804).

(b) *Buffer.*—0.01*M* sodium borate. Dissolve 38.1 g $Na_2B_4O_7.10H_2O$ in 1 L water. Take 100 ml aliquot and dilute to 1 L with water.

(c) *Eluant.*—0.20*M* sodium perchlorate-0.10*M* sodium borate. Dissolve 351.6 g $NaClO_4.H_2O$ in 500 ml water (5*M*). Mix 40 ml aliquot of this solution and 100 ml aliquot of 0.1*M* sodium borate, and dilute to 1 L with water.

(d) *Standard solution.*—Prepare DMMA according to Bailey and Cox (1). Dissolve 10 mg in 1 L eluant, and determine absorptivity at 385 nm.

Procedure

Add 500 mg FD&C Red No. 40 to 10 ml volumetric flask, dissolve by adding ca 7 ml buffer and swirling, and dilute to volume with buffer.

Prepare slurry of 3.0 g Cellex D and ca 50 ml buffer, and pour into chromatographic tube. Let column form and all liquid enter column. Clean column by carefully adding ca 50 ml eluant. When all eluant has entered column, carefully add ca 100 ml buffer and let this enter column.

With column clamped off, add 1 ml (50 mg) sample solution to top of column. Let sample enter column, and wash sides of tube twice with ca 10 ml buffer until all sample has entered column. Carefully add ca 10 ml eluant to top of column and let all liquid enter column. Fill reservoir with eluant and let column develop.

Discard first 75 ml, and collect next ca 150 ml, which contains DMMA. Measure exact volume of DMMA fraction, and determine absorbance at 385 nm, using 5 cm or longer cell.

Calculation

$$\% \text{ DMMA} = (A \times V \times 100\%)/(W \times L \times A')$$

where A = sample absorbance at 385 nm; V = volume of sample fraction, ml; W = sample weight, g; L = cell length, cm; and A' = absorptivity of standard at 385 nm, A-ml/mg-cm.

Results and Discussion

Table 1 gives the recoveries of DMMA added to a sample of FD&C Red No. 40 containing no detectable DMMA and analyzed by the anion

Table 1. Recoveries of DMMA added to 50 mg samples of FD&C Red No. 40 and analyzed by ion exchange chromatography

Added, %	Found, %	Rec., %
0.20	0.18	90
	0.19	95
	0.18	90
	0.19	95
0.10	0.11	110
	0.095	95
	0.10	100
	0.10	100
0.05	0.048	96
	0.047	94
	0.052	104
	0.049	98
0.02	0.025	125
	0.023	115
	0.025	125
	0.025	125

exchange chromatographic method. Recoveries of DMMA added at levels of 0.05 to 0.20% ranged from 90 to 110%. The high recoveries at the 0.02% level seem to be due to interference, which increases as the levels of DMMA decrease. The precision at low levels, however, is good.

Table 2 gives results of analyses of 5 commercial samples of FD&C Red No. 40 for DMMA by HPLC and by ion exchange chromatography. The results from the 2 methods are in good agreement. Sample E showed interference from a component absorbing at 475 nm. Samples with no peak at 385 nm can be assumed to contain <0.02% DMMA.

The columns cannot be reused because of the high affinity of FD&C Red No. 40 for cellulose.

This paper was presented at the 89th Annual Meeting of the AOAC, Oct. 13–16, 1975, at Washington, DC.

Table 2. DMMA found (%) in commercial samples of FD&C Red No. 40, using high-pressure liquid chromatography (HPLC) and ion exchange chromatography (IEC)

Sample	HPLC	IEC
A	0.105	0.099, 0.105, 0.095
B	0.054	0.055, 0.057
C	0.082	0.070, 0.075
D	0.025	0.024, 0.028
E	0.01	<0.02, <0.02

The dye cannot be washed completely off the column.

There is some variance between columns made by the same procedure with Cellex D, particularly the flow rate. If a particular column is flowing at a rate of <1.5 ml/min, the column flow can be increased by applying a small amount of suction to the bottom of the column for about 1 sec.

It is essential that the samples be dissolved only in buffer, as the pH range of FD&C Red No. 40 samples includes values low enough to destroy DMMA (M. Singh, 1975, Food and Drug Administration, Washington, DC, unpublished report).

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VITAMINS AND OTHER NUTRIENTS

Determination of Vitamin B_{12} in Dry Feeds by Atomic Absorption Spectrophotometry

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Vitamin B_{12} was determined in dry feeds by atomic absorption spectrophotometry (AAS). Samples containing B_{12} were extracted with an assay solution, 5 g EDTA was added to the filtrate, the pH was adjusted to 7 with NH_4OH, and 5 g charcoal was added. The charcoal was removed by filtering through ashless paper which was then placed in a beaker and ashed at 600°C. After dissolving the cobalt oxide from the ash in 5*N* HNO_3, cobalt content was determined by using AAS. To determine mg B_{12}/lb feed, ppm cobalt in the feed is multiplied by 10.43. The sensitivity of the proposed procedure is 1 mg vitamin B_{12}/lb. The procedure is rapid and precise, and results compare favorably with AOAC method 43.109.

Vitamin B_{12}, cyanocobalamin, can be assayed by biological and chemical methods. The method of analysis recognized by the Association of Official Analytical Chemists is a microbiological assay (1). Since vitamin B_{12} contains 4.35% cobalt, several investigators have employed atomic absorption spectrophotometry (AAS) to determine cobalt and have used this value to calculate vitamin B_{12}. Some investigators (2–4) determined cobalt by directly aspirating solutions of pharmaceutical vitamin preparations. These AAS methods, however, are not applicable for determining vitamin B_{12} in feeds, since the level of this vitamin in most feeds is too low for direct aspiration of extracts. In addition, some feeds contain supplemental inorganic cobalt which must be separated from the vitamin. Therefore, a method was developed which eliminates inorganic cobalt and concentrates vitamin B_{12} for AAS analysis.

METHOD

Apparatus

(a) *Atomic absorption spectrophotometer.* — Techtron Model AA-5 with Beckman Model 1005 linear-log 10″ potentiometric recorder, or equivalent, cobalt cathode tube, and equipment for air-acetylene operation.

(b) *Filter tube.*—36 × 160 mm (Corning).

(c) *Büchner funnel.*—Modified California, polyethylene, 9 cm.

(d) *Filter disks.*—Asbestos, 36 mm diameter, 2 μm pore size.

(e) *Filter paper.*—15 cm, ashless (Whatman No. 42, or equivalent).

Reagents

(a) *Vitamin B_{12} extraction solution.*—*See* 43.108 (1).

(b) *Filter aid.*—Celite 545.

(c) *Charcoal.*—Darco G-60 activated carbon, or equivalent.

(d) *Cobalt standard solutions.*—(*1*) *Stock solution.*—100 μg Co/ml. Dissolve 0.4769 g $CoSO_4.7H_2O$ in 500 ml 10*N* HNO_3. Dilute to 1 L with water. (*2*) *Working solutions.*—Dilute stock solution to 0.2, 0.5, 1, 5, and 10 μg Co/ml 5*N* HNO_3.

Preparation of Sample

Weigh feed sample to contain from 15 to 500 μg vitamin B_{12} into 125 ml Erlenmeyer flask and add 75 ml B_{12} extraction solution. Let stand 5 min, swirling occasionally. Join filter tube to 500 ml filter flask. Insert asbestos disk into filter tube and add ca 2 cm layer of filter aid onto asbestos. Apply vacuum. Use water to transfer Erlenmeyer contents onto filter aid. Wash residue with additional water (ca 25 ml portions) until filtrate volume is ca 200 ml. Add 5 g EDTA to filtrate and adjust to ca pH 7 with NH_4OH. Swirl to dissolve EDTA. Add 5 g charcoal and mix well. Press filter paper into Büchner funnel to cover sides and bottom. Transfer contents of Erlenmeyer flask with water onto paper and wash 5 times with 70°C water (ca 20 ml portions). If >100 μg inorganic cobalt is present in contents of Erlenmeyer flask, wash 25 times. After washing charcoal, transfer filter and contents to beaker, and ash at 600°C ca 12 hr. Cool and add 3.0 ml 5*N* HNO_3 to ash. Swirl, tilt, and aspirate upper fine suspension from beaker into atomic absorption spectrophotometer.

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Determination

Analyze cobalt by AAS at 240.725 nm with slightly reducing air-acetylene flame and slit width of 25 μm. Calculate mg B_{12}/lb feed, using the following equation:

$$\text{mg } B_{12}\text{/lb feed} = (\text{ppm Co} \times \text{ml } 5N \text{ HNO}_3 \times (0.001 \text{ mg}/\mu\text{g}) \times (22.99 \text{ units } B_{12}\text{/units Co}) \times (453.59 \text{ g/lb}))/\text{g feed}$$

or

$$\text{mg } B_{12}\text{/lb feed} = (\text{ppm Co} \times \text{ml } 5N \text{ HNO}_3 \times 10.43)/\text{g feed}$$

Results and Discussion

Several commercial feeds containing no detectable vitamin B_{12} were composited. To 5 g portions of this mixed feed, 6 levels of vitamin B_{12} standard ranging from 23 to 340 μg were added. The values in Table 1 indicate 96–103% recovery of supplemental vitamin B_{12} when samples were analyzed by the proposed method.

To determine contamination from inorganic cobalt, three 5 g samples were spiked with 100 μg cobalt (as cobalt chloride) and 3 with 1000 μg cobalt. The samples were extracted with vitamin B_{12} extraction solution. EDTA and charcoal were added as proposed. After charcoal filtration, one charcoal filter and contents exposed to each inorganic cobalt level were washed 5, 15, and 25 times each with about 20 ml 70°C water. Contamination from elemental cobalt was below detectable levels after 5 and succeeding washings of the samples containing 100 μg cobalt (Table 2). However, 25 washings were necessary to eliminate detectable cobalt from feeds containing 1000 μg cobalt/sample.

Commercial feeds or premixes containing from 1.2 to 1000 mg vitamin B_{12}/lb were analyzed by AOAC method **43.109** (1) and the proposed method. The quantity of vitamin B_{12} determined by the proposed method compared favorably with that of the AOAC method (Table 3). The results of these experiments validate the proposed procedure for the determination of vitamin B_{12} in mixed feeds.

Table 1. Recovery of vitamin B_{12} added to 5 g feed[a]

Added, μg	Recd., μg	Rec., %
23	22±3	96
69	71±2	103
138	141±6	102
207	213±8	103
345	352±6	102

[a] Mean and standard deviation based on 5 analyses.

Table 2. Washing necessary to prevent contamination from inorganic cobalt added to 5 g feed

No. of water washings	Co added, μg	Co contam., μg
5	100	<0.4
15	100	<0.4
25	100	<0.4
5	1000	1.2
15	1000	0.6
25	1000	<0.4

Table 3. Comparison of AOAC and proposed methods for determination of vitamin B_{12} (mg/lb) in commercial feeds

Guarantee	AOAC[a]	Proposed[b]
1.2	1.1± 0.2	1.5± 0.2
10.0	9.3± 0.5	10.4± 1.5
33.0	32.1± 4.0	32.4± 1.9
600.0[c]	557.0± 44.4	553.0±59.3
1000.0[c]	985.0±155.3	962.0±50.3

[a] Mean and standard deviation based on 3 analyses.
[b] Mean and standard deviation based on 5 analyses.
[c] Vitamin B_{12} premix.

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Relationship Between the Biological Availability and Solubility Rate of Reduced Iron

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Solubility rate determinations were conducted on electrolytic iron and hydrogen reduced iron and carbonyl iron samples to study the correlation of biological availability and solubility. There was excellent correlation for the hydrogen reduced iron and electrolytic iron samples. The carbonyl iron samples showed a direct relationship between solubility and bioavailability, but these data were not numerically equivalent to data from other types.

Iron deficiency anemia has continued in the United States at a high rate of incidence (1, 2) in spite of the cereal enrichment program. This has prompted renewed interest in the iron sources that have been used for enrichment purposes. Reduced iron, which includes several types of metallic iron powders such as hydrogen reduced iron, electrolytic iron, and carbonyl iron, has attracted much interest because it is frequently used for enrichment. The present Food Chemicals Codex specifications (3) state that 100% of hydrogen reduced iron must pass through a 100 mesh sieve and 95% of electrolytic iron must pass through a 325 mesh sieve.

Recent reports have shown a wide variation in the bioavailability of reduced iron, based on the hemoglobin repletion method (4, 5). Some of this variation has been attributed to particle size (5), with those samples of fine particle size having the highest bioavailability. Other reports have suggested that factors other than particle size may be responsible for the variable bioavailability of elemental iron samples (6–8). Because of this variability between reduced iron samples and also between fractions of different particle size within the specifications, it became apparent that a simple chemical or physical test to predict bioavailability would be most useful.

To this end, work was begun on a solubility method which would predict bioavailability. Early attempts (4, 5) to correlate solubility in hydrochloric acid with availability in biological systems were not encouraging. In those studies samples were suspended in dilute acid for 3 hr with mechanical shaking, or for 72 hr with occasional manual shaking. The purpose of this study was to determine if the rate of solution could be used to predict the bioavailability of electrolytical iron, hydrogen reduced iron, and carbonyl iron powders.

METHOD

Single lots of electrolytic iron and hydrogen reduced iron samples were selected and each was separated into 4 fractions based on particle size (from ca <20 to >38 μm) by air elutriation. Three carbonyl iron samples were used, each containing ≥ 2 fractions of 5 different particle sizes.

Bioavailability was measured by the hemoglobin repletion method described previously (5). Solubilities were determined for 25 mg samples suspended in 25 ml 0.1*N* HCl at 37°C. These conditions were chosen to simulate, as nearly as possible, conditions in the stomach. The suspension was shaken manually for 1 min, and transferred rapidly to a 0.2 μm filter unit to which suction was attached. The filtrate was analyzed for iron by the *o*-phenanthroline method (9).

Results and Recommendations

The data in Table 1 show the relative biological values and solubility rates for the electrolytic iron and hydrogen reduced iron fractions. For all the fractions tested, there was a direct relationship between the solubility data and bioavailability, and an inverse relationship between particle size and bioavailability. Similar results have been reported previously (6, 8, 10).

Ratios of electrolytic-to-hydrogen reduced iron for relative biological value and solubility data show excellent correlation of biological availability and solubility between types of samples

This report of the Associate Referee, J. C. Fritz, was presented at the 89th Annual Meeting of the AOAC, Oct. 13–16, 1975, at Washington, DC.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee D and was accepted by the Association. *See* (1976) *JAOAC* **59**, 390.

Table 1. Relative biological values (RBV) and solubility rates of reduced iron samples of different particle sizes

Particle size, μm[a]	RBV	Solubility, μg/min	% of total/min
Electrolytic Iron			
<20	56	3409.0	13.6
20–30	48	1647.7	6.6
30–38	38	1500.0	6.0
>38	31	1159.1	4.6
Total[b]	44	1875.0	7.5
Hydrogen Reduced Iron			
<20	24	1527.3	6.1
20–30	22	1090.8	4.4
30–38	17	785.4	3.1
>38	17	610.8	2.4
Total[b]	18	1243.5	5.0

[a] Fractions obtained from 1 sample of each type.
[b] Not fractioned.

Table 2. Comparison of relative biological values (RBV) and solubility rates for electrolytic:hydrogen reduced iron ratio

	Electrolytic:hydrogen reduced iron	
Particle size, μm[a]	RBV	Solubility rate
<20	2.33	2.23
20–30	2.18	1.50
30–38	2.24	1.94
>38	1.82	1.92
Total[b]	2.44	1.50

[a] Fractions obtained from 1 sample of each type.
[b] Not fractioned.

Table 3. Relative biological values (RBV) and solubility rates for carbonyl iron samples of different particle size

		Solubility	
Particle size,[a] μm	RBV	μg/min	% of total/min
Carbonyl A			
4–6	64	643.9	2.6
3–4	69	2289.0	9.2
3–5	62	1818.2	7.3
Carbonyl B			
5–7	43	1920.0	7.7
2–4	57	2565.0	10.3
Carbonyl C			
2–4	48	850.8	3.4
3–5	48	872.8	3.5

[a] Fractions obtained from 1 sample of each lot.

(Table 2). The best correlation was obtained with the fraction <20 μm, and the fractions most suitable for enrichment are undoubtedly within that range.

The data from the 3 lots of carbonyl iron (Table 3) also indicate a direct relationship between solubility and bioavailability within lots, but these data are not interchangeable with data from other types of iron. This may be related to the unique chemical and physical properties of these samples.

Solubility rate determinations show good promise as predictors of biological availability. Further standardization is necessary to provide application to all types of reduced iron samples.

It is recommended that study on this topic be continued.

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A Screening Method for Protein Characterization and Differentiation

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A circular paper chromatographic method was developed for the separation of the amino acids in proteins into 7 subgroups. Butanol-acetic acid-water (4+1+1) was used as the developing solvent. Eluted ninhydrin-stained aminograms gave rise to graphic profiles or numerical indexes based on absorbance percentages. The profiles can be used to compare protein-containing samples. Twenty different samples were studied through 190 comparisons of graphic profiles and coefficients of correlation, with only 4% misleading results. The method showed excellent reproducibility for the identification or differentiation of proteins and has the advantage of being performed with low-priced apparatus and reagents.

In various fields of research a simple and inexpensive method would be desirable for the identification or differentiation of protein systems or amino acid mixtures. Several areas of research have used the survey of amino acid profiles or images concerning protein mixtures for clarifying nutritional problems (1–6), studying microbial metabolism (7, 8), classifying species (9, 10), and diagnosing amino acid metabolism disorders (11–14). There are 2 reasons for the development of methods for the survey of aminograms: (*1*) it is not always essential to know the absolute content of all amino acids in a sample, as given by amino acid analyzers, and (*2*) such surveys are often needed in laboratories not equipped with amino acid analyzers, or in work carried out in the field.

Theoretically, pure proteins, systems totally or partially composed of proteins, and proteinaceous materials obtained after total hydrolysis give rise to a mixture of amino acids which reflects their characteristics. Such mixtures, when submitted to a separation process, can be converted into subgroups which have characteristics that can be related to the original material. The extent of separation may vary from obtaining 2 subgroups to the isolation of all individual amino acids.

Thus we have the detection of subclinical kwashiorkor by grouping plasma amino acids into 2 subgroups; the amounts of grouped amino acids allow the establishment of ratios that indicate the protein nutritional state of individuals (1). Braga *et al.* (15), in estimating nutritional status, have used circular paper chromatography for the separation of 10 subgroups. The technique presented in this paper is derived from Giri and Rao's circular chromatograms (16) and further takes advantage of improvements introduced by Braga *et al.* (15) and Martins-Campos *et al.* (17). The present method leads to the separation of 7 subgroups, thereby reducing cuttings and elutions while maintaining an excellent power of identification or differentiation. It provides an easily understood graphic or numerical image, obtainable with low-priced apparatus and without specialized technicians. Evaluation of the method's reproducibility and power of identification or differentiation shows that it appears to be highly useful for simplifying the analysis of amino acid mixtures, pure proteins, and proteinaceous material mixtures.

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METHOD

Hydrolysis of Samples

To sample containing 4.5–5 mg Kjeldahl nitrogen in 5–10 ml vial add 3 ml 6*N* HCl (prepared from double distilled analytical grade HCl), being sure to remove all sample particles adhering to vial walls. Shake gently and heat sample 5 min in 100°C water bath. Seal vial immediately after removing it from bath and maintain in 105°C oven for 24 hr; open vial after it cools to room temperature. Centrifuge acid hydrolysate at $800 \times g$ for 10 min, transfer 2 ml supernate to watch glass, and dry 24 hr in vacuum desiccator over NaOH pellets and silica gel. Suspend dry residue in 0.5 ml distilled water and dry again as indicated above. Resuspend residue in 2 ml distilled water and centrifuge 10 min at $800 \times g$. Save supernate and

store at <0°C. This final solution contains ca 0.1 mg crude protein (N × 6.25)/10 μl.

In applying the present method to systems containing only free amino acids, the hydrolysis step should obviously be omitted. For complex samples, when free amino acids are the only components of interest, peptides, proteins, and salts, if present, should be eliminated, since large concentrations of these substances may alter the chromatographic development.

Chromatography and Quantitative Evaluation of Spots

On circular 29 cm diameter Whatman No. 1 chromatographic paper micropipet 10 μl samples of diluted hydrolysate. Avoid spots >0.5 cm diameter. After 6–7 hr run (butanol-acetic acid-water, 4+1+1) in circular chromatographic jar, dry paper overnight at room temperature and then spray with 0.4% (w/v) ninhydrin solution in acetone. Carry out color development in 75°C oven for 20 min. Separation of amino acid subgroups is obtained according to Fig. 1. Cut subgroups out according to shape of spots; for quantitative evaluation elute each subgroup with 5 ml 10 mg% alcoholic solution of cupric sulfate. Add aqueous solution of copper salt to ethanol immediately before use. Elute piece of chromatographic paper stained without revealing any reactive compound for use as blank. Elute spots by shaking copper solution in the dark for 30 min. Read eluates at 520 nm.

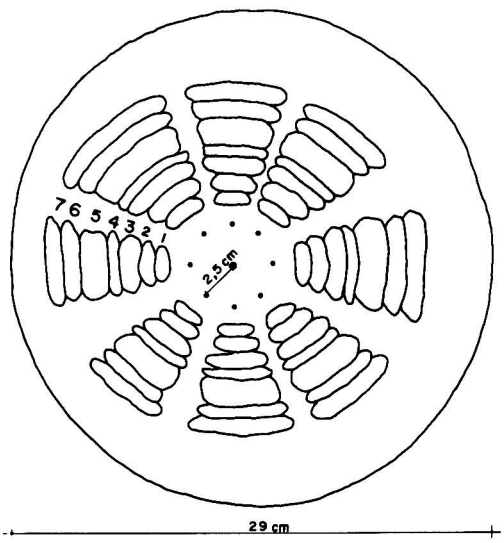

FIG. 1—Typical aminogram after development and staining.

Identification of Subgroup Constituents

In order to characterize the amino acids present in each subgroup, 2 chromatograms were developed as described above: (*1*) with 8 samples of a common cornmeal hydrolysate, and (*2*) with 8 samples of a casein hydrolysate. After chromatographic development, only 4 of the samples (corresponding to half of the paper) were stained with ninhydrin. This staining allowed the superposition of the stained subgroups over those diametrically opposite which had not been stained with ninhydrin. This procedure permits location of subgroups without staining; unstained subgroups were eluted 30 min with 75% ethanol. The solution was evaporated and the amino acids were submitted to a complete analysis by means of a Beckman Model 121 amino acid analyzer.

For statistical analysis separate samples of casein and common cornmeal were analyzed several times.

Construction of Graphic Profiles

The sum of absorbances found for each sample is taken as 100 and the absorbance for each subgroup is expressed as a percentage of the total. This calculation allows for the construction of tables and graphic profiles consisting of 7 bars (ordered according to increasing R_f value) drawn with heights proportional to their percentage values (*see* Table 1 and Fig. 2).

For illustrating the graphic profile method and in order to evaluate its resolving power, 18 other samples were analyzed (≥3 hydrolysates, ≥24 aminograms).

Statistical Studies

Due to low numerical values and high standard deviations, subgroup 1 has only been used as a helpful semiquantitative aid. In estimating the accuracy of the method, we have used the following approximation: variations found for casein were

Table 1. Construction of an amino acid profile for trypsin hydrolysate[a]

Eluate (subgroup)	Absorbance × 10^3 (520 nm)	%
1	145	3.93
2	395	10.72
3	835	22.66
4	565	15.33
5	420	11.40
6	510	13.84
7	815	22.12
Total	3685	100.00

[a] The figures in this table refer to one chromatographic run of a single application of hydrolyzed sample. The data are the mean of 24 determinations.

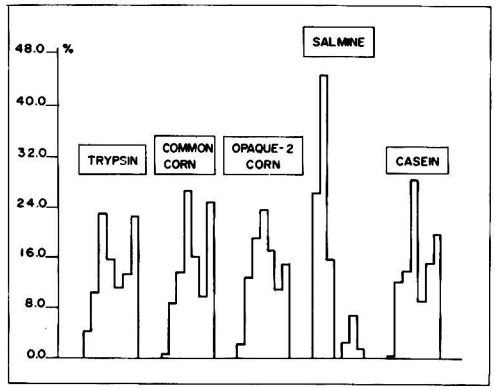

FIG. 2—Profiles for trypsin, common corn, opaque-2 corn, salmine, and casein (data for the construction of these profiles are in Table 4).

extrapolated for all pure protein analyses and those found for common cornmeal were used for determinating the precision of analysis for various systems containing non-proteinaceous components.

To affirm that 2 identical samples are equal or not or that 2 different samples are equal or not, we use the coefficient of correlation, defined as:

$$r = (\Sigma\ (x - \bar{x}) \times (y - \bar{y}))/((N - 1) \times \sigma_x \times \sigma_y)$$

where r = coefficient of correlation; σ_x = standard deviation for aminogram x; σ_y = standard deviation for aminogram Y; N = number of subgroups considered ($N = 6$); x = value of the subgroup in aminogram X; y = value of the subgroup in aminogram Y; $\bar{x}$ and $\bar{y}$ = arithmetic average of the values of the subgroups in aminograms X and Y, respectively.

The values of r for various systems known to be different are shown in Fig. 3 and those for samples known to be identical in Fig. 4.

We may also compare samples by superposing their graphic profiles. Profiles of all samples analyzed in which each subgroup was graphed as its mean value $\pm 2\sigma$ were superposed in such a way that every profile was checked against all others. Each comparison between samples was evaluated in relation to the number of subgroups with coincident values in a range of $\pm 2\sigma$. Data on the superposition of values for the subgroups in several samples known to be different and in samples known to be identical are shown in Figs. 3 and 4, respectively.

Results

Good resolving power and excellent reproducibility are obtained when sample hydrolysates

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
A		685	810	389	663	722	698	-672	637	794	714	282	455	123	765	-074	-077	567	762	768
B	2		700	812	582	790	906	-401	808	886	742	757	312	442	566	551	656	497	816	582
C	0	3		654	962	968	842	-319	923	940	863	678	482	481	669	196	167	925	945	949
D	3	3	2		512	815	901	150	877	774	514	932	001	335	122	650	744	667	834	523
E	1	4	4	2		928	755	-212	891	884	891	691	590	650	673	259	141	931	853	908
F	2	3	5	2	5		931	-175	989	968	845	831	410	522	565	398	372	916	974	877
G	2	5	6	4	4	4		-132	951	950	781	859	364	441	504	583	512	728	918	677
H	0	0	1	0	0	1	0		-074	-337	-423	191	-343	-085	-720	359	123	-016	-246	-425
I	2	3	4	4	3	6	4	0		955	819	901	316	553	495	513	475	888	956	808
J	2	5	6	2	5	6	6	0	4		915	792	525	570	705	448	396	797	939	826
K	0	3	5	2	4	4	5	0	3	6		656	799	778	883	436	239	685	751	749
L	1	4	3	4	3	4	4	0	4	4	4		227	636	242	772	754	710	781	537
M	1	0	2	0	2	2	1	0	1	1	2	2		721	867	328	-172	275	242	331
N	0	2	1	0	2	0	1	0	1	0	3	2	0		598	635	413	430	363	384
O	1	2	3	0	2	3	2	0	1	3	3	3	4	0		127	-076	385	490	603
P	2	1	1	4	0	0	2	0	1	1	2	2	1	2	0		724	167	285	-048
Q	1	3	3	0	3	3	4	0	2	3	3	3	2	0	3	0		178	398	102
R	1	1	6	1	3	5	4	1	3	5	4	3	2	1	3	1	3		888	909
S	2	2	4	1	1	5	4	1	4	3	3	1	1	0	1	1	1	4		893
T	2	1	2	1	4	2	0	0	2	2	3	0	2	3	0	0	0	4	5	

FIG. 3—Coefficients of correlation ($r \times 10^3$) among the several samples known to be different and number of overlapping subgroups in a range of $\bar{x} \pm 2\sigma$. Letters refer to samples listed in Table 4.

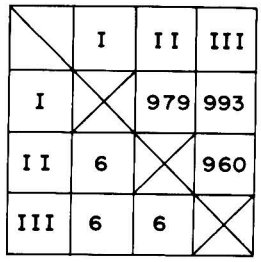

FIG. 4—Coefficient of correlation ($r \times 10^3$) and number of overlapping subgroups for 3 different sets of aminograms derived from one sample of common corn. Roman numerals correspond to sets referred to in Table 5.

are analyzed up to 3 days after hydrolysis. Table 2 shows the standard deviations for casein and common corn which have been extrapolated for pure protein and proteinaceous material analyses, respectively. As demonstrated by the low dispersion of the results (Table 2), small amounts of contaminants found in some subgroups (Table 3) do not interfere with the adequacy of the method when applied to the construction of reliable graphic profiles.

According to the technique, 20 graphic profiles have been drawn from the numerical values presented in Table 4. In the graphs these numerical values are represented with the addition of $\pm 2\sigma$. Table 5 shows data arising from 3 independent analyses of one sample of common cornmeal; each profile is based on the mean values of 8 aminograms.

From Figs. 3 and 4 we may conclude that under the conditions described samples known to be identical give a coefficient of correlation $\geqslant 0.96$. Of 190 coefficients of correlation for samples known to be different, only 5 (2.6%) produced values >0.96. Thus, it is demonstrated that $r = 0.96$ would be a limit to indicate whether samples are identical or not. Therefore, identical samples should have $r \geqslant 0.96$, whereas different samples should have $r < 0.96$. Samples of common corn and opaque-2 corn show a coefficient of correlation ($r = 0.68$) below the value taken as the limit and we may use this criterion to differentiate between the 2 varieties of corn.

Table 6 (derived from Fig. 3) shows that among 190 tests only 7 (3.7%) total superpositions (superposition of 6 subgroups) can be found. On the other hand, comparisons of profiles known to be equal have shown complete superposition in every case (Fig. 4).

Table 2. Reproducibility of separation of the 7 subgroups in hydrolysates analyzed 1, 2, and 3 days after hydrolysis

Subgroup	% of absorbance (mean = $\bar{x}$)	2σ	2σ as % of $\bar{x}$[a]
Common Corn (72 Chromatograms)			
1	0.59	0.452	76.6
2	8.70	0.838	9.6
3	13.58	1.168	8.6
4	26.48	1.608	6.1
5	16.08	1.692	10.5
6	9.73	1.262	13.0
7	24.83	1.646	6.6
Casein (64 Chromatograms)			
1	0.26	0.322	123.8
2	12.34	0.764	6.2
3	14.09	0.682	4.8
4	28.65	2.176	7.6
5	9.18	0.674	7.3
6	15.45	1.216	7.9
7	19.94	1.668	8.4

[a] Values have been used for the estimation of the variation in the values of the subgroups for all other samples.

Table 3. Amino acid distribution among the 7 proposed subgroups, according to data obtained using an amino acid analyzer

Subgroup	Major constituents	Contaminants[a]
1	Ammonia, ½ Cys	—
2	Arg, His, Lys	Ammonia
3	Gly, Ser, Asp	Glu, Met sulf, Cyst acid
4	Glu, Thr	Ala, Gly, Asp
5	Pro, Ala, Tyr	Glu
6	Val, Met, Phe	—
7	Leu, Ile, Phe	—

[a] The analysis of each subgroup revealed small amounts of contaminating amino acids derived, in general, from neighboring groups.

Discussion

After studying the resolving power of the method, it is evident that excellent information can be obtained from the separation of amino acids into 7 subgroups instead of the 10 considered in previous papers (15, 17). Transformation of absorbances for each subgroup into percentages of the total absorbance enables one to use the method within certain limits irrespective of the mass of protein hydrolysate to be analyzed. If the amount of hydrolysate to be developed is too large, the resolution of the subgroups is impaired, due to their interpenetration. When the quantity of hydrolysate is too small, the accuracy of the method is lower.

Table 4. Percentage of absorbance found for the 20 samples analyzed

	Sample[a]	Subgroup 1	2	3	4	5	6	7
A	Common corn	0.59	8.70	13.58	26.48	16.08	9.73	24.83
B	Opaque-2 corn	2.20	12.70	19.00	23.50	17.00	10.70	15.00
C	Fish meal	1.09	14.90	16.52	22.02	12.86	14.04	18.55
D	Cassava meal	0.00	19.12	18.66	26.46	14.54	8.52	12.25
E	Enriched cassava meal[b]	1.32	14.03	18.48	25.03	7.51	13.52	20.05
F	Castor bean meal (defatted)	2.38	15.40	17.80	25.52	11.42	11.48	17.91
G	Baker's yeast	0.87	15.89	18.20	22.39	14.92	10.87	16.84
H	Salmine	26.30	44.90	15.80	0.20	2.50	6.90	1.60
I	Soybean meal	1.00	16.10	18.90	26.50	10.80	10.10	16.80
J	Torula yeast	0.92	14.19	18.42	22.93	13.89	12.33	17.85
K	Hen's egg	2.08	12.72	19.67	20.51	12.84	13.66	18.48
L	Papain	3.70	17.29	19.94	22.26	12.33	11.54	12.97
M	Trypsin	4.10	10.40	22.90	15.65	11.17	13.28	22.52
N	γ-Globulin	2.54	11.72	26.70	19.19	8.02	15.58	14.80
O	Pepsin	1.09	2.06	23.02	22.72	12.02	12.78	26.30
P	Collagen	1.91	18.60	27.54	18.07	14.57	8.09	11.22
Q	Ribonuclease	4.20	16.20	22.20	23.10	17.40	12.20	4.40
R	Cashew nut meal (defatted)	2.60	16.90	15.60	22.40	10.70	14.60	17.50
S	Lupine	0.90	14.40	15.60	32.00	10.80	8.90	17.50
T	Casein	0.26	12.34	14.09	28.65	9.18	15.45	19.94

[a] Origin of complex samples: purchased at local supermarket: A, D, I, K, and S; prepared in our laboratory: F, G, and R; Federal University of Viçosa, Viçosa, Brazil: B; Navy Research Institute, Rio de Janeiro, Brazil: C; Instituto Açúcar Álcool, Rio de Janeiro, Brazil: J.

[b] Enriched cassava meal composition (%) 83.8 cassava meal, 10 soy protein isolate-Sanbra, 6 dried skim milk, 0.2 DL-methionine.

Table 5. Percentage of absorbance found for 3 different sets of aminograms derived from one sample of common corn

Set	Subgroup 1	2	3	4	5	6	7
I	0.45	8.63	13.14	26.47	16.99	9.82	24.60
II	0.96	8.71	13.28	25.99	14.08	10.29	26.59
III	0.36	8.76	14.31	26.99	17.16	9.07	23.31

Table 6. Summary of results of the comparisons of each one of the 20 profiles with the other 19, known to be different ($P \leq 0.05$)

No. of overlapping subgroups	Frequency[a]
—	38 (20.0%)
1	35 (18.4%)
2	37 (19.5%)
3	33 (17.4%)
4	29 (15.3%)
5	11 (5.8%)
6	7 (3.7%)

[a] Total of superpositions carried out = 190.

High reproducibility of the method requires application of spots $\leqslant$0.5 cm diameter, and concentrations $\leqslant$10 μg hydrolysate/μl for the application of 10 μl.

In some cases, it may be difficult to eliminate the strong contamination by certain amino acids belonging to another subgroup. This occurs with proteins rich in basic amino acids such as protamines and histones, where the application of 0.1 mg hydrolyzed crude protein may give rise to a subgroup 2 with a high absolute value for absorbance, causing a very expanded subgroup which may penetrate the neighboring subgroups.

As can be seen from Table 2, the reproducibility of the profiles is quite satisfactory. For casein as well as for common corn, standard deviations derived from 64 and 72 chromatograms, respectively, are very low. Extrapolation of the standard deviations for casein and common corn to the remaining samples has been carried out tentatively.

The numerical or graphic profile of a protein, featuring its amino acid composition, allows interesting observations concerning its composition. Pepsin, an acidic protein with a low percentage of basic amino acids, shows a profile that points out this characteristic. Collagen, showing a high percentage (about 25) of glycine

and a low proportion of neutral amino acids, has a large amount of subgroup 3 and relatively small amounts of subgroups 6 and 7. Although collagen contains a large amount of proline (belonging to subgroup 5), this amino acid develops a yellowish color with ninhydrin, giving a very low contribution to the absorbance read at 520 nm.

Opaque-2 corn, extremely important for the production of protein-rich mixtures, has a higher lysine content than common corn. Our method, showing higher values for subgroup 2, provides profiles which may differentiate quite definitely the 2 varieties deriving from the same botanic species.

Although enriched cassava meal is composed of about 80% cassava meal, the addition of soybean protein concentrate (at such an amount that this material becomes the major protein constituent), dried skim milk, and methionine makes the profile for enriched cassava meal sufficiently different from those obtained for common cassava meal and soybean meal, so that they can be distinguished by the present method.

Yet proteins with distinct origins and different activities may show identical profiles. Such is the case with castor bean meal and soybean meal, torula and baker's yeast, etc. Nevertheless, the percentage of coincidences, within the limits of this work, is very low (<4).

Conclusion

In many of the references cited as evidence of the multidisciplinary interest of subgroup amino acid analysis, we can point out several examples of the application of the present method: The comparison of amino acid pools of microorganisms under different physiological conditions (7, 8); the eventual support to systematic paleobiochemistry, repeating fossil identification by aminograms of its protein fractions, without using amino acid analyzers (10); and the construction of profiles of free amino acids in organic fluids (application of the method without previous hydrolysis) with the aim of detecting anomalous states of amino acid metabolism (11–13, 15, 17).

Several branches of the pharmaceutical, biotechnological, and food industries may find advantageous applications of the present method. Quality control of native raw materials, quite often without a defined chemical composition, may be improved by using graphic profiles of their amino acid components. For government audit, a file with graphic profiles of several food and pharmaceutical products practically beyond control by chemical analysis (vegetal extract mixtures, microorganism lysates, mixtures with antigenic properties, etc.) would be very useful for testing the composition reproducibility of individual batches. In such cases, it is advisable to have standards derived from the analysis of several reference samples of raw materials or from several batches specifically produced from materials already tested. From these data, standard deviations can be determined under the same conditions of samples and standard profiles of various systems may be developed and filed. Profiles of each new batch of raw material received or meal produced could be compared with the standards.

Finally, it must be emphasized that all graphic profiles derive from relative values (absorbance percentage values of all eluted spots), so that all identification and differentiation assays are independent of accurate measures during sampling, hydrolysis, and chromatogram application.

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ATTENTION

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Authors should provide an informative, carefully prepared abstract typed on the special form, plus 2 additional copies (carbon copies or photocopies). This form includes general rules for preparing satisfactory abstracts. Improperly prepared abstracts received too late to allow their return will not appear in the Abstract Bulletin.

In addition to the abstract, *six* complete copies (including abstract) of all Associate Referee reports (1 copy for each member of the Subcommittee, the Statistical Consultant, and the Editorial Office) and three complete copies of all contributed papers must be submitted to the AOAC office. The manuscripts should be double-spaced throughout. Unless otherwise specified, all manuscripts received will be considered for publication in *JAOAC*, pending satisfactory review. Each Associate Referee should also send one copy of his report to his General Referee.

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Write for standard abstract forms (one required for each presentation). Mail your abstract in early.

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DISINFECTANTS

Semiautomated Ring Carrier to Facilitate and Expedite Disinfectant Testing by the AOAC Use-Dilution Method

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The AOAC use-dilution test requires the replicate examination of a number of contaminated metal rings. Ten metal rings may be tested within the 20 min structure of the test. From a statistical and economical viewpoint, it would be highly desirable to increase the number of rings examined in a single test. A semiautomated ring carrier permits testing 200 rings within a 20 min test.

The AOAC use-dilution test (1) is used to substantiate disinfectant claims for disinfectant products registered with the Environmental Protection Agency. It is now generally recognized that the extent of replication required in the test is large, and several hundred contaminated rings are required to adequately develop efficacy data as a part of the registration of a given disinfectant product. For example, the replication specified for a hospital-type disinfectant, as summarized by Brown (2) and Beloian (3), involves a minimum of 420 rings for a particular product.

The rank score approach proposed by Walter and Scheusner (4) for monitoring phenol resistance of test cultures requires a minimum of 30 rings to determine the rank score for an organism. If the logic proposed by Walter and Scheusner is extended to develop control charts for a product system, then an approximate minimum of 10 replicate scores would be required to develop a model control chart against which oncoming production samples could be compared. Ten replicate scores to form the model control chart would require 300 rings.

Following the method as stated in the AOAC test (1), a single operator can examine 10 rings within the 20 min framework of the test. Using 20 sec transfers, rather than the stated 1 min intervals, a trained operator can examine 30 rings per test.

The manual operation involved does not permit the degree of replication desired from an economical viewpoint. If rank scores are used as the test response in more complicated experimental designs, the limitation of only approximately 30 rings per test presents a serious restriction on designs and requires extensive blocking. It would, therefore, be highly desirable from both an economical as well as an experimental design viewpoint to greatly increase the number of rings that may be examined within the 10 min exposure time of the test.

One approach to the solution of this problem would be to apply the principle of "ganging" whereby multiples of cylinders may be examined without destroying the basic structure of the bacteriological aspects of the test. The devices shown in Fig. 1 accomplish this end result.

Experimental

The semiautomated ring carrier (SARC) (available from Bosley, Inc., 1220 Washington Ave, Racine, WI 53403) is a device which groups 10 stainless steel wires in a row. A contaminated steel ring is placed on each wire so that 10 rings are handled as easily as 1 by the operator.

The SARC device consists of an 11½″ long section of aluminum angle (Reynolds aluminum angle No. 2410, ¾ × ¾ × ⅛″) in which 10 equally spaced 3/16″ holes are drilled along one side.

The same size holes are drilled in the centers of stainless steel Morton closures (Bellco Glass, Vineland, NJ, steel closures, without fingers, 25 mm, No. 2006-00025).

Starting with 9″ of 0.045, Type 309 stainless steel wire, a circular 3/16″ diameter loop is formed on one end and bent perpendicular to the remainder of the wire.

The wire loops and Bellco closures are attached to the aluminum angle with ⅛″ diameter ¼″ grip range aluminum pop rivets with a stainless steel flat washer covering the loop (Shadbolt & Boyd Co., Milwaukee, WI, Pop Rivet AD44ABS and No. 6 stainless steel washer Type 18-8). A Marson HP-2 pop riveter is used for this purpose.

The hook on the lower end of the wire is formed to hold the rings such that a minimum contact area between the wire and the ring is assured.

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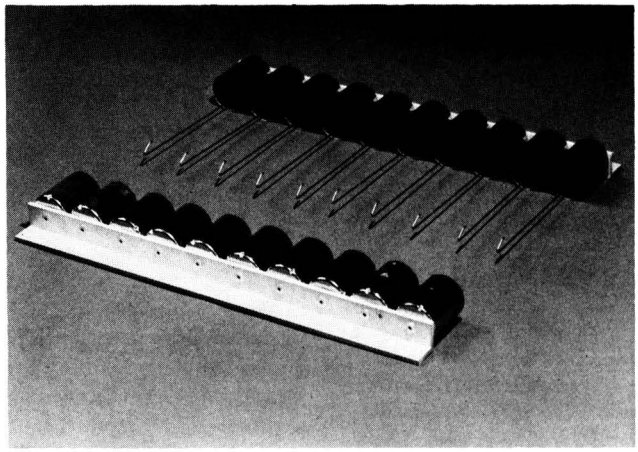

FIG. 1—SARC unit with wires for holding rings and SARC cover for medicant and subculture broth tubes.

Wires are of sufficient length to immerse rings without touching the bottoms of the tubes.

Also shown in Fig. 1 is a second SARC device without the stainless steel wires which serves to cover the medicant tubes and subculture tubes.

The dimensions of all test tubes used for holding the SARC device, disinfectant solutions, and subculture broth are 25 × 150 mm. These tubes are held in polypropylene racks, capacity 40 tubes (Dynalab Corp., Rochester, NY, No. 5928).

Six hundred tubes are required to test 200 rings rather than the 400 in the current test procedure; 200 for 20 wire SARCs, 200 for SARC covers on medicant tubes, and 200 for SARC covers on broth subculture tubes.

To perform tests, dried contaminated stainless steel rings are prepared by the AOAC method and placed on the SARC wires with sterile forceps. The assemblies are replaced within the sterile tubes until the test is started. Racks containing the disinfectant solutions are held at 20°C in a circulating water bath.

The racks of subculture broth are placed directly behind the medicant tube racks and outside the water bath. The racks of SARCs holding rings are placed in front of the test solution rack.

At zero time, the SARC covering the first row of medicant tubes is removed and set aside and the first SARC device with 10 contaminated rings is placed over this row of tubes, immersing the rings in the disinfectant test solution. A slight rocking of the SARC assures that the entire ring comes in contact with disinfectant solution with good mixing.

It has been customary in our laboratories to perform use-dilution tests with the SARC devices at 30 sec intervals. Using this interval, 200 contaminated stainless steel rings may be tested during a 20 min test period.

At the end of 10 min contact with the disinfectant test solution, the SARC is carefully raised, shaken slightly to remove excess disinfectant solution, and inserted halfway into a row of tubes containing subculture broth. A quick snapping movement will force the rings off the wires into the subculture broth. The cover for the culture tubes is quickly replaced and the SARC device is replaced on its original row of sterile tubes, and decontaminated later by autoclaving.

It should be stressed that only minor modifications of the technique of the AOAC test have been made to greatly increase the number of rings that may be tested during a 20 min period. These modifications have not affected the results of disinfectant test responses in our hands.

Discussion

Use of the foregoing described device permits the routine examination of 200 rings per 20 min test, using 30 sec transfer intervals. This is accomplished simply by a ganging factor of 10. Ten cylinders may be examined at each time interval rather than the usual one.

The utility of the device becomes obvious from a quality control or monitoring viewpoint. If rank scores are used as the test response, 6 disinfectant samples may be tested in the single 20 min test. If a binomial approach is preferred, then better estimates of the probability of failure at a fixed label use-dilution are achievable.

The intangible savings in manpower simply to cap and uncap culture tubes proves substantial

in the routine preparation of culture media and glassware washing.

One decided improvement possible by use of the device is that more complex experimental designs are feasible. Using tests as blocks and a response of 4 rank scores obtained per block (120 rings) (4), factorial designs are routinely possible. In a study designed to elucidate the significance of factors influencing the test response, for example, we are able to routinely conduct ½ replicate fractional factorial (2^5) designs confounded into 4 blocks or 4 tests.

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MICROBIOLOGICAL METHODS

Collaborative Study of the Serological Identification of Staphylococcal Enterotoxins by the Microslide Gel Double Diffusion Test

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A collaborative study was conducted, using the microslide gel double diffusion test for enterotoxin detection by determining the staphylococcal enterotoxigenicity of 7 strains of *Staphylococcus aureus*. Two strains produced staphylococcal enterotoxin A (SEA), 2 strains produced staphylococcal enterotoxin B (SEB), one strain produced both SEA and SEB, and 2 strains produced neither SEA nor SEB. To ascertain the effectiveness of this method for the detection of enterotoxins, strains of enterotoxigenic staphylococci which produced small or relatively large amounts of toxin were used in the study. The cultures were grown on semisolid brain-heart infusion agar and the culture fluids were assayed serologically for SEA and SEB. The qualitative results of this study by 16 collaborators indicated that this technique, in general, demonstrated a high degree of specificity as well as simplicity and reproducibility in the identification of the enterotoxins. Of the 16 participating laboratories, 14 reported the correct diagnoses for the 7 staphylococcal strains studied and 2 laboratories determined 6 of the 7 strains correctly. Based on these results, this method has been adopted as official first action.

Of the methods proposed for the serological detection and identification of the staphylococcal enterotoxins, antigen precipitation by its specific antibody in gels (1) has been the most frequently employed. This method utilizes either the linear (2, 3) or radial migration phenomenon for enterotoxin detection, although the radial system design initially described by Ouchterlony (4) and later miniaturized by Crowle (5) has proved to be the most specific because it provides for the identification of a specific antigen-antibody complex through its coalescence with the known reference antigen-antibody line of precipitation. This specificity is necessary, since antisera to the enterotoxins frequently contain antibodies to other antigens. The microslide gel double diffusion test (6) has been useful in fulfilling the need of specificity as well as simplicity in design and use. A preliminary evaluation of this method was previously reported (7) and has been used by a large number of laboratories in quality control and regulatory programs.

This collaborative study was conducted to evaluate the microslide gel double diffusion test by a cross-section of investigators representing State and municipal health facilities, industry, academic institutions, and both Federal and State regulatory agencies. Of the 21 laboratories contacted as potential collaborators, 16 participated in this study.

Collaborative Study

This method was evaluated for enterotoxin detection and identification by determining the enterotoxigenicities of 7 strains of *Staphylococcus aureus*. These strains of staphylococci produced one or more of the serologically identifiable enterotoxins or were nonenterotoxigenic according to monkey feeding studies (8). The strains used in this study consisted of 2 enterotoxin Type A producers (Food and Drug Administration (FDA) accession Nos. 246-3A (American Type Culture Collection (ATCC) 14459) and 743); 2 enterotoxin Type B producers (FDA Nos. 243 (ATCC 14458) and 778); one enterotoxin Type A and Type B producer (FDA No. 485); and 2 strains which produced neither enterotoxin A nor B (FDA Nos. D87 and D184). These particular enterotoxigenic strains were selected for their capabilities in producing different quantities of enterotoxin, thus providing collaborators

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with greater variation in the observation of qualitative assay results. Each of the collaborators was provided with the 7 strains of staphylococci on nutrient agar slants, lyophilized antisera and reference enterotoxin preparations, dehydrated brain-heart infusion (BHI) broth, templates for assembling the slide test, other convenience items, and the method (9). The collaborators were instructed to grow the cultures by the semisolid BHI agar plate method of Casman and Bennett (10); however, several other methods which use various kinds of media have been proposed for the production of the enterotoxins (11). Some additional methods which have been employed for the routine laboratory production of the enterotoxins are the flask dialysis culture method of Donnelly *et al.* (12), the cellophane-over-agar method (13), and a scaled-down version of the shake flask method (11) described by Kato *et al.* (14).

METHOD

Staphylococcal Enterotoxin

Microslide Gel Double Diffusion Test
Official First Action

(Detects 0.1–0.01 μg enterotoxin/ml and is applicable to detection of enterotoxin in culture fluids and concd food exts)

46.B07 ***Principle***

Pptn line occurs when serological type of enterotoxin diffuses thru gel and reacts with its specific antibody. Coalescence with ref. pptn line which results from serological reactivity of enterotoxin serotype and specific antibody confirms identity.

46.B08 ***Apparatus***

(a) *Debubblers.*—Fine glass rods. Prep. by pulling glass tubing very fine, as in making capillary pipets. Break into ca 6 cm lengths and seal ends in flame.

(b) *Electrical tape.*—Insulating tape, 0.25 × 19.1 mm (Scotch Brand, 3M Co., Electro-Products Division, St Paul, MN 55110, or equiv.).

(c) *Microscope slides.*—Plain glass, pre-cleaned, 3 × 1" (7.62 × 2.54 cm), 0.96–1.06 mm thick.

(d) *Pasteur pipets.*—Prep. by drawing out ca 7 mm od glass tubing or use disposable 30 or 40 μl pipets (Kensington Scientific Corp., 1399 64th St, Emeryville, CA 94608, or equiv.).

(e) *Petri dishes.*—20 × 150 mm and 15 × 100 mm.

(f) *Plastic templates.*—*See* Fig. **46:B1**. (Available from Division of Microbiology, Food and Drug Administration, 200 C St, SW, Washington, DC 20204.)

(g) *Silicone lubricant.*—High vac. grease (Dow Corning Corp., or equiv.).

(h) *Staining jars.*—Coplin or Wheaton jars.

(i) *Sterile bent glass spreaders.*—Bend glass rods like hockey sticks and fire polish.

(j) *Water-saturated synthetic sponge strips.*—Approx. 1.5 × 1.5 × 6.5 cm H_2O-satd absorbent cotton is also satisfactory.

46.B09 ***Media and Reagents***

(a) *Agar soln for coating slides.*—0.2%. Add 2 g bacteriological grade agar to 1 L boiling H_2O and heat until agar dissolves. Pour 20–30 ml portions agar into 6 oz (180 ml) prescription bottles or equiv. containers and store at room temp. Remelt when needed for coating slides.

(b) *Brain-heart infusion (BHI) agar.*—0.7% (w/v). Adjust BHI broth to pH 5.3; add bacteriological grade agar to prep. 0.7% concn and dissolve by boiling gently. Distribute in 25 ml portions into 25 × 200 mm

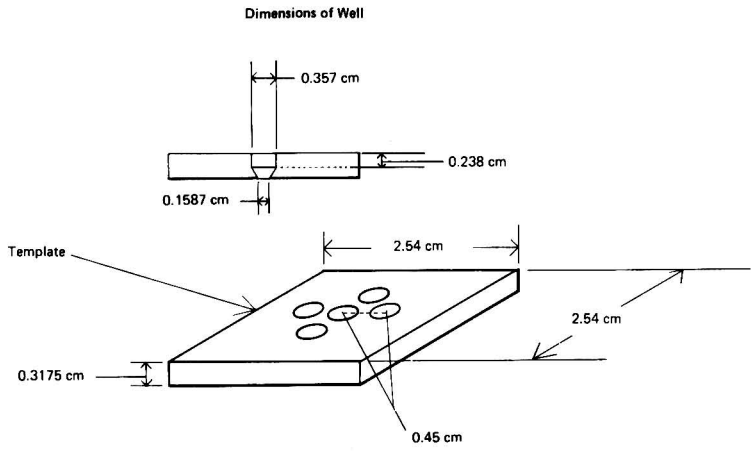


FIG. 46:B1—Plastic template schematic for microslide assembly

test tubes, and autoclave 10 min at 121°. Immediately before use, aseptically empty tubes of sterile medium into 15 × 100 mm petri dishes.

(c) *Enterotoxin antisera.*—Dil. lyophilized sera (Gallard-Schlesinger Chemical Manufacturing Co., 584 Mineola Ave, Carle Place, Long Island, NY 11514, or Division of Microbiology, Food and Drug Administration, 200 C St, SW, Washington, DC 20204) with normal physiological saline according to specific instructions of supplier. Store liq. stocks (highly concd) and working dilns of antisera at 4°; for long term storage, freeze-drying or freezing is recommended.

(d) *Enterotoxin references.*—Rehydrate lyophilized enterotoxin prepns, (c), according to specific instructions of supplier.

(e) *Gel diffusion agar.*—Add 1.2% purified agar (Noble special agar, Difco Laboratories) to boiling fluid base (0.85% NaCl–0.80% Na barbital with final concn of 1:10,000 merthiolate (Eli Lilly and Co., or equiv.) adjusted to pH 7.4. Filter hot agar thru 2 layers of anal. grade paper and store in 15–25 ml portions in screw-cap bottles.

(f) *Staining soln.*—0.1% Thiazine Red R stain (Allied Chemical Corp., or equiv.) in 1% HOAc.

(g) *Sterile distilled water.*—Dispense 5 ml distd H_2O into tubes and autoclave 15 min at 121°. Normal physiological saline may be substituted for H_2O.

(h) *Turbidity std.*—1% $BaCl_2$–1% H_2SO_4 (1+99) (No. 1 of McFarland nephelometer scale).

46.B10 *Preparation of Sample*

Select ≥4 isolated staphylococcal colonies from enumeration and recovery media, and streak nutrient media agar slants, or equiv. Incubate slants 18–24 hr at 35–37°. Add loopful of growth from agar slants to 5.0 ml sterile distd H_2O or saline and prep. aq. suspension of organisms from each slant which is equiv. to turbidity of No. 1 tube of McFarland nephelometer scale (ca 3×10^8 organisms/ml). Inoculate surface of semisolid BHI agar with 4 drops aq. suspension of organisms delivered from sterile 1.0 ml pipet. Spread drops of aq. culture suspension over entire surface of semisolid agar with sterile glass rod and incubate plates upright 48 hr at 35–37°. Transfer contents of petri dish to 50 ml centrf. tube with aid of wood applicator stick and centrf. 10 min at 32,800 *g* to remove agar and organisms. Examine culture fluid for presence of serologically identifiable enterotoxins.

46.B11 *Preparation of Slides*

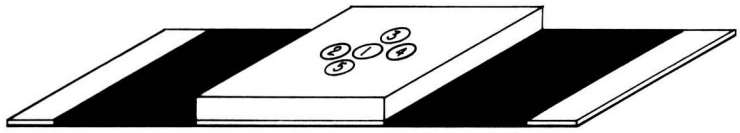
Wrap double layer of elec. tape around pre-cleaned microscope slide, leaving 2.0 cm space in center, as follows: Start piece of tape ca 9.5–10 cm long ca 0.5 cm from edge of bottom surface of slide and wrap tightly around slide twice. Wipe area between tapes with cheesecloth soaked with alcohol, and dry with dry cheesecloth. Coat upper surface area between tapes with 0.2% bacteriological grade agar as follows: Melt 0.2% agar, and maintain at ≥55° in screw-cap bottle. Hold slide over beaker on hot plate adjusted to 65–85° and pour or brush 0.2% agar over slide between 2 pieces of tape. Let excess agar drain off, wipe bottom surface of slide, and collect agar in beaker for reuse. Place slide on tray and dry in dust-free atmosphere (e.g., incubator). If slides are not clean, agar will not coat slides uniformly.

46.B12 *Preparation of Slide Assemblies*

Prep. plastic templates according to specifications in Fig. **46:B1**. Spread *thin* film of silicone grease on side of template that will be placed next to agar (i.e., side with smaller holes). Place ca 0.4 ml melted and cooled (55–60°) 1.2% gel diffusion agar between tapes. Immediately lay silicone-coated template on melted agar and edges of bordering tapes. Place 1 edge of template on 1 piece of tape, and bring opposite edge to rest gently on other piece. Sat. strips of synthetic sponge (ca 1.5 × 1.5 × 6.5 cm) with H_2O, and place 2 strips on periphery of each 20 × 150 mm petri dish. Place slide in prepd petri dish (2–4 slide assemblies/dish) soon after agar hardens, and label slide.

46.B13 *Slide Gel Diffusion Test*

To prep. record of assay, draw hole pattern of template on record sheet and indicate number (same as that used for slide) and contents of each well. Place suitable diln of antiserum or sera in central well, homologous ref. enterotoxin in peripheral well(s), and material under examination in well adjacent to that contg ref. enterotoxin. See Fig. **46:B2(*1*)** for reagent arrangement for simultaneous detection of 2 enterotoxin types (bivalent detection system). Prep. control slide with only ref. toxin and antienterotoxin serum to det. proper reactivity of reagents. Fill wells to convexity with reagents, using Pasteur or disposable 30 or 40 μl pipet. Partially fill capillary pipet with soln and remove excess liq. by touching pipet to edge of sample tube. Slowly lower pipet into well until it touches agar surface, and fill to convexity. Remove trapped air bubbles from *all* wells by probing with debubbler, (a), against dark background. Let slides incubate 48–72 hr at room temp. in covered petri dishes contg moist sponge strips (24 hr slide incubation at 25° or 35° is generally sufficient for testing of culture fluids). Carefully remove template by sliding it to 1 side. If necessary, clean slide by dipping in H_2O and wiping bottom of slide. Enhance lines of pptn by immersing slide in staining soln, (f), 5–10 min. To preserve slide as permanent record, rinse any reactant liq. remaining on slide by dipping in H_2O and then immerse slide in each of following baths 10 min: staining soln, 1% HOAc, 1% HOAc, and 1% HOAc contg 1% glycerol.



(1) Bivalent
1. Combination Antisera (e.g., Anti A and B)
2. Prepn under test
3. Ref. enterotoxin (e.g., Anti A)
4. Prepn under test
5. Ref. enterotoxin (e.g., Anti B)

(2) Monovalent
1. Antiserum (e.g., Anti A)
2. Dilns of prepn under test
3. Ref. enterotoxin (e.g., type A)
4. Dilns of prepn under test
5. Dilns of prepn under test

FIG. 46:B2—Arrangement of antisera and homologous reference enterotoxins (1) when assaying preparation(s) under test for presence of 2 staphylococcal enterotoxins simultaneously (bivalent detection system) or (2) when assaying dilutions of preparation under test with apparent enterotoxin excess (monovalent detection system)

Drain excess fluid from slide and dry in 35° incubator. After prolonged storage, lines of pptn may not be visible until slide is immersed in H_2O.

46.B14 *Slide Interpretation*

Examine slide for lines of pptn by holding at oblique angle to light source against dark background. Coalescence of test sample lines of pptn with ref. line(s) of pptn indicates pos. reaction. Fig. **46:B3** shows microslide gel diffusion test as bivalent detection system: Antisera to enterotoxins A and B are in well 1; known ref. enterotoxins A and B are in wells 3 and 5, resp., to produce ref. lines of A and B; prepns under test are in wells 2 and 4. Interpret 4 reactions as follows: (1) No line development between test prepns—absence of enterotoxins A and B; (2) coalescence of test prepn line from well 4 with enterotoxin A ref. line (inter-

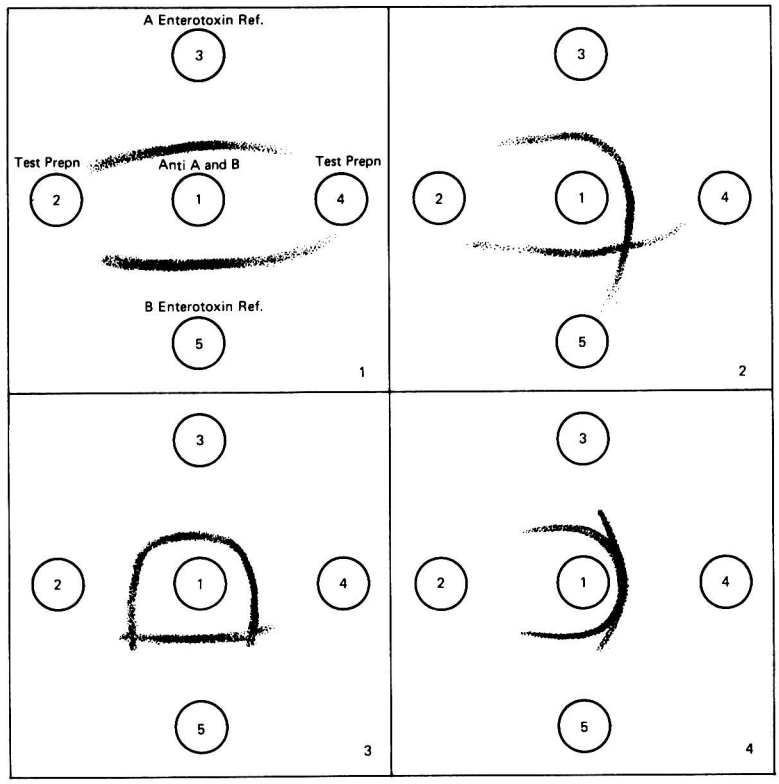


FIG. 46:B3—Examples of 4 possible reactions in bivalent detection system. See 46.B14 for explanation of reactions.

section of test prepn line with enterotoxin B ref. line) —absence of enterotoxins A and B in well 2, presence of enterotoxin A and absence of enterotoxin B in well 4; (3) presence of enterotoxin A and absence of enterotoxin B in both test prepns; and (4) absence of enterotoxins A and B in test prepn in well 2, presence of enterotoxins A and B in well 4. Operator can simplify assay by testing only 1 prepn for presence of 2 different enterotoxins on same set of slides.

If concn of enterotoxin in test material is excessive, formation of ref. line will be inhibited because of fast migration of toxin thru gel, thus localizing antibody in its well. Fig. **46:B4**(*A*) shows this inhibition of ref. line formation when 10 and 5 μg enterotoxin/ml, resp., are used. Figs. **46:B4**(*B*)–**46:B4**(*F*) show ppt patterns when successively less enterotoxin is used. If test prepn inhibits formation of ref. line as in Fig. **46:B4**(*A*), dil. test material, utilizing monovalent system shown in Fig. **46:B5**. Reactant arrangement for assaying dilns of prepn under test is shown in Fig. **46:B2**(*2*). Figure **46:B5** shows microslide gel diffusion test as monovalent system in which antiserum is placed in well 1; ref. enterotoxin in well 3; and dilns of test prepn in wells 2, 4, and 5. Do not make starting diln of culture fluid (test material) so high as to dil. beyond reactive concn of enterotoxin.

Occasionally, atypical ppt patterns form which may be difficult for inexperienced analysts to interpret. One of most common atypical reactions is formation of lines not related to toxin, but caused by other antigens in test material. Examples of such patterns are given in Fig. **46:B6**, which shows microslide gel diffusion test as bivalent detection system. (*See* reactant arrangement in Fig. **46:B2**(*1*).) In ppt pattern **46:B6**(*1*), test prepn in well 4 produced atypical reaction indicated by nonspecific line of pptn (lines of nonidentity with enterotoxin refs A and B), which intersects enterotoxin ref. lines. In ppt pattern **46:B6**

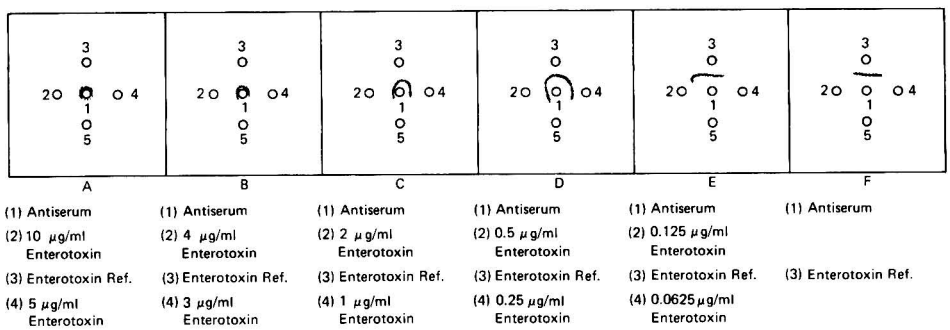


FIG. 46:B4—Effect of amount of enterotoxin in test preparation on development of reference line of precipitation. See 46.B14 for explanation of reactions.

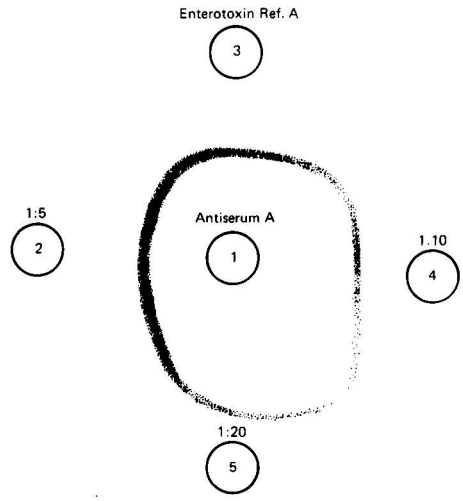


FIG. 46:B5—Appearance of microslide gel diffusion test as monovalent system

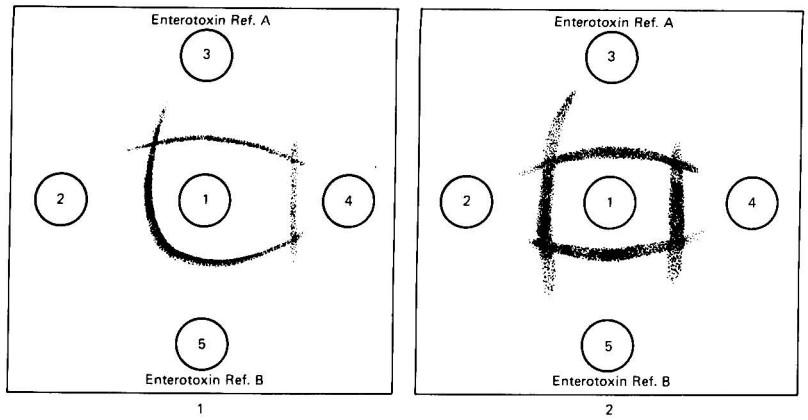


FIG. 46:B6—Precipitate patterns in microslide gel diffusion test demonstrating nonspecific (atypical) lines of precipitation

(*2*), both test prepns (wells 2 and 4) are neg. for enterotoxins A and B but produce nonspecific lines of pptn which intersect enterotoxin A and B ref. lines of pptn.

46.B15 *Slide and Template Recovery*

To recover slides for reuse, clean without removing tape. Rinse slides with tap H_2O to remove agar gel, boil 3–5 min in tap H_2O contg mild detergent, rinse in tap H_2O and then in distd H_2O, immerse momentarily in alcohol, and wipe dry with cheesecloth. Wash templates with hot (not boiling) H_2O contg moderately strong detergent, using cheesecloth to remove silicone film. Rinse templates with tap H_2O, distd H_2O, and alcohol; dry with cheesecloth, and tap alcohol out of wells. In cleaning plastic templates, avoid exposure to excessive heat or plastic-dissolving solvs. Templates and especially wells must be dry before reuse.

Results and Recommendation

The general design and conduct of this study generated qualitative-type data. Table 1 shows the results of the collaborators involved in the study. While 14 collaborators determined the correct enterotoxigenicities of all 7 strains of *S. aureus*, Collaborator 6 indicated that strain 485 was negative, while it actually produces both enterotoxins A and B. Similarly, Collaborator 14 experienced some difficulty in the detection of enterotoxin A with strain 485; however, the collaborator indicated that poor growth was achieved with this particular strain. This possibly explains why he did not detect enterotoxin A, which is produced in much smaller amounts than enterotoxin type B with this strain.

Statistical evaluation of this study, employing Cochran's chi-square test (15), indicated that there was no significant change in the percentage of cases where enterotoxin types A and B were properly identified as being present or absent among the laboratories ($P > 0.25$ for both toxins). For the application of the method to enterotoxins A and B over all of the laboratories and samples involved, the probability that the method indicates enterotoxin A or B as being present when, in fact, it is present is 0.958 and 0.979, respectively. Thus this method appears to be sensitive for the detection of enterotoxins A and/or B. In addition, the probability that the method would indicate that enterotoxins A and/or B are present when, in fact, they are absent, is effectively zero, thus indicating that the test is also specific in the detection of these toxins. Therefore, there should be very few false positive and false negative results generated by the method if it is properly applied.

Each collaborator was instructed to perform duplicate assays on each of the culture fluids in order to ascertain if the method produced consistent serological reactions. Repeatability analyses of the microslide gel double diffusion test for the identification of staphylococcal enterotoxin types A and B are shown in Table 2. Most of the collaborators (Nos. 1–4, 7–13, and 16) performed duplicate assays, while Collaborators 5 and 14 performed either duplicate or double-duplicate assays. Collaborator 15 performed

The recommendation of the Associate Referee, R. W. Bennett, was approved by the General Referee and by Subcommittee F and was adopted by the Association. *See* (1976) *JAOAC* **59**, 397.

Table 1. Collaborative results for the serological identification of staphylococcal enterotoxin by the microslide gel double diffusion test

	Staphylococcal strains													
	243 (A−, B+)[a]		246-3A (A+, B−)		485 (A+, B+)		743 (A+, B−)		778 (A−, B+)		D87 (A−, B−)		D184 (A−, B−)	
Coll.	A	B	A	B	A	B	A	B	A	B	A	B	A	B
1–5, 7–13, 15, 16	−	+	+	−	+	+	+	−	−	+	−	−	−	−
6	−	+	+	−	−	−	+	−	−	+	−	−	−	−
14[b]	−	+	+	−	−	+	+	−	−	+	−	−	−	−

[a] Enterotoxigenicities of the staphylococcal cultures.
[b] Collaborator 14 reported poor culture growth with *S. aureus* strain 485.

Table 2. Repeatability of the microslide gel double diffusion test for the identification of staphylococcal enterotoxin types A and B

	Staphylococcal strains													
	243 (A−, B+)[a]		246-3A (A+, B−)		485 (A+, B+)		743 (A+, B−)		778 (A−, B+)		D87 (A−, B−)		D184 (A−, B−)	
Coll.	A	B	A	B	A	B	A	B	A	B	A	B	A	B
1–4, 7–13, 16	2[b]	2	2	2	2	2	2	2	2	2	2	2	2	2
5	4[c]	4	4	4	4	4	2	2	2	2	4	4	4	4
6	—[d]	2	3[e]	3	—	—	3	3	—	—	—	—	2	2
14	4	4	4	4	2	4	4	4	4	4	2	4	2	4
15	4	4	4	4	4	4	4	4	4	4	4	4	4	3(1)[f]

[a] Enterotoxigenicities of the staphylococcal cultures.
[b] Duplicate assays (both slides showed the same serological results).
[c] Double-duplicate assays.
[d] Single assays not applicable to repeatability analysis.
[e] Triplicate assays.
[f] Single assay difference in double-duplicate set.

double-duplicate tests on all of the samples assayed with only a single assay which differed in the double-duplicate set with *S. aureus* strain D184. The formation of a faint nonspecific line of precipitation accounted for the difference in the double-duplicate assay. Collaborator 6 performed only single assays on some of the samples (culture fluid 243 for the detection of enterotoxin A and culture fluids 485, 778, and D87 for the detection of both enterotoxins A and B). Therefore, these were not applicable for repeatability analysis. The remaining 7 assays performed by Collaborator 6 were done either in duplicate (culture fluid 243 for enterotoxin B presence and culture fluid D184 for both enterotoxins) or in triplicate (culture fluids 246-3A and 743). In general, excellent repeatability appeared to be demonstrated within the laboratories. Also, there was a high degree of reproducibility among the laboratories.

The method is equally applicable in the identification of other enterotoxins which have been established as serological entities, since no variation in the methodology exists other than in the use of the specific antibody and homologous reference enterotoxins.

The directions for performing the microslide gel double diffusion test are adequate and the method is applicable to the serological identification and differentiation of the staphylococcal enterotoxins. Based on the results of this study, it is recommended that this method be adopted as official first action.

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Raymond Bryant, State of California Department of Health, Berkeley, CA

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James Davidson and Natalie Huminiak, Chicago Board of Health, Chicago, IL

Evelyn D. Forney, FDA, Cincinnati, OH

Gayle Lancette, Minneapolis Center for Microbiological Investigations, FDA, Minneapolis, MN

Richard Mageau, U.S. Department of Agriculture, Beltsville, MD

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Terry E. Munson, FDA, Brooklyn, NY

Barry L. Reese, Nestle Co., Inc., Marysville, OH

Richard Ruby, FDA, Los Angeles, CA

Janet Spitz, University of Wisconsin, Madison, WI

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Sita Tatini, University of Minnesota, St. Paul, MN

Robert R. Williams, State Department of Agriculture, Salem, OR

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Phosphorus Pentoxide as a Drying Agent for Bacterial Culture Extracts Analyzed by Gas-Liquid Chromatography

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The procedure for gas chromatographic analysis of metabolic products of microbial fermentation includes solvent extraction of the aqueous growth media, drying of the extract, and direct chromatographic analysis of the solvent. In this study, 2 drying agents, magnesium sulfate and phosphorus pentoxide, were compared. Both were effective in removing water; however, phosphorus pentoxide removed water more completely and at a faster rate than magnesium sulfate. When a thermal conductivity detector is used, it is important to completely remove water from the solvent to prevent interference with volatile acids and alcohols. When water is present, short-chain alcohols (C_2–C_5) are eluted together with the water, causing peak overlap and shoulder separations. Phosphorus pentoxide quickly and effectively removed water so that a baseline was established following the solvent front on the chromatogram. The use of phosphorus pentoxide is particularly advantageous for identification or fermentation studies on *Clostridium* and *Propionibacterium* when rapid identification is desired or when large numbers of cultures are to be tested.

Gas chromatographic techniques have been used to identify metabolic products formed by cultures of *Clostridium* and as an aid in differentiating species of this genus (1–3). All the clostridia, as well as many of the non-spore-forming anaerobic bacteria, produce well defined fermentation patterns on a specific medium. In our laboratory, hundreds of cultures of *Clostridium* have been tested for the presence of both short-chain alcohols and acids for the identification of the organisms (4). In addition, several thousand cultures of *Propionibacterium* have also been tested for production of volatile acids under varying conditions of temperature and nutrients (5). The method used (1) specifies extraction of the culture medium with ether and drying of the ether extract with magnesium sulfate before chromatographic analysis. Because of overlap in the retention times of water and alcohols, it is important to remove water from the ether extract, as traces of water will interfere with the elution of alcohols. Also, since water has a very broad elution peak, it can interfere with elution of acetic acid, the first acid eluted from the column. This paper describes the comparison of phosphorus pentoxide and magnesium sulfate as drying agents for the chromatographic determination of acids and alcohols produced by cultures of *Clostridium* and *Propionibacterium*.

Experimental

Growth of Organisms and Extraction of Metabolic Products

The media and methodology used in this study were those reported by Finne and Matches (4). Small volumes of culture medium can be used for analysis; however, in these studies 9 ml cultures were used. After growth of the organisms, the culture medium was acidified to pH 2.0 with 50% H_2SO_4 and shaken with 1.5 ml ethyl ether to extract both volatile alcohols and acids. Tubes were centrifuged briefly to break the ether-culture emulsion, allowing the ether with extracted products to collect on the surface. Tubes were then placed in a freezer until the aqueous phase in each tube was frozen and the ether layer could be decanted into a small screw-cap tube or vial. For rapid analysis, the ether layer can be removed from the tube immediately after centrifugation with a disposable glass Pasteur pipet and transferred to a vial.

Drying of the Ether Extracts

Anhydrous $MgSO_4$ was added to the vials at ca ½ the volume of the ether extract in the tube. P_2O_5 was added at ca 1/10 the volume of the ether. Samples dried by these 2 methods were then chromatographed after various time intervals.

Chromatographic Analysis

Ether extracts were analyzed on a Dohrman 15C-3 gas chromatograph, using a ¼″ × 6′ stainless steel column packed with Resoflex LAX-1-R 296 (Burrell Corp., Pittsburgh, PA). Chromatographic conditions were: column 110°C, injection port and detector 115°C, helium flow 120 ml/min, thermal conductivity detector current 100 ma. Sample size

injected varied between 5 and 14 μl, depending on metabolic concentration.

A standard volatile acid mixture was prepared by dissolving 1 mequiv. each of acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic, and caproic acids in 100 ml water. Ten ml of this mixture was acidified to pH 2.0 with 50% H_2SO_4, extracted with 2 ml ether, and analyzed as described above. The standard alcohol mixture was prepared by dissolving 1.7 mequiv. ethanol, 0.5 mequiv. propanol, and 0.1 mequiv. each of isobutanol, butanol, and pentanol in 100 ml water. Ten ml of this mixture was extracted and analyzed as described above.

Results

In this study, phosphorus pentoxide was superior to magnesium sulfate as a rapid drying agent. Water present in the ether extracts was completely removed within 5 min with phosphorus pentoxide, while magnesium sulfate required $\geqslant$15–20 min to achieve an acceptable water concentration. The standard acid mixture used for identifying volatile acids produced by clostridial fermentation is shown in Fig. 1. The standard acid mixture shown in Fig. 1A was dried 5 min with 1/10 its volume of phosphorus pentoxide, while the standard mixture shown in Fig. 1B was dried 10 min with ½ its volume of magnesium sulfate. The more effective drying properties of the former are evident; only traces of water are present after 5 min as compared with samples dried with magnesium sulfate, where water concentrations after 10 min drying are still high enough to cause interference. However, a longer drying time with magnesium sulfate will reduce the water concentration to acceptable levels.

Figures 2A and 2B show the standard alcohol mixture used in these studies. The alcohols elute between 1 and 3 min, which coincides with the

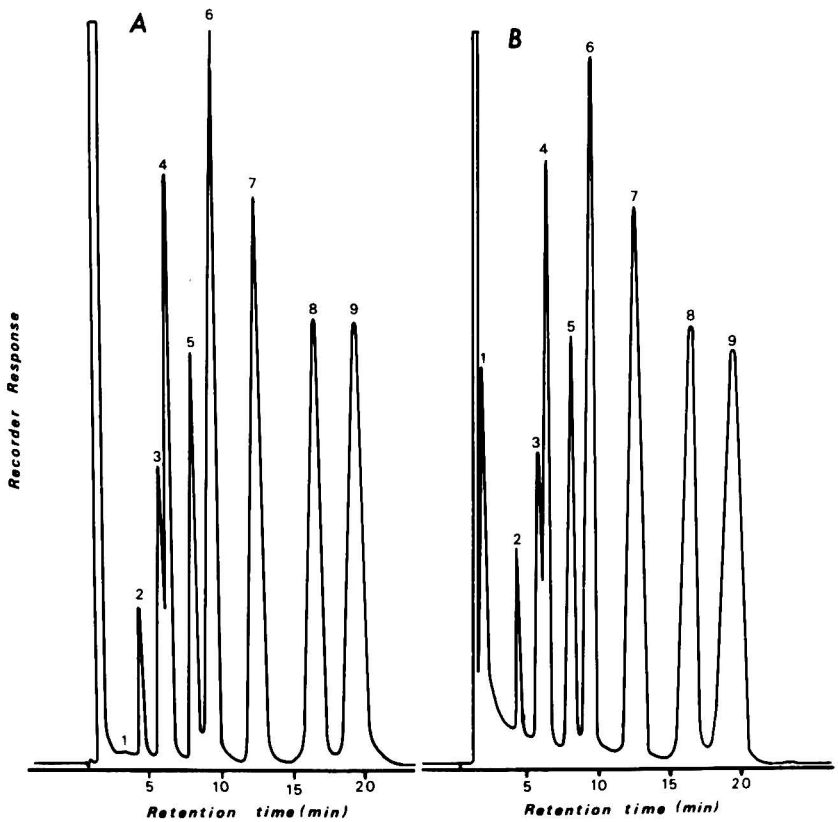

FIG. 1—GLC spectra of standard acid mixture: A, dried 5 min with 1/10 of its volume of P_2O_5; B, dried 10 min with 1/2 its volume of $MgSO_4$. Peaks: 1, water; 2, acetic acid; 3, propionic acid; 4, isobutyric acid; 5, butyric acid; 6, isovaleric acid; 7, valeric acid; 8, isocaproic acid; 9, caproic acid.

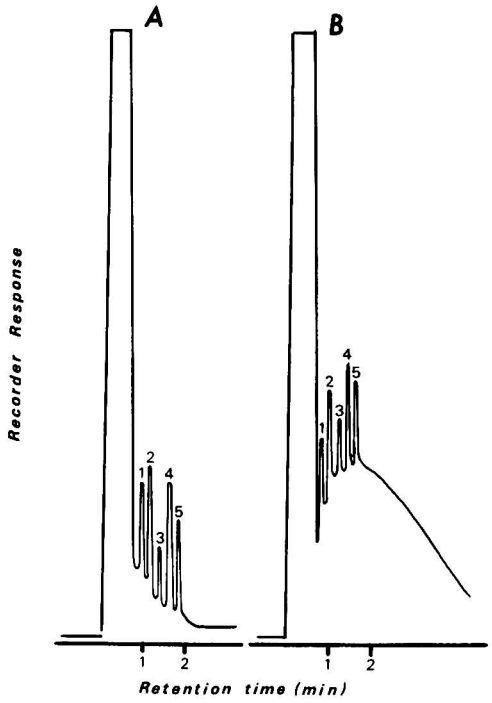

FIG. 2—GLC spectra of standard alcohol mixture: A, dried 5 min with 1/10 its volume of P_2O_5; B, dried 10 min with 1/2 its volume of $MgSO_4$. Peaks: 1, ethanol; 2, propanol; 3, isobutanol; 4, butanol; 5, pentanol.

wide water peak, eluting between 1 and 4 min. Figure 2A shows a standard alcohol mixture dried 5 min with phosphorus pentoxide, and Fig. 2B shows the same mixture dried 10 min with magnesium sulfate. These data show that the water peak was completely removed after drying with phosphorus pentoxide, while it was clearly present under the alcohol peaks after drying with magnesium sulfate.

When phosphorus pentoxide is used as a drying agent, the drying time must be kept at a minimum because secondary products may be formed or alcohol levels may be reduced. However, neither of these situations occurred for any combination dried 30 min with 1/10 volume of phosphorus pentoxide.

When acid concentrations are low, it is often necessary to inject large volumes of solvent into the chromatograph. This gives a wide solvent peak and total or partial masking or shoulder separation of alcohol peaks. Therefore 2 μl of the ether extracts is injected for alcohol determination, followed by injection of 14 μl for detection of acids. Figures 3A and 3B show the chromatographic spectra of the metabolic products formed by a psychotrophic *Clostridium*, No. 13, described earlier by Finne and Matches

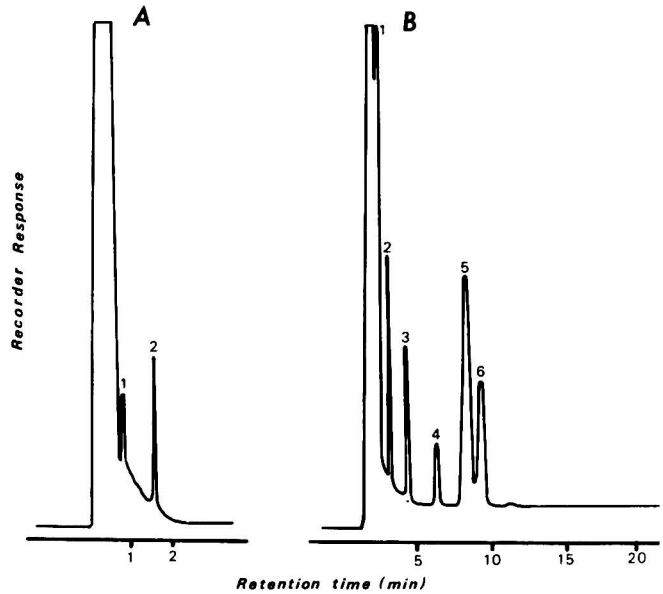

FIG. 3—GLC spectra of metabolic products produced by organism No. 13: A, alcohol spectrum at 2"/min chart speed; B, total spectrum at 1"/min chart speed. Peaks: 1, ethanol; 2, butanol; 3, acetic acid; 4, isobutyric acid; 5, butyric acid; 6, isovaleric acid.

(4). The alcohols ethanol and butanol are evident when 2 μl is injected and a 2″/min chart speed is used (Fig. 3A). These alcohols also appeared on the acid spectrum (Fig. 3B), with a 14 μl injection, but they appeared as shoulder peaks on the solvent peak and were close together because of the slower chart speed. The acids acetic, isobutyric, butyric, and isovaleric are products of fermentation of the psychrotrophic organism.

Discussion

By testing a number of standard *Clostridium* cultures, chromatographic patterns similar to those reported by Cato *et al.* (6) were obtained. The method permits the assignment of unknowns to groups containing a maximum of 10 organisms and, in some cases, even a specific organism, thus permitting a quick presumptive identification. A number of anhydrous inorganic salts such as magnesium sulfate have been used to remove water from organic systems. For this reagent to be effective, only low levels of water may be present in the ether extracts. To achieve such low levels of water, the sample may be frozen before the ether layer is decanted (1). For this study, we found that phosphorus pentoxide is more effective, and the freezing step can be omitted.

A major problem with the use of magnesium sulfate is that large concentrations of finely divided solid particles in the ether layer can easily plug the chromatographic syringe. This problem does not exist with phosphorus pentoxide. However, the latter compound is corrosive and an irritant to the skin; therefore, extreme caution must be exercised in its use.

Because of the high reactivity of phosphorus pentoxide with possible components of the sample, the drying time must be kept short. A prolonged drying reduces both alcohols and acids, and volatiles other than those normally present are formed. If only acids are present, phosphorus pentoxide will catalyze anhydride formation. If both acids and alcohols are present, esters will be formed and additional chromatographic peaks will be produced. If only alcohols are present, their levels will be reduced, presumably because phosphoric esters are formed. However, no adverse effects were noted for any combination dried 30 min with 1/10 volume of phosphorus pentoxide. This method is especially applicable when a rapid analysis is desired or when large numbers of samples must be analyzed. With large numbers of samples, the extracts can be prepared, dried, and injected at a rapid pace, without a long drying time. The complete removal of water permits detection of very low levels of alcohols, which would be difficult in samples dried with magnesium sulfate.

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Collaborative Study of an Improved Method for the Enumeration and Confirmation of *Clostridium perfringens* in Foods

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A collaborative study was conducted in 10 laboratories to evaluate the performance of a new method for the enumeration of vegetative cells of *Clostridium perfringens* in foods. Results obtained by the new method were compared with results from the official first action method, 46.049–46.053. Per cent recoveries of 4 *C. perfringens* strains from inoculated roast beef samples were higher and more consistent in tryptose-sulfite-cycloserine (TSC) agar with or without added egg yolk than in sulfite-polymyxin-sulfadiazine (SPS) agar, specified in the official first action method. The confirmatory technique utilized in the new method was also found to be more reliable than the technique described in the official first action method. Based on the collaborative results, the new method with TSC agar for enumeration and a modified motility-nitrate medium together with a lactose-gelatin medium for confirmation of *C. perfringens* has been adopted as official first action to replace 46.049–46.053.

In recent years, several new plating media have been developed for the enumeration of *Clostridium perfringens* in foods (1–4). These media were developed in an attempt to eliminate some serious shortcomings of plating media such as the sulfite-polymyxin-sulfadiazine (SPS) agar recommended in the official first action method, **46.049–46.053**. The most serious problems which have been encountered with SPS agar are (*1*) the frequent failure of *C. perfringens* colonies to reduce sulfite in this medium, and (*2*) the lack of selectivity which prevents the isolation of pure cultures for the required confirmation tests. The commercial preparations of SPS agar have proved to be particularly unsatisfactory, as indicated by different reports in the literature (5). The results of comparative studies recently conducted in different laboratories indicate that tryptose-sulfite-cycloserine (TSC) agar is probably the most satisfactory plating medium available at the present time for enumerating *C. perfringens* in foods (2, 3). This medium permits essentially quantitative recovery of *C. perfringens* and is very effective in preventing outgrowth of competing bacteria which may be present in foods. Furthermore, commercial preparations of the basal medium used for TSC agar have proved to be very satisfactory. A modification of this medium has recently been recommended in which it is suggested that egg yolk be omitted from TSC agar and that the sample dilutions be cultured in pour plates (3). Because use of TSC agar in this manner was shown to be quite effective, this variation was included in the collaborative testing.

Some problems have also been reported with media and testing procedures recommended in the official first action method for confirming isolates from the plating medium. These include poor growth response of some strains in nitrate-motility medium, failure of isolates to produce detectable nitrite, and difficulty in demonstrating sporulation with many strains. Several investigators have made suggestions for improving the performance and reliability of the confirmation tests (3, 4). The most promising of the improved confirmatory media were chosen for collaborative testing.

A method incorporating what appeared to be the best media and techniques for enumeration of *C. perfringens* was drafted in May 1974 and submitted to 10 laboratories for a collaborative study. The purpose of the study was to compare the performances of the new media and testing techniques with those recommended in the official first action method.

Collaborative Study

Ten microbiologists, each from a different laboratory, agreed to serve as collaborators. Each collaborator received a complete set of instructions, a copy of the proposed examination method, and data report forms. All media and other materials needed for the collaborative study were supplied by the Associate Referee.

This report of the Associate Referee was presented at the 89th Annual Meeting of the AOAC, Oct. 13–16, 1975, at Washington, DC.

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The plating media used were prepared from the same lots of commercial medium recommended for each method, and the confirmatory media were prepared from the ingredients specified. Each collaborator examined four 50 g samples of canned roast beef hash after inoculating each with 10^5–10^6 vegetative cells of a different *C. perfringens* strain/g. Spore stocks of the same 4 strains used for inoculating the test samples were supplied to each collaborator by the Associate Referee. The *C. perfringens* cultures used were grown under specified conditions in each laboratory in the same lot of a supplemented commercial cooked meat medium, and 0.5 ml culture of the strain was added to a pre-weighed 50 g beef hash sample. This method of sample preparation was considered to be necessary because of the very substantial reduction in the number of viable *C. perfringens* vegetative cells which occurs during storage and shipment of food contaminated with this organism.

The general techniques outlined in the proposed method and in the official first action method were followed throughout, except that peptone water was used for homogenizing and diluting the samples. Sample dilutions were plated in duplicate in SPS agar, TSC agar, egg yolk-free TSC agar, and TSC agar base from which egg yolk and D-cycloserine were omitted. The TSC agar basal medium, which is nonselective, was included to provide a common basis for comparing the performance of the selective plating media. Colony counts were determined in each of the 4 plating media, and 10 colonies were selected from each medium and subjected to the confirmation tests recommended in the proposed method and those specified in the official first action method.

METHOD

CLOSTRIDIUM PERFRINGENS OFFICIAL FIRST ACTION

(Applicable to examination of outbreak foods in which relatively large numbers of vegetative cells are expected to be present)

46.B01 *Apparatus*

(a) *Pipets.*—1.0 ml serological with 0.1 ml graduations and 10.0 ml with 1.0 ml graduations.

(b) *Colony counter.*—Quebec, or equiv., dark field model.

(c) *High-speed blender.*—Waring Blendor, or equiv., 2-speed std model, with low-speed operation at 8000 rpm, and 1 L glass or metal blender jars with covers. One jar is required for each sample.

(d) *Anaerobic jars.*—BBL Gas-Pak jars equipped with Gas-Pak H + CO_2 generator envelopes are recommended. Anaero-jar (Pfizer Diagnostics, 1407 N Dayton St, Chicago, IL 60622) with replacement of air by purified N or N-CO_2 (9+1) is satisfactory.

46.B02 *Reagents*

(a) *Peptone dilution water.*—Dissolve 2.0 g peptone (Difco B118) in 2 L H_2O for each sample, and adjust to pH 7.0±0.1. Dispense enough vol. in 6 oz bottles to give 90±1 ml and in 750 ml erlenmeyers to give 450±5 ml after autoclaving 15 min at 121°.

(b) *Nitrite test reagents.*—(*1*) *Reagent A.*—Dissolve 8 g sulfanilic acid in 1 L 5*N* HOAc (2+5). (*2*) *Reagent B.*—Dissolve 5 g α-naphthol in 1 L 5*N* HOAc.

46.B03 *Culture Media*

(Sizes of culture media containers (test tubes, flasks, and petri dishes) are specified for each medium. All media except tryptose-sulfite-cycloserine (TSC) agar are incubated in air at 35°. Media not used ≤4 hr after prepn must be heated 10 min in boiling H_2O or flowing steam to expel O and cooled rapidly in tap H_2O without agitation just before use.)

(a) *Tryptose-sulfite-cycloserine agar.*—15.0 g tryptose, 20.0 g agar, 5.0 g soytone, 5.0 g yeast ext, 1.0 g Na metabisulfite, and 1.0 g ferric ammonium citrate (NF Brown Pearls) dild to 1 L with H_2O (SFP agar base, Difco 0811-01, is satisfactory). Adjust to pH 7.6±0.1, dispense 250 ml portions into 500 ml flasks, and sterilize 15 min at 121°. Before plating, add 20.0 ml 0.5% filter-sterilized soln of D-cycloserine to each 250 ml sterile melted medium at 50°. To make egg yolk-contg plates, add 20 ml 50% egg yolk emulsion, (c), to 250 ml sterile medium contg D-cycloserine. Dispense 15 ml portions into 100 × 15 mm sterile petri dishes. Cover plates with towel and let dry overnight at room temp. before use.

(b) *D-Cycloserine soln.*—Dissolve 1 g D-cycloserine (Calbiochem No. 23983; ICN Pharmaceuticals, Inc., Life Sciences Group No. 100535; or Serva Feinbiochemicia, Heidelberg, West Germany) without heating in 200 ml 0.05*M* phosphate buffer (pH 8.0±0.1) and sterilize with 0.45 μm membrane filter.

(c) *Egg yolk emulsion.*—Wash fresh eggs with stiff brush and drain. Soak 1 hr in 70% alcohol. Aseptically remove yolk and mix with equal vol. sterile 0.85% NaCl soln. Store at 4°.

(d) *Buffered motility-nitrate medium.*—3.0 g beef ext, 5.0 g peptone, 5.0 g KNO_3, 2.5 g Na_2HPO_4, 3.0 g agar, 5.0 g galactose, and 5.0 g glycerol dild to 1 L with H_2O. Adjust to pH 7.3±0.1, dispense 11 ml portions into 150 × 16 mm tubes, and sterilize 15 min at 121°.

(e) *Lactose-gelatin medium.*—15.0 g tryptose, 10.0

g yeast ext, 10.0 g lactose, 5.0 g Na_2HPO_4, 0.05 g phenol red, and 120.0 g gelatin dild to 1 L with H_2O. Adjust to pH 7.5±0.1 before adding lactose and phenol red. Dispense 10 ml portions into 150 × 16 mm screw-cap tubes and sterilize 15 min at 121°.

(f) *Sporulation broth.*—15.0 g polypeptone, 3.0 g yeast ext, 3.0 g sol. starch, 0.1 g $MgSO_4$, 1.0 g Na thioglycolate, and 11.0 g Na_2HPO_4 dild to 1 L with H_2O. Adjust to pH 7.8±0.1, dispense 15 ml portions into 150 × 20 mm screw-cap tubes, and sterilize 15 min at 121°.

(g) *Polypeptone-yeast extract (PY) medium.*—20.0 g polypeptone, 5.0 g yeast ext, and 5.0 g NaCl dild to 1 L with H_2O. Adjust to pH 6.9±0.1, dispense 9 ml portions into 125 × 16 mm screw-cap tubes, and sterilize 15 min at 121°.

(h) *Fluid thioglycolate medium.*—(BBL-01-140, Difco B256, Oxoid CM173). Dispense 10 ml portions into 150 × 16 mm screw-cap tubes. Sterilize 15 min at 121°, and cool quickly. Final pH is 7.1±0.1.

46.B04 Preparation of Food Homogenate

Using aseptic technic, weigh 50 g food sample into sterile blender jar. Add 450 ml peptone diln H_2O and homogenize 2 min at low speed (8,000–10,000 rpm). Use this 1:10 diln to prep. serial dilns from 10^{-2} to 10^{-6} by transferring 10 ml of 1:10 diln to 90 ml diln blank, mixing well with gentle shaking, and continuing until 10^{-6} diln is reached.

46.B05 Plate Count Technic

Pour ca 5 ml TSC agar without egg yolk into each of ten 100 × 15 mm petri dishes and spread evenly by rapidly rotating dish. When agar has solidified, label plates and aseptically pipet 1 ml of each diln of homogenate in duplicate onto agar surface in center of dish. Pour addnl 15 ml TSC agar without egg yolk into dish and mix well with inoculum by gently rotating dish.

Alternatively, with sterile glass rod spreader, spread 0.1 ml diln over previously poured plates of TSC agar contg egg yolk emulsion. Let plates absorb inoculum 5–10 min; then overlay with 10 ml TSC agar without egg yolk. (TSC agar contg egg yolk is preferred for foods which may also contain other sulfite-reducing *Clostridium* sp.)

When agar has solidified, place plates in upright position in anaerobic jar. Produce anaerobic conditions, and incubate jar 20 hr at 35° for TSC agar without egg yolk and 24 hr at 35° for TSC agar with egg yolk. After incubation, remove plates from jar and observe macroscopically for growth and black colony production. Select plates showing estd 20–200 black colonies. Using Quebec colony counter with piece of white tissue paper over counting area, count black colonies and calc. number of *Clostridium* sp./g food. *C. perfringens* colonies in medium contg egg yolk are black and usually surrounded by 2–4 mm zone of white ppt due to lecithinase activity. However, since a few strains are weak or neg. for lecithinase, count any black colonies suspected to be *C. perfringens* and confirm identity as in **46.B06**.

46.B06 Confirmation Technic

Select 10 characteristic colonies from countable plates (20–200 colonies), inoculate each into tube of fluid thioglycolate medium, and incubate 18–24 hr at 35°. Make Gram-stained smear of fluid thioglycolate cultures and check for purity and presence of short, thick, Gram-pos. bacillus characteristic of *C. perfringens*. Streak contaminated cultures on TSC agar contg egg yolk and incubate plates anaerobically 24 hr at 35° to obtain pure cultures. Stab-inoculate buffered motility-nitrate and lactose gelatin media with 2 mm loopfuls of pure fluid thioglycolate culture or portion of isolated colony from TSC agar plate. Inoculate sporulation broth with 1 ml fluid thioglycolate culture and incubate 24 hr at 35°. Examine tubes of buffered motility-nitrate medium by transmitted light for type of growth along stab. Nonmotile organisms produce growth only in and along line of stab. Motile organisms produce diffuse growth out into medium away from stab.

Test buffered motility-nitrate medium for presence of nitrite by adding 0.5 ml Reagent A and 0.2 ml Reagent B. Orange which develops within 15 min indicates presence of nitrites. If no color develops, add few grains of powd Zn metal, and let stand 10 min. No color change after addn of Zn indicates that nitrates are completely reduced; change to orange indicates that organism is incapable of reducing nitrates.

Examine lactose-gelatin medium for gas and color change from red to yellow, indicating that lactose is fermented with production of acid. Chill tubes 1 hr at 5° and check for gelatin liquefaction. If medium solidifies, reincubate addnl 24 hr at 35° and repeat test for gelatin liquefaction. Make Gram-stained smear from sporulation broth and examine microscopically for spores. Report whether or not spores are produced. Store sporulated cultures at 4° if further testing of isolates is desired.

Nonmotile, Gram-pos. bacilli which produce black colonies in TSC agar, reduce nitrates to nitrites, produce acid and gas from lactose, and liquefy gelatin within 48 hr are provisionally identified as *C. perfringens*.

Organisms suspected to be *C. perfringens* that do not meet criteria stated above must be confirmed by further testing. Subculture into fluid thioglycolate medium isolates that do not liquefy gelatin or which are atypical in other respects. Incubate 24 hr at 35°, make Gram-stained smear, and check for purity. Inoculate 1 tube of PY medium, (g), contg *1% salicin* and 1 tube contg *1% raffinose* with 0.1 ml fluid thio-

glycolate culture. Incubate media 24 hr at 35° and check PY-salicin for acid and gas. Transfer 1.0 ml culture to test tube and add 1–2 drops *0.04% phenol red.* Yellow indicates acid is produced from salicin. (Salicin is not usually fermented by *C. perfringens* but is rapidly fermented with production of acid and gas by closely related species.) Reincubate media addnl 48 hr and test both media for production of acid. Acid is usually produced from raffinose by *C. perfringens* but not by closely related species. Acid is produced from salicin in PY medium by a few strains of *C. perfringens.*

Calc. number of *C. perfringens* in sample on basis of % colonies tested that are confirmed as *C. perfringens.* (*Example*: If av. plate count of 10^{-4} diln was 85, and 8 of 10 colonies tested were confirmed as *C. perfringens,* number of *C. perfringens*/g food is 85 × (8/10) × 10,000 = 680,000.) (*Note:* Diln factor with plates contg egg yolk is 10-fold higher than diln plated.)

Results and Discussion

The collaborative results presented in Table 1 show the relative overall per cent recoveries in the selective plating media compared to plate counts obtained with the nonselective control medium TSC agar base. For the purpose of evaluating performance of the selective media, recovery in TSC agar base without antibiotics was considered to be 100%. These results show that higher overall recoveries were obtained in egg yolk-free TSC agar than in the other selective plating media. Average recoveries in this medium ranged from 83.0 to 104.8% with an overall mean of 93.3% compared to colony counts in TSC agar base. Overall per cent recoveries in TSC agar containing egg yolk were somewhat lower than in the egg yolk-free medium. The recoveries reported in TSC agar containing egg yolk ranged from 60.8 to 96.8% with an overall mean of 78.2% compared to the nonselective control medium.

The unsatisfactory performance of the commercial SPS agar recommended in the official first action method, which has long been recognized, was amply demonstrated by the collaborative results. Four of the 10 collaborators reported that no black colonies were produced by *C. perfringens* in SPS agar with any of the samples examined and all of the collaborators reported the absence of black colonies with 1 or more samples. This probably accounts for the low recoveries reported with SPS agar, as shown in Table 1. Average recoveries in SPS agar on the basis of sulfite-reducing colonies varied from

Table 1. Collaborative results (per cent recovery)[a] with 4 *C. perfringens* strains[b] in different plating media

	TSC agar without egg yolk					TSC agar contg egg yolk					SPS agar				
Coll.	S1	S2	S3	S4	Av.	S1	S2	S3	S4	Av.	S1	S2	S3	S4	Av.
1	89	103	109	95	99.0	78	105	104	90	94.3	98	0	52	0[c]	37.5
2	83	87	97	89	89.0	74	94	103	61	83.0	0	0	0	0	0.0
3	98	113	99	109	104.8	96	86	88	59	82.3	84	0	67	0	37.8
4	93	92	108	77	92.5	46	73	89	35	60.8	102	106	107	0	78.8
5	89	85	76	89	84.7	59	70	58	93	70.0	0	0	0	0	0.0
6	87	100	112	113	103.0	86	77	69	55	71.8	61	83	85	0	57.3
7	100	112	100	89	100.3	41	105	114	56	79.0	80	88	0	0	42.0
8	93	71	78	95	84.3	74	69	66	84	73.3	0	0	0	0	0.0
9	95	80	100	93	92.0	97	73	112	105	96.8	108	79	0	0	46.8
10	65	103	83	81	83.0	48	98	85	52	70.8	0	0	0	0	0.0
Overall mean					93.3					78.2					30.2
Coeff. of var., % (transformed data)															
reproducibility			15.50					22.14					139.83		
repeatability			11.42					2.89					5.36		
Coeff. of var., % (per cent recovery data)															
reproducibility			12.92					25.92					130.09		
random or exper. error			11.21					24.73					116.17		

[a] Compared with colony counts in TSC agar base without cycloserine = 100%.
[b] Strains tested: FD-1 (S1), FD-2 (S2), CDC 1861 (S3), and NCTC 8797 (S4).
[c] No black colonies produced.

0.0% in 4 laboratories to 78.8% in 1 laboratory with an overall mean of only 30.2% compared with results with TSC agar base. Black colonies were produced by *C. perfringens* with only 14 of the 40 samples examined in the commercial SPS agar recommended.

The 4 different *C. perfringens* strains tested showed considerable variation in their response to the different selective plating media as shown by the collaborative results in Table 1. These results show that the per cent recovery of all 4 strains from the inoculated beef hash in TSC agar without egg yolk was the most similar to that obtained in TSC agar base. Since these 2 media differed only in the addition of D-cycloserine to TSC agar basal medium to provide selectivity, such a result might be expected. With TSC agar containing egg yolk, the per cent recoveries reported were often lower and more variable than with the egg yolk-free medium. However, in comparing these results, it should be kept in mind that, because TSC agar containing egg yolk is surface plated, a smaller sample volume and different sample dilutions were used. This undoubtedly would affect, to some extent, colony counts obtained by the 2 plating techniques. Other factors which may also have adversely affected results in TSC agar with egg yolk are: (*1*) use of a plating technique unfamiliar to some of the collaborators, and (*2*) storage of the plated TSC agar medium in air prior to use. Studies conducted previously in our laboratory had shown that storage of plating media in air prior to use resulted in a small decrease in colony counts of *C. perfringens*.

The collaborative results with the plating media were transformed into logarithms and were analyzed statistically to determine the precision of results in the different laboratories and to measure variability for replicate determinations within a laboratory. The effects of media and strain interactions were also determined. Results of these analyses showed that the difference in overall recovery in TSC agar without egg yolk, which was 93.3% compared with the control medium TSC agar base, was of only borderline significance ($P = 0.05$). Overall per cent recoveries in TSC agar with egg yolk and in SPS agar differed significantly from that of the control medium, TSC agar base ($P < 0.01$). However, from a practical standpoint, the difference in overall recovery in TSC agar with or without added egg yolk was quite small. The weighted overall per cent recovery in TSC agar with egg yolk was 79.1 compared with 89.3 recovery in the egg yolk-free medium. Strain and media interactions were also found to differ significantly, i.e., $P < 0.01$. This was due primarily to variability in response of the 4 strains to SPS agar.

The coefficients of variation for results between laboratories (reproducibility) were 11.42% with TSC agar base, 15.50% with egg yolk-free TSC agar, and 22.14% with TSC agar containing egg yolk. The lack of reproducibility with SPS agar resulted in a very large coefficient of variation (139.83%) for this medium. The coefficients of variation for replicate determinations within a laboratory (repeatability) were quite small for an anaerobic plate count method. These values were 2.77% for TSC agar base, 2.89% for TSC agar containing egg yolk, 5.36% for SPS, and 11.42% for egg yolk-free TSC agar. The coefficients of variation for reproducibility and random or experimental data, based on per cent recovery rather than transformed data, are also given in Table 1.

The collaborative results indicate that TSC agar medium is superior to the SPS agar recommended in the official first action method for enumeration of *C. perfringens*. Sulfite-reducing colonies were produced in TSC agar with or without added egg yolk by all 40 of the test samples, whereas only 14 samples yielded positive results in SPS agar. Comments solicited from the collaborators indicated that most of them would prefer to use the egg yolk-free modification of TSC agar. The advantages of this medium are ease of preparation and a slightly higher rate of recovery with less variation between replicate tests. The disadvantages of the egg yolk-free TSC agar are its inability to distinguish colonies of *C. perfringens* from those of other sulfite-reducing organisms and the requirement for an inconvenient (18–20 hr) incubation period. Otherwise, excessive blackening of the medium may prevent accurate colony counts.

Even though the collaborative results indicate that a lower overall per cent recovery may be obtained with some *C. perfringens* strains in TSC agar containing egg yolk, the advantages of using this medium should be pointed out. Because no suitable naturally contaminated foods

were available for collaborative testing, these advantages could not be effectively evaluated in the present study. Results from previous comparative studies with naturally contaminated food have shown that *C. perfringens* colonies are easier to recognize in TSC agar containing egg yolk because of their lecithinase activity. Therefore, fewer colonies had to be isolated for confirmation tests. These data were presented at the 87th Annual AOAC Meeting in 1973 (6). Because *C. perfringens* colonies are easier to recognize and isolate from TSC agar containing egg yolk, the use of this medium for foods which may contain other sulfite-reducing bacteria is a very desirable alternative.

The confirmatory tests recommended in the proposed method and in the official first action method were also evaluated. Collaborative results obtained with the confirmatory media are presented in Table 2. These results show that growth response was better, and that higher nitrite levels were present, with the majority of isolates of each of the test strains from plating media in the modified motility-nitrate medium. Overall results with this medium were much better than in the motility-nitrate medium recommended in the official first action method. Because of the higher nitrite level present in the modified medium, α-naphthol could be substituted for α-naphthylamine in conducting the test for presence of nitrites. This change seems highly desirable in view of the specific request of the AOAC to eliminate the use in official methods, whenever possible, of reagents such as α-naphthylamine which are known to be potentially carcinogenic. Substitution of α-naphthol for α-naphthylamine would not affect specificity of the nitrate reduction test for *C. perfringens*. No particular difficulties in preparation or use of either the confirmatory medium or the test reagents were reported.

All of the *C. perfringens* strains used in the study fermented lactose and liquefied gelatin in lactose-gelatin medium within 48 hr as shown in Table 2. Profuse gasing of the medium and production of acid usually occurred within 24 hr at 35°C and gelatin was liquefied by all strains after 48 hr incubation. Recent reports in the literature indicate that a gelatin liquefaction test is needed to differentiate *C. perfringens* from closely related organisms (7, 8). These organisms, currently classified as *C. paraperfringens*, *C. absonum*, and *C. celatum*, may be differentiated from *C. perfringens* by their inability to liquefy gelatin and by tests for fermentation of salicin and raffinose in polypeptone-yeast extract medium. These species have been isolated from soil, human feces, and spices, so it seems likely that they would also be present in some foods.

Because of the many reports which have been made concerning the difficulty of demonstrating sporulation with isolates of *C. perfringens* from foods, no attempt was made in the present study to evaluate the performance of the many different sporulation broths which have been recommended. Since the demonstration of sporulation per se does not contribute further to identification of isolates as *C. perfringens*, it is suggested that demonstration of spores be considered an optional part of the confirmation technique. A sporulation broth is included in the proposed method for this purpose and for maintaining the cultures in a viable state in case further testing is desired (9).

Recommendation

It is recommended that the proposed method for the enumeration and confirmation of *C. perfringens* be adopted as official first action to replace the official first action method **46.049–46.053**, which should be repealed.

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The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee E and was adopted by the Association. *See* (1976) *JAOAC* **59**, 397.

Table 2. Collaborative results with 180 isolates in confirmatory media[a]

	Reaction in[b]							
	Motility-nitrate (MN) medium			Modified MN medium			Lactose-gelatin medium	
Strain	Growth	Motility	Nitrite	Growth	Motility	Nitrite	Lactose	Gelatin
FD-1	fair-good	–	++	good	–	+++	AG	L
FD-2	fair-good	–	(+)	good	–	+++	AG	L
CDC 1861	poor	–	+	fair	–	+++	AG	L
NCTC 8797	poor	–	(+)	fair-good	–	+++	AG	L

[a] Results reported for at least 35 of the 45 isolates of each strain tested.
[b] – = negative test; (+) = weak or trace; + = positive test; ++ = higher NO_2 reaction; +++ = strong NO_2 reaction; AG = acid and gas; L = liquefaction.

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DECOMPOSITION IN FOODS (CHEMICAL METHODS)

Lactic and Succinic Acid Levels and Refractive Indices in the Determination of the Age of Eggs

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The increase in the concentration of lactic and succinic acids during storage was measured in an attempt to determine the age of eggs. The increase in succinic acid alone is not sufficient to indicate egg age. Although lactic acid concentrations increase more rapidly, the levels are still very low, the increase is not linear, and concentrations in fresh eggs vary widely. On the other hand, refractive indices showed a nearly linear correlation with age and very little variation between eggs. These measurements are reproducible and easy to perform and, with further study, should provide an alternative to the AOAC method.

Lactic acid and succinic acid determinations were proposed several years ago as indices of egg decomposition. Early methods specified titration to measure these acids, but, following extensive studies (1–4), a gas chromatographic method was adopted as official final action by the AOAC (3, 5). In fresh eggs, lactic acid may be as high as 4.2 mg/100 g egg (6, 7), a value which is generally considered as passable.

Refractive indices may also be used to calculate the so-called value number (Wertzahl) and ageing number (Alterungszahl). This method, basically developed by Janke and Jirak (8), is still used in Switzerland and some other European countries as an official analysis procedure for the estimation of egg quality (9).

The present study compares both methods for eggs destined for consumption. Furthermore, both methods were tested for determination of egg age. Lactic acid and succinic acid content is usually determined in a mixed pool of several eggs. We preferred to analyze eggs individually. Accordingly we used a slight modification of the official AOAC method (5).

Experimental

Seven series of 11 fresh, unfertile eggs each from a controlled flock of white Leghorn hens were used for this study. On alternate days and then at regular intervals, one egg from each of 6 series was analyzed for its lactic and succinic acid content. During the experiment, eggs were protected from sunlight and stored at a constant temperature (24°C). The remaining series was used for refractive index measurements.

Reagents

(a) *Boron trichloride-1-propanol solution.*—Prepare as follows: Bubble BCl_3 (Air Products) through ice-cooled 1-propanol until saturated. This solution contains ca 12% BCl_3.

(b) *Calcium lactate standard solution.*—Dissolve 340.5 mg calcium-L(+)-lactate (purum grade, Fluka, Buchs, Switzerland) in 100 ml water. Prepare fresh daily.

(c) *Succinic acid standard solution.*—Dissolve 40.6 mg succinic acid (E. Merck, Darmstadt, W. Germany, No. 682) in 100 ml water.

(d) *Acetophenone internal standard solution.*—(E. Merck, No. 800028). 100 mg/100 ml methylene chloride.

Apparatus

(a) *Continuous liquid extractor.*—40 ml capacity; *see* Fig. 1.

(b) *Gas chromatograph.*—Varian Model 1400, or equivalent, with flame ionization detector and 1.8 m × 2 mm id glass column packed with 15% DEGS on 80–100 mesh Gas-Chrom Q. Operating conditions: column temperature programmed from 90 to 150°C at 4°C/min and then operated isothermally; injector and detector temperatures 180°C; gas flows (ml/min)—nitrogen carrier gas 15, hydrogen 15, air 300.

(c) *Digital integrator.*—Kipp, The Netherlands.

(d) *Abbe refractometer.*—Model 60 ED (Bellingham and Stanley Ltd., London, England) with sodium spectral lamp.

Extraction and Esterification

Place contents of 1 egg in preweighed 600 ml beaker with magnetic stirring bar. Weigh again,

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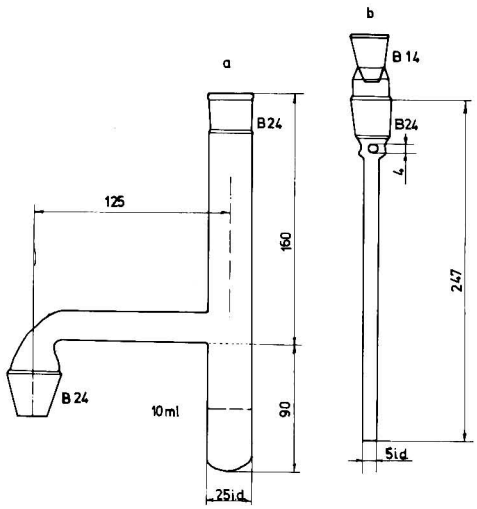

FIG. 1—Continuous extraction apparatus: a, 40 ml extraction tube; b, inner tube—ether vapors pass through 4 mm holes to condenser (all measures in mm).

and add 125 ml water and 20 ml 1*N* H_2SO_4. To break egg yolk, place beaker on a magnetic stirrer and mix thoroughly, adding 30 ml 10% phosphotungstic acid solution while mixing. Dilute contents to 250 g on balance, mix again, and filter through 11 cm folded rapid paper (Whatman No. 1) into pre-weighed 250 ml beaker. Weigh filtrate and evaporate on steam bath to ca 5 ml. Transfer concentrated extract to 40 ml extractor, rinse beaker twice with 2 ml water, and pour rinsings into extractor. Adjust volume in extractor to 10 ml with water, and add 4 g $(NH_4)_2SO_4$ and 1 drop concentrated H_2SO_4. Put 2 mm diameter glass beads into extractor to 10 ml calibration mark. After inserting inner tube, connect efficient condenser together with external 100 ml flask containing 70 ml ethyl ether to extractor. Continue extraction 5 hr. Remove internal tube of extractor and decant remaining ether into attached 100 ml flask. Evaporate organic extract on steam bath until nearly dry and add 1 ml boron trichloride-1-propanol reagent.

Esterify 15 min under reflux on steam bath. Cool, and add 4 ml saturated $(NH_4)_2SO_4$ solution and 5 ml methylene chloride. Transfer contents to 50 ml separatory funnel, shake 1 min, and let layers separate. Filter organic layer into 10 ml volumetric flask through ca 0.5 g Na_2SO_4 in small funnel with glass wool plug. Use 3 ml methylene chloride to rinse round-bottom flask and extract lower aqueous layer a second time. Filter upper organic layer over same anhydrous Na_2SO_4 into the volumetric flask. Rinse Na_2SO_4 with 1 ml methylene chloride and add rinse to flask. Add 1 ml internal standard solution and adjust volume to 10 ml. Inject 1 μl of this solution into gas chromatograph.

Calibration.—Combine 1, 2, 3, and 4 ml each of calcium lactate and succinic acid stock solutions in 50 ml round-bottom flasks and evaporate solvent to dryness on steam bath. Add 1 ml boron trichloride-1-propanol reagent and esterify as in second paragraph above. Calibration solutions are stable several weeks when stored in freezer. Prepare calibration curves before every series of analyses (10).

Recoveries

Homogenize contents of 2 fresh eggs and divide into 2 parts of equal weight in separate 600 ml beakers with magnetic stirring bars. To one beaker add known amount of calcium lactate and succinic acid. Use other portion as a control. Treat both samples similarly. Add 125 ml water and 20 ml 1*N* H_2SO_4 and mix. Proceed with *Extraction and Esterification,* beginning ". . . add 30 ml 10% phosphotungstic acid solution . . .".

Determination of Refractive Indices

Calibrate refractometer according to operating instructions of manufacturer. Maintain temperature of prisms at 20°C, using water circulation from connected ultra thermostat. Make all readings relative to sodium D_1 line.

Break egg shell and separate egg white from yolk in two 100 ml beakers. Homogenize egg white by sucking material up and down several times through narrow pipet to prevent foaming. Using same pipet, spread 1 drop of sample in center of polished surface of prism. Repeat same procedure for yolk, using one drop of sample sucked from the center of egg yolk. Calculate value numbers and ageing numbers as follows:

$$\text{Value number} = 1000\ (n_D^y - n_D^w)$$
$$\text{Ageing number} = 1000\ (n_D^s - n_D^y)$$

where n_D^y = refractive index of yolk; n_D^w = refractive index of egg white; n_D^s = refractive index of standard fresh yolk at same temperature. Its value at 20°C was 1.4195 (9).

Results and Discussion

Recoveries were satisfactory and agreed with earlier studies (2). Lactic acid recoveries varied between 95 and 103%; succinic acid recoveries varied between 93 and 97% (*see* Table 1). For incubator reject eggs, with the embryo growth terminated on the third day, Salwin *et al.* (11) found a steady increase in the formation of β-hydroxybutyric acid. Although they did not use

Table 1. Recovery of lactic and succinic acids added to egg (mg acid/100 g egg)

Lactic acid			Succinic acid		
Added	Recd	Rec., %	Added	Recd	Rec., %
4.0	4.1	103	1.00	0.97	97
8.0	7.9	99	2.00	1.88	94
16.0	16.3	102	3.00	2.87	96
32.0	30.7	96	4.00	3.73	93
64.0	60.8	95	6.00	5.71	95

it to determine the time of incubation, β-hydroxybutyric acid content could be used for this purpose. We wished to test whether variations in lactic and/or succinic acid concentrations could be used to determine the age of eggs.

Lactic acid and succinic acid concentrations did increase with storage time (Tables 2 and 3). However, succinic acid concentration alone is not sufficient to determine age because the increase is too small. Although lactic acid concentration increases more rapidly, it alone cannot be used as a parameter of age, for 3 reasons: First, the increase in lactic acid content as a function of time was much lower than expected. The maximum concentration after 35 days in a completely decomposed egg did not exceed 18.2 mg/100 g egg. Although selected literature references mention values up to 100 mg/100 g (1, 2), it may be that lactic acid concentration was influenced by microbial contamination of the eggs. Second, a plot of the data in Table 2 shows several irregularities. Finally, lactic acid contents of fresh eggs can very widely. We observed this in some experiments on eggs of other poultry breeds (concentrations in fresh eggs vary between 0.3 and 4.0 mg/100 g). Nevertheless, the method seems to be satisfactory in order to determine the freshness of an egg.

Refractive index measurements, when carefully performed, could be used to determine the age of eggs, although further studies need to be done. Table 4 shows a nearly linear correlation with age, especially for the value indices. Our results do not completely correspond to the Swiss values (8). We found an ageing value of 5.8 after 8 days, while they claim that an egg 8 days old or more is still fresh if the ageing number is not higher than 5. However, it is well known that refractive indices of eggs stored at high temperature (24°C) change much faster than those of eggs stored at low temperature (12). In our opinion, this very simple method

Table 2. Lactic acid content in egg as a function of time

Storage time, days	Lactic acid, mg/100 g egg						Mean
0	4.3	4.0	3.4	3.6	3.5	3.5	3.7
2	4.0	3.6	3.4	3.5	4.2	3.5	3.7
4	4.2	3.9	4.1	4.2	3.9	4.1	4.1
6	5.2	4.9	5.5	4.8	4.9	5.0	5.1
8	6.4	6.5	6.7	6.4	6.5	5.9	6.4
10	6.4	6.4	6.1	5.6	6.3	6.4	6.2
15	7.8	8.9	9.3	8.9	8.5	9.0	8.7
21	12.5	11.9	11.5	13.5	13.9	12.6	12.7
25	13.0	12.8	14.1	13.4	13.6	12.7	13.3
30	12.6	11.8	13.6	13.8	12.7	13.5	13.0
35	17.7	16.7	18.2	17.5	16.9	17.3	17.4

Table 3. Succinic acid concentrations in egg as a function of time

Storage time, days	Succinic acid, mg/100 g egg						Mean
0	0.9	2.2	1.3	1.2	1.3	1.3	1.4
2	1.4	1.3	0.9	1.1	1.2	1.3	1.2
4	1.4	1.2	1.1	1.3	1.4	1.2	1.3
6	1.3	1.2	1.3	1.4	1.4	1.5	1.4
8	1.6	1.5	1.2	1.2	1.6	1.2	1.4
10	1.5	1.3	1.1	1.3	1.3	1.7	1.5
15	1.2	1.2	1.6	1.6	1.6	1.7	1.5
21	3.1	1.8	2.5	2.8	1.1	2.4	2.3
25	1.7	1.6	1.2	1.6	1.5	1.4	1.5
30	1.8	1.7	1.2	1.4	2.6	3.2	2.0
35	2.1	1.9	2.3	2.4	2.1	2.0	2.1

Table 4. Refractive index measurements as a function of time

Storage time, days	d_D^{20} yolk	n_D^{20} white	Value indices	Ageing No.
0	1.4198	1.3547	65.1	0
2	1.4184	1.3562	62.2	1.10
4	1.4177	1.3573	60.4	1.80
6	1.4153	1.3557	59.5	4.2
8	1.4137	1.3550	58.7	5.8
10	1.4130	1.3562	56.8	6.5
15	1.4119	1.3566	55.3	7.6
21	1.4106	1.3585	52.1	8.9
25	1.4113	1.3608	50.5	8.2
30	1.4086	1.3632	45.4	10.9
35	1.4076	1.3616	46.0	11.9

can be a valuable alternative to the AOAC method to determine whether an egg is fresh because of the rather good reproducibility of refractive index measurements. Triple determinations on the same egg showed variations of $n_D^{20} = \pm 0.0002$. Furthermore, refractive indices between different eggs of the same hen are not significantly different (12).

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PESTICIDE RESIDUES

Gas-Liquid Chromatographic Analysis of Ethephon and Fenoprop Residues in Apples and Their Decline Before and After Harvest

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Ethephon (2-chloroethylphosphonic acid) and fenoprop (2-(2,4,5-trichlorophenoxy) propionic acid) may be determined in the same apple sample. After extraction with methanol, 2 separate methylation procedures were required to quantitatively convert each compound. Ethephon was esterified with diazomethane and analyzed by a flame photometric detector in the P-mode. Fenoprop was esterified with boron trifluoride/methanol and analyzed by electron capture gas chromatography. Average recoveries were about 95% at 0.05 ppm for both compounds. The limit of detection was 0.05 ppm for ethephon and 0.01 ppm for fenoprop in a 1 g sample. The persistence of both compounds before and after harvest was studied. Ethephon and fenoprop were applied simultaneously to apple trees at the recommended concentrations of 300 and 20 ppm, respectively. Ethephon residues averaged 1.6, 0.75, and 0.4 ppm at 2 hr, 10 days, and after washing at 13 days, respectively. The corresponding fenoprop residues were 0.70, 0.025, and 0.024 ppm.

Ethephon (2-chloroethylphosphonic acid) is a plant growth regulator used extensively in agriculture to promote ripening of fruits (1). To achieve early ripening of apples it is applied, at a recommended concentration of 300 ppm, 2 weeks before harvest together with an auxinic "stop-drop" material. Fenoprop (2-(2,4,5-trichlorophenoxy) propionic acid) is presently the most effective auxin used to prevent fruit drop.

Bache (2) described a gas chromatographic method for ethephon residues in fruits and vegetables, and some residue data have already been reported for free and conjugated fenoprop residues in apples (3), orange peel (4), and sugarbeets, soybeans, and corn (5). Recently Leidy *et al.* (6) analyzed residues on fenoprop-treated apples, using a modification of the method of Chow *et al.* (7). Of 59 apple samples analyzed, no residues >0.05 ppm were detected; this is the limit of detection of the method employed. This paper describes a method for the gas chromatographic determination of both fenoprop and ethephon in the same sample. The method is used to study the persistence of these plant growth regulators before and after harvesting.

Experimental

Apparatus and Reagents

All glassware used in this study was acid-washed.

(a) *Gas chromatograph.*—(*1*) *Ethephon analysis.*—Pye GCV equipped with flame photometric detector (P-mode, 534 nm) and 3′ × ¼″ od glass column containing 10% DEGS on 100–120 mesh Gas-Chrom Z, conditioned 2 days at 200°C. Operating conditions: injection port 110°C, column 125°C, detector 200°C, nitrogen carrier gas flow 75 ml/min. Under these conditions, 250 pg dimethyl ethephon gave ½ full-scale deflection at a retention time of 5.2 min (Fig. 1).

(*2*) *Fenoprop analysis.*—Hewlett-Packard Model 5700A equipped with linearized ^{63}Ni electron capture detector and 4′ × ¼″ od glass column containing 4% OV-101/6% OV-210 on 80–100 mesh Chromosorb W (HP). Operating conditions: injection port 200°C, column 185°C, detector 250°C, argon-methane (9+1) carrier gas flow 55 ml/min. Under these conditions, 260 pg fenoprop methyl ester gave ½ full-scale deflection at a retention time of 4.3 min (Fig. 2).

(b) *Solvents.*—Distilled-in-glass solvents (Fisher Scientific Chemical Co., Fair Lawn, NJ 07410 or Caledon Laboratories Ltd., Georgetown, Ontario, Canada) were used throughout, except for methanol, reagent grade.

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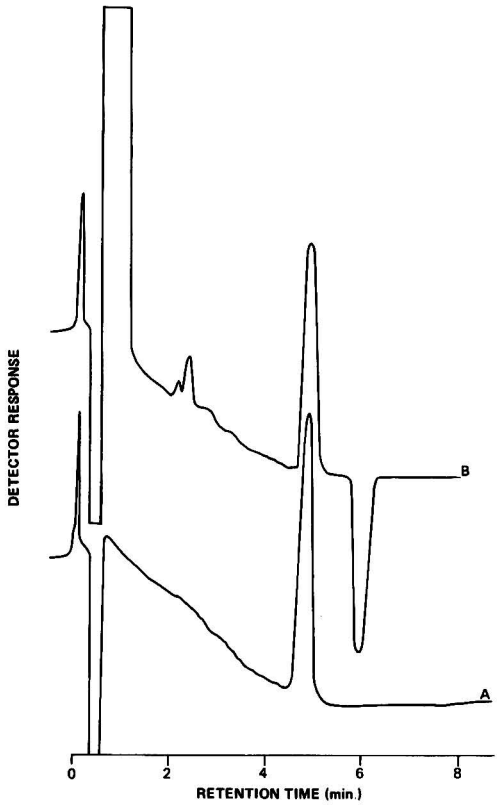

FIG. 1—Gas chromatograms of 250 pg dimethyl ethephon standard (A) and an apple extract containing 0.05 ppm ethephon (B), using flame photometric detection (P-mode).

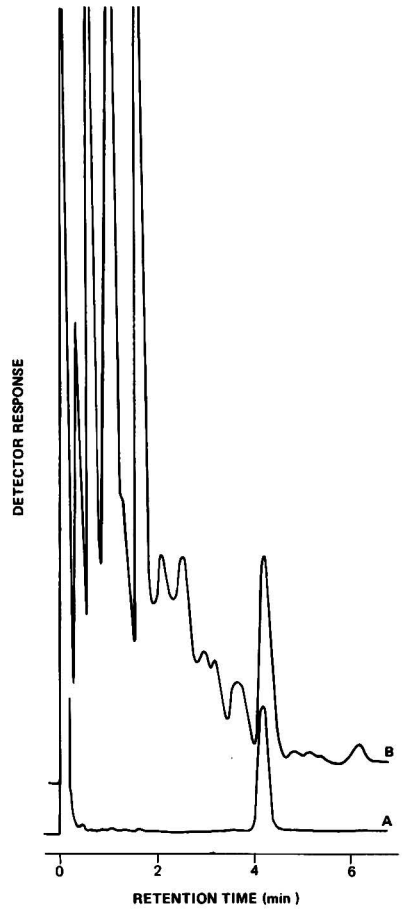

FIG. 2—Gas chromatograms of 190 pg fenoprop methyl ester standard (A) and an apple extract containing 0.068 ppm fenoprop, using electron capture detection.

(c) *Reaction reagents.* — BF_3/MeOH (14%, w/v), BF_3/BuOH (14%, w/v), and methanolic HCl (used at 3%, w/v) (Applied Science Laboratories, State College, PA).

(d) *Ethephon (95%).*—Provided by Amchem Products Inc., Ambler, PA.

(e) *Fenoprop (99%).*—Provided by Dow Chemical Co., Midland, MI.

(f) *Diazomethane.* — Prepared from Diazald (Aldrich Chemical Co.) according to procedure of de Boer and Backer (8).

Extraction

Blend 10 g ground apple pulp with 75 ml methanol 5 min at medium speed in a Waring Blendor. Filter extract through Celite, using Büchner funnel and filter flask. Reblend filter cake and debris with another 75 ml methanol, filter, combine filtrate and washing, and reduce volume to 100 ml with rotatory evaporator. Further reduce 10 ml aliquots (equivalent to 1 g apple sample) to 1 ml in 15 ml centrifuge tube with a stream of dry nitrogen.

Esterification

(a) *For ethephon.*—Add ethereal solution of diazomethane (9 ml) to 1 ml apple extract. Stopper tube, shake, and let stand 30 min. A brown oil may form following addition of ethereal diazomethane. Evaporate contents to 1 ml (brown oil will redissolve). Inject sample directly into gas chromatograph without further cleanup (Fig. 1).

(b) *For fenoprop.*—Add 1–2 ml BF_3/MeOH solution to 1 ml apple extract. Stopper tube and place in 60°C water bath 20 min. Remove, cool, and add 10 ml water and 2 ml benzene. Shake, let stand 10 min, and analyze organic phase directly on gas chromatograph (Fig. 2).

(c) *For ethephon and fenoprop in same sample.*

—Determine ethephon first; then dilute sample with 10 ml water and extract fenoprop residue with 2 ml benzene. Proceed with fenoprop analysis.

Spraying and Sampling

A number of McIntosh trees planted in 1950 and spaced 22 × 22′ apart at Kelowna, British Columbia were hand-sprayed with ethephon (300 ppm) and fenoprop (20 ppm) 10 days before harvest.

Apples were harvested 2 hr and 1, 2, 3, 5, and 10 days after spraying. Samples consisted of 12 apples (3 apples/tree quadrant) from each of 3 different trees. Apples from the major harvest at 10 days were divided into 3 equal lots and stored at 0°C. On day 13, a local packing house carried out a commercial detergent wash on one-third of the fruit from each tree and a detergent wash and wax on another one-third, leaving the remaining one-third unprocessed. Subsequent samples were taken at 2 weeks and 2 and 4 months from 0°C storage.

Each sample was ground in a Hobart stainless steel hammer mill. Ground apple samples (about 250 g each) were spooned into plastic bags, frozen in Dry Ice, and stored at −18°C until analyzed.

Results and Discussion

We attempted to find a common reagent for the methylation of ethephon and fenoprop in the same sample. Four different reagents were tried but none gave quantitative results for both compounds. Table 1 gives the per cent recoveries for ethephon and fenoprop at 0.05 ppm, using the various esterification procedures. Methanolic hydrochloric acid (HCl) gave quantitative recoveries for fenoprop standards but only 32–38% recovery of fenoprop present in apple extract. At the concentration used (3%, w/w), ethephon was not methylated. Similarly, ethephon was not methylated with methyl fluorosulfonate (Magic Methyl®, Aldrich Chemical Co.). Fenoprop was methylated to some extent with Magic Methyl, but recoveries from apple extracts were drastically reduced. Boron trifluoride/methanol gave the best and most consistent recovery for fenoprop but no methylated product was isolated for ethephon. Diazomethane gave quantitative results for ethephon standards and samples, but only 64–73% recovery of fenoprop. The workup of the diazomethane esterification for mixed standards and samples involved determination of ethephon first, and then dilution of the solution with water and extraction with benzene for the analysis of fenoprop. The 2-step partition is required when both residues are present because the diazomethane solution gave unacceptable chromatograms with electron capture detection. We attempted to simplify this workup by evaporating the first esterifying solution to dryness and adding benzene. However, the recovery of fenoprop was only 50%. Also, for fenoprop-fortified samples, if the solutions were taken to dryness before the addition of diazomethane the average recovery was only 25%. Hence, the final procedure involved 2 separate methylation steps.

There is no single set of conditions for which both ethephon and fenoprop can be chromatographed simultaneously. Both are detectable by an electron capture detector, but 150 ng dimethyl ethephon is required for ½ full-scale recorder deflection. Therefore, the amount required for detection of ethephon is far greater than the amount found in apple samples. Also, an elevation of temperature from 130 to 185°C and a decrease in attenuation are necessary for the determination of fenoprop. By comparison, only 250 pg dimethyl ethephon is needed for

Table 1. Per cent recovery of fenoprop and ethephon after various methylation procedures[a]

Substrate	BF_3/MeOH (14%, w/v)	HCl/MeOH (3%, w/w)	Methyl fluorosulfonate	Diazomethane
Fenoprop standard	98	108	60	64
Ethephon standard	0	0	0	92
Mixed standard: fenoprop	95	96	32	66
ethephon	—	—	—	107
Fenoprop in apples	92	38	5	73
Ethephon in apples	—	—	—	95
Mixed apple samples: fenoprop	89	32	4	67
ethephon	—	—	—	92

[a] Averages of 3–5 replicates.

½ full-scale deflection, when the phosphorus-selective flame photometric detector is used.

A cleanup procedure was initially developed for ethephon, using a Dowex 1 × 8 ion exchange column. The extract was applied to the column with water; the column was washed with 0.01*N* HCl and eluted with 0.1*N* HCl. Although the method was successful in cleaning up apple extracts, removal of the final traces of water prior to methylation with diazomethane appeared to affect the success of the final step. This led to nonreproducibility when the method was subjected to an interlaboratory study and the method was not utilized in the final procedure.

Bache's method (2) was adopted for this study and simplified by omitting the precipitation step. This resulted in contamination of the gas chromatographic column, which was alleviated by replacing the first 1″ of the column on a regular basis. A glass injector insert could be used for this purpose. The time saved was thought to warrant this step.

For apple extracts fortified at 0.05 ppm, the average recovery of ethephon was 95%. It was difficult to determine ethephon at lower concentrations because of background interference. The average recovery of fenoprop at 0.05 ppm in apple extract was 96%. The corresponding figure at the detection limit of 0.01 ppm was 84%. It is necessary to analyze the fenoprop sample on the same day, because fenoprop hydrolyzes to the free acid in the 2-phase extract. After 6 days at room temperature, only 56% of the original fenoprop methyl ester was present. If analysis is to be performed later, the organic layer must be separated and dried to prevent hydrolysis. The alkylation procedure for fenoprop could also be used to confirm residue identity. Using 14% boron trifluoride/butanol, the butyl ester is formed in high yield and has a retention time 2.3 times that of the fenoprop methyl ester.

The levels of ethephon residues present in the apple samples are shown in Table 2. The series shows a steady decline from the time of spray-

Table 2. Decline of fenoprop and ethephon residues on apples up to harvest

		Residues, ppm					
Tree	Compound	2 hr	Day 1	Day 2	Day 3	Day 5	Day 10
1	fenoprop	0.069	0.059	0.080	0.056	0.030	0.026
	ethephon	1.42	0.85	1.55	0.76	0.88	0.66
2	fenoprop	0.110	0.080	0.049	0.049	0.023	0.023
	ethephon	1.55	1.36	1.20	0.97	0.80	0.57
3	fenoprop	0.026	0.037	0.045	0.035	0.027	0.018
	ethephon	1.80	1.57	1.35	0.97	1.58	0.72
Av.	fenoprop	0.068	0.059	0.058	0.047	0.027	0.022
	ethephon	1.59	1.26	1.37	0.90	1.09	0.65

Table 3. Persistence of fenoprop and ethephon residues after processing and storage

		Residues, ppm											
		Day 14[a]			2 Weeks			2 Months			4 Months		
Tree	Compound	U	W	W/W	U	W	W/W	U	W	W/W	U	W	W/W
1	fenoprop	0.029	0.019	0.029	0.095	0.03	NS[b]	—	—	—	0.102	0.051	0.039
	ethephon	0.080	0.56	0.30	0.73	0.30	NS	0.75	0.32	0.45	0.65	0.35	0.30
2	fenoprop	0.025	0.033	0.048	0.041	0.042	0.026	—	—	—	0.041	0.037	0.026
	ethephon	0.78	0.37	0.62	0.32	0.30	0.16	0.65	0.37	0.20	0.50	0.15	0.27
3	fenoprop	0.033	0.020	0.027	0.048	0.034	0.031	—	—	—	0.027	0.021	0.035
	ethephon	0.96	0.73	0.35	0.71	0.51	0.25	0.62	0.33	0.48	0.60	0.45	0.37
Av.	fenoprop	0.029	0.024	0.035	0.061	0.035	0.028	—	—	—	0.057	0.036	0.033
	ethephon	0.85	0.55	0.42	0.59	0.37	0.21	0.67	0.34	0.38	0.58	0.30	0.31

[a] At 14 days the apples were analyzed as unwashed (U); washed with detergent and then water-rinsed (W); washed and then waxed (W/W).
[b] NS = no sample.

ing to that of harvest. The half-life for ethephon averaged 6 days. The effects of washing and of washing and waxing are shown in Table 3. At 0°C, the half-life appeared to be greater than 4 months, so the rate of decomposition is much slower in storage. Both packing house treatments affected the level of ethephon residues. Washing alone reduces the level to approximately 60% while washing and waxing reduces it to approximately 50% of the original level. These results suggest that ethephon has penetrated the apple skin, because the highly polar nature of ethephon would cause it to preferentially partition into the water phase.

Results of the fenoprop analysis from spraying until harvest (Table 2) reveal a steady decline with a half-life for fenoprop of approximately 5 days. The levels found on harvesting were all below 0.05 ppm. This is in accord with the findings of Leidy *et al.* (6), whose lower limit of detection was 0.05 ppm. The effects of post-harvest treatments and subsequent storage are shown in Table 3. Washing and waxing reduced the fenoprop residue level to approximately 70% of the original level. There was very little further decline in cold storage for 4 months.

Acknowledgments

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Mass Spectra of Sodium *N*-Alkyl and *N,N*-Dialkyl Dithiocarbamates and Some Related Compounds

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Electron impact mass spectra of 8 sodium alkyl and dialkyl dithiocarbamate salts are reported with the spectra of 2 dithiocarbamate zinc salts and *N',N'*-dimethyl-*N,N*-dimethylthiocarbamylsulfenamide. The investigated compounds all gave molecular ions, and generalized descriptions of their fragmentation behavior are presented.

Mass spectral data for a number of thiocarbamates, carbamates, thiocarbonates, ureas (1, 2), ethyl *N*-ethylcarbamate, ethyl *N*-phenylcarbamate (3), and *S*-*n*-propyl, *N*-monoalkyl, and *N,N*-dialkyl dithiocarbamates (4–5) have been reported, but to date no information has been presented for their sodium salt derivatives.

The mass spectrometric behavior of various sodium *N*-monoalkyl and *N,N*-dialkyl dithiocarbamates was examined by direct inlet introduction. Although considered non-volatile, they were stable enough to form a number of intense characteristic ions.

The hydrated portion of the molecules posed no problems at relatively low temperatures and high vacuum. No hydrated sodium salt molecular ions were observed. The water molecules are comparatively weakly bonded to the molecule and the process of dehydration preceded vaporization. Simple dehydration was not a serious complication, although care had to be exercised to avoid the possibility of thermal decomposition and hydrolysis at elevated temperatures before vaporization. Electron impact spectra were recorded at 20 ev to determine if thermal cracking contributed to the spectral patterns; no evidence of this effect was found.

Relatively short-term stability was experienced with the sodium salts examined. After 6 months, most compounds contained decomposition products. The compounds investigated are given in Table 1.

Experimental

(a) *Mass spectrometer.*—Hitachi Perkin-Elmer Model RMU-6L interfaced to Varian MAT 620L computer. Introduce sample at ambient temperature by direct inlet technique using standard probe. Increase source temperature to optimum value and record spectrum at 70 ev on Statos 21 printer/plotter. Measure metastable ions on ultraviolet (UV) recorder trace.

(b) *Sample preparation.*—Prepare sodium salts in manner described by Delepine (6) and Klopping and Van Der Kerk (7). Zinc dimethyl dithiocarbamate (Chemical Services Inc.) and zinc dibenzyl dithiocarbamate (British Drug Houses) were analytical grade chemicals. All compounds were recrystallized from absolute ethanol and used as such.

Table 1. Identity and experimental conditions for investigated dithiocarbamates

Fig. No.	Chemical name	Mol. wt	Mol. formula	MS ion source temp.,[a] °C
1	sodium *N*-methyl dithiocarbamate	129	CH_4NCS_2Na	200
2	sodium *N*-ethyl dithiocarbamate	143	$C_2H_6NCS_2Na$	210
3	sodium *N*-*n*-propyl dithiocarbamate	157	$C_3H_8NCS_2Na$	220
4	sodium *N*-*n*-butyl dithiocarbamate	171	$C_4H_{10}NCS_2Na$	220
5	sodium *N,N*-dimethyl dithiocarbamate	143	$C_2H_6NCS_2Na$	220
6	sodium *N,N*-diethyl dithiocarbamate	171	$C_4H_{10}NCS_2Na$	230
7	sodium *N,N*-di-*n*-propyl dithiocarbamate	199	$C_8H_{14}NCS_2Na$	230
8	sodium *N,N*-di-*n*-butyl dithiocarbamate	227	$C_8H_{18}NCS_2Na$	230
9	zinc *N,N*-dimethyl dithiocarbamate (ziram)	304	$C_2H_6NCS_2ZnS_2CNC_2H_6$	220
10	zinc dibenzyl dithiocarbamate	608	$[(C_6H_5.CH_2)_2N.CS.S]_2Zn$	250
11	*N',N'*-dimethyl-*N,N*-dimethylthiocarbamylsulfenamide	164	$C_2H_2NCS_2NC_2H_6$	180

[a] Optimum temperature conditions for obtaining spectrum.

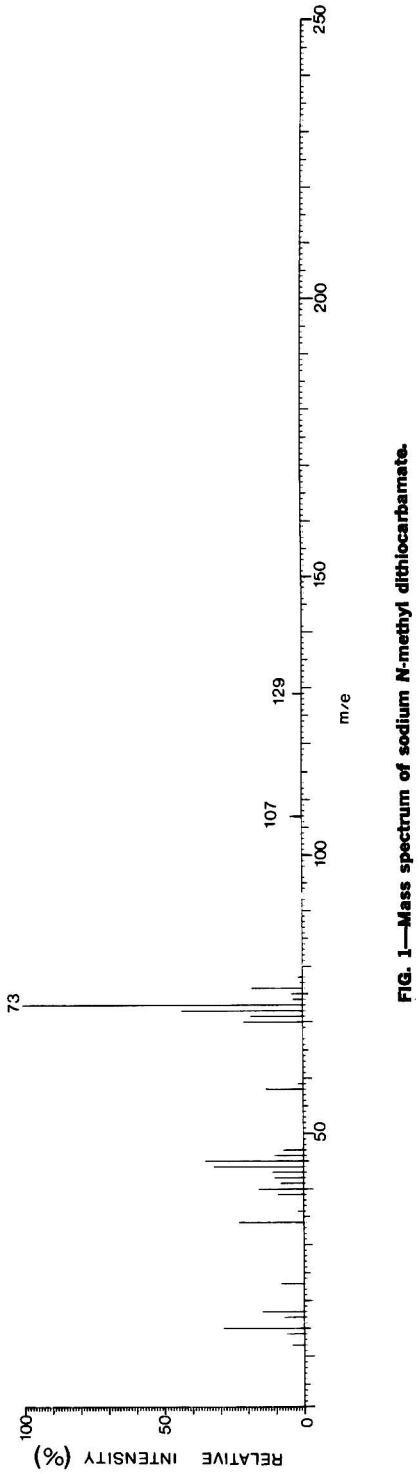


FIG. 1—Mass spectrum of sodium *N*-methyl dithiocarbamate.

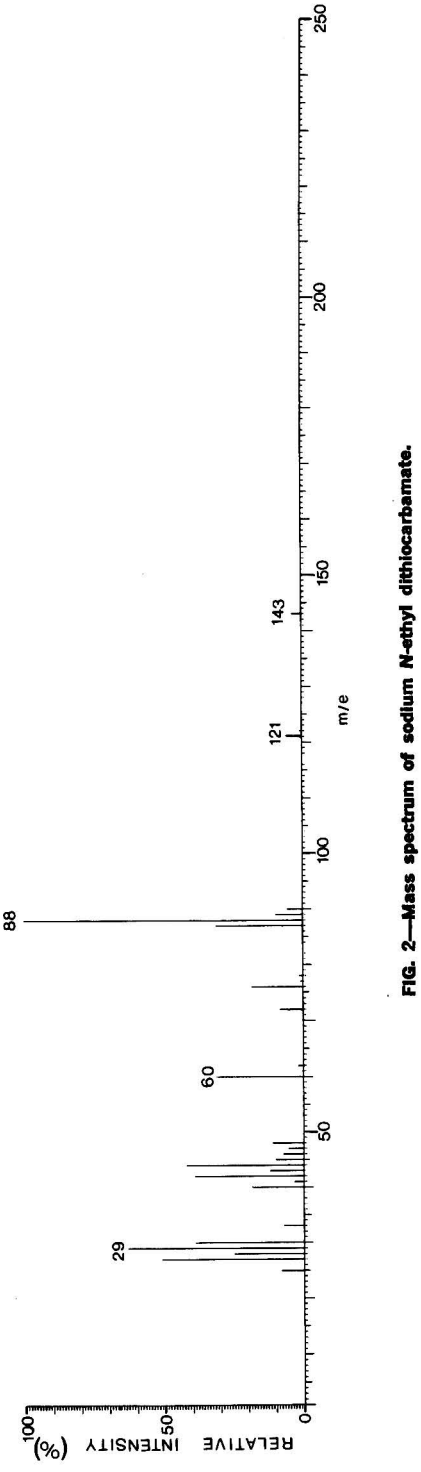


FIG. 2—Mass spectrum of sodium *N*-ethyl dithiocarbamate.

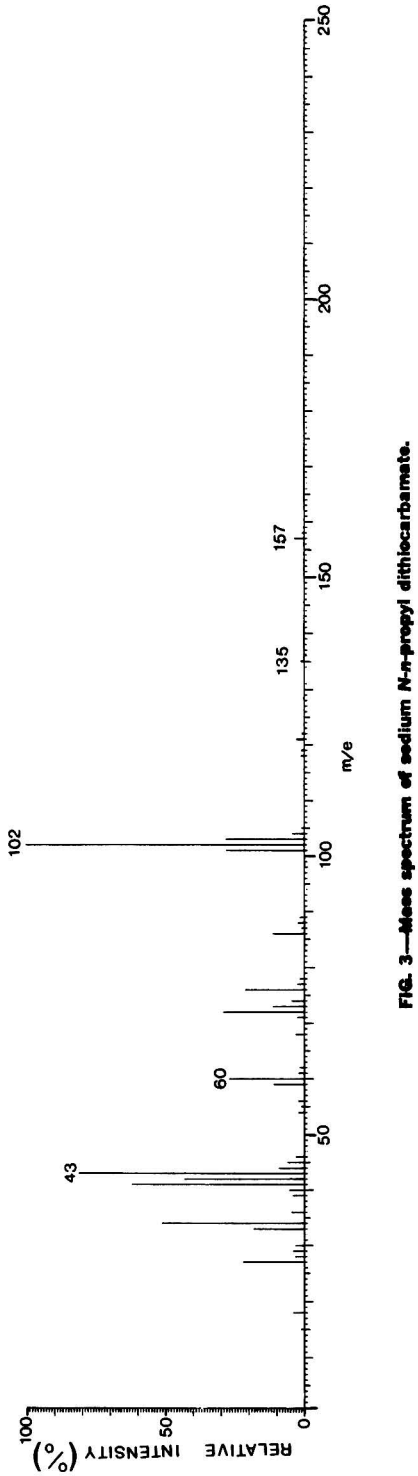


FIG. 3—Mass spectrum of sodium *N*-*n*-propyl dithiocarbamate.

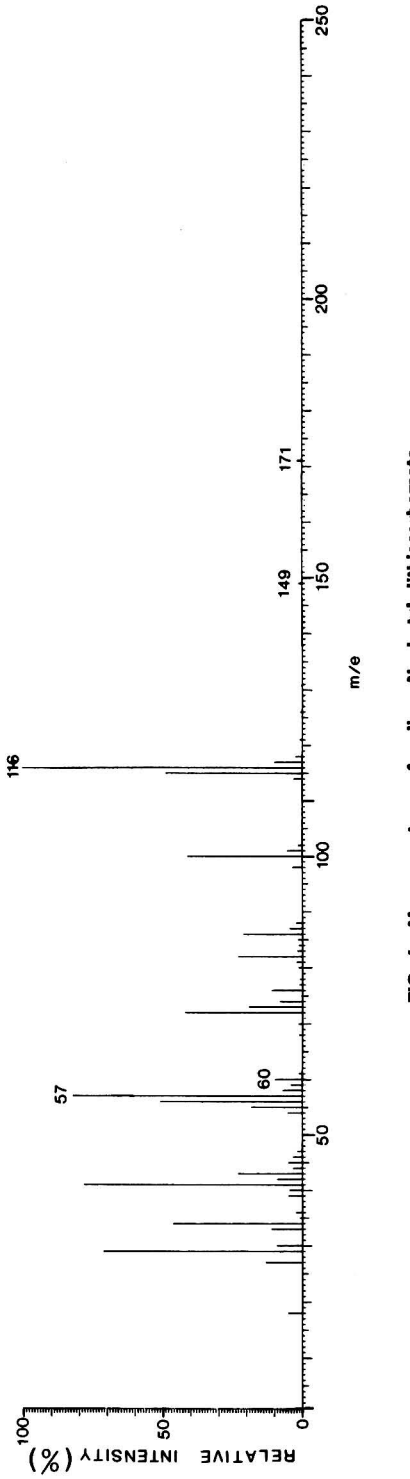


FIG. 4—Mass spectrum of sodium *N*-*n*-butyl dithiocarbamate.

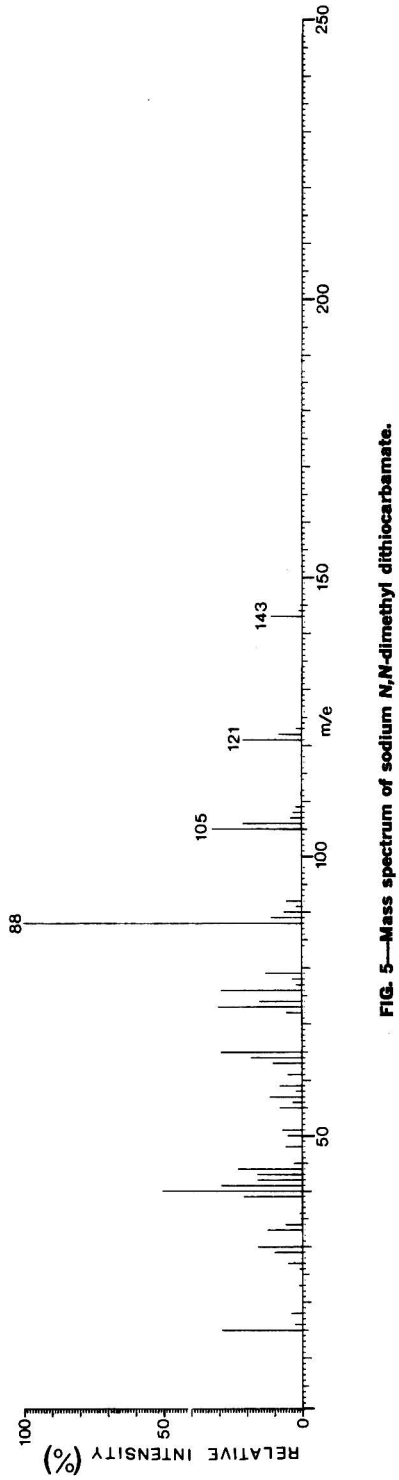


FIG. 5—Mass spectrum of sodium *N,N*-dimethyl dithiocarbamate.

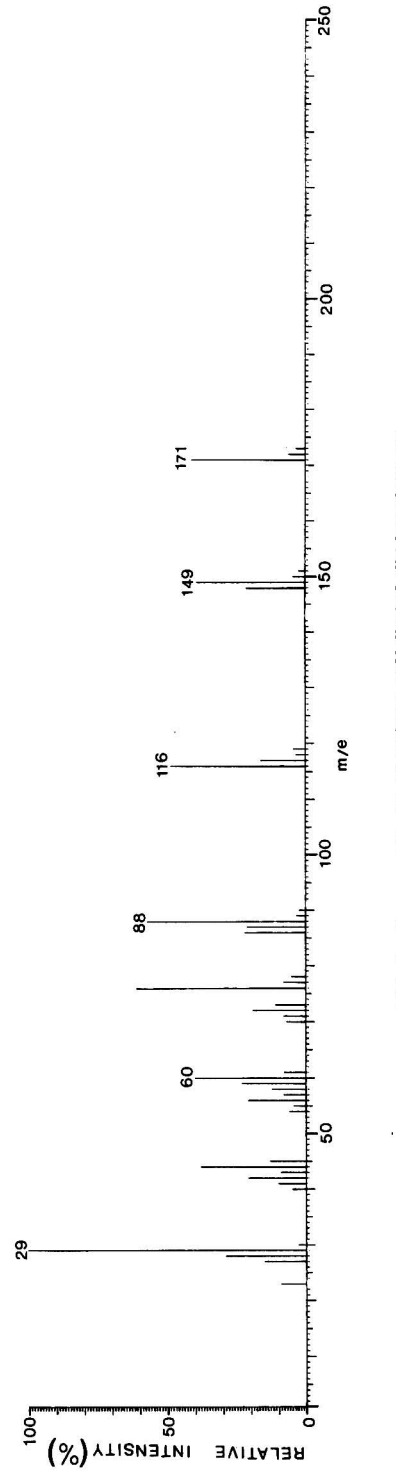


FIG. 6—Mass spectrum of sodium *N,N*-diethyl dithiocarbamate.

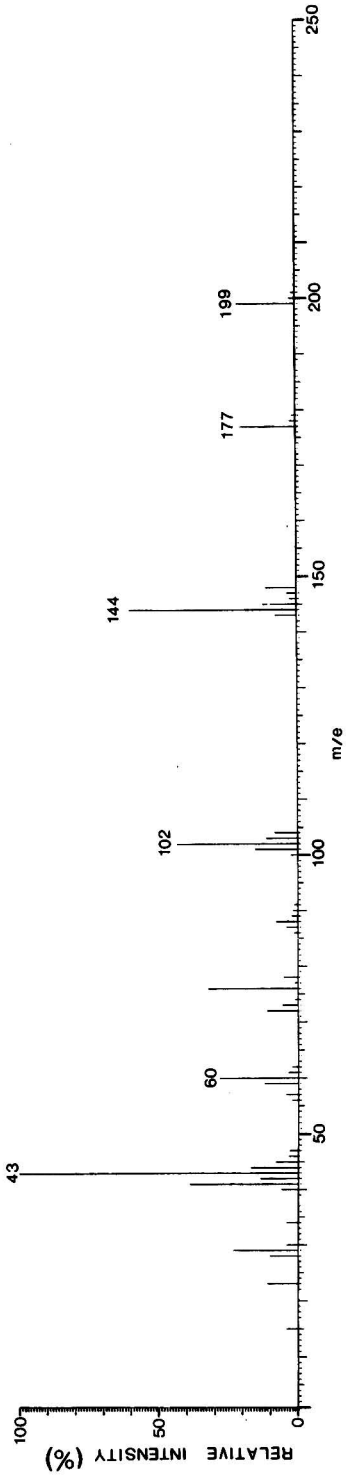


FIG. 7—Mass spectrum of sodium *N,N*-di-*n*-propyl dithiocarbamate.

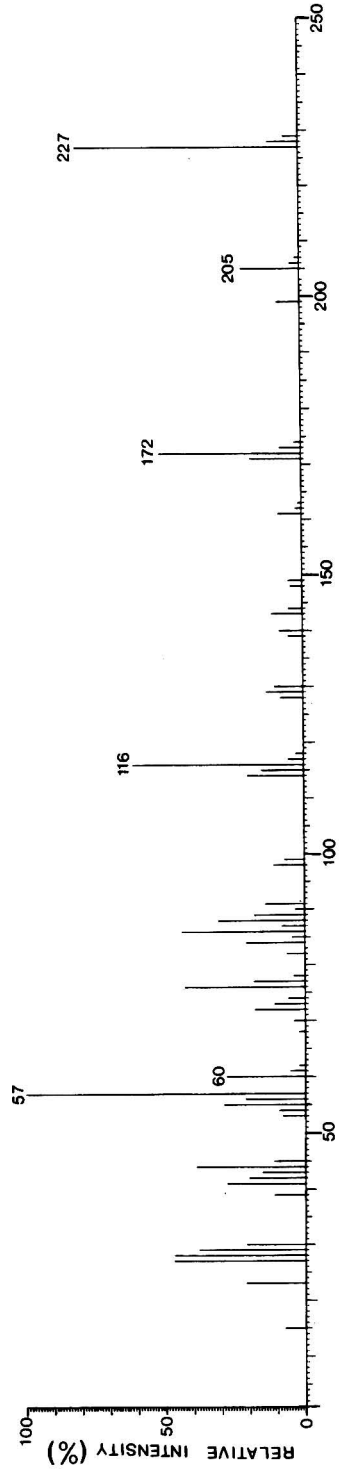


FIG. 8—Mass spectrum of sodium *N,N*-di-*n*-butyl dithiocarbamate.

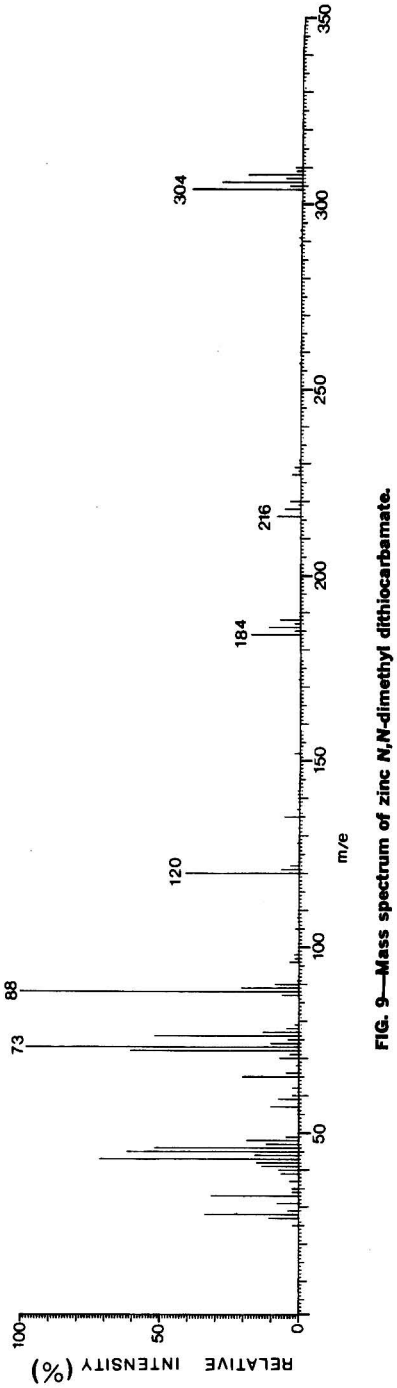


FIG. 9—Mass spectrum of zinc *N,N*-dimethyl dithiocarbamate.

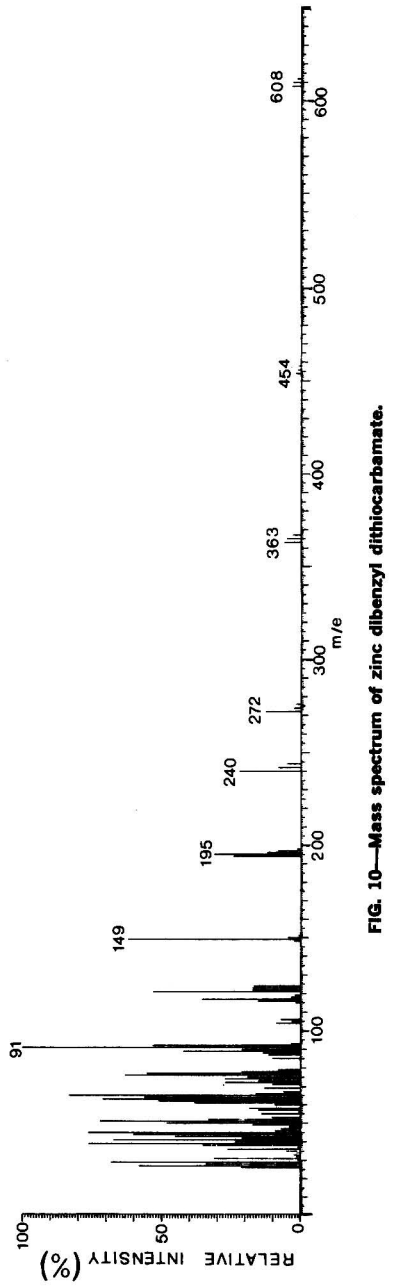


FIG. 10—Mass spectrum of zinc dibenzyl dithiocarbamate.

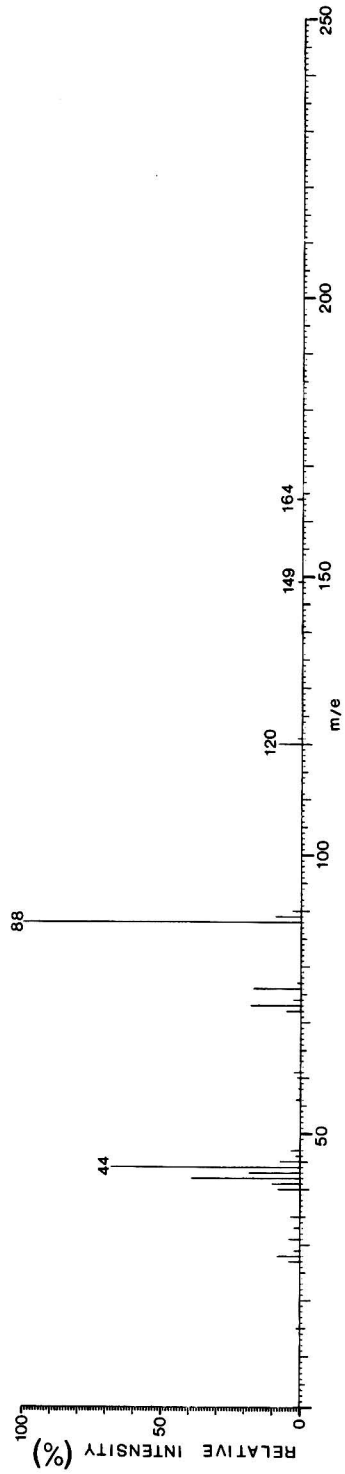


FIG. 11—Mass spectrum of *N',N'*-dimethyl-*N,N*-dimethylthiocarbamylsulfenamide.

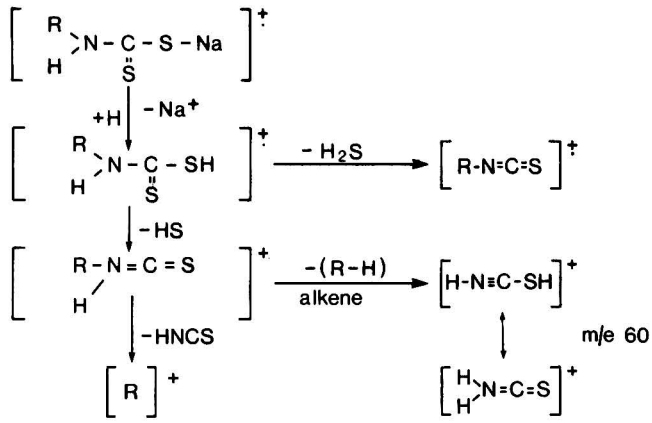
Results and Discussion

The mass spectra of the sodium *N*-methyl, *N*-ethyl, *N*-*n*-propyl, and *N*-*n*-butyl dithiocarbamate salts, Figs. 1 to 4, respectively, exhibited weak molecular ions for the dehydrated molecules. Of particular interest was the ion abundance observed for the initial expulsion of the sodium atom from the molecule, which was represented by a $[M - 22]^+$ loss rather than the expected $[M - 23]^+$ loss for sodium. This implied that, after formation of the alkyl dithiocarbamate ion, protonation occurred. This can be justified because a source of free hydrogen was available from the ionized water molecules, produced by the evaporation of the hydrated sodium salts examined.

Except for *N*-methyl, the base peaks in the spectra of the other monoalkyl dithiocarbamates resulted from the formation of the protonated alkyl isothiocyanate ion by elimination of SH· radical from the molecule. *N*-Methyl, however, exhibited a base peak at m/e 73, arising from the loss of a neutral H_2S molecule. Although less prominent, the formation of the ion at m/e 60 is important for structural confirmation and is characteristic of the configuration of the alkyl dithiocarbamates. It involves a 6-membered ring hydrogen rearrangement to the sulfur atom, with elimination of the corresponding neutral alkene moiety. This may also occur via a 4-membered ring rearrangement to the nitrogen atom, but is less favorable. In both cases the final resonance structure, for stability, should be the protonated hydrogen isothiocyanate ion. The absence of the m/e 60 ion in the spectrum of sodium *N*-methyl dithiocarbamate coincides with the structural restrictions governing the rearrangement.

Substantial ion abundances were observed in the spectra of sodium *N*-methyl, *N*-ethyl, *N*-*n*-propyl, and *N*-*n*-butyl dithiocarbamates at m/e values of 15, 29, 43, and 57 for cleavage of the alkane group from the alkyl isothiocyanate ion by donation of an electron pair to the nitrogen atom. Characteristic ion abundances were also observed for the loss of carbon disulfide (CS_2) from the alkyl dithiocarbamate ion and the concurrent m/e 76 ion for CS_2. Fragmentation of the sodium *N*-alkyl dithiocarbamates is summarized in Scheme 1.

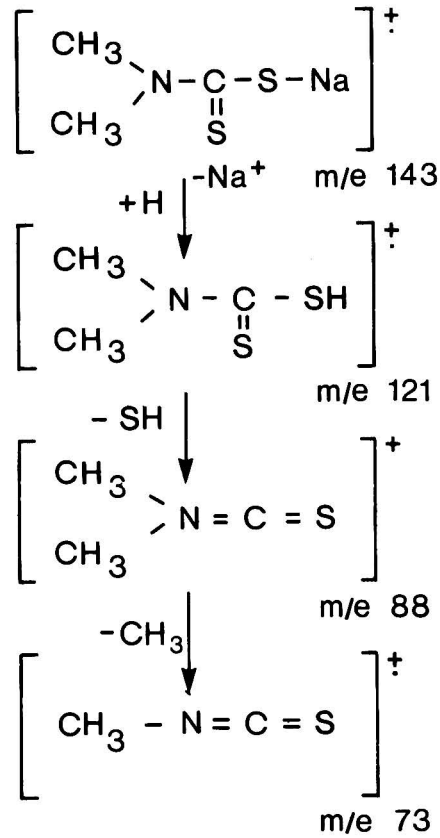
As expected, stronger molecular ions were observed for the dialkyl dithiocarbamates in con-



Scheme 1.

junction with increased structural stability of the molecules. As found for monoalkyl dithiocarbamates, cleavage of the sodium atom from the molecule resulted in a $[M - 22]^+$ loss and formation of the protonated dialkyl isothiocyanate ion by elimination of a SH• radical. Whereas this pathway represented the major fragmentation process for monoalkyl dithiocarbamates, the base peaks for the dialkyl homologs corresponded to the cleavage of an alkyl group from the protonated dialkyl isothiocyanate ion, with the charge being retained on the alkane chain. The exception is dimethyl dithiocarbamate because elimination of a methyl group was less favorable. The mass spectra (Fig. 5) of sodium *N,N*-dimethyl dithiocarbamate exhibited successive losses of sodium and hydrogen sulfide from the molecular ion. As outlined in Scheme 2, the formation of the protonated dimethyl isothiocyanate ion gave the base peak at m/e 88. The absence of the protonated hydrogen isothiocyanate ion at m/e 60 confirmed the fact that dimethyl dithiocarbamate cannot undergo rearrangement.

In contrast, the other dialkyl dithiocarbamates gave intense ion fragments for consecutive hydrogen rearrangements, accompanied by the elimination of the corresponding alkene molecules from the alkyl isothiocyanate products. These were observed at m/e values of 80, 60; 102, 60; and 116, 60 in the spectra of sodium *N,N*-diethyl, *N,N*-di-*n*-propyl, and *N,N*-di-*n*-butyl dithiocarbamates, Figs. 6–8, respectively. Metastable ions [m*] for the above transition states are given in Table 2. Fragmentation mechanisms for the sodium diethyl, *N,N*-dialkyl, dipropyl, and dibutyl dithiocarbamates are presented in Scheme 3.



Scheme 2.

Isotopic distribution patterns were apparent

Table 2. Metastable ions formed from *N,N*-dialkyl dithiocarbamates, m/e values

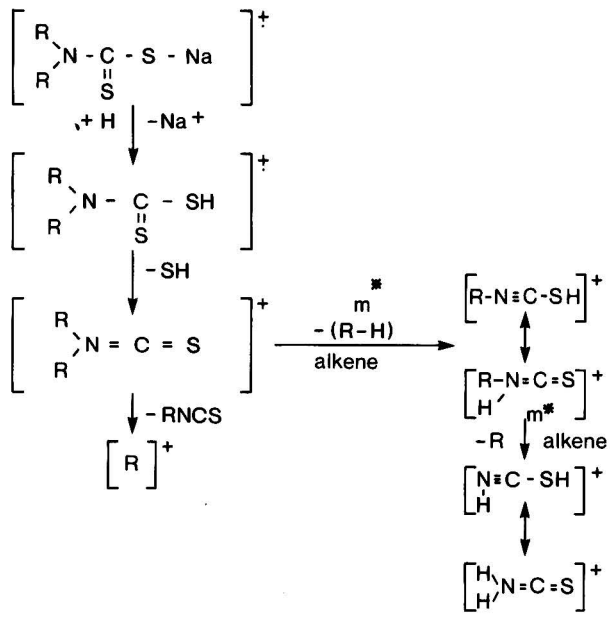
Compound	Precursor[a]	Daughter[b]	m*[c]	Precursor	Daughter	m*
Sodium *N,N*-dimethyl dithiocarbamate	—	—	—	—	—	—
Sodium *N,N*-diethyl dithiocarbamate	116	88	66.8	88	60	40.9
Sodium *N,N*-di-*n*-propyl dithiocarbamate	144	102	71.3	102	60	35.3
Sodium *N,N*-di-*n*-butyl dithiocarbamate	172	116	78.3	116	60	78.3

[a] Initially formed product ion (m_1).
[b] Ion decomposition product (m_2).
[c] Metastable ion ($m^* = m_2^2/m_1$).

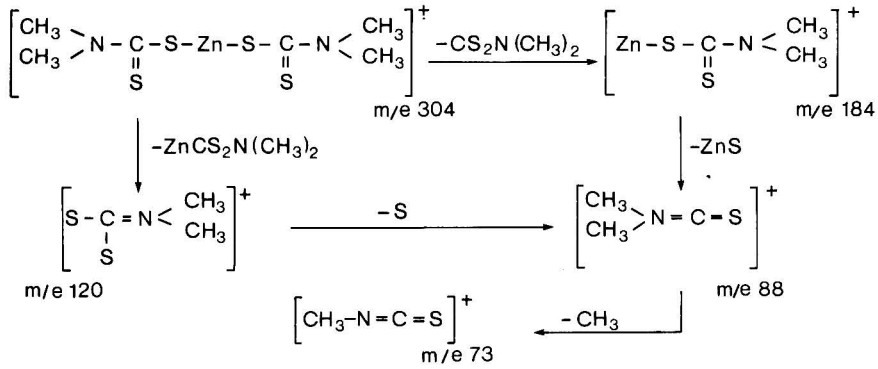
in the spectra of zinc *N,N*-dimethyl (Fig. 9) and zinc dibenzyl dithiocarbamates (Fig. 10). The fragmentation of the *N,N*-dimethyl zinc salt, outlined in Scheme 4, corresponded to data obtained using a time-of-flight instrument (2). A weak ion abundance at m/e 289 was observed for the elimination of a methyl radical from the molecular ion, which decomposed to the m/e 88 dimethyl isothiocyanate ion base peak and the concurrent m/e 216 ion for the $[M - 88]^+$ loss. In contrast, zinc dibenzyl dithiocarbamate fragmentation (Scheme 5) was highly complex due to the competitive pathways involved. Initial cleavage of a phenyl radical was not observed, although successive cleavage of a second phenyl from the molecule yielded an ion cluster at m/e 454, which further decomposed by loss of a benzyl radical to m/e 363. Multiple fragmentation reactions contributed to intense ion formations at the lower mass scale; the m/e 91 benzyl ion was the base peak.

N',N' - Dimethyl - *N,N* - dimethylthiocarbamylsulfenamide was an impurity associated with the preparation of the sodium monoalkyl and dialkyl dithiocarbamate derivatives. As with the other dimethyl compounds, decomposition of the molecular ion to the protonated dimethyl isothiocyanate (m/e 88) was the predominant reaction; the other fragmentation products in the spectrum (Fig. 11) were comparatively weak.

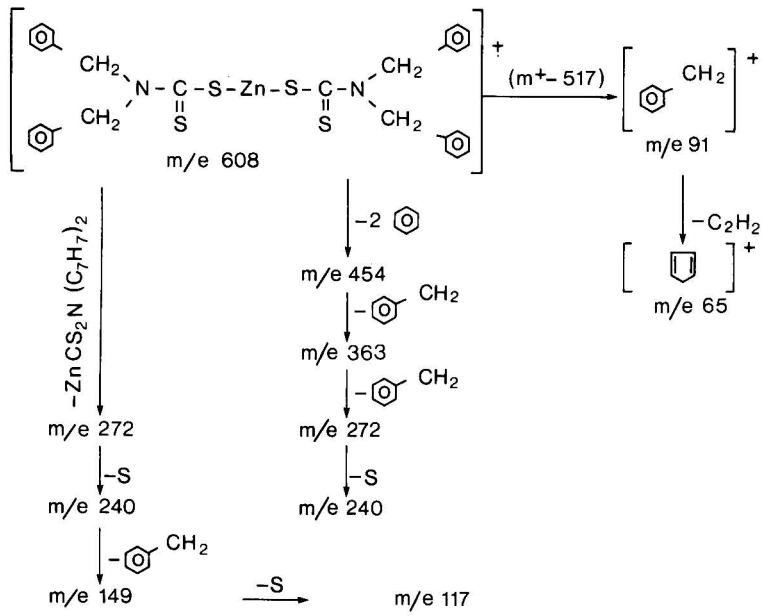
Scheme 6 gives the major ion fragments observed.



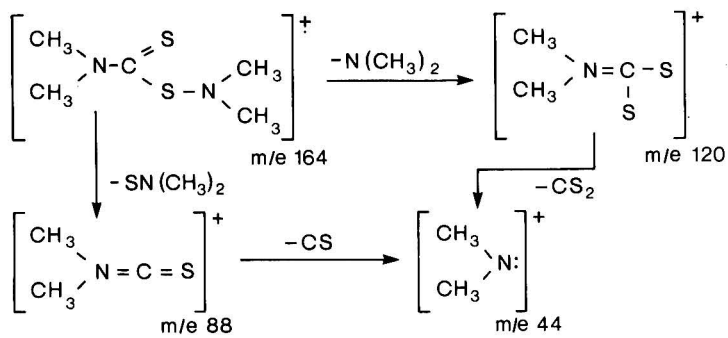
Scheme 3.



Scheme 4.



Scheme 5.



Scheme 6.

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Analysis of Pesticides by Chemical Derivatization. III. Gas Chromatographic Characteristics and Conditions for the Formation of Pentafluorobenzyl Derivatives of Ten Herbicidal Acids

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The reaction conditions to form pentafluorobenzyl derivatives of 10 herbicidal acids, at pesticide residue levels, were studied. The optimum conditions were reaction at room temperature for at least 5 hr (or overnight), using potassium carbonate solution as catalyst. The gas chromatographic characteristics of these derivatives were also studied. Nine of 10 of these derivatives can be separated on an OV-101/OV-210 gas chromatographic column.

The high polarity or low volatility of phenoxyalkanoic acids makes direct gas chromatographic analysis impractical and requires derivatization to more volatile derivatives. The most commonly prepared derivatives are the alkyl esters (1–8), among which the methyl esters (3–8) are the most widely used. Recently, the use of 2-chloroethyl esters has been advocated (9–11). Pentafluorobenzyl bromide (12–14) derivatives of phenols and some carboxylic acids are particularly amenable to electron capture detection. The versatility of this reagent has recently been demonstrated for the confirmation of organophosphorus pesticides (15, 16), in the analysis of carbamates (17), in the analysis of 2,4-D herbicide (18), and, as originally described by Kawahara (12, 13), in the characterization of phenolic and carboxylic compounds in the analysis of oils. These investigations indicated the 2 most desirable general characteristics of these derivatives, namely, generally longer retention times and greatly increased electron capture detector responses compared with the parent compounds or with other derivatives such as methyl ester, acetate, and silyl derivatives.

Experimental

Reagents

Pesticide residue grade solvents were used throughout.

(a) *Herbicidal acids.*—The following analytical grade acids were supplied by the manufacturers: dicamba (2-methoxy-3,6-dichlorobenzoic acid), Velsicol Chemical Corp.; MCPA (4-chloro-2-methylphenoxyacetic acid) and MCPB (4-(4-chloro-2-methylphenoxy) butyric acid), May and Baker (Canada) Ltd.; picloram (4-amino-3,5-dichlorobenzoic acid), Dow Chemicals. Technical grade 2,4-D (2,4-dichlorophenoxyacetic acid), silvex (2-(2,4,5-trichlorophenoxy) acetic acid), 2,4,5-T (2,4,5-trichlorophenoxy acetic acid), 2,4-DB (2,4-dichlorophenoxy butyric acid), 2,3,6-TBA (2,3,6-trichlorobenzoic acid), and 2,4-DP (2,4-dichlorophenoxy propionic acid) were purchased commercially and purified as described previously (11).

(b) *Pentafluorobenzyl bromide reagent.*—Transfer 1 ml reagent (Pierce Chemical Co.) to low actinic 100 ml volumetric flask and dilute to volume with acetone. (*Caution:* Reagent is strong lachrymator.)

(c) *Standard solutions.*—Prepare stock solutions of individual acids except MCPB by dissolving 100 mg acid in 100 ml benzene. Prepare 1000 mg MCPB in 100 ml ethyl acetate. Prepare standard solutions in acetone from stock solutions as follows: Standard solution I (ng/μl)—dicamba, 40; 2,4-DP, 40; 2,4-D, 80; silvex, 80; 2,4,5-T, 100; MCPB, 100; 2,4-DB, 200; picloram, 160. Standard solution II (ng/μl)—2,3,6-TBA, 20; MCPA, 36.

Apparatus

(a) *Gas chromatographs.*—Tracor Micro-Tek Model 222 with two ^{63}Ni electron capture detectors and two 6′ × ¼″ od U-shaped glass columns was used to obtain retention time data of derivatives and for early investigations. Operating conditions: injector 205°C, column 200°C, detector 280°C, nitrogen carrier gas 50–55 ml/min with 30–40 ml/min purge for both columns.

For the latter part of the investigation, a Hewlett-Packard Model 5710A gas chromatograph, equipped with ^{63}Ni detector, 6′ × ¼″ od coiled glass column, and automatic sampler Model 7671A, was connected to an Autolab System IV computing integrator for data processing.

(b) *Columns.*—The following 3 columns were installed in the Micro-Tek as required. Column I was used for the Hewlett-Packard chromatograph at all times. *Column I.*—3.6% (w/w) OV-101 and 5.5% (w/w) OV-210 on 80–100 mesh Chromosorb

W, acid-washed and dimethylchlorosilane-treated, obtained from Chromatographic Specialties Ltd., Brockville, Ontario, Canada, prepared as described by Chau and Wilkinson (19). *Column II.*—3% (w/w) OV-225 on 80–100 mesh Chromosorb Q (HP) (Chromatographic Specialties Ltd.). *Column III.*—11% (w/w) OV-17 + QF-1 on 80–100 mesh Chromosorb Q (Applied Science Laboratories, Inc.). According to the manufacturer, the exact w/w per cent of this packing is 1.8% OV-17 plus 4.0% QF-1 but it is designated as "11%".

Derivatization Procedures

Add aliquot ($\geq$20 μl) of appropriate standard solution to 15 ml glass-stoppered centrifuge tube, and add 200 μl pentafluorobenzyl bromide (PFBBr) solution, 30 μl 30% K_2CO_3 solution, and 4 ml acetone. Agitate contents ca 1 min. Let mixture react at room temperature $\geq$5 hr. If more convenient, reaction may be left overnight. After reaction, add 3–4 ml water and 10 ml benzene. Shake vigorously. Let layers separate. Using disposable pipet, withdraw benzene and dry over Na_2SO_4. Benzene extract, with or without concentration, is ready for gas chromatographic examination for pentafluorobenzyl (PFB) esters of herbicidal acids.

Results and Discussion

As reported earlier (11), the 2-chloroethyl ester derivatives are preferable to the alkyl esters for the analysis of the 10 herbicidal acids studied. Nine of the ten 2-chloroethyl esters studied have significantly different retention times under the conditions commonly used for the gas chromatographic analysis of organochlorine pesticides. On the other hand, several alkyl esters of these acids were poorly separated under the same conditions (11). The electron capture detector shows good sensitivity toward the 2-chloroethyl esters.

Reaction Conditions

In the initial investigations, the reaction conditions were studied to achieve maximum derivatization yields. Acetone was the best solvent among the several tested for the derivatization procedure. Few or no derivatives were formed in methanol, ethanol, or benzene with potassium carbonate or hydroxide.

After establishing acetone and potassium carbonate (K_2CO_3) as the desirable solvent and reagent for the derivatization, the reaction conditions, i.e., time and temperature, were investigated in more detail. Maximum yield of the derivatives was obtained for all 10 acids after reaction at room temperature for 5 hr (*see* Fig. 1). High temperature reactions, i.e., at 60 and 100°C (sealed tube) did not improve yield and were difficult to perform because of the low boiling point of acetone. The detector response to the derivatives obtained at the higher temperature was not improved over that for derivatives obtained under the same conditions at room temperature. In fact, additional peaks were observed in the chromatograms for these extracts from higher temperature reactions. For convenience, overnight reaction is recommended. An extended period of reaction, such as reaction over a weekend (70–75 hr) or even over a week, did not cause any noticeable changes in peak heights of the PFB derivatives.

Figure 1 shows the effect of reaction time on the formation of the PFB derivatives of the herbicidal acids investigated in this study. Figure 2 depicts a chromatogram of all 10 herbicidal acids after derivatization.

Lesser amounts of K_2CO_3 and PFBBr reagents had no effect on the yield of derivatives; however, the amounts of K_2CO_3 and PFBBr reagents specified in the procedure are recommended to increase the tolerance of this method to the presence of extraneous materials such as other acidic compounds, water, and co-extractives that may be present in actual lake sample extracts. Reaction with these materials can decrease the amount of K_2CO_3 and PFBBr reagents available for derivatization.

The presence of traces of water can affect the

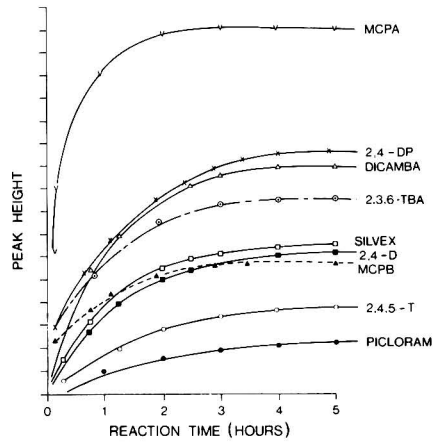

FIG. 1—Effect of reaction time on peak heights of PFB derivatives of 10 herbicidal acids.

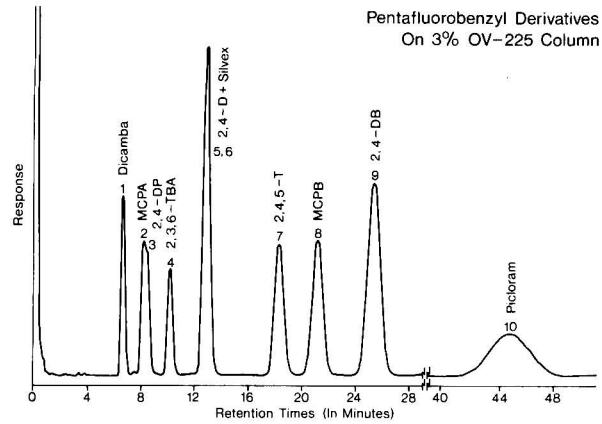

FIG. 2—Gas chromatogram of PFB derivatives of 10 herbicidal acids on OV-101/OV-210 column, attenuation 256: 200 pg dicamba, 100 pg MCPA, 200 pg 2,4-DP, 400 pg 2,4-D, 100 pg 2,3,6-TBA, 400 pg silvex, 500 pg 2,4,5-T, 500 pg MCPB, 1000 pg 2,4-DB, 800 pg picloram.

yield of the derivatives (H. Agemian, Canada Centre for Inland Waters, Burlington, Canada, 1974). The derivatization procedure was tested with 20, 40, 60, 100, 150, and 200 μl water added. Up to 150 μl water did not significantly affect the yield of derivatives for all acids studied except 2,4-D, 2,4,5-T, and 2,3,6-TBA. For these acids, the derivative yield was affected by as much as 30% by the presence of 100 μl water. To ensure reproducible results, traces of water (other than the 30 μl used in the K_2CO_3 solution) should be eliminated.

Preliminary investigations of the reaction in 1, 2, 5, and 10 ml acetone showed slight decrease in derivative yield in less acetone, which was insignificant except at 1 and 2 ml. However, 4 ml was chosen because it minimized any possible solvent effect from traces of co-extractives in some sample extracts, and at the same time it is more convenient to evaporate than a large volume of acetone.

In the analysis of herbicidal acids, the sample is extracted with organic solvent which is evaporated to a low volume or to dryness before esterification. We investigated the effects of ethyl acetate, chloroform, ethyl ether, and benzene solvents on the derivatization procedure. No significant solvent effect was observed except for 2,4-DP and 2,3,6-TBA. The derivative yield for these acids was decreased (15–25%) in the presence of these solvents.

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The retention times of the 10 PFB derivatives on 3 gas chromatographic columns are given in Table 1. The column which gave the best separation is the OV-101/OV-210 column (19); 9 derivatives were separated (*see* Fig. 2). Although the separation characteristics of the PFB derivatives and the 2-chloroethyl esters are comparable, other factors such as sensitivity and convenience must also be considered. A detailed comparison is under investigation and will be reported later.

Acknowledgment

The authors are grateful to Mikio Chiba of Research Station, Agriculture Canada, Vineland, Ontario, Canada, for his critical review of the manuscript.

Table 1. Retention times of 10 PFB esters on 3 GLC columns

Esters	Relative retention times (arbitrary units)		
	OV-17/QF-1	OV-101/OV-210	OV-225
Dicamba	2.2	5.3	4.3
2,3,6-TBA	3.3	7.8[a]	6.4
MCPA	2.5	5.75	5.2[b]
2,4-DP	2.4	6.0	5.3[b]
2,4-D	3.5	7.4[a]	8.3
Silvex	3.9	8.9	8.3
2,4,5-T	5.5	11.3	11.7
MCPB	5.7	12.4	13.5
2,4-DB	7.5	15.3	16.2
Picloram	14.9	22.6	54.4

[a] When together, these 2 peaks are unresolved at 7.6.
[b] When together, these 2 peaks become a doublet.

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Gas-Liquid Chromatographic Analysis and Chemical Confirmation of Azodrin (Monocrotophos) Residues in Strawberries

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A gas-liquid chromatographic (GLC) method is described for the analysis and confirmation of azodrin (monocrotophos, 3-(dimethoxyphosphinyl)-*N*-methyl-*cis*-crotonamide) residues in strawberries. The strawberries are extracted with acetone, and the filtrate is partitioned with a mixture of methylene chloride and petroleum ether followed by further extraction with methylene chloride. The organic phases are combined, dried with anhydrous sodium sulfate, and concentrated to a small volume for GLC analysis on a 3% OV-210 column with flame photometric detection. Identity of the compound is confirmed by chromatography on the same column after trifluoroacetylation of an aliquot of the strawberry extract. The detection limit is about 2 ppb. The types of strawberry samples analyzed were fresh, frozen, pureed, and jam.

Azodrin (monocrotophos, 3-(dimethoxy-phosphinyl) - *N* - methyl - *cis* - crotonamide) residues have been determined by several techniques including enzyme inhibition (1, 2), bioassay (3), colorimetry (4, 5), thin layer chromatography (6, 7), and gas-liquid chromatography (GLC) (8–10). Of these, the GLC methods which use phosphorus-specific detectors (8, 9) are the simplest and most convenient because very little cleanup is required for quantitative analyses.

Azodrin is not registered for use and no tolerances for azodrin residues in foods have been established in Canada. However, the recent problem of azodrin residues in strawberries on the Canadian market indicated that a simple, reliable, and rapid method for this compound is required for screening purposes. The extraction method found most useful in the present study was a modification of the Luke *et al.* method for multiresidue screening for pesticides (M. A. Luke, H. T. Masumoto, & J. E. Froberg, 1974, Food and Drug Administration, Los Angeles, CA). No chemical confirmation methods have yet been reported for azodrin. This paper describes a simple and sensitive derivatization technique applicable to azodrin residues in strawberries at levels as low as 1–2 ppb.

Received August 29, 1975.

METHOD

Reagents and Apparatus

All solvents were analytical or pesticide grade.

(a) *Azodrin.*—Analytical standard (Shell Chemical Co.).

(b) *Homogenizer.*—Sorval, with 250 ml capacity steel cups.

(c) *Gas chromatography.*—Micro-Tek Model MT220 (Tracor Inc.) equipped with phosphorus flame photometric detector (input attenuation, 10^3; output attenuation, 4×), and 2′ × ¼″ od borosilicate glass column packed with 3% OV-210 on 80–100 mesh Chromosorb W (HP). Operating conditions: temperatures (°C)—column 185, injector 210, detector block 265; gas flows (ml/min): helium 85, hydrogen 250, compressed air 100.

(d) *Centrifuge.*—Capable of holding 5 ml centrifuge tubes and attaining 2000 rpm.

Extraction

Blend 25 g strawberries 4 min with 75 ml acetone in homogenizer. Suction-filter the macerate through medium porosity 150 ml fritted glass funnel. Rinse cup with 20 ml acetone and collect rinse through same funnel. Quantitatively transfer filtrate to 500 ml separatory funnel and partition with 150 ml methylene chloride-petroleum ether (1+1). Transfer aqueous (bottom) layer to second separatory funnel and add 10 ml saturated (aqueous) NaCl solution. Partition with two 50 ml volumes of methylene chloride. Collect organic (bottom) layer and combine with organic extract from first funnel. Dry extract 15 min over 5 g anhydrous Na_2SO_4. Filter with suction through medium porosity fritted glass funnel into 500 ml round-bottom flask. Evaporate to 1–2 ml by rotary vacuum evaporation at 30°C. Transfer residue to 5 ml glass-stoppered centrifuge tube and dilute to 2.5 ml with acetone. Inject aliquot of the clear solution into gas chromatograph. Inject standards as frequently as necessary.

Confirmation

Evaporate 0.5 ml of above GLC extract to near dryness in 5 ml centrifuge tube under nitrogen at 50°C. Add 0.2 ml trifluoroacetic anhydride (TFA) (reagent grade), followed by ca 0.6 ml benzene so that homogeneous solution is produced. Stopper tube and let reaction proceed 15 min at room temperature. Evaporate solvent and dilute residue to 0.5 ml with acetone for GLC analysis on same column at 170°C.

Results and Discussion

Several extraction procedures reported in the literature were evaluated for azodrin in strawberries. These included chloroform or methylene chloride extraction, which has been used for azodrin in tomatoes (11), beans (12), oranges (13), lettuce (10), and sweet corn (8), methanol extraction (14), and acetone extraction described herein. The acetone extraction was the most useful. It is simple and provided no technical problems during the analyses of strawberries whether they were fresh, frozen, pureed, or jam. The saturated sodium chloride used was an expediency. The sodium chloride used in the original method should be equally effective. Recoveries (>90%) were quantitative for samples fortified at 10, 1.0, 0.1, and 0.01 ppm. Figure 1 shows a typical chromatogram of fresh strawberries spiked with azodrin at 0.1 ppm. No interfering peaks were obtained for any of the samples analyzed. The detection limit was about 2 ppb (2:1 signal-to-noise ratio). The analytical column performed well for more than 50 sample injections after which only the glass wool and first few inches of column material were replaced for further use. One analyst can analyze as many as 6 samples per day.

Several derivatization techniques were attempted for confirmation of azodrin in strawberries. These included 2 alkylation reactions (methyl iodide-sodium hydride (15, 16) and on-column alkylation with Methelute®) to convert azodrin to bidrin, a related organophosphate which is separable from azodrin by GLC. The Methelute reaction failed and the methyl iodide-sodium hydride produced only about 10% yield with background interference. Silylation was also attempted with *N,O*-bis-(trimethylsilyl)-trifluoroacetamide, *N,O* - bis - (trimethylsilyl) - acetamide, and *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide without success. Trifluoroacetylation proved to be the most successful approach examined. Azodrin was converted to TFA-azodrin quantitatively in 10–15 min at room temperature. Figure 2 shows the probable structure as indicated by mass spectrometry with m/e values at 223 and 319 for parent and derivative, respectively. No interferences were obtained from the reaction. As little as 1–2 ppb azodrin in strawberries was confirmed. Figure 3 shows typical chromatograms obtained for the confirmation of azodrin at 0.0, 0.03, and 0.4 ppm. The tailing solvent peaks shown here and in Fig. 1 are due to benzene, which was originally used for final dilution before GLC. This has since been changed to acetone, which is mentioned in the text. Equivalent sample quantities injected were 100 mg for jam and fresh strawberries, and 20 mg for frozen strawberries. It is of some interest to note that azodrin was detected and confirmed in the jam samples. This indicates that it can persist through some degree of processing.

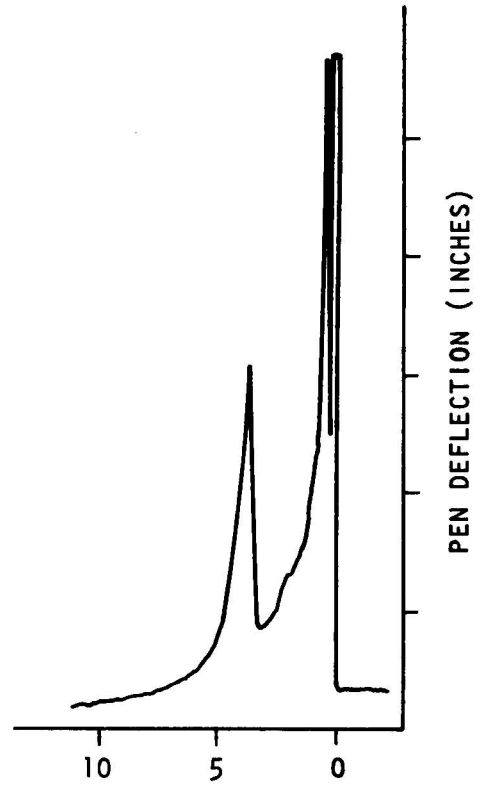

FIG. 1—Chromatogram of fresh strawberry sample fortified at 0.1 ppm azodrin. GLC conditions as described in text; 5 μl injected (abscissa in minutes).

This method was successfully applied to the

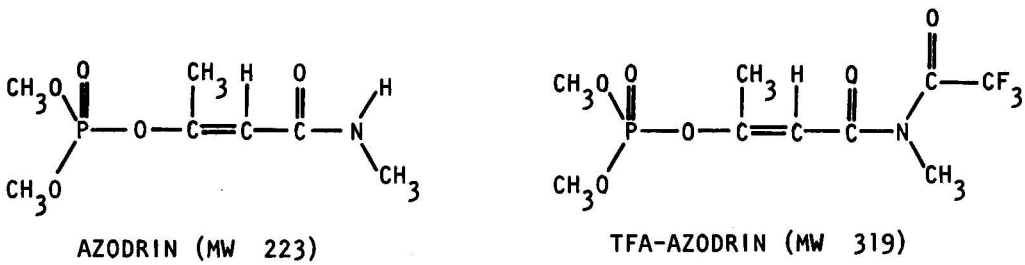

FIG. 2—Structures of azodrin and TFA-azodrin.

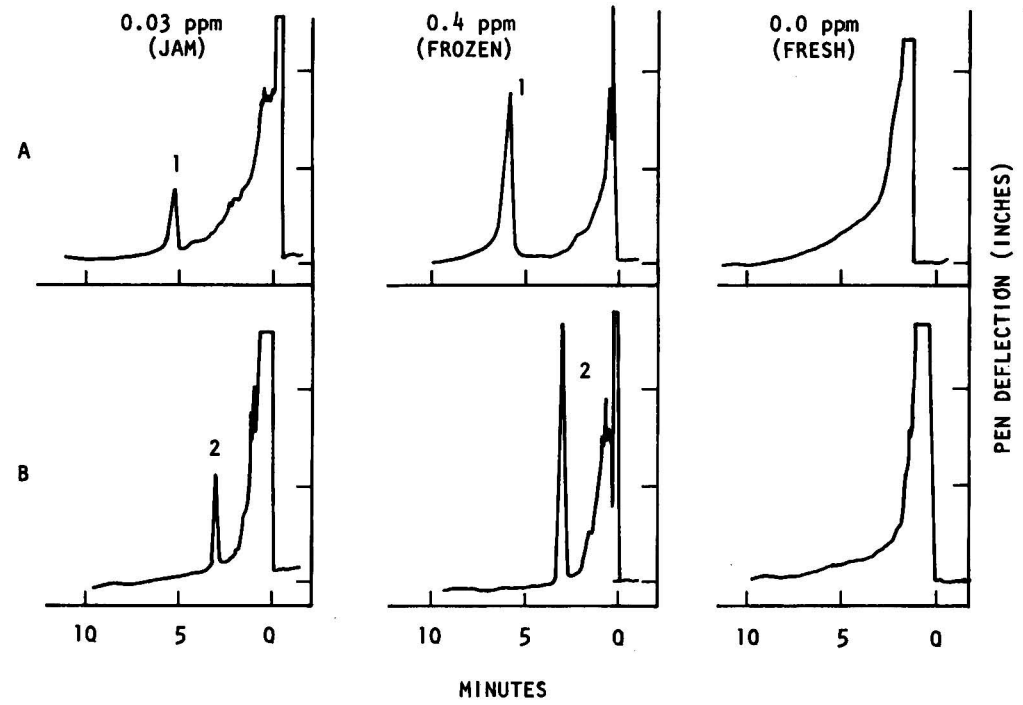

FIG. 3—Confirmation of azodrin residues found in imported strawberries at various levels. A, sample analyses; B, same sample extracts after trifluoroacetylation; 1, azodrin peak; 2, TFA-azodrin. Column temperature 170°C.

quantitative analysis and confirmation of azodrin residues in a variety of imported strawberry products.

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Thin Layer Chromatography of Parathion as Paraoxon with Cholinesterase Inhibition Detection

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A simple, sensitive, and rapid method is described for the quantitative estimation of ng amounts of parathion (*O,O*-diethyl *O*-*p*-nitrophenyl phosphorothioate) as paraoxon (*O,O*-diethyl *O*-*p*-nitrophenyl phosphate) on thin layer chromatograms. Paraoxon is detected by inhibition, using *p*-nitrobenzenediazonium fluoroborate as the chromogenic reagent. This chromogenic reagent is more sensitive than Fast Blue B or indoxyl acetate; 0.1 ng may be detected and amounts from 5 to 50 ng may be estimated. The method is a viable alternative to gas chromatographic analysis for parathion.

The application of the cholinesterase inhibition technique in the thin layer chromatography (TLC) of organophosphorus pesticides has led to substantial progress in toxicological analysis. The enzymatic techniques are very sensitive and permit the detection of nanogram amounts of organophosphate insecticides (1–8). Previous workers have also attempted to quantitate the insecticide detected by TLC by nonenzymatic methods such as gas-liquid chromatography (GLC) (9–14), chromatogram spectrophotometry, and fluorometry (15). But no attempts have been made to simplify the TLC procedures for the quantitative estimation of organophosphorus compounds. Although the above instruments are ideal for detection and estimation, their use is often limited by unavailability and cost. The method presented here offers a viable alternative to gas chromatography that is simple, sensitive, and selective, and does not require sophisticated facilities. The chromogenic reagent *p*-nitrobenzenediazonium fluoroborate has many advantages over the other chromogenic reagents currently used.

Fast Blue B has been used as a chromogenic reagent for the detection of organophosphorus compounds on TLC plates by cholinesterase inhibition (5) but simple methods were not available in the literature for simultaneous quantitation. We reported a rapid and simple technique (7, 8) but the colorless inhibition zones were masked within a fraction of a minute, thereby rendering the subsequent experimental operation difficult. This has been overcome by using *p*-nitrobenzenediazonium fluoroborate as the chromogenic reagent; it reacts with 2-naphthol in a diazo coupling reaction. Rat liver was used as the cholinesterase source.

METHOD

Reagents and Apparatus

All reagents are analytical reagent grade.

(a) *Parathion (O,O-diethyl O-p-nitrophenyl phosphorothioate).*—100% pure. Prepare different concentrations of parathion (Ciba-Geigy Ltd., Basel, Switzerland) in acetone.

(b) *2-Naphthyl acetate substrate solution.*—Dissolve 20 mg 2-naphthyl acetate (Kochlight Laboratories. Colnbrook, Bucks, England) in 4 ml ethanol.

(c) *p-Nitrobenzene diazonium fluoroborate.*—(Eastman Organic Chemicals, New York, NY). Prepare 0.4% in acetone.

(d) *Silica gel G.*—National Chemical Laboratory (CSIR), Poona, India.

Preparation of Rat Liver Homogenate

Excise fresh liver lobes from 6-month-old albino Wistar female rats and freeze at −10°C immediately. Prepare 10% homogenate of liver in ice cold pH 7.0 sodium phosphate buffer in glass homogenizer at 0°C. Filter homogenate through 4 layers of cheese cloth. Keep homogenate filtrate at 0°C before use.

Thin Layer Chromatography

Coat 20 × 9.5 cm glass plates with 450 μm layer of silica gel G in water slurry. Air-dry plates and activate in 110°C oven 1 hr. Store plates in desiccator before use.

Analyze surface residues of cauliflower, paddy straw, and paddy grain by adapting cleanup procedure of Mendoza and Shields (6). Concentrate aliquots to obtain equivalent amounts of residues in each extract. Apply different concentrations of parathion standards (1–10 μl), and sample extracts, with micropipet. Expose plate 30 sec to evenly

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distributed bromine vapor for oxidation of parathion by keeping plate in closed glass jar (25 × 25 cm) containing 0.1 ml liquid bromine. Remove plate and let bromine evaporate in air for 5 min.

Develop plate 15 cm in acetone-hexane (1+4). Air-dry plate 5 min. Spray liver enzyme solution uniformly over plate, thoroughly wetting the gel (enzyme should not be sprayed in excess). About 5–10 ml liver enzyme solution is required for 20 × 9.5 cm silica gel plate 450 μm thick. Keep plate 20 min in moist atmosphere at 37°C. Spray plate with 2-naphthyl acetate substrate solution. Replace plate 2 min in moist atmosphere at 37°C. Uniformly spray plate with 0.4% *p*-nitrobenzenediazonium fluoroborate in acetone and let stand at room temperature. The parathion inhibition of cholinesterase appears as clear white spot in orange background. Lower limit of detection is 0.1 ng.

Quantitative Estimation of Parathion

(a) *Colorimetry.*—Scrape off 1.5 sq. cm of orange background from 3 random areas on developed plate. Extract color with 5 ml acetone in test tube, stirring with glass rod. Filter extract through Whatman No. 1 paper and measure mean absorbance of clear solution at 460 nm. These values serve as controls where zero concentration of parathion represents 100% activity.

Scrape exactly 1.5 sq. cm from the TLC plate around each inhibition zone for standards and samples. Extract each zone with acetone as described above, and measure absorbance at 460 nm in spectrophotometer. Area of inhibition is proportional to parathion concentration; area of activity is inversely proportional to parathion concentration. Subtract activity value from control value and normalize units to 100 to obtain per cent inhibition of cholinesterase. Determine concentration of parathion as paraoxon (*O,O*-diethyl (*O*-*p*-nitrophenyl phosphate) by comparing with standard curve of per cent inhibition vs. concentration.

(b) *Area measurement method.*—Place sheet of thin, centimeter graph paper against back of TLC plate (side not coated with silica gel G), trace area of inhibited zone, and count area in millimeter squares. Number of millimeter squares in standard unit area of 1.5 sq. cm (225 sq. mm) is control representing 100% cholinesterase inhibition. Calculate per cent of cholinesterase inhibition and compare with standard curve.

(c) *Area weight method.*—Trace area of inhibition zone on uniformly thick tracing paper, cut out, and weigh. Weigh also standard unit area of 1.5 sq. cm which represents 100% cholinesterase inhibition, calculate per cent inhibition of cholinesterase activity, and compare with standard curve.

Results and Discussion

The substrate, 2-naphthyl acetate, is hydrolyzed to 2-naphthol by cholinesterase. *p*-Nitrobenzenediazonium fluoroborate reacts with 2-naphthol to form an orange diazo compound. The colored area on the chromatogram represents cholinesterase activity. Organophosphates inhibit the cholinesterase hydrolysis of the substrate and the subsequent formation of the colored product. *p*-Nitrobenzenediazonium fluoroborate is preferable to other chromogenic reagents (1–8) because the chromatogram spots remain clear indefinitely and 0.1–1 ng parathion can be detected. Chromatogram spots formed with indoxyl acetate (6) were either barely visible or disappeared in 5 min at 5 ng parathion. Pig liver, beef liver, or beeheads were the enzyme source (6).

The amount of insecticide material on a thin film chromatogram can be determined from the size of the spot it forms (16–18). The square root of the area is a linear function of the logarithm of the weight of the material in the spot (16–18). Evidently, the area of the spot increases proportionately with the concentration of the organophosphorus insecticide and this function is taken as a measure for the quantitation of the insecticide concentration on TLC in the present investigation. A few methods based on this principle have been developed but with Fast Blue B as the chromogenic reagent (7, 8).

In all 3 techniques reported here for the estimation of parathion as paraxon, a standard unit area of 1.5 sq. cm was taken. This was the smallest standard unit area which could accommodate the maximum area representing the highest quantity of insecticide in the linearity curve. A plot of inhibition zone area in millimeter squares vs. parathion concentration or weight of inhibition zone area in mg vs. parathion concentration shows a straight line relationship between 5 and 50 ng (Table 1). The colorimetric method also obeyed Beer's law between 5 and 50 ng (Table 1).

The above method was applied to the quantitative analysis of the surface deposits of parathion as paraoxon in cauliflower, paddy straw, and paddy grain, cleaned up according to the method of Mendoza and Shields (6) (Table 2).

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Table 1. Relationship of cholinesterase inhibition with parathion concentration as determined by colorimetric, area measurement, and area weight methods (values are mean of 6 observations ± standard deviation of the mean)

Parathion concn, ng	Area measurement method		Area weight method		Colorimetric method
	Inhibit. zone, sq. mm	Cholinest. inhibit., %	Inhibit. zone, mg	Cholinest. inhibit., %	Cholinest. inhibit., %
5	27.00	11.88±0.35	3.00	15.00±0.67	14.00±2.18
10	48.86	21.41±0.68	4.16	21.62±0.89	23.00±1.91
20	69.22	30.45±1.50	6.40	33.28±1.48	32.24±3.10
30	90.50	39.60±2.16	7.50	39.00±1.21	39.00±2.61
40	99.50	44.00±2.60	8.25	42.90±2.06	45.50±4.01
50	116.20	52.80±1.89	10.80	56.16±2.21	58.60±4.81

Table 2. Surface residues of paraoxon on various samples determined by area measurement and area weight methods

Sample and wt, g	Area measurement method		Area weight method	
	Cholinest. inhibit., %	Parathion, ng	Cholinest. inhibit., %	Parathion, ng
Cauliflower, 500[a]	27.00	20.00	29.00	19.00
Paddy straw, 7[a]	19.00	12.50	21.00	12.00
Paddy grain, 40[b]	11.88	5.00	15.00	4.50
Paddy grain, 40[b]	30.00	24.00	32.00	23.50

[a] Samples bought from market.
[b] Samples were sprayed under laboratory conditions and analyzed after 36 hr. The concentration of the solutions spotted was 5 μl/5 ml hexane.

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FISH AND OTHER MARINE PRODUCTS

Collaborative Study of the Determination of the Amount of Shrimp in Shrimp Cocktail

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An acceptable method to determine shrimp in shrimp cocktail is needed to determine compliance with proposed labeling regulations. Our method employs a sieve and washing technique to separate shrimp from other ingredients. The percentage of shrimp is calculated from its weight and the declared weight of the total content. For the collaborative study, duplicate samples from 3 different formulations were selected randomly. Eighteen collaborators estimated the amount of shrimp in shrimp cocktail with a standard deviation of 2. A simplified version of the collaborative method has been adopted as official first action.

In October 1974, the National Fisheries Institute and the National Marine Fisheries Service sponsored an industry-wide meeting to discuss declaration of per cent seafood ingredient in shrimp and other seafood cocktails. The nature of this problem among seafood processors is 2-fold: (*1*) The scientific community does not generally agree on any current standard method to measure compliance with proposed regulations in the *Federal Register* (1–3). (*2*) To determine amount of container overfill to meet final content requirements, weight losses due to material or processing variables such as pH, seafood surface-to-weight ratio, precook treatment, storage conditions, and use of condensed phosphates must be investigated. Such a study requires an acceptable method of analysis to determine amount of seafood ingredient.

A recommendation was made at this meeting to develop an acceptable method for determining amount of shrimp or other seafood ingredient in shrimp or other seafood cocktails. Our previous work on shrimp products has resulted in specifying a U.S. Standard No. 20 sieve to measure shrimp material in raw headless shrimp (4) and raw breaded shrimp (5). For the present study, we included a U.S. Standard No. 8 sieve in the procedure to separate shrimp material from smaller pieces of horseradish which may be a component of the sauce. We used the proposed procedure to analyze commercial samples of shrimp cocktail (Table 1).

A test of the proposed procedure indicated that recovery of shrimp from shrimp cocktails tested immediately after preparation varied from 99.3 to 99.8% (R. A. Hixon, 1975, personal communication, Safeway Stores, Inc., Oakland, CA). A related test included horseradish and 200–300 count shrimp treated with sodium tripolyphosphate and cooked. The sauce was adjusted to pH 3.7 immediately before adding shrimp to a final level of 30±1%. Shrimp recovery was tested 45 sec after preparation. In 6 jars without added horseradish, the average recovery of shrimp was 102.71±0.26% of the amount added, and <0.01 g material was removed from the No. 20 sieve. In 6 jars containing added grated horseradish (10% by weight of sauce), the average recovery of shrimp was 102.98±0.59% of the amount added. No material was recovered from the No. 20 sieve because it was difficult to differentiate shrimp and horseradish even under a 4-power magnification. Recovery values in excess of 100% may have resulted from using treated shrimp which picked up moisture during washing. Untreated shrimp were used in subsequent tests.

The problem of visual differentiation of shrimp and horseradish on the No. 20 sieve led us to propose a supplementary, chemical method. Horseradish is exceptionally rich in peroxidase (6). *p*-Aminodimethylaniline oxalate is used to detect oxidase enzymes (6–8).

Preliminary tests also indicated that shrimp lost a significant amount of moisture when com-

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bined with cocktail sauce (Table 2). Results of other tests indicated a retarded rate of weight loss from frozen stored shrimp cocktails compared with cocktails stored at 40°F (Table 3). Lerke (9) has published data showing the effects of storage on pH of shrimp and crabmeat cocktails. Apparently, transfer of water and water-soluble compounds from the seafood flesh into the sauce contributed to the storage changes that Lerke (9) observed.

Collaborative Study

Three lots of authentic shrimp cocktail samples were prepared. Two types of frozen, raw, peeled, and deveined shrimp were purchased from commercial sources: 130–200 and 90–110 count/lb, both without prior treatment with sodium tripolyphosphate or other condensed phosphates. After cooking, the shrimp were weighed by hand into 4 oz jars containing ca 1 oz cocktail sauce. More sauce was added to fill the jars and to leave adequate headspace for expansion due to freezing. The sauce contained grated horseradish. The amount and size of shrimp used to make each lot of shrimp cocktail was as follows:

Lot	Wt of cooked shrimp/4 oz jar	Comments
1	1.50 oz, 130–200 count	estimated to contain ≥30% shrimp, using a size that is normal for shrimp cocktail
2	1.20 oz, 130–200 count	estimated to contain ≥25% shrimp, using a size that is normal for shrimp cocktail
3	1.50 oz, 90–110 count	estimated to contain ≥30% shrimp, using a larger (and more expensive) size that would be expected in higher priced shrimp cocktail

Jars of shrimp cocktail were capped in an automatic, steam-vacuum sealer, held 48 hr at 35–38°F, and then quick-frozen at 0°F. Two days later, frozen samples were packed in insulated shipping containers with Dry Ice and instructions, and shipped by air express or the equivalent.

Table 1. Shrimp material in samples of commercial brands of shrimp cocktail[a]

Brand[b]	Av. net wt of contents, oz	Av. count of shrimp/jar	Av. % shrimp[c]
A	4.35±0.05	23.5±6.9	30.7±3.7
B	4.41±0.05	25.5±3.0	31.2±1.0
C	4.49±0.004	17.3±1.3	25.9±1.7
D	4.07±0.11	16.5±2.3	30.7±1.8
E	5.80±0.20	17.5±0.9	31.7±2.1
F	4.18±0.06	13.3±1.9	21.5±2.0

[a] Four jars of each brand obtained from retail stores in September 1974. No shrimp material was found on No. 20 sieve for any jars used.
[b] Declared net weight of 4 oz/jar, except for brand E, which was 6 oz.
[c] Per cent shrimp = weight shrimp (× 100)/declared net weight of contents.

Table 2. Moisture and weight losses of shrimp cocktail stored at 36–38°F[a] (C. M. Hayek, 1974)

Days stored	Moisture content of shrimp,[b] %	Shrimp yield, %	pH of sauce
0	81.91	—	3.63
1	71.91	81.96	3.98
7	70.88	83.84	4.07
14	70.87	83.88	4.07
41	70.77	80.96	4.07

[a] Shrimp were 120–200 count and had been treated with tripolyphosphate. When shrimp cocktails were prepared, they contained 30±1% shrimp (w/w) with individual percentage recorded to nearest 0.01%.
[b] Each moisture content listed is the average of 3 aliquots taken from a composite sample of 6 cocktails.

Table 3. Weight loss of shrimp in shrimp cocktails stored at 40 or 0°F[a] (C. M. Hayek, 1974)

	Samples stored at 40°F		Samples stored at 0°F	
Days stored	Wt loss, %	pH	Wt loss, %	pH
0	0	3.86	0	3.86
1	13.36	4.36	7.84	4.34
4	13.78	4.49	7.92	4.34
7	16.06	4.25	14.33	4.10
14	13.20	4.31	10.57	4.17
21	15.29	4.27	11.06[b]	4.29[b]
29	14.21	4.20	12.57[b]	4.33[b]
36	14.49	4.27	10.39	4.26
42	13.86	4.36	11.96[b]	4.28[b]
49	15.35	4.27	—	—
56	16.52	4.30	—	—
63	—	—	11.09	4.11
63	—	—	9.20[b]	4.37[b]

[a] Shrimp were 120–200 count and had been treated with tripolyphosphate. When shrimp cocktails were prepared, they contained 30±1% shrimp (w/w) with individual percentage recorded to nearest 0.01%. Each weight loss listed is the average of 3 shrimp cocktails.
[b] Samples frozen and stored for number of days indicated, then stored at 40°F for 8 more days before analysis.

Each of 18 collaborators received duplicate samples from each of the 3 lots and a seventh, unnumbered jar to determine thawing time. Samples for analysis were numbered by 3-digit code numbers which were selected randomly. Collaborators were given the detailed information and instructions below. The simplified method adopted as official first action follows.

Collaborative Method

Note: Preliminary information indicates that combining shrimp or crabmeat with cocktail sauce results in significant loss of moisture from the flesh within first 24 hr. This method should *not* be used to analyze samples having a total unfrozen storage life <48 hr.

Thaw unopened jars in 60±10°F water bath until product is defrosted (42±10°F). Lids of jars should be above water level to prevent migration of water into jars. A test run is advisable to determine time required to thaw given quantity of jars under conditions actually used.

Assemble two 8″ diameter sieves by placing No. 8 U.S. standard sieve on top of No. 20 U.S. standard sieve. Sieve specifications are contained in U.S. Department of Commerce, National Bureau of Standards' Specifications for Sieves, U.S. Standard Sieve Series. Set assembled sieves under cold tap water faucet, preferably with spray attachment.

Weigh unopened full jar. If contents were thawed in water bath, wipe outside of jar before weighing it. Record all weights to 0.01 oz or 0.1 g on balance having this accuracy.

Empty thawed contents of jar onto No. 8 sieve. Wash jar and lid with water and pour washings onto sieve until jar is clean. Save jar plus lid, dry, and weigh. Rinse material on sieves without rubbing flesh. Keep all rinsings over sieves. Have only gentle stream of water hit flesh directly. Use rubber spatula to remove adhering material.

Place a cover or piece of moisture-barrier film over top sieve. Incline sieves on 17–20° angle, and let drain exactly 2 min. Transfer flesh on No. 8 sieve to previously tared container and cover. Determine weight of contents. If flesh appears on No. 20 sieve, add it to previously tared container and cover before weighing. If pieces of horseradish are retained on No. 20 sieve, do *not* include these pieces in weighing. If pieces of horseradish and fragments of seafood flesh cannot be distinguished visually use chemical test below.

If seafood cocktail is being analyzed, also weigh amount of each seafood component. After weighing total amount of seafood flesh, transfer identifiable pieces of flesh into as many previously tared containers and covers as necessary to separate seafood components (such as shrimp, crabmeat, or clammeat) by species of origin. Leave unidentifiable pieces of flesh in original container and cover. Weigh and record amount of flesh in each covered container.

If shrimp cocktail or seafood containing shrimp is being analyzed, count number of shrimp obtained. Also count number of shrimp pieces obtained. "Shrimp piece" is defined (from U.S. Standards for Grades of Frozen, Raw, Headless Shrimp): Piece refers to any portion of shrimp that contains less than 5 segments.

Do not count number of flesh pieces derived from crabs, clams, or other non-shrimp species if product being analyzed contains such ingredients. Repeat analysis for other jars in sample.

Per cent seafood ingredient(s) = net weight of seafood ingredient(s) × 100/weight of total contents. If actual net weight of total contents is greater than declared weight, use declared weight for weight of total contents. If actual net weight of total contents is less than declared weight, use actual net weight for weight of total contents. Express to nearest 0.1%.

Chemical Test

Horseradish is exceptionally rich in peroxidase. *p*-Aminodimethylaniline oxalate or related compound is used to detect oxidase enzymes.

Dissolve 1 g *p*-aminodimethylaniline oxalate in 100 ml water by heating gently. Solution is pink. It can be refrigerated 2–3 days, but for best results, prepare fresh solution daily.

Collect suspected material on No. 20 sieve into dish or beaker. Flood with reagent solution. Horseradish particles will decolorize solution after 2–3 sec, but shrimp pieces will not change the color.

METHOD

Shrimp in Shrimp Cocktail—Official First Action

18.B02 *Preparation of Sample*

Thaw unopened jars in 16±5° H_2O bath until product is defrosted (6±5°). Keep jar lids above H_2O level. Alternatively, place frozen jars in refrigerator until contents have thawed.

18.B03 *Determination*

Empty thawed contents of jar onto No. 8 sieve. Wash jar and lid with H_2O and pour washings onto sieve until jar is clean. Rinse shrimp on sieve with gentle stream or spray of cold tap H_2O. Use rubber spatula to remove adhering material. Cover sieve with metal cover or moisture barrier film, incline at 17–20° angle, and let drain exactly 2 min. Transfer shrimp to

container previously tared with cover and weigh to ±0.1 g.

% Shrimp
= wt shrimp × 100/*declared wt* total contents

Results and Discussion

Results from the collaborative study (Table 4) suggest that the amount of shrimp in shrimp cocktail can be estimated with a standard deviation of 2. Assuming a shrimp weight loss of 10% after combining shrimp with cocktail sauce, we had estimated that Lots 1 and 3 should yield 33.8% shrimp and that Lot 2 should yield 27% shrimp. The collaborators' results for Lot 1 (mean of 32.1%) and Lot 2 (mean of 26.5%) suggest that our weight loss assumption was reasonable. Both of these lots contained 130–200 count shrimp which is a normal size for commercial packs. In contrast, Lot 3 contained larger shrimp (90–110 count) which would be expected only in a more expensive item. The collaborators' results for this lot are about 5% higher than our estimate, which suggests that these large shrimp lost less moisture than anticipated when marinated in the sauce.

The greatest difficulty the collaborators encountered was use of the No. 20 sieve. Eight collaborators commented that it would be difficult to separate small pieces of shrimp that passed through the No. 8 sieve from grated horseradish, and that the amount of shrimp material involved should be negligible compared with the amount retained by the No. 8 sieve. None of them reported shrimp material from our samples retained by the No. 20 sieve or grated horseradish retained by the No. 8 sieve. The proposed chemical test to distinguish grated horseradish and small pieces of shrimp on the No. 20 sieve was stated to be unreliable and the chemical reagent was difficult to obtain. This

Table 4. Results from collaborative study of proposed method to determine amount of shrimp in shrimp cocktail[a]

	Lot 1		Lot 2		Lot 3	
Coll.	Sample A	Sample B	Sample A	Sample B	Sample A	Sample B
1	32.2	33.1	28.2	27.0	39.0	37.9
2	33.0	37.2	27.2	26.2	38.0	37.8
3	30.8	33.7	26.5	24.9	36.6	37.0
4	33.8	30.9	25.7	26.1	35.2	37.4
5	32.2	33.6	26.7	26.4	38.7	37.7
6	33.3	33.0	27.3	27.7	39.6	41.0
7	31.7	34.0	25.9	25.0	36.1	37.9
8	35.7	34.4	27.0	25.7	34.2	38.5
9	32.4	31.0	29.3	29.8	37.9	39.7
10	31.2	31.4	25.5	25.0	34.9	36.7
11	26.6	25.0	23.4	21.9	34.4	31.2
12	30.7	30.4	26.8	25.4	31.6	35.4
13	32.5	33.3	27.6	26.7	37.9	35.6
14	30.7	26.5	22.0	24.0	35.4	31.5
15	31.2	—[b]	25.8	25.5	31.2	35.5
16	30.3	30.5	25.7	27.7	36.2	36.2
17	32.3	35.3	28.8	28.8	41.0	38.3
18	31.7	32.1	25.5	27.1	36.0	33.3
No. of samples	18	17	18	18	18	18
Mean	31.8	32.1	26.4	26.2	36.3	36.6
Std dev.	1.8	2.9	1.7	1.8	2.5	2.5
No. in lot	35		36		36	
Mean	31.9		26.3		36.5	
Std dev.	2.4		1.7		2.7	

Var. between lots, σ_L^2	25.49275
Var. between colls. within lots, σ_C^2	8.89032
Var. between replicate samples within colls., σ_E^2	2.2392[c]

[a] Results expressed in per cent. True amount of shrimp in these samples is not known because shrimp mixed with cocktail sauce loses varying amounts of moisture. Some collaborators reported that their samples were thawed on arrival, but their results are not significantly different from those whose samples were still frozen.

[b] Accident.

[c] The variance for any sample average taken under the same conditions can be computed by the formula: $\sigma^2 = (\sigma_L^2/lcr) + (\sigma_C^2/cr) + (\sigma_E^2/e)$, where l = number of lots included in the average, c = number of collaborators included in the average, r = number of replicates, and e = number of replicate samples included in the average.

chemical test is not needed to distinguish shrimp pieces retained by the No. 8 sieve.

Nine collaborators reported their results based on the actual net weight of total contents instead of the declared net weight of 4 oz. This error was presumably an oversight in reading the instructions. In commercial practice, it is customary to base amount of shrimp used on a declared weight and to give consumers extra sauce to allow for production variables.

Three collaborators commented on counting and differentiating shrimp and shrimp pieces. This part of the procedure was designed to estimate size, not weight, of the shrimp. One collaborator mentioned breaking shrimp during handling and counting, making it difficult to determine which shrimp contained at least 5 segments. Two others commented on the extra time taken to obtain these data. None of the collaborators said that this estimate of shrimp size was sufficiently important to be retained in the proposed method.

Although the collaborative method was written to cover all types of seafood cocktails, the study included only shrimp cocktail. There is an immediate need for a tested method to study the effects of material and process variables on labeling and overfill requirements for shrimp cocktail. We are also planning a collaborative study of the proposed method for other types of seafood cocktails such as crabmeat, oyster, or mixed types.

Recommendations

It is recommended that the collaborative method be revised as follows and adopted as official first action:

(1) Eliminate the No. 20 sieve and the chemical test to distinguish very small pieces of shrimp material and grated horseradish.

(2) Eliminate determination of actual net weight of total contents, and specify more clearly that declared net weight is to be used in calculating results.

(3) Eliminate counting shrimp or shrimp pieces.

(4) Limit coverage of the method to shrimp cocktail.

The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee C and were adopted by the Association. *See* (1976) *JAOAC* **59**, 385.

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The following participated in the collaborative study. Their efforts and comments are greatly appreciated:

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G. V. Reddy, Fishking Processors, Inc., Los Angeles, CA

Don Toloday and Adrian Vela, Singleton Packing Corp., Tampa, FL

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METALS AND OTHER ELEMENTS

Determination of Arsenic and Selenium in Foods By Electroanalytical Techniques

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Arsenic and selenium are determined in foods by differential pulse polarography and cathodic stripping voltammetry. The sample is digested with nitric acid and magnesium nitrate and then dissolved in dilute hydrochloric acid. An aliquot is removed, the arsenic is chemically reduced to the trivalent state, and interferences are removed by ion exchange before polarography. Selenium is determined in a second aliquot by cathodic stripping voltammetry. Recoveries for both elements in several foods were from 90 to 110%. The relative standard deviations for arsenic at 5 ppm and selenium at 0.48 ppm were 5.8 and 7.3%, respectively.

The most commonly used methods for determining low levels of arsenic and selenium in foods include colorimetry after generation and reaction of arsine with silver diethyldithiocarbamate or ammonium molybdate (1) and fluorometry of selenium 2,3-diaminonaphthalene complex (2). For the arsenic determination, the sample is either wet-digested with nitric and sulfuric acids or dry-ashed in the presence of magnesium nitrate. For the selenium determination, the sample is digested with nitric, sulfuric, and perchloric acids. More recently, atomic absorption spectrophotometry has been used as the determinative step after generation of gaseous arsine and hydrogen selenide (3–5). Various gas sampling techniques have been developed for this purpose, including a liquid nitrogen trap for arsine (3), direct introduction of arsine into the atomic absorption spectrophotometer flame (4), and a rubber balloon trap (5) for arsine, selenium hydride, and other hydrides. These special techniques are necessary because conventional atomic absorption spectrophotometry is relatively insensitive to these elements. Although the above methods are very useful for the analysis of arsenic and selenium in foods, some drawbacks still exist. For example, the methods involving the generation of arsine are subject to interferences from excessive salts, acids from the digestions (1), and certain ions including copper and others (6). The fluorometric method for selenium is lengthy and tedious.

Among the electrochemical techniques in the literature are anodic stripping of arsenic from a gold electrode (7, 8) and cathodic stripping of selenium complex from a hanging mercury drop electrode (9). Anodic stripping voltammetry of arsenic or selenium from a hanging mercury drop electrode is not feasible because these elements do not form amalgams. Differential pulse polarography of arsenic(III) in 1*M* HCl (10) and selenium diaminobenzidine complex in 1*M* $HClO_4$ (11) are 2 of the polarographic methods recently described. With the exception of anodic stripping of arsenic from a gold electrode, we have not seen any practical applications of the above techniques to the determination of low levels of arsenic and selenium in foods. From information in the literature, it appeared that differential pulse polarography of arsenic and cathodic stripping voltammetry of selenium could be valuable techniques for the determination of these elements in foods if methodology were developed. Because inexpensive commercial instruments are available, these methods could be reasonable alternatives to other procedures.

METHOD

Apparatus

(a) *Polarograph.*—Princeton Applied Research (Princeton, NJ 08540) Model 174, or equivalent, equipped with stripping accessories.

(b) *Cells.*—Standard cell bottom with saturated calomel reference electrode, carbon rod counter electrode, dropping mercury electrode, and hanging mercury drop electrode (Metrohem Model E 410).

(c) *Pipets.*—20, 50, and 100 µl Eppendorf micropipets, or equivalent.

Reagents

All reagents are reagent grade unless otherwise indicated.

(a) *Ion exchange resin.*—Amberlite IRA-400, 20–50 mesh analytical reagent, or equivalent.

(b) *Standard arsenic solution.*—1000 µg As(III)/ml. Dissolve 1.320 g As_2O_3 in ca 25 ml 1*N* NaOH

in 1 L volumetric flask, acidify with 1*N* HCl, and dilute to volume with water. Make other dilutions, as required, with 1*N* HCl.

(c) *Standard selenium solution.*—1000 μg Se(IV)/ml. Dissolve 1.638 g selenious acid (H_2SeO_3) in 0.2*N* HCl in 1 L volumetric flask and dilute to volume with 0.2*N* HCl. Make other dilutions, as required, with 0.2*N* HCl.

Digestion of Samples

Accurately weigh representative 1 g sample into 100 ml borosilicate beaker and add 10 ml HNO_3 and 4 g $Mg(NO_3)_2.6H_2O$. Heat on hot plate at low heat until mixture is completely dry; then raise heat to maximum setting until brown fumes are no longer evolved. Put beaker in 500°C muffle furnace 30 min (longer if carbon particles are still present). Let cool, add 10 ml 6*N* HCl, cover beaker with watch glass, and place on steam bath until residue dissolves. Transfer solution with water to 25 ml volumetric flask and dilute to volume. Analyze reagent blank in same manner as sample.

Determination of Selenium

Pipet 10 ml sample solution into polarographic cell, bubble nitrogen through solution 5–10 min, and then direct nitrogen over solution. Dial out mercury drop by turning micrometer 4 divisions. Stir solution with magnetic stirrer at constant and reproducible rate. Stirring rate must be such that mercury drop is not disturbed. Slide selector switch to "External Cell," and time for 60–120 sec (depending on concentration) with stop watch. Turn off stirrer and let stand 30 sec. Press "Scan" button to obtain peaks due to selenium. Remove mercury which has fallen to bottom of cell and add 50 μl saturated $KMnO_4$ solution. Stir, and bubble nitrogen through solution 2 min. Remove excess permanganate by decolorizing with ca 5 mg hydrazine sulfate. Perform deposition and stripping operations as before to ascertain that selenium in sample has been completely oxidized. Add more $KMnO_4$ if necessary. Add several aliquots of standard selenium solution to cell by means of Eppendorf pipet. Amounts of standard added should bracket selenium peak current in sample. After each addition, bubble nitrogen through solution briefly and perform deposition and stripping operations for each aliquot added exactly as for sample. Plot calibration curve of amount of selenium added vs. peak current and obtain selenium concentration in sample from this curve. Use selenium peak at ca −0.54 v vs. saturated calomel electrode.

Determination of Arsenic

Pipet 10 ml solution into 50 ml beaker, add 1 ml HCl and 1 g anhydrous Na_2SO_4, cover with watch glass, and put beaker on steam bath 20 min. Cool, transfer solution to polarographic cell, and rinse beaker with ca 2 ml 1*N* HCl. Bubble nitrogen through solution 10 min; add 2 g ion exchange resin, bubble nitrogen through another 5 min, and then direct nitrogen over solution. Switch selector to "Cell," and let recorder pen come to rest. Depress "Scan" pushbutton and record polarogram from −0.15 to 0.9 v. Using Eppendorf pipet, add standard arsenic solution to cell to approximately double amount present, bubble nitrogen through solution 2 min, direct nitrogen over solution, and record polarogram as before. Repeat this procedure on 2 additional successive aliquots of the standard arsenic solution. Plot standard addition curve of amount of arsenic added vs. peak current and obtain arsenic concentration from this curve. Use arsenic peak at ca −0.35 v vs. saturated calomel electrode.

Results and Discussion

Before arsenic and selenium can be determined in foods by polarographic techniques the sample must be subjected to the proper pretreatment procedures. First, the food sample must be digested to destroy the organic matter without loss of either element, a rather difficult task since many arsenic and selenium compounds are quite volatile. Second, these elements will be oxidized to the electrochemically inactive As(V) and Se(VI) as the result of the digestion; therefore, they must then be chemically reduced to the electrochemically active As(III) and Se(IV). It is important that the materials used in these manipulations do not interfere with the determinations.

The typical conditions for both arsenic and selenium determinations are listed in Table 1. These, of course, can be varied and are given only as a guide.

In polarography and stripping voltammetry it is desirable to perform quantitative measurement by a method of standard addition in order to compensate for matrix differences. This is not possible for selenium because the calibration curve is not linear. In our procedure, however, selenium standards are added to the sample to obtain a calibration response. Se(IV) is oxidized to inactive Se(VI) after analysis of the former, and added Se(IV) standards are analyzed subsequently.

Figures 1 and 2 are examples of cathodic stripping scans obtained according to the method. The peaks are due to the reduction of HgSe(solid) species deposited on the mercury

Table 1. Typical instrument settings

Parameter	Arsenic	Selenium
Working electrode	dropping mercury electrode	hanging mercury drop electrode
Scan rate, mv/sec	5	20–50
Scan direction	"–"	"–"
Range, v	1.5	1.5
Initial potential	−0.15	0
Modulation amplitude	100	—
Operation mode	diff. pulse	dc
Current range, μa	0.5–2	1–2
Output offset	as needed	as needed
Display direction	"+"	"+"
Drop time, sec	1	—
Low pass filter	off	0.3
Selector	off	off
Deposition, sec	—	60–120 stirred, 30 unstirred
Pushbutton	initial	initial
Recorder X-axis, v/in.	0.1	0.1
Recorder Y-axis, v/in.	1	1

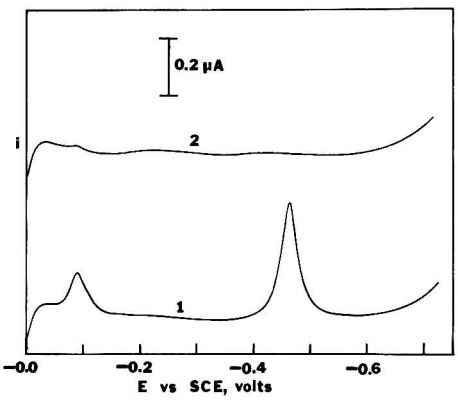

FIG. 1—Cathodic stripping scans of tuna fish sample for Se(IV).

Scan rate, 50 mv/sec; deposition, 60 sec stirred, 30 sec unstirred; 1, sample, 0.4 g/10 ml, before addition of $KMnO_4$; 2, sample after addition of $KMnO_4$.

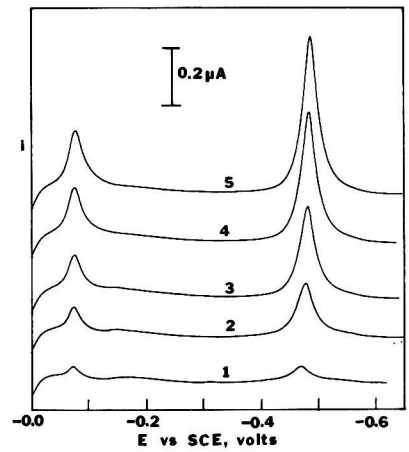

FIG. 2—Cathodic stripping scans of Se(IV) standards.

Scan rate, 50 mv/sec; deposition, 60 sec stirred, 30 sec unstirred; Se concentration, ng/ml: 1 = 10, 2 = 20, 3 = 30, 4 = 40, 5 = 50.

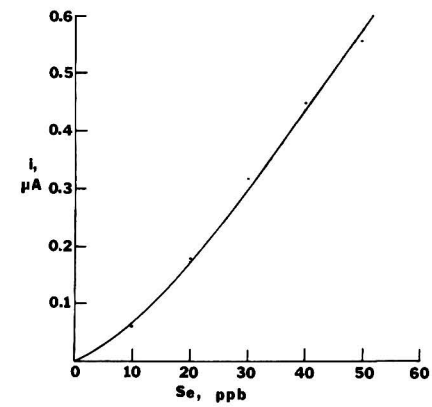

FIG. 3—Typical calibration curve for 0–50 ng Se(IV)/ml.

drop electrode. Note the scan after $KMnO_4$ is added. Selenium was quantitatively oxidized to the electrochemically inactive Se(VI) by one addition of 2–3 mg $KMnO_4$. $KMnO_4$ should not be added to the cell when mercury is present because mercury will be oxidized and will contribute to the background signal. Figure 3 shows a typical calibration curve.

Table 2 summarizes the recovery results obtained for several food commodities which were fortified with the indicated amounts of selenium. Each value represents one determination. An estimate of the precision of the method was obtained by multiple analyses of a canned tuna fish sample which contained measurable quantities of selenium. The relative standard deviation at the 0.48 μg/g level was 7.3%. As little as 0.1 ppm selenium in a sample can be quantitated if a 1 g sample is analyzed. The most important parameters which affect sensitivity in stripping voltammetry include the deposition time and the stirring rate. Deposition times longer than 1.5 min may be used for better sensitivity. The stirring rate, however, can only be increased to the point that the hanging mercury drip is not disturbed.

Table 3 shows a comparison of the results by the proposed method and by the established

Table 2. Recovery data for selenium in several foods

Food	Amt found,[a] μg/g	Amt added, μg/g	Rec., %
Raw eggs	0.00	1.00	91
Frankfurters	0.00	0.50	100
Lobster	0.00	1.51	90
Lake perch	0.00	4.00	91
Canned tuna 1	0.30	1.00	94
Canned tuna 2	1.00	1.00	112
Canned tuna 3	0.16	2.00	94
Canned tuna 4[b]	0.48	0	—

[a] Before fortification.
[b] Average of 6 determinations. Standard deviation = ±0.035; relative standard deviation = 7.3%.

Table 3. Selenium found (μg/g) in fish by cathodic stripping and fluorometry

Sample	Cathodic stripping	Fluorometry
	Cod Fillet	
1	0.25	0.21
2	0.38	0.47
3	0.33	0.37
	Flounder Fillet	
4	0.35	0.30
5	0.43	0.46
6	0.25	0.30
	Perch Fillet	
7	0.35	0.42
8	0.23	0.03
9	0.44	0.53

fluorometric (1) method. The agreement, in general, is good. The advantage of the proposed method is that it is considerably less time consuming and requires very little attention from the analyst before the determinative step.

A very serious drawback in arsenic determination by differential pulse polarography in foods has been interferences due to reduction currents of lead and tin which occur at approximately the same potential as arsenic. In many cases, the determination of arsenic in foods would be impossible for this reason. We have developed a purification technique with an ion exchange resin which is convenient and effective. In an HCl medium, lead, tin, and many other metals (12) exist as negatively charged complexes which are adsorbed by a strongly basic anion exchange resin. Under the same conditions, arsenic, which is present as uncharged H_3AsO_3, is not adsorbed. Magnesium and other alkaline earths and alkalies are also not adsorbed.

In a complexing medium such as HCl, the background current due to capacitance in differential pulse polarography can be significant. Ce(IV) (3) was used to oxidize As(III) after the analysis to the polarographically inactive As(V). The polarogram was recorded again, giving the current due to capacitance as well as to ions such as lead and tin. The difference in the 2 currents is assumed to be due to arsenic. In addition to Ce(IV) we have used oxidizing agents such as $KMnO_4$, chlorine water, and bromine water in an attempt to measure the background current accurately. Unfortunately, at high sensitivity settings (needed to detect low levels of arsenic) all these reagents alter the background current significantly.

Figure 4 shows some typical As(III) scans of sample and added As(III) standards obtained according to the method. The peak shown (ca −0.35 v) is due to the reduction of As(III) to As(0). Arsenic in 1*N* HCl also produces a peak at about −0.75 v which is due to the reduction of As(0) to AsH_3, but it is not proportional to the concentration. A polarographic maximum appears at about −0.60 v at arsenic concentrations above approximately 0.3 μg/ml.

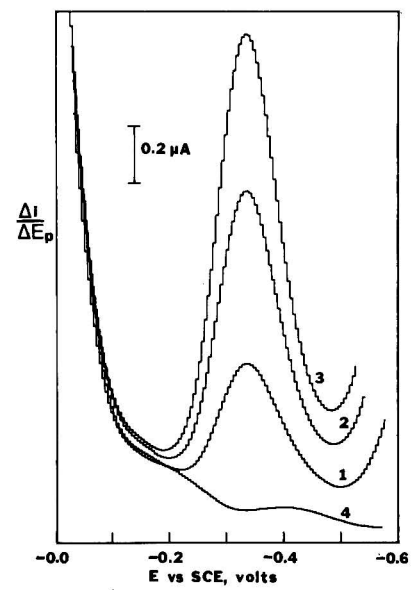

FIG. 4—Differential pulse polarograms of tuna fish sample plus added standards including background current for As(III).

Scan rate, 5 mv/sec; drop time, 1 sec; pulse amplitude, 100 mv; 1, sample, 0.4 g/10 ml; 2, 1 + 0.2 μg As(III)/ml; 3, 2 + 0.2 μg As(III)/ml; 4, 3, after addition of $KMnO_4$.

The background current which was recorded after oxidizing As(III) to As(V) with $KMnO_4$ is also shown. Figure 5 shows a polarogram of a sample which was not treated with an ion exchange resin to remove interferences and another polarogram of the same sample after such treatment. Note the absence of interferences, which presumably are due to lead and tin.

Recovery results for several fortified food commodities are shown in Table 4. The precision of the method was estimated by analyzing one sample 6 times. At 5.0 μg arsenic/g, the relative standard deviation was 5.8%. Approximately 0.4 μg arsenic/g can be quantitated if a 1 g sample is taken. Larger samples may be used for samples containing lower arsenic levels. Higher modulation amplitude and slower scan rate will increase sensitivity with the system described. However, some compromise may be necessary; for example, high modulation amplitude in differential pulse polarography may result in lower resolution (broadening of the peak). It is best to determine the conditions by experiment to suit a particular requirement.

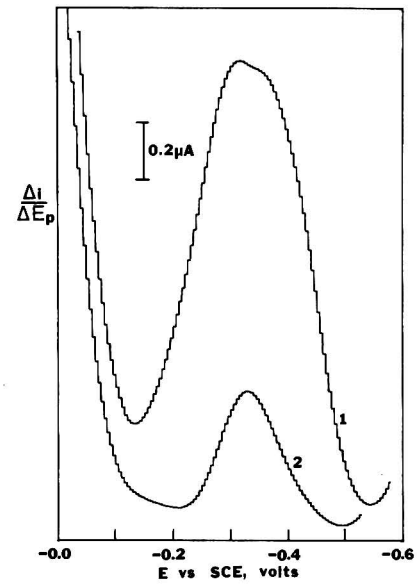

FIG. 5—Differential pulse polarograms of unpurified and purified tuna fish sample for As(III).

Scan rate, 5 mv/sec; drop time, 1 sec; pulse amplitude, 100 mv; 1, sample, 0.4 g/10 ml, before ion exchange treatment; 2, sample after ion exchange treatment.

Table 4. Recovery data for arsenic in several foods

Food	Amt found,[a] μg/g	Amt added, μg/g	Rec., %
Oranges	0.00	2.00	110
Frankfurters	1.75	4.00	97
Perch	0.00	2.00	109
Halibut	0.50	2.50	110
Shrimp	0.47	6.66	102
		3.33	90
		0.67	99
Shrimp	0.00	1.00	100
Trout[b]	0.00	5.00	97

[a] Before fortification.

[b] Average of 6 determinations. Standard deviation = ±0.28; relative standard deviation = 5.8%.

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Digestion of Fish Samples for Mercury Determination by Flameless Atomic Absorption Spectrophotometry

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Methods of digestion of fish samples for mercury determination by flameless atomic absorption spectrophotometry have been investigated. Digestion in Teflon bombs, Kjeldahl flasks, or borosilicate test tubes gives comparable precisions and mercury recoveries. Because of their cost and effect in limiting productivity, Teflon bombs were considered unnecessary for analysis of fish samples. Experiments with cooked and uncooked fish samples have confirmed that no appreciable loss of mercury occurs during baking 30 min at 170°C.

The analytical chemistry of mercury has been advanced in recent years by the development of the flameless atomic absorption spectrophotometric (AAS) method. This field has been the subject of a comprehensive review by Ure (1). In the analysis of fish samples, many workers use procedures based on the method of Hatch and Ott (2).

The reproducibility of the AAS method is strongly influenced by instrumental parameters (3) and by problems associated with sample digestion. Digestion methods have included refluxing the sample with various acid mixtures and oxidants in flasks fitted with condensers (4–6) and in Kjeldahl flasks (3, 7). Teflon bombs have been described (8, 9) and used for the digestion of fish samples (10–13), ensuring that no mercury is lost during digestion. Such bombs must be capable of withstanding the pressures (several atmospheres) that develop in an enclosed system when nitric acid reacts with organic matter. The high cost of the Teflon bombs ($50–100 each) places an apparatus limitation on the number of analyses that can be performed in one batch.

We have studied the parameters of sample digestion by various procedures, including the Teflon bomb, to determine mercury losses and to develop a simple digestion method which will give satisfactory precision, accuracy, and productivity. The results of this investigation are presented in this paper.

METHOD

Reagents

All reagents were analytical grade. Stock solutions were prepared with deionized water and were stored in polyethylene bottles. Nitrogen gas was standard, dry, oxygen-free grade supplied by New Zealand Industrial Gases Ltd., Auckland.

Apparatus

Apparatus was based on Techtron AA3 atomic absorption spectrophotometer with mercury electrodeless discharge tube as source of mercury resonance line at 253.7 nm. Discharge tube was excited with EMS Microtron 200 microwave power generator operating at 25 watt output. Absorption signals were obtained on Varian Model A4027 25 cm chart recorder operated at 2.5 cm/min.

Teflon bombs were constructed in our laboratory according to specifications of Rantala and Loring (12), using 7.2 cm diameter Teflon rod for top and 5.2 cm diameter rod for base. Bomb capacity was 21 ml. Initial work showed that leakage or explosions could occur as a result of distortion of the Teflon above 130°C, and units were therefore strengthened with stainless steel ring (4.3 cm high, cut from 7.2 cm diameter tubing) around screw top. Stainless steel plates (8.8 × 8.8 × 0.5 cm) on top and bottom of bomb were used for further strengthening. Plates were joined by 4 stainless steel bolts (1 cm diameter).

Material for Analysis

In order to ensure homogeneity, material for analysis consisted of edible muscle tissue from single fillet from each of 2 specimens of rainbow trout (*Salmo gairdneri*) caught in Lake Rotorua, North Island, New Zealand. Fillets were taken from just behind the head. Because of adjacent geothermal activity, fish from this lake contain unusually high levels of mercury, often exceeding 1.0 μg/g (wet weight basis) in edible muscle tissue (14). Selected specimens had a mercury content of ca 1.2 and 1.8 μg/g, respectively.

Basic Procedure for Solution-Reduction Technique

Place ca 50 ml solution containing ≤100 ng mercury (as Hg^{2+} in 10% (w/v) HNO_3) in 150 ml

Erlenmeyer flask fitted with a fritted glass nitrogen distributor. Add 2 ml 10% (w/v) $SnCl_2$ solution, and stir magnetically 2 min at 1250 rpm. Stop stirring and direct mercury vapor into absorption cell (17 × 1.4 cm id), using stream of nitrogen at 5 L/min. To avoid supersaturation of nitrogen with water vapor after passage through solution, introduce additional flow of dry nitrogen into carrier gas via by-pass. Prepare fresh standards daily by diluting stock solution containing 100 μg/g.

This procedure has sensitivity of 5 ng Hg^{2+} (i.e., corresponding to 0.005 μg mercury/g fish sample).

Since all work was reported on comparative basis (i.e., precision rather than accuracy was required), relationship between samples and standards was not studied.

Digestion

For all procedures, analyses were carried out on same day to avoid loss of mercury by adsorption on container walls. Fresh standards were also prepared daily.

Bomb digestion.—Place 1 g fish sample in bomb and add 7 ml concentrated HNO_3. Seal bomb, place in its metal casing, and heat in drying oven for specified time at specified temperature. Cool bomb in cold running water 1 hr before opening. Remove digest and dilute to 50 ml with deionized water. Take appropriate aliquots for analysis.

Kjeldahl flask digestion.—Place 1 g fish sample in Kjeldahl flash with 5 ml concentrated H_2SO_4 and 2 ml concentrated HNO_3. Heat flask on its side, with tube acting as air condenser, at specified temperature. After cooling, dilute digest with deionized water to 50 ml, and take suitable aliquots for analysis.

Test tube digestion.—Place 1 g fish sample in borosilicate glass test tube (15 × 1.2 cm id) and add 7 ml concentrated HNO_3. Place test tube in boiling water bath for up to 3 hr. Dilute digest to 50 ml, and analyze aliquots as before. Many more samples can be digested in single batch by this method, as no special apparatus is required.

Cooking procedures.—Wrap 1 g fish sample in aluminum foil and bake in muffle furnace 30 min at 170°C. Bake additional 10 samples 10 min at 115°C. Analyze by test tube digestion procedure. As control, analyze uncooked samples from the same fillet at same time.

Results and Discussion

Effects of Varying Digestion Time and Temperature

Bomb Digestion.—Figure 1 shows the times needed to completely digest sample at various temperatures. The limiting mercury value of about 0.6 μg/g was found after 2–3 hr digestion

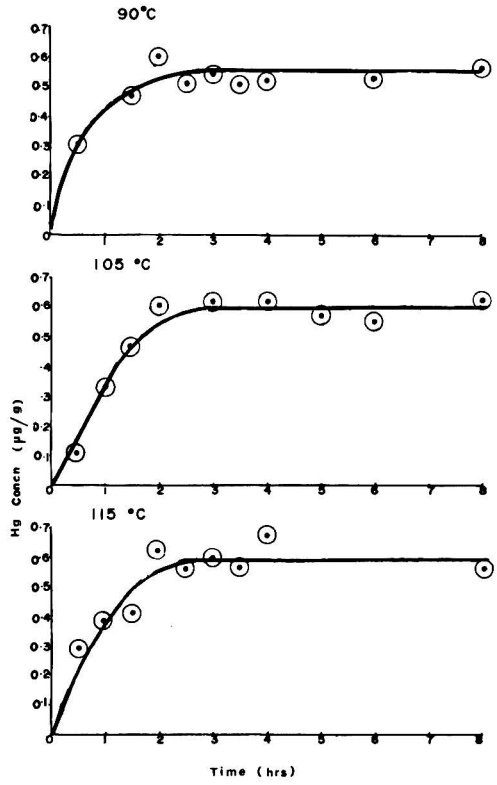

FIG. 1—Mercury concentrations (μg/g wet weight) found in 1 g fish samples for different digestion temperatures in Teflon bomb, using 7 ml concentrated HNO_3 as digestant.

at each temperature (90, 105, and 115°C). At 140°C, distortion of the bombs caused leakage of the digests and produced erratic results. The clarity of the digests was strongly dependent on the digestion temperature. The solutions became clear after 3.5 hr at 115°C, or 5 hr at 105°C, but remained turbid after 8 hr at 90°C. However, provided the digestion time exceeded 2 hr, the recovery of mercury was unrelated to the turbidity or clarity of the solution.

Adding additional HNO_3 and analysis after heating 3 hr at 105°C showed no detectable

Table 1. Mercury levels found in identical 1 g fish samples: Kjeldahl flask digestion, 5 ml concentrated HNO_3 and 2 ml concentrated H_2SO_4

Digestion temperature program	Mercury found, μg/g
130–140°C, 20 min, 210°C, 40 min	0.71
130–140°C, 20 min, 180°C, 40 min	0.86
130–140°C, 20 min, 160°C, 40 min	1.13
130–140°C, 60 min	1.18
100°C, 60 min	1.17

Table 2. Reproducibilities of different digestion procedures for 1 g fish samples

Digestion app.	Acid[a]	Temp, °C	Time, hr	Mercury concns, μg/g Mean	Std dev.	Rel. std dev., %
Kjeldahl flask	A	135	1	1.17	0.10	8.6
Teflon bomb	B	105	3	1.11	0.09	8.1
Test tube	B	100	3	1.15	0.09	7.8

[a] A = 7 ml concentrated HNO_3 + 2 ml concentrated H_2SO_4; B = 7 ml concentrated HNO_3.

mercury and indicated the absence of any memory effect in the Teflon containers.

Kjeldahl Flask Digestion.—Table 1 shows the recovery of mercury for samples of fish digested as previously described at various temperatures. It is clear that losses of mercury are appreciable when digestion is allowed to proceed for 40 min above 160°C.

Test Tube Digestion.—The digests (*see* above) usually cleared within 3 hr, and analysis gave mercury values equal to the limiting values found by the other 2 digestion techniques.

Reproducibility of Digestion Procedures

The reproducibilities of the 3 procedures were compared by using each technique to analyze 15 samples of edible muscle tissue from the same fish. The results are shown in Table 2. The Student's *t*-test showed no significant difference between the means at the 90% probability level. Percentage relative standard deviations were also very similar, between 7.8 and 8.6 in all cases.

Recovery of Added Mercury

Analyses for 500 g mercury added to a series of Teflon bombs, Kjeldahl tubes, and test tubes (10 of each) and digested under the conditions shown in Table 2 showed mercury recoveries of 90–94% for bombs, 90–93% for Kjeldahl flasks, and 90–102% for test tubes. Hence the recoveries were comparable and exceeded 90% in all cases.

Effect of Cooking Fish Samples

It has been noted that significant mercury is lost during Kjeldahl flask digestion at temperatures above 160°C. This prompted a study of mercury loss from fish samples during cooking procedures (Table 3). From results of *t*-tests, it was concluded that there was no significant difference between the means at the 70% probability level. The statement of Westöö (15) that

Table 3. Mercury concentration in fish after baking

No.	Baking temp., °C	Baking time, min	Mean Hg concn, μg/g wet wt	Std dev.	Rel. std dev., %
9	170	30	1.77	0.13	7.4
10	115	10	1.91	0.22	11.5
Control (uncooked)			1.82	0.20	11.0

boiling or frying fish causes no mercury loss through volatilization is therefore supported by these experiments with a baking procedure. It is clear that simple cooking processes cannot be used as a means of reducing the mercury content of fish samples containing excessive amounts of this element.

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OILS AND FATS

Methods of Analysis Approved by the Codex Alimentarius Commission. I. Acid Value

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Numerous variations exist for determining the acidity of fats and oils by titration. A Youden ruggedness test was performed, using extreme conditions existing in the methods, endorsed by various international organizations: aqueous and alcoholic standard alkali, 5 or 56 g sample, 50 or 150 ml alcohol or alcohol-ether solvent, titration at room temperature or 60°C with 2 ml or 8 drops of phenolphthalein indicator, to the first color change or to a pink persisting for 60 sec. The titration method was found to be extremely rugged at the 1% oleic acid level with none of the variables influencing the result. At the 0.15% level, only the sample size affected the result.

The acid value or acidity of a fat or oil is a characteristic required for assessing the quality of the initial and final product and for technical control of the refining process. The determination consists of the relatively simple procedure of direct titration of the dissolved or dispersed (usually in alcohol) sample. If the result is expressed as mg potassium hydroxide required to neutralize the fatty acids of 1 g fat, it is called the *acid value*; if it is expressed in terms of a specific fatty acid, usually oleic, it is called *acidity*. The relationship between the 2 constants is: Acid value = 1.99 × % oleic acid.

The Codex Alimentarius Commission of the Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO) have submitted a number of recommended standards for fats and oils to governments for acceptance (1). The commodities involved and the specified constants are given in Table 1. The usual acid value limits are 4 (2% oleic acid) for the raw oil and 0.6 (0.3% oleic acid) for the refined oil.

The methods of analysis for acid value originally considered for use with these standards were those of the International Union of Pure and Applied Chemistry (IUPAC) (2), American Oil Chemists' Society (AOCS) (3), and Association of Official Analytical Chemists (AOAC) (4). After several stages of comments by governments and interested international organizations, and discussions in both the Codex Committee on Fats and Oils and the Codex Committee on Methods of Analysis and Sampling, the IUPAC method was accepted as the Codex (referee) method.

A discussion of various methods for the determination of acidity is given by Mehlenbacher (5). A number of internationally recognized methods for the determination of acidity are compared in Table 2. The fifth method in this table is accepted by the FAO/WHO Code of Principles Concerning Milk and Milk Products and Associated Standards (6), and by the International Dairy Federation (IDF) (7), International Organization for Standardization (ISO) (8), and AOAC (9), for butterfat.

Very little collaborative or comparative data exist on which a choice of method of analysis can be based. Mehlenbacher (10) supplies data

Table 1. Acid value (or acidity) specifications in Codex Alimentarius Commission Recommended Standards (CAC/RS)

CAC/RS No.	Commodity	Acid value		
		Virgin	Unspecified	Nonvirgin
19-1969	general standard for edible fats and oils	4		0.6
20-1969	soya bean oil		0.6	
21-1969	arachis (peanut) oil	4		0.6
22-1969	cottonseed oil		0.6	
23-1969	sunflower seed oil	4		0.6
24-1969	rapeseed oil	4		0.6
25-1969	maize (corn) oil	4		0.6
26-1969	sesame seed oil	4		0.6
27-1969	safflower seed oil		0.6	
28-1969	lard		1.3	
29-1969	pork fat		2.5	
30-1969	premier jus		2	
31-1969	edible tallow		2.5	
33-1970	olive oil (expressed as acidity, % oleic acid)	3.3%		0.3%
34-1970	mustard seed oil	4		0.6

Table 2. Comparison of methods for determining free fatty acids and acid value

Variable	Free fatty acids, AOCS **Ca 5a-40**	Acid value, AOCS **Cd 3a-63**	IUPAC, **II.D.1**	AOAC (1975), **28.029**	AOAC (1975), **16.193–16.195**
Prepn of alkali	presumably aq., 0.1, 0.25, or 1.0*N* accurately stdzd	carbonate-free aq. KOH; KH phthalate stdzn	aldehyde-free 0.1 or 0.5*N* alc. KOH	0.25*N* aq.	0.1*N* alc.
Sample wt, g	3.5–56.4	0.1–20	5–10	7.05 or 56.4	5–10
Solv., preneutzd to phthln	50–100 ml hot 95% alcohol (or contg 5 or 10% MeOH); 99% isoPrOH may be used with vegetable oils	125 ml isoPrOH and toluene (1+1); warm if necessary to dissolve sample	50–150 ml alcohol-ether (1+1)	50 ml warm alcohol	50–100 ml alcohol (or contg 5 or 10% MeOH)-ether (1+1)
End point (phthln)	2 ml 1% alc.; pink of same intensity as neutzd solv.; persists 30 sec	2 ml 1% isoPrOH; pink of same intensity as neutzd solv.; persists 30 sec	5 drops 1% alc./100 ml; to indicator color change	2 ml 1% alc.; faint pink persisting 1 min	0.1 ml 1% alc. (or with 5 or 10% MeOH); faint pink persisting 10 sec
Scope	0–100%; crude and refined; vegetable, marine, and animal	0–>75%; crude and refined; vegetable, marine, animal, and derivatives	coconut, palm kernel, palm, and other fats	crude and refined	butter

from 14 to 47 collaborators on 6 samples with a range of 0.11–3.5% fatty acids, showing a coefficient of variation of 5–10% by AOCS method **Ca 5a-40**. The current general AOAC method (4), based on that of the National Cottonseed Products Association, was adopted as a result of the collaborative study of McKinney (11) in which 4 samples containing 0.03–5.3% fatty acids were analyzed by 6 collaborators. The coefficients of variation were from 1% (5% acid level) to 13% (0.03% acid level). McKinney also compared 2 other variants of the method on 2 low-acid samples. These variations, which included a hot alcoholic titration (12) and a cold titration (details not given), showed slightly, although probably not significantly, greater variation at the low level.

Data in the files of the AOCS (kindly made available by Jack W. McEwan, Central Soya Co., Inc., 1200 N Second St, Decatur, IN) showed that absolute ethanol and denatured alcohols formulae 30 and 3-A could be used interchangably over a range of 0.01–81% oleic acid, but that methanol gave significantly ($P \leq 0.01$) lower values.

The purpose of this investigation was to determine through the application of the Youden ruggedness technique (13) those variables which are significant for the determination of acidity in fats and oils and thereby recommend the most suitable combination of variables for this test.

Experimental

Design

Table 2 was examined to extract from these methods reasonable extreme values which could serve as the experimental conditions for the test. These variables are listed in Table 3 and distributed into the 16 combinations given in Table 4.

A 16-experiment design, rather than the 8-experiment design recommended by Youden, was used in order to clearly separate the main effects from 2-factor interactions. Even so, the larger design still assumes that the only interactions which exist are 2-factor interactions between no more than 4 of the main 7 variables. The 8-observation design assumes that all interactions are nonexistent.

Reagents and Apparatus

(a) *Alkali.*—Alcoholic KOH solution was prepared according to the IUPAC method (2). The alcohol was refluxed 1 hr with 8 g KOH and 5 g aluminum, and distilled. Then 6.8 g KOH was dissolved in 1 L of the aldehyde-free alcohol, the solution was allowed to stand several days, and the clear liquid was decanted. Aqueous NaOH solution was prepared from carbonate-free NaOH

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Table 3. Assigned values for the variables and the combinations for the Youden ruggedness test for acidity of fat

Variable	Values assigned	
	Capital letter	Lower case letter
Standardized 0.1*N* alkali	A = aqueous	a = alcoholic
Sample size, g	B = 5	b = 56.4
Solvent, preneutralized to phenolphthalein	C = alcohol	c = alcohol-ether (1+1)
Volume solvent, ml	D = 50	d = 150
Temperature, °C	E = room	e = 60[a]
Volume 1% alcoholic phenolphthalein solution	F = 2 ml	f = 8 drops
End point	G = first color change	g = faint pink persisting 60 sec

[a] When this variable was combined with c, the maximum temperature attainable was the boiling point of ether.

Table 4. Combinations of variables and % oleic acid found for 3 samples of oleic acid in corn oil

Titration	Combination	Sample 1	Sample 2	Sample 3
1	abcdefg	1.102	1.038	0.142
2	AbcdEfG	1.073	1.030	0.144
3	aBcdEFG	1.045	1.016	0.192
4	ABcdeFg	0.987	1.099	0.196
5	abCdEFg	1.059	1.025	0.151
6	AbCdeFG	1.036	1.016	0.130
7	aBCdefG	1.143	0.981	0.187
8	ABCdEfg	1.155	1.059	0.183
9	abcDeFG	1.060	1.035	0.139
10	AbcDEFg	1.066	1.014	0.143
11	aBcDEfg	1.000	0.989	0.162
12	ABcDefG	1.023	1.038	0.119
13	abCDEfG	1.034	1.030	0.137
14	AbCDefg	1.024	1.022	0.134
15	aBCDeFg	1.064	1.026	0.208
16	ABCDEFG	1.144	1.101	0.197
Av.		1.063	1.032	0.160
Std dev.		0.050	0.032	0.029
Rel. std dev., %		4.7	3.1	18.1

(1+1) and boiled water. Both solutions (0.1*N*) were standardized against potassium acid phthalate as in **50.035** (4).

(b) *Oil samples.*—A commercial oleic acid which assayed 80% by gas chromatography and 101.3% as oleic acid by titration was used to formulate Sample 1. A second commercial oleic acid which assayed 92% by gas chromatography and 98.1% as oleic acid by titration was used to formulate Sample 2. A corn oil which had been stored in the laboratory at room temperature in a 1 gal. metal can for several years was used directly as Sample 3 and it was also used as the diluent for Samples 1 and 2.

(c) *Solvents.*—USP alcohol and absolute ether. The actual solvents, alcohol and alcohol-ether (1+1), must be preneutralized to phenolphthalein with 0.1*N* alkali.

(d) *Phenolphthalein indicator.*—1% in alcohol.

(e) *Buret.*—Since, theoretically, the fatty acid content was unknown, for the purpose of this experiment, a 50 ml buret was used throughout.

Determination

All samples in a series were weighed consecutively. The reagents were added; the conditions were established for each determination individually, in random order; and each solution was titrated. For example, Determination 8 (ABCdEfg) consisted of a 5 g sample dispersed in 150 ml alcohol which was titrated at room temperature with aqueous 0.1*N* NaOH, using 8 drops of phenolphthalein, to a faint pink persisting 60 sec.

Results

The results, calculated as per cent oleic acid, are given in Table 4. Samples 1 and 2, although calculated to contain 1% oleic acid, are independent samples, using different oleic acids but the same corn oil diluent. The same corn oil was used as Sample 3. Its acid value, although within Codex specifications, reflected its storage history.

Conducting the 16 determinations consecutively in a single series immediately reveals why numerous modifications of this basic method exist—to suit individual preferences as determined by the usual fat or oil examined. Those combinations of variables using a large sample and a small volume of alcohol solvent resulted in an inconveniently slow titration. A small weight of a low acid sample gives a low volume of titrant solution. A large volume of solvent, addition of ether, and heat give a clear solution and an unambiguous end point. The volume of indicator and persistence of end point did not influence the results, operationally or statistically, on these light-colored samples. The smaller volume and appearance of the first pink are more desirable. Where the sample is dispersed, rather than dissolved (50 ml alcohol at room temperature), the end point is more difficult to perceive, but this still did not affect the results.

The only variable which was significant was the sample weight, and this was significant only

at the low acid value. Here the larger sample resulted in lower acid values. The effect was negligible at the 1% oleic acid level. Otherwise the method is extremely rugged, since the final values are not influenced by substantial changes in operating conditions. This probably accounts for the numerous variations available for the determination. The variables can be altered to suit the nature of the sample ordinarily analyzed and the convenience of the operator.

For a general purpose laboratory examining samples for compliance with Codex specifications, with no specialization with regard to commodity, the following conditions would probably be most convenient and efficient:

Alkali: 0.1*N*, aqueous, prepared from carbonate-free sodium hydroxide.

Sample size: 5–10 g.

Solvent and volume: 100 ml alcohol (formulae 30 and 3-A which contain 5 and 10% methanol, respectively, are satisfactory).

Indicator: 10 drops of 1% alcoholic phenolphthalein and titration to the first color change at room temperature.

Further work is required to establish the optimum sample size.

Acknowledgments

Foster McClure, Food and Drug Administration, Washington, DC, provided the essential statistical design and analysis of this study. William A. Hallam, Food and Drug Administration, Boston, MA, did some preliminary work on this subject but was unable to complete the project.

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MYCOTOXINS

Aflatoxin Occurrence on Raw and Cooked York Soybeans Inoculated With Three *Aspergillus* Isolates

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Raw and cooked soybean media were inoculated in separate experiments with *Aspergillus flavus* NRRL 3251, *A. flavus* ATCC 15548, and *A. parasiticus* NRRL 2999 isolates. The total quantity of aflatoxins produced and the percentage distribution of aflatoxins B_1, B_2, G_1, and G_2 varied with the state of the medium (raw or cooked) and with the fungal isolate used. Cooked soybean medium supported higher aflatoxin production by *A. flavus* NRRL 3251 and *A. parasiticus* NRRL 2999 than did raw medium. Larger quantities of aflatoxins B_1, B_2, G_1, and G_2 were produced by *A. flavus* ATCC 15548 on raw soybean medium than were produced by any of the isolates on cooked medium. Application of these data is discussed briefly in relation to possible use of soybean media as a source of aflatoxin production.

Aflatoxins have been found in many agricultural products such as stored peanuts (1, 2), corn (3, 4), oats, sorghum, and wheat (5), cottonseed (6), hazelnuts (7), and haricot beans (8). Also, aflatoxins have been produced on cured wet tobacco leaves (9) and crushed black sunflower seeds (10) inoculated with *Aspergillus flavus*.

It has been reported that soybeans have become naturally contaminated with aflatoxins (11) but studies concerning aflatoxin production on soybeans are contradictory. Certain investigations show that soybeans may be a poor substrate for the production of aflatoxins (12). Very low toxin production (0.03 μg/ml) was obtained on pearled soybeans var. Hawkeye by Hesseltine *et al.* (13) using an *A. flavus* strain. However, Davis and Diener (14) obtained much higher toxin levels (41–138 μg/ml) on the Bragg variety of soybeans using a strain of *A. parasiticus*. Nagarajan *et al.* (15) observed that soybeans support the production of aflatoxins under optimal laboratory conditions but that the amount of toxin production was determined by both the fungal isolate and the variety of soybean used.

The present investigation was undertaken to obtain information concerning aflatoxin production on raw and cooked soybean media by different *Aspergillus* isolates. In this study the toxin was produced on one of the more recently developed varieties of soybeans, York.

Experimental

Preparation of Cultures

Cooked and raw soybeans (*Glycine max* var. York) were washed 3 times in sterile distilled water and then were crushed using a sterile blender. Each soybean culture (7 g) was mixed with 20 ml sterile distilled water and placed in a previously autoclaved 250 ml Erlenmeyer flask. Flasks were sealed with sterile cotton plugs. Cooked soybean samples were then prepared by autoclaving for 15 min (121°C, 15 psi) and after cooling were manually shaken to loosen the medium.

Inoculation

A. flavus ATCC 15548, *A. flavus* NRRL 3251, and *A. parasiticus* NRRL 2999 cultures maintained on potato dextrose agar were used in separate experiments to inoculate cooked and raw soybean cultures. Aseptic inoculation techniques were employed. Seven flasks with cooked soybeans and 3 flasks with raw soybeans were prepared for each fungal isolate. Control flasks contained cooked or raw samples but were not inoculated with fungi. After inoculation, samples were cultured without light at 27±3°C for 30 days.

Analysis

Cultures were then attenuated by addition of chloroform. Samples were extracted from the

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flasks and analyzed qualitatively and quantitatively for the presence of aflatoxins by using thin layer chromatography (TLC) and visual determinations under longwave ultraviolet light (16). Extraction and TLC preparation procedures were completed at the Mycotoxin Laboratory, Virginia Division of Consolidated Laboratory Services.

Other Media Evaluated

Polished rice served as a medium for *A. flavus* ATCC 15548. Six cultures containing 7 g rice and 7 ml water each were prepared, inoculated, and harvested the same as the soybean cultures previously mentioned.

Five agar slants containing potato dextrose and yeast were also extracted and analyzed for aflatoxin levels. These slants had served as the original growth medium for *A. flavus* ATCC 15548 which was used to inoculate the rice and soybean cultures reported on here.

Results

Cooked Soybeans

Table 1 summarizes the amounts of aflatoxins produced in cooked and raw soybeans. *A. flavus* NRRL 3251 produced a larger percentage of aflatoxin B_1 (97.10%) than of the other toxins, B_2, G_1, and G_2, although the amount was less than that produced by the *A. parasiticus* strain. No aflatoxin G_1 or G_2 was detected in the *A. flavus* NRRL 3251 flasks. A higher amount and percentage of aflatoxin G_1 (2345 μg/sample, 87.53%) was produced by *A. flavus* ATCC 15548 than by *A. flavus* NRRL 3251 or *A. parasiticus* NRRL 2999. *A. parasiticus* NRRL 2999 produced a higher percentage of aflatoxin G_1 (54.19%) than of aflatoxins B_1, B_2, or G_2. The total aflatoxin yield on cooked soybeans was greater from *A. parasiticus* NRRL 2999 cultures than from the *A. flavus* cultures. *A. parasiticus* also produced larger amounts of individual aflatoxins B_1, B_2, and G_2 as compared with *A. flavus* cultures.

Raw Soybeans

A. flavus NRRL 3251 produced less B_1, B_2, and G_2 on raw than on cooked soybeans but produced more aflatoxin G_1 on raw soybeans. Greater amounts of all aflatoxins were produced by *A. flavus* ATCC 15548 on raw than on cooked soybeans. These amounts also exceeded those obtained from cultures of *A. flavus* NRRL 3251 and *A. parasiticus* on either cooked or raw media. Aflatoxins B_1 and G_1 formed the largest percentage of aflatoxin produced by *A. flavus* ATCC 15548. Quantities of all aflatoxins formed by *A. parasiticus* were much reduced on raw as compared with cooked soybeans but as with

Table 1. Aflatoxins produced on cooked and uncooked York soybeans by *A. flavus* and *A. parasiticus* isolates

	Mean yield				Maximum yield, μg/culture[a]	
	Cooked		Uncooked			
Aflatoxin	μg/culture[a]	% of total	μg/culture[a]	% of total	Cooked	Uncooked
			A. flavus NRRL 3251			
B_1	1104	97.10	9	50.00	1521	15
B_2	33	2.90	1	5.56	36	1
G_1	—	—	8	44.44	—	8
G_2	—	—	—	—	—	—
Total	1137	100.00	18	100.00	1557	24
			A. flavus ATCC 15548			
B_1	251	9.37	41882	51.2	298	47601
B_2	—	—	1113	1.4	—	1260
G_1	2345	87.53	35350	43.2	3042	47252
G_2	83	3.10	3466	4.2	118	4096
Total	2679	100.00	81811	100.00	3458	100209
			A. parasiticus NRRL 2999			
B_1	1501	39.31	10	45.45	2124	16
B_2	72	1.89	2	9.09	78	3
G_1	2069	54.19	9	40.91	2756	12
G_2	176	4.61	1	4.55	182	1
Total	3818	100.00	22	100.00	5140	32

[a] Cultures contained 7 g media and 20 ml water.

Table 2. Comparison of aflatoxin production by *A. flavus* ATCC 15548 on various substrates

Media	Mean aflatoxin levels, μg/g solid media					Culture time, days	Temp., °C	Water in culture, %
	B_1	B_2	G_1	G_2	Total			
York soybeans (crushed, uncooked)	5983.20	159.05	5050.09	495.19	11687.53	30	27±3	74
York soybeans (crushed, cooked)	35.85	—	355.00	11.86	402.71	30	27±3	74
Agar slants[a]	1.29	—	1.88	—	3.17	30	24±3	96
Rice (polished, cooked)	951.38	—	1907.38	75.00	2933.76	30	25±3	50
Sunflower seeds[b] (crushed, cooked)	79.73	—	113.40	10.00	203.13	25	27±3	40

[a] Potato dextrose and yeast.
[b] Data from ref. 10.

the *A. flavus* cultures the percentages of aflatoxins B_1 and G_1 produced on raw media were higher than those of aflatoxins B_2 and G_2.

Other Media

Table 2 shows that cooked polished rice medium inoculated with *A. flavus* ATCC 15548 produced no aflatoxin B_2, low levels of G_2, higher levels of B_1, and still higher levels of G_1. This medium ranked between crushed raw soybeans and crushed cooked soybeans in total aflatoxin production based on μg toxin/g solid medium. The agar slants from which the original inoculum was taken for the rice and soybeans used showed very low levels of B_1 and G_1 and no B_2 or G_2.

Discussion

It appears from the results obtained that York soybeans will support the production of aflatoxins by *Aspergillus* isolates. *A. flavus* NRRL 3251 and *A. parasiticus* NRRL 2999 both produce higher quantities of aflatoxin on cooked than on raw soybeans. Thus, cooking seems to enhance the production of aflatoxins by these isolates, especially by *A. parasiticus*, possibly by changing the composition of nutritional constituents available to the fungus.

When raw soybean medium is compared with other substrates (Table 2) in relationship to the aflatoxins produced, it appears that the total aflatoxin levels synthesized exceed those for both rice and crushed sunflower seeds. Probably such levels also exceed the 8 mg aflatoxin/g substrate produced on coconut medium (17). It is possible therefore that either *A. parasiticus* NRRL 2999 grown on cooked York soybean medium or *A. flavus* ATCC 15548 grown on raw York soybean medium could be used to produce large quantities of aflatoxins B_1, B_2, G_1, and G_2 for experimental purposes.

The medium used in these experiments has a high water content (70%). Our results may thus be applicable to the conditions reported by Bean *et al.* (11) following the harvest and subsequent storage of soybeans and associated products under conditions of extreme precipitation. Under such similar conditions it is possible but not probable that quantities of aflatoxins approaching the levels we reported here could occur naturally.

It is evident from these studies that York soybeans can be used as a medium for production of aflatoxins by *Aspergillus* species. However, the total amounts and percentages of distribution of the aflatoxins vary with the state of the medium (cooked or raw) and with the fungal isolate used. The latter also could partially explain the apparent discrepancies between results obtained by various investigators using different strains of *Aspergillus* to produce aflatoxins on soybeans. These data are also in agreement with those of Nagarajan *et al.* (15) in that the amount of aflatoxin produced varied with the strain of *Aspergillus*.

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Determination of Zearalenone in Corn: Collaborative Study

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Corn samples spiked at levels of 100, 300, 1000, and 2000 μg zearalenone/kg were sent to 22 collaborators for analysis by the Eppley method. All samples were yellow corn except one white corn sample spiked at 2000 μg/kg. Results from 16 collaborators were statistically analyzed. Only 4 of 16 collaborators detected zearalenone in the sample containing 100 μg/kg, but 11 detected the toxin in the sample containing 300 μg/kg. Average recoveries from all samples were 129% at 300 μg/kg, 101% at 1000 μg/kg, and 88% at 2000 μg/kg. The between-laboratory coefficients of variation were 53.0% at 300 μg/kg, 38.2% at 1000 μg/kg, and 27.0% at 2000 μg/kg. Five naturally contaminated corn samples, one in triplicate, were also provided. The mean level of zearalenone in the naturally contaminated samples ranged from 431 to 7622 μg/kg. The mean coefficient of variation for all samples was 40.5%. Two collaborators measured quantities of zearalenone on thin layer chromatographic plates densitometrically. Their results were not included in the statistical analysis, but the results indicated that densitometric measurement, given proper dilutions of solutions, could be used. The method has been adopted as official first action.

This study was conducted to determine whether a method developed by Eppley (1) for the screening of agricultural commodities for zearalenone, aflatoxin, and ochratoxin could be used to determine levels of zearalenone in white and yellow corn. The method, slightly modified, had been applied to the screening of 567 corn samples from commercial markets for the determination of zearalenone, aflatoxin, and ochratoxin (2, 3). The method had also been used to analyze 223 samples of the 1972 crop corn collected from terminal elevators or from stocks on hand at food processing establishments (4).

Collaborative Study

Description of Samples

Naturally contaminated lot samples of corn were ground to pass a U.S. standard No. 20 sieve, using a 6″ Raymond hammer mill equipped with a screen containing ⅛″ diameter round-hole perforations. Each ground sample (2–4 kg) was blended 15–30 min with a flat paddle at slow speed in a Hobart planetary mixer, Model A200, 12 qt capacity. All analytical samples (50 g) were preweighed into wide-mouth, 100 ml polyethylene bottles. Spiked samples were prepared by adding known amounts of zearalenone in benzene by syringe to preweighed portions of "clean" corn in individual bottles. The collaborators were instructed to use the entire contents of each bottle for analysis.

Description of Study

Twenty-two laboratories each received a practice sample with a noted level of zearalenone, 7 naturally contaminated samples, and 7 spiked samples. All but the practice sample were randomly coded. The samples of yellow corn were spiked to contain 100, 300, and 1000 μg/kg. The spiked white corn sample contained 2000 μg/kg. The laboratories were instructed to use all of the sample in a bottle for an analysis.

Zearalenone Reference Standard

Zearalenone dissolved in benzene was supplied in sealed ampoules; the concentration was 50 μg/ml. The ultraviolet absorption spectrum in methanol solution of the zearalenone used to prepare the standard solution was $\lambda_{max.}$ 314, 274, and 236 nm ($\varepsilon_{max.}$ 6240, 13,370, and 29,930). Reported values of ε at these wavelengths for crystalline zearalenone were 6000±5%, 13,900±5%, and 30,000±5% (5). The molecular absorptivity of zearalenone in benzene used to prepare the reference standard was found to be 6050 at 317 nm. Flame ionization gas chromatography of the trimethylsilyl derivative of the zearalenone used indicated a purity of >98%.

METHOD

ZEARALENONE

Corn—Official First Action

26.B01 ***Apparatus***

See **26.014.**

26.B02 ***Reagents***

See **26.002**, **26.015(a)** and **(b)**, and in addn:

(a) *Alcohol-chloroform mixt.*—5+95.

(b) *Aluminum chloride soln.*—Dissolve 20 g $AlCl_3 .6H_2O$ in 10 ml alcohol.

(c) *Zearalenone std soln.*—Det. chromatgc purity of cryst. zearalenone (available from Commercial Solvents Corp., Terre Haute, IN 47808) as in **26.011**. UV absorption in benzene: max. *A* 317 nm; ϵ 6060± 5%. UV absorption spectrum in MeOH: max. *A* 314, 274, and 236 nm; MW 318; ϵ 6000±5%, 13,900±5%, 30,000±5%, resp.; GLC purity of trimethylsilyl derivative >98%. Prep. soln contg 50 μg/ml benzene.

26.B03 ***Preparation of Sample***

Proceed as in **26.037**.

26.B04 ***Extraction***

Proceed as in **26.017(a)**.

26.B05 ***Column Chromatography***

(*Caution: See* **51.011**, **51.043**, **51.045**, **51.046**, and **51.061**.)

Prep. column, and add 50 ml $CHCl_3$ ext and 150 ml hexane wash as in **26.018(a)**. Wash column with 150 ml hexane and elute zearalenone with 250 ml acetone-benzene (5+95).

26.B06 ***Liquid-Liquid Partition***

Add few SiC chips to eluate contg zearalenone and evap. to near dryness on steam bath, preferably under gentle stream of N. Transfer residue to 60 ml separator with four 10 ml hexane washes. Finally, rinse with 10 ml CH_3CN and transfer to separator. Shake, and let phases sep. Sep. CH_3CN (lower) phase and ext hexane layer with 5 ml CH_3CN. Combine CH_3CN fractions and evap. to dryness in rotary vac. evaporator. Transfer to vial with $CHCl_3$. Evap., preferably under gentle stream of N. Seal with polyethylene stopper and cap. Save for TLC.

26.B07 ***Preparation of Plates for Thin Layer Chromatography***

Proceed as in **26.019(a)**, except that zearalenone replaces aflatoxin as test mycotoxin.

26.B08 ***Thin Layer Chromatography***

To residue, **26.B06**, add 500 μl benzene, seal with stopper, and shake vigorously on tube shaking machine to dissolve. For preliminary plate, apply 10 μl benzene soln to 2 spots. On one spot superimpose 5 μl zearalenone std soln, **26.B02(c)**, for internal std, and apply 5 μl zearalenone std soln to third spot.

Develop plate with alcohol-$CHCl_3$ (5+95), alcohol-$CHCl_3$ (3.5+96.5), HOAc-benzene (5+95), or HOAc-benzene (10+90), in lined, equilibrated tank ca 40 min. Compare spots presumed to be zearalenone with std. Zearalenone has greenish-blue fluorescence under shortwave UV (256 nm) at R_f ca 0.5 and is not visible under longwave UV light except at high concns. Examine sample spot contg internal std to verify identity of zearalenone. When presence of zearalenone is suspected, spray plates with $AlCl_3$ soln, heat 5 min at 130°, and examine under longwave UV light (365 nm). Zearalenone fluoresces blue under longwave UV light after spraying with $AlCl_3$ soln.

If zearalenone is detected in sample soln, perform quant. TLC. Spot 3, 5, and 7 μl zearalenone std soln and 4, 6, and 8 μl sample soln, and develop plate with alcohol-$CHCl_3$ (5+95) or other appropriate solvs as in par. 2. Compare fluorescent intensities of zearalenone spots of sample with those of std and det. which sample spot matches that of std. If spots of smallest portion of sample are too intense to match stds, dil. sample soln and rechromatograph.

26.B09 ***Calculations***

Calc. concn of zearalenone in μg/kg or ppb corn:

$$\mu g/kg = (S \times Y \times V)/(X \times W),$$

where S = μl zearalenone std soln equal to unknown; Y = concn of zearalenone std soln, μg/ml; V = μl of final diln of sample ext; X = μl sample ext spotted giving fluorescent intensity equal to S (zearalenone std soln); and W = g sample applied to column (10 g). If final ext diln does not represent 10 g, calc. correct sample wt and substitute.

Results and Discussion

The analytical results reported by 16 of the collaborators for the spiked corn samples are presented in Table 1; those for naturally contaminated corn are in Table 2. Two collaborators measured amounts of zearalenone on thin layer chromatographic (TLC) plates fluorodensitometrically, and their results are shown in Table 3.

Inspection of the study results (Tables 1 and 2) indicates that the Eppley method is suitable for determining zearalenone in corn. The limit of detection for the method is not much under 300 μg/kg, and one collaborator estimated that it was 250 μg/kg. The use of the aluminum chloride spray did not seem to increase the sensitivity. Only 4 of the 16 collaborators were able to detect zearalenone in the sample spiked at 100 μg/kg, and 5 did not detect the mycotoxin in the sample spiked at 300 μg/kg (Table 1). Recoveries were satisfactory at the levels at which zearalenone could be detected (300, 1000, and 2000 μg/kg).

Table 1. Collaborative results (μg zearalenone/kg sample) for visual analysis of zearalenone in spiked corn

Coll.	Sample 1[a] (0)	Sample 2 (100)	Sample 3 (300)	Sample 4 (1000)	Sample 5 (1000)	Sample 6 (1000)	Sample 7[b] (2000)
1	0	<125	(0)[c]	750	750	1709	1250
2	0	0	333	938	1250	938	2083
3	0	281	269	750	938	656	1880
4	1563	0	625	1333	833	625	833
5	0	0	375	938	938	1250	1250
6	0	0	100	800	625	625	1560
7	0	0	488	1300	1250	938	2500
8	0	trace	(0)	936	2000	900	1875
9	0	188	312	1000	875	875	1750
10	0	0	363	524	757	969	2200
11	0	0	(0)	624	417	625	1250
12	0	0	800	1500	1700	1700	2500
13	trace	trace	(4700)	1040	(0)	1560	1820
14	0	0	(0)		950	1250	1400
15	0	0	(0)	750	1000	750	2100
16	0	200	200	2000	800	1000	2000
Av.			386	1012	1005	1023	1766
Range: high			800	2000	2000	1709	2500
low			100	524	417	625	833
Std dev.			205	383	406	370	476
Coeff. of var., %			53.0	37.9	40.4	36.2	27.0
Av. rec., %			129	101	100	102	88.2
N[d]			10	15	15	16	16

[a] Values in parentheses under sample numbers are amounts (μg/kg) in sample.
[b] Spiked white corn sample.
[c] Values in parentheses were not included in calculations.
[d] N = number of values used to determine average.

In Tables 1 and 2, it is clear that the standard deviation increases as the mean level increases. When the same analyses were computed by using the logarithm of the zearalenone level, the standard deviation was more constant between samples. The logarithm transformation would be useful for a statistical analysis of zearalenone data with a wide range in values. A 2-way analysis of variance indicated that the variation between laboratories was significant at the 1% level. The overall precision estimates showed that the coefficient of variation on spiked samples was 31% within a laboratory and 44% between laboratories. The coefficient of variation for naturally contaminated samples within a laboratory was 35% and between laboratories, 52%. Differences in the means over all laboratories between the triplicate samples were not significant.

The least significant differences were calculated (6). The ratios of 2 values for spiked and naturally contaminated samples would have to be <2.16 and 2.31, respectively, within a laboratory to conclude that the results agreed. The respective ratios between laboratories would have to be 2.78 and 3.53. To attain a coefficient of variation or relative standard deviation of 20% based on the mean, at least 3 independent analyses of a given sample would have to be made. In the analysis of spiked corn samples, 64% of the variability is attributed to errors within the laboratory and 36% to between laboratories. For naturally contaminated samples, 51% of the variability of the analysis is caused by errors within the laboratory, and 49% by factors between laboratories.

Results obtained by the 2 investigators using fluorodensitometry to measure zearalenone on TLC plates are given in Table 3. Collaborator 18 listed his apparatus as a Zeiss; the excitation wavelength was 313 nm and fluorescence was measured at 443 nm. Collaborator 17 did not list his conditions. Also included in Table 3 are the results obtained at the Northern Regional Research Laboratory (NRRL) for the naturally contaminated corn samples, using the Eppley method (1) and measuring amounts of zearalenone on TLC plates densitometrically.

The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

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Table 2. Collaborative results (μg zearalenone/kg sample) for visual analysis of zearalenone in naturally contaminated corn

Coll.	Sample 8	Sample 9	Sample 10[a]	Sample 11[a]	Sample 12[a]	Sample 13	Sample 14
1	375	375	938	886	917	2952	7500
2	333	1250	750	833	833	3125	7500
3	269	833	550	656	656	1690	7500
4	(0)[b]	1875	625	625	833	3125	10,000
5	500	938	938	625	938	3125	7813
6	200	312	470	468	312	1560	4050
7	488	938	938	1250	1250	3125	12,500
8	(0)	900	1550	(trace)	(trace)	2650	7300
9	625	750	875	750	750	2500	7500
10	398	732	534	570	535	1813	6583
11	125	312	312	417	625	937	4167
12	800	1100	1500	800	1000	3300	12,000
13	(0)	1000	1383	760	390	1380	2420
14	(0)	375	625	780	780	1875	7500
15	625	625	875	500	500	2500	10,000
16	250	800	400	400	400	800	6000
Av.	416	820	829	688	715	2278	7521
Range: high	800	1875	1550	1250	1250	3300	12,500
low	125	312	312	400	312	800	2420
Std dev.	199	400	379	219	260	842	2705
Coeff. of var., %	47.9	48.9	45.7	31.8	36.4	36.9	36.0
N[c]	12	16	16	15	15	16	16

[a] Triplicate series.
[b] Values in parentheses were not included in calculations.
[c] *N* = number of values used to determine average.

Table 3. Densitometric determination of zearalenone in corn

Spiked corn				Naturally contaminated corn				
		Found				Found		
Sample	Added, μg/kg	Coll. 17	Coll. 18	Sample	Av.,[b] μg/kg	Coll. 17	Coll. 18	NRRL[c]
1	0	0	0	8	431	275	500	
2	100	0	0	9	821	355	700	662
3	300	265	310	10[d]	858	435	900	815
4	1000	575	850	11[d]	708	685	900	
5	1000	750	1100	12[d]	737	435	900	
6	1000	850	1100	13	2377	1250	2400	1690
7	2000	1100	2200	14	7622	3000	5500	6850

[a] Excitation was measured at 313 nm, and fluorescence at 443 nm.
[b] As determined by other collaborators visually.
[c] After information was received from Collaborator 18, samples were assayed at NRRL densitometrically.
[d] Triplicate series.

To determine the effect of substances in corn extracts on visual and densitometric measurements of zearalenone zones, partially purified extracts of zearalenone-free corn were prepared for TLC by the Eppley method. Residues of the extracts were spiked before TLC with quantities of crystalline zearalenone in benzene to represent different levels of contamination. After development, the TLC plates were read both visually and densitometrically; *see* Table 4. Results obtained densitometrically were more consistent than those obtained visually.

Collaborator 9 suggested that the liquid-liquid partition step with acetonitrile-hexane be omitted initially to save time. The partition would be performed to obtain cleaner extracts only if preliminary TLC indicated that zearalenone was present. Collaborator 12 did not think the liquid-liquid partition was effective in removing impurities from the extracts.

Most of the comments concerned the TLC step. Four collaborators suggested either applying less zearalenone standard solution to the plates and in smaller increments or diluting the standard. Concentrations mentioned for the standard were 10 or 25–30 μg zearalenone/ml. The collaborators reported that acetic acid-benzene (10+90), ethanol-chloroform (3.5+

Table 4. Effect of substances in corn extracts on determination of zearalenone levels (μg/kg)[a]

Level in original corn represented by spiked ext	Levels determined on TLC plates	
	Visually	Densitometrically
300	625	327
	625	298
500	703	424
	625	504
1000	1094	730
	1250	850
3000	4375	2690
	2500	2615
5000	5410	5445
	5000	4850
8000	10,000	9545
		9155

[a] Excitation was measured at 313 nm, and fluorescence at 443 nm.

96.5), or acetic acid-benzene (5+95) were also effective solvent systems. Other than the Adsorbosil-1 plates described, the following TLC plates were used with success by one or more collaborators: precoated kieselgel G (0.25 mm), precoated Brinkmann Silplate-22 (0.25 mm), precoated Brinkmann G-25-HR (No. 6614600-6), Mallinckrodt-7G, and precoated Merck silica gel 60 plates.

Recommendation

Results of the collaborative study indicate that the Eppley method, modified as described in this report, is applicable to the determination of zearalenone in corn. It is recommended that the modified method (1; R. M. Eppley, 1968, Food and Drug Administration, Washington, DC) be adopted as official first action for the determination of zearalenone in corn.

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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* (1976) *JAOAC* **59**, 386–387.

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ALCOHOLIC BEVERAGES

Clarity Evaluation of Distilled Alcoholic Products with a Particle Counter

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An HIAC particle counter is used to measure clarity of distilled alcoholic products. The number of particles is measured in 5 size ranges from 2 to 90 μm. Average data are given for whiskies, brandy, rum, white gin, and vodka. The highest counts are obtained for aged products and most of the particles are 2–5 μm. The particle counts of different brands of blended whiskies varied from 62 to 5265 particles/ml in the 2–90 μm range. There is no apparent relationship between nephelometer values and particle counts.

A Coleman nephelometer is used routinely in our laboratories to measure the clarity of alcoholic products and demineralized water used for reducing proof. This instrument measures the amount of light reflected by haze and suspended particles at a 90° angle to the incident light beam. A nephelometer operated at the highest sensitivity frequently cannot detect differences in such samples and the results often do not relate to visual observations. Also, nephelometer readings are subject to errors caused by temperature, color, density of sample, criticality of sensing angle, and inability to detect large particles which settle below the light path before a reading is recorded.

An instrument that will give quantitative data by sizing and counting small amounts of particulate material and is unaffected by the conditions given above should be a useful processing aid. Relatively few such instruments are suited to all types of alcoholic products.

This paper describes an HIAC Model 305 particle counter that can be used for sizing and counting particles in liquids or gases (1–4). Other investigators have reported applications for intravenous solutions (5), hydraulic or transmission fluids (6–8), and petroleum fuels.

The HIAC particle counter operates on the principle of light-blocking or interruption. The sample is forced through a sensor containing a small rectangular cell with windows on opposite sides. Standard sensors are available for measuring particles in different ranges; the smallest range is 2–60 μm and the largest is 85–2500 μm.

A light beam from a tungsten lamp is directed through the sample in the sensing zone onto a photodetector. Each particle passing between the windows interrupts a portion of the light beam according to its size, causing a momentary reduction (or pulse) in the amount of light reaching the photodetector. The specific reduction in the phototube output signal (voltage) is proportional to the particle size.

The instrument used for this study is equipped with a D-3-90 sensor for detecting particles 3–90 μm at a maximum concentration of 4700 particles/ml. However, calibration with standard solutions indicates that this sensor measures particles as small as 2 μm. Particles smaller than 2 μm are not measured because they do not block enough light to cause a change in the signal; particles larger than 90 μm block the sample flow through the sensing zone. It is important that the sample contain less than 4700 particles/ml because the phototube provides no way of distinguishing light blockage caused by one particle or a group of particles passing through together.

All particles block light according to size only, regardless of color, shape, brightness, translucence, surface roughness, or other physical characteristics. In order to be counted, it is only necessary for the particle to have an index of refraction different from that of the fluid. This feature is important when determining the particle count of some products, such as colored liqueur. Five counting circuits or channels with setable thresholds record the number of particles by size. This instrument has a built-in calibration pulse generator which produces reference pulses to simulate any particle size, with which the operator adjusts and verifies the size range

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of each channel. The HIAC company also markets other units with 2–12 channels.

A switch permits recording particle count for each channel in either the delta or total mode. All data in this report were obtained using the delta setting, which records the number of particles in each channel by size according to the channel threshold setting. Total count is obtained by adding counts of all channels. In the total mode, a cumulative count is registered in each channel of all particles larger than the size for which the channel has been set.

Automatic control of the light intensity produced by the tungsten lamp compensates for sample color. This feature permits accurate sizing of the particles at all times with no change required in calibration settings regardless of the sample color intensity.

Standard solutions containing a known number and size of particles (latex, glass beads, or fine dust) can be used to calibrate and check the accuracy of the unit (4, 9, and G. C. West (1975) HIAC Instruments Division, Montclair, CA, private communication).

Consistent particle counts can only be obtained by adhering to standard techniques, including cleaning and storing of beakers, use of filtered demineralized rinse water, calibration with standards, and cleaning the sensor when it is plugged by backwashing.

METHOD

Apparatus and Reagents

(a) *HIAC particle counter.*—Model 305 SSTA, equipped with D-3-90 sensor and automatic bottle sampling system (HIAC Instruments Division, Pacific Scientific Co., PO Box 3007, Montclair, CA 91763).

(b) *Beakers.*—200 ml, glass, Berzelius tall-form.

(c) *Demineralized water.*—Filtered through 0.45 μm Gelman filter, No. 12571.

(d) *Ultrasonic bath.*—1 qt (Cole-Parmer Model 8845-3).

(e) *Wetting agent.* — Kodak Photo-Flo 200 (Eastman Kodak Co.).

(f) *Field calibration kit.*—HIAC Model H5606.

Cleaning of Glassware

Submerge each 200 ml beaker 30 sec in 0.2% hot detergent solution (Cascade, Micro, or equivalent) in ultrasonic bath. Remove beaker with tongs and rinse inside and outside with membrane-filtered (0.45 μm) demineralized water. Finish rinsing by inverting beaker over sprayer supplied with filtered water. Let clean beakers drain and dry on rack equipped with stainless steel rods. Store dry beakers inverted on Saran Wrap in drawer.

Calibration

Determine correct calibration number for each channel by using standard solution containing known size and number of particles. Use calibration procedure included with the HIAC calibration kit (9, 10).

Operation of Particle Counter

Mix sample by inverting container 3 times and rinse lip of sample bottle by pouring ca 5 ml to waste. Pour ca 10 ml sample into clean 200 ml beaker, rinse, and discard. Pour 100 ml sample into beaker without touching beaker with sample container. Place beaker containing sample in ultrasonic bath containing ca 3″ clean water for 1 sec. This removes air bubbles instantly. Dry bottom of beaker with lint-free towel and place in HIAC bottle sampler. With bottle sampler switches in "pump on" and "auto" positions and selector valve in "pressure" position, push "recycle" button. This starts pump which forces liquid through sensor. Adjust pressure regulator to give flow of 10 ml/30±1 sec. Pump and counter automatically stop after 10 ml sample is collected in graduated tube. Divide particle count displayed on each channel by 10 to obtain results on per ml basis.

Drain graduated tube, close valve, and make duplicate run by pressing "recycle" button.

Cleaning System Between Samples

If a large particle plugs sensor, push it in reverse direction with special wire supplied by manufacturer. Also, if previous sample contained >200 particles/ml in 2–5 μm range, backflush system as follows with filtered demineralized water. Place clean beaker in bottle sampler. Disconnect slip joint at Luer fitting above sensor, connect 20 ml syringe without plunger, and fill it with filtered demineralized water. Turn bottle selector valve to "vacuum" and mode switch to "manual" position to backflush water through sensor.

Remove syringe and connect slip joint. Turn selector to "pressure" and mode switch to "auto" position before proceeding with next sample.

Shut-Down of Instrument

Place instrument on standby by rinsing system with filtered water and 1:500 dilution of Kodak

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wetting agent, which is left in the lines. On bottle sampler, turn pump switch to "off" and mode switch to "manual". Turn selector and count switches on particle counter to "null" and "stop" positions, respectively. Leave main power switch on at all times.

Results and Discussion

The HIAC particle counter was acquired because of its potential for measuring the size and number of small amounts of suspended solids in white, aged, and highly colored alcoholic products and water. Previously, all clarity evaluations were determined exclusively by nephelometer and visual observation.

Accuracy of the HIAC particle counter is acceptable as determined by Hopkins and Young (5), using microscopic techniques.

Before a standard procedure could be developed for the HIAC particle counter, it was necessary to prepare a liquid blank and rinsing fluid containing virtually no particles. Alcohol or demineralized or distilled water was not satisfactory "as is." A typical laboratory-demineralized water sample contains approximately 78 particles/ml in the 2–90 μm range. Filtration of this water through a 0.45 μm Gelman line filter reduces the count to 1–3/ml. Therefore, filtered demineralized water was used to develop the procedures and "clean" techniques are required to obtain consistent results with this instrument.

The particle counter has been used to test distilled alcoholic products from various distillers. These data are presented along with the corresponding nephelometer values. Table 1 shows the average particle profile for 5 kinds of whisky products. In these and most other products tested, the highest counts are obtained in the 2–5 μm range. These particles probably consist largely of lipids, fine char, and other barrel-derived materials. Note that bourbon, which is usually chill-filtered to remove lipids, has a relatively low particle count. Some of the products in the other categories also may have been chill-filtered or specially processed. Bourbon that has been primary filtered and then reduced in proof will contain approximately 8000 particles/ml before secondary or final filtration. This illustrates both the need for and effectiveness of the final filtration step. A lack of agreement with nephelometer readings is apparent, especially for the Irish whisky, which would appear to contain a relatively high concentration of particles smaller than 2 μm.

Table 2 shows a similar analysis of nonwhisky distilled products. Gin and vodka are distilled at high proof and are generally not aged in wood, so the particle counts are low.

The number of particles found in each type of product varies widely, depending on several factors, e.g., type of product, filtration procedure,

Table 1. Average particle count of randomly selected whiskies produced by different distillers

Whiskey	HIAC, particles/ml in micron range						Nephelometer SCF units[a]
	2–5	5–10	10–15	15–20	20–90	Total	
Blend	408	75	12	3	2	500	0.9
Bourbon	145	22	4	2	2	175	0.7
Canadian	235	38	6	2	2	283	0.9
Scotch	645	52	5	3	2	707	1.2
Irish	80	14	3	2	3	102	1.4

[a] Seagram scale.

Table 2. Average particle count of randomly selected distilled products produced by different distillers

Distd prod.	HIAC, particles/ml in micron range						Nephelometer SCF units[a]
	2–5	5–10	10–15	15–20	20–90	Total	
Brandy	298	54	8	3	3	366	0.9
Rum	369	47	7	3	3	429	0.9
Clear gin	89	15	2	1	1	108	0.7
Vodka	85	14	3	1	1	104	0.7

[a] Seagram scale.

Table 3. Variation in particle count of blended whiskies produced by different distillers

Brand	HIAC, particles/ml in micron range						Nephelometer SCF units[a]
	2–5	5–10	10–15	15–20	20–90	Total	
A	49	9	2	1	1	62	0.9
B	80	25	5	1	1	112	0.8
C	350	116	37	15	15	533	0.8
D	1293	692	216	32	8	2241	1.4
E	3466	1549	224	20	6	5265	1.3

[a] Seagram scale.

cleanliness of processing equipment, cleanliness of bottles and caps, storage time, and temperature.

The wide variation that can be encountered in individual brands of blended whisky is illustrated in Table 3. The lack of good agreement with nephelometer readings is again apparent. The SCF units reported for the Coleman nephelometer represent a Seagram scale utilizing the highest instrument sensitivity.

The nephelometer, operating at a 90° angle, measures colloidal (milky) haze effectively, while the HIAC will not. The nephelometer will also measure high concentrations of particles 2–90 μm, but not low concentrations, e.g., counts in the low hundreds/ml. On the other hand, the particle counter apparently quantifies all particles within the size limits of the sensor, which excludes colloidal haze. The smaller particles detected by the HIAC, when observed visually in a beam of light, appear as a "granular" haze. The degree to which visual observation and particle counts agree involves acquiring the ability to describe the different characteristics observed visually in a sample.

Clarity specifications can be based on any one kind of measurement or on a combination of measurements, the latter being the most satisfactory. However, because of the higher particle counts in the 2–5 μm range, the ability to measure smaller particles would be useful as a bridge between the HIAC and the nephelometer in quantitating visual characteristics.

Work is in progress to develop techniques and procedures for application of the particle counter for process control, e.g., filtration experimentation, demineralized water management, bottle cleaning operations, and other areas.

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Gas-Liquid Chromatographic Determination of β-Asarone in Wines and Flavors

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Wine samples containing β-asarone (*cis*-2,4,5-trimethoxy-1-propenylbenzene) are distilled; β-asarone is extracted by hexane and then quantitatively determined by gas-liquid chromatography (GLC), using ethyl palmitate as the internal standard. The GLC procedure is rapid and yields precise and accurate results. Mass spectrometery confirmed the identity of the GLC peak as β-asarone. The ultraviolet spectra of β-asarone and its isomer were also determined.

Calamus comes from *Acorus calamus* L. (family *Araceae*), common sweet flag, a wild perennial which grows along or in the swamps of North America, Europe, and Asia (1). Traditionally, the dried root (rhizome) has been used as a medicine and as a flavor for alcoholic beverages. The volatile oil, straw to yellow-brown in color, with a camphor-like odor and a spicy burning flavor, can be separated from the rhizome by steam distillation. In his rather extensive treatise on essential oils, Guenther (1) presents data from various researchers on the composition of calamus oils of both Indian and European origin. β-Asarone (*cis*-2,4,5-trimethoxy-1-propenylbenzene) is one of the major constituents of the oil of calamus, with other aromatic hydrocarbon compounds such as asarone, eugenol, methyleugenol, and pinene also present. The Food and Drug Administration has prohibited the use of calamus in any form, such as the root, extract, or oil, in any food or drug as a result of toxicological studies (2).

2,4,5-Trimethoxy-1-propenylbenzene can occur in the 2 isomeric forms, *cis* (β-asarone) and *trans* (asarone). Asarone (*trans*) is a white crystalline solid and β-asarone (*cis*) is a yellow liquid with a specific gravity of 1.0933. Both isomers are readily soluble in ethanol and common organic solvents; neither isomer is soluble in water.

The purpose of this paper is to continue the work initiated by Larry (3) on a quantitative determination of β-asarone in flavors and beverages, using a steam distillation and solvent extraction of the sample prior to a gas-liquid chromatographic (GLC) determination. In an attempt to improve, simplify, and reduce the time of the analysis, several modifications are presented here: A simple distillation is used in place of the steam distillation; a single solvent and one extraction procedure are used; an internal standard has been added, a series of standards is analyzed, and a standard curve is prepared; and more selective chromatographic conditions are used, achieving complete separation of β-asarone from interfering compounds. A further purpose of the work is to confirm the identity of β-asarone by mass spectrometry (MS).

This paper also includes a discussion of the nomenclature of 2,4,5-trimethoxy-1-propenylbenzene including the *cis* and *trans* relationship; the ultraviolet (UV) absorbance maxima of asarone and β-asarone; and the results of the analysis of several vermouths and other samples for β-asarone. We have identified the *trans* isomer of 2,4,5-trimethoxy-1-propenylbenzene as asarone and the *cis* isomer as β-asarone.

METHOD

Reagents and Apparatus

(a) *Ethanol.*—USP grade.

(b) *Standard solutions.*—Ethyl palmitate (No. 1575, Eastman Kodak Co., Rochester, NY), 1 mg/ml hexane; β-asarone (No. TT 7150, Fritzsche Brothers, New York, NY), 1 mg/ml ethanol; asarone (No. A-8259, Sigma Chemical Co., St. Louis, MO), 1 mg/ml; oil of calamus (No. 052/686202, Fritzsche Brothers), 1 mg/ml ethanol.

(c) *Gas chromatograph.*—Micro-Tek Model 220, with flame ionization detector and 6′ × 2 mm id glass column packed with 10% SP-1000 on 80–100 mesh Chromosorb W (HP). Operating conditions: column 180°C, detector and injection port 220°C; helium carrier gas (with purifier filter) 40 ml/min.

Preparation of Standard Curve

Prepare standards containing 1, 2, 3, 4, and 5 mg β-asarone/L by adding 100, 200, 300, 400, and 500 μl β-asarone standard solution to separate 100

ml flasks, each containing ca 90 ml 20% ethanol. Mix, dilute to volume with 20% ethanol, and mix again. Transfer 100 ml of each standard (with ca 50 ml water to rinse out flask) to simple distilling apparatus. Distill and collect 100 ml. Transfer distillate to 250 ml separatory funnel and add 100 ml saturated NaCl solution and mix. Add 10 ml hexane and extract β-asarone by shaking vigorously 2 min. Let layers separate; then drain and discard aqueous solution. Wipe inside of drain tube of separatory funnel dry with tissue and collect hexane in calibrated centrifuge tube (10±0.5 ml should be recovered). Add 200 μl ethyl palmitate internal standard solution and mix well. Chromatograph 5 μl and plot standard curve of mg β-asarone/L vs. peak height ratio.

Determination

Distill and collect 100 ml sample in same manner as 100 ml standard. Transfer 100 ml distillate and extract as described above. Chromatograph 5 μl extract and check for presence of β-asarone and ethyl palmitate. Absence of β-asarone peak indicates there is no β-asarone at 0.5 mg/L level, since this is least amount that can be measured under conditions described.

If peak has same retention time as ethyl palmitate, use peak height measurements directly to determine concentration of β-asarone. If β-asarone is present in range of standard curve and no ethyl palmitate is present, add 200 μl ethyl palmitate internal standard solution, mix, and chromatograph again. Use peak height ratio to determine β-asarone concentration from prepared standard curve. If β-asarone peak is off scale, dilute sample with hexane to contain 1–5 mg β-asarone/L and continue analysis.

Confirmation

For confirmation of asarone and β-asarone, a gas-liquid chromatograph-mass spectrometer coupled to a dual stage Biemann-Watson molecular separator to remove the carrier gas was employed. The chromatograph was operated at similar conditions as described above. Effluent peaks from the gas chromatograph were introduced into a Varian Model CH7, single focusing mass spectrometer with an electron bombardment source. The ionizing section of the mass spectrometer was operated at 200°C. The mass spectra were generated with an electron impact energy of 70 ev. Each spectrum was scanned by varying the magnetic field. The output was recorded on a Varian Statos I, Model 150 high-speed electrostatic recorder.

Ultraviolet Spectrophotometry

A Beckman DU-2 spectrophotometer was used to determine the UV absorption spectra of asarone and β-asarone. Absorbance readings were taken, using ethanol as the blank and a sample concentration of 10 mg compound/L ethanol.

Results and Discussion

After the previous method for the determination of β-asarone was evaluated, the distillation, extraction, and chromatographic steps were modified. Work with the mass spectrometer and the UV spectrophotometer was included in order to further investigate the compound.

A simple distillation rather than a steam distillation can be employed to separate the volatile materials, including β-asarone. One extraction with hexane was sufficient to extract the β-asarone from the distillate when an equal volume of saturated sodium chloride solution was present. No efforts were made to concentrate the hexane, since the extraction conditions presented a 10-fold concentration factor of the β-asarone. If additional concentration of the sample extract is necessary, a Kuderna-Danish apparatus can be used to concentrate the hexane extract to the desired level. The choice of ethyl palmitate as the internal standard was based on the usual criteria of its retention time being close to that of the desired component, similar response level, and the general absence of ethyl palmitate in vermouth samples and flavors. The selection of column packing material (10% SP-1000 on 80–100 mesh Chromosorb W (HP)) was based on efficiency and the resolution of the β-asarone from interfering components. β-Asarone is completely resolved from interfering compounds and the chromatogram shows sharp peaks. All of the above modifications improve the method of analysis for β-asarone in terms of speed, precision, and accuracy.

To ascertain the quantity of β-asarone lost due to distillation and extraction, 20% ethanol-water samples were prepared with known amounts of added β-asarone (1–5 mg/L). These samples were analyzed and the amount of β-asarone was determined from a standard curve obtained from β-asarone prepared in hexane directly, without going through the distillation and extraction steps. An average of 10 determinations indicated that a 10% loss of β-asarone resulted from the distillation and extraction process.

A vermouth sample was analyzed to ensure the absence of β-asarone and ethyl palmitate and then known amounts of β-asarone (1–5

mg/L) were added. The sample was then analyzed by the described method. The average recovery for 10 determinations was 100.3% of the added β-asarone with a standard deviation of 2.94%. The data from these 2 experiments indicate that accurate results can be obtained when the β-asarone standards are carried through the distillation and extraction procedure described.

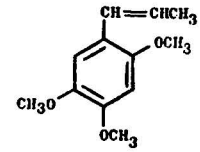
Our laboratory has analyzed 19 samples of flavors and vermouths, including imported and domestic as well as sweet and dry types, and no β-asarone was detected. The limit of detection using the conditions described is 0.5 mg β-asarone/L.

A series of mass spectrometer tests were carried out to confirm the identity of the major component of the oil of calamus. Oil of calamus samples, asarone, and β-asarone were injected in the gas chromatograph directly coupled to the mass spectrometer. The identification of the GLC peaks was verified by their characteristic mass spectra. Comparison with MS data obtained by the direct introduction of the reference standards of the samples indicated close agreement of all the samples injected. The mass spectra indicated a strong response due to ions of mass number 208, which agrees with both the molecular weight and the stability of the parent molecular ion resulting from the aromatic nature of 2,4,5-trimethoxy-1-propenylbenzene.

In GLC of the isomers, the rule of Shulgin (4) is followed whereby the *cis* isomer precedes the *trans* isomer on a GLC column. For the GLC conditions described in this paper, the retention times for these isomers are 325 sec for β-asarone and 483 sec for asarone. The data for the GLC of the *cis-trans* isomers agree with the results reported in the work of Baxter (5).

A spectrophotometric curve for asarone and β-asarone was plotted (absorbance vs. wavelength) to determine the UV absorbance characteristics of the *cis-trans* isomers. The absorbance maximum for the *cis* form, β-asarone, is 254 nm, while the absorbance maximum for the *trans* form, asarone, is 259 nm. The difference in UV spectra of these 2 isomers is due to the *cis-trans* relationship, and, as expected, the absorbance maximum of the *cis* isomer is at the lower wavelength (6).

The structural formula (7) for 2,4,5-trimethoxy-1-propenylbenzene is given below:



One reference (8) suggests another name, isoasarone, for the compound 2,4,5-trimethoxy-1-propenylbenzene. The name isoasarone is consistent with the other common names for phenol ether-type compounds, e.g., eugenol (1-hydroxy-2-methoxy-4-allylbenzene), and isoeugenol (1-hydroxy-2-methoxy-4-propenylbenzene), and safrole (1,2-methylenedioxy-4-allylbenzene) and isosafrole (1,2-methylenedioxy-4-propenylbenzene).

In view of consistency with the names of similar compounds, isoasarone certainly seems more appropriate. In this case, the *cis* isomer of isoasarone would refer to β-asarone and the *trans* isomer to asarone. The iso compound can occur as *cis-trans* isomers.

Recommendation

It is recommended that the GLC method described for the determination of β-asarone (*cis*-2,4,5-trimethoxy-1-propenylbenzene) be collaboratively studied.

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Methods for the Determination of Calcium in Beer

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The American Society of Brewing Chemists (ASBC) has submitted 2 new analytical methods for the colorimetric determination of calcium in beer to the AOAC. These 2 methods give similar results and have been tested by an ASBC technical committee for 3 years. The methods have been adopted as official first action.

The American Society of Brewing Chemists (ASBC) has released 2 methods for the determination of calcium in beer to the Associate Referee for presentation to the AOAC. These 2 colorimetric calcium methods were tested for 3 years by the collaborators of a technical committee of ASBC and have been used by industry laboratories for 2 additional years. The 2 methods give equivalent values so the choice of procedure is left to the user.

The determination of calcium ion is an important control factor in brewing. It is important for the action and stability of malt enzymes and also has an effect on the taste properties of finished beer. Calcium is introduced into brewing by the cereal grains used and by the water used in the mashing process.

In 1970 (1), 6 laboratories collaboratively analyzed 4 beer samples containing from 58 to 108 ppm calcium. The 1971 testing (2) was done on 7 samples with 7 collaborating laboratories. In 1972, 8 collaborators analyzed 7 samples. In the 1972 study (3) the difference in means between the 2 methods for samples with <100 ppm calcium was <1 ppm and for samples with >100 ppm calcium, $\leqslant 4$ ppm. The standard deviations for both methods were low. The statistical analyses of the results are given in Table 1, along with results from an atomic absorption method (4).

Recommendation

It is recommended that the following 2 methods for calcium in beer be adopted as official first action: calcium by eriochrome black T (chrome black T) indicator method and calcium by calcein indicator method.

METHODS

Calcium—Official First Action

ASBC Method I

10.B01 ***Reagents***

(a) *Ammonium oxalate soln.*—Satd soln (ca 6%) of $(NH_4)_2C_2O_4.H_2O$ in H_2O.

(b) *Buffer soln.*—pH 10.0. Dissolve 67.5 g NH_4Cl in 200 ml H_2O. Measure pH, and add NH_4OH (ca 200 ml) to pH 10.0. Dil. to 1 L.

(c) *Eriochrome black T indicator soln.*—Dissolve 0.1 g indicator (Eastman Kodak Co., No. P6361, or equiv.) in 25 ml MeOH contg 1 g $H_2NOH.HCl$. Store <2 months.

(d) *Sodium sulfide soln.*—2%. Dissolve 2 g Na_2S in 100 ml H_2O.

(e) *Magnesium std soln.*—1.00 mg/ml. Dissolve 1.00 g Mg turnings in 100 ml 0.1*N* HCl and dil. to 1 L with double distd H_2O.

(f) *Disodium dihydrogen EDTA std soln.*—0.1%. Dissolve 1 g Na_2H_2EDTA in 1 L H_2O. Stdze against Mg std soln.

10.B02 ***Standardization***

Pipet 5 ml Mg std soln into 250 ml erlenmeyer and add 50 ml H_2O and 1 ml Na_2S soln. Prep. blank soln with 50 ml H_2O and 1 ml Na_2S soln. Add 5 ml buffer soln and 10 drops indicator soln to each erlenmeyer. Titr. flask contg Mg with EDTA std soln until permanent blue color exactly matches blank. Titr. 3 aliquots and use av. to calc. titer Mg soln, M = 5/ml EDTA std soln.

Calc. Ca factor, $C = (40.08/24.32) \times M$.

10.B03 ***Determination***

(a) *Total calcium and magnesium.*—Pipet 5 ml prepd sample, **10.001**, into 250 ml erlenmeyer and add 40 ml H_2O, 1 ml Na_2S soln, 5 ml buffer soln, and 10 drops indicator soln. Titr. immediately with EDTA std soln. ml EDTA std soln = X.

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The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee D and was adopted by the Association. *See* (1976) *JAOAC* **59**, 388.

Table 1. Determination of calcium by 3 methods

Sample	Calcein			Eriochrome black T			Atomic absorption
	Mean[a]	Std dev.	Coeff. of var., %	Mean[a]	Std dev.	Coeff. of var., %	Mean[a]
1	60.28	2.82	4.67	59.64	2.34	3.92	59.55
2	69.64	2.30	3.30	69.35	3.29	4.74	69.18
3	88.35	3.80	4.30	87.21	3.09	3.54	88.08
4	109.78	3.12	2.84	106.07	7.55	7.11	108.25
5	75.14	1.99	2.64	74.35	3.17	4.26	73.50
6	95.16	2.41	2.53	95.00	1.65	1.73	92.55
7	110.57	2.68	2.42	106.42	7.11	6.68	107.73

[a] Mean results from 8 collaborators.

(b) *Magnesium.*—Pipet 25 ml prepd sample, **10.001**, into 125 ml erlenmeyer and add 0.5 ml $(NH_4)_2C_2O_4.H_2O$ soln and 2 drops buffer soln. Refrigerate 2 hr at 0–2°. Filter thru Whatman No. 40, or equiv., paper. Add 1 drop HCl to filtrate. Pipet 5 ml filtrate into 250 ml erlenmeyer and add 40 ml H_2O, 1 ml Na_2S soln, 5 ml buffer soln, and 10 drops indicator soln. Titr. immediately with EDTA std soln. ml EDTA std soln = Y.

$$\text{ppm Ca} = [X - (25.5Y/25)] \times C \times 200$$

ASBC Method II

10.B04 ***Reagents***

(a) *Calcein indicator soln.*—Dissolve 0.2 g indicator (G. Frederick Smith Chemical Co., Eastman Kodak Co., or equiv.) in 100 ml H_2O contg 1 ml 5*N* NaOH.

(b) *EDTA std soln.*—1 ml = 1 mg $CaCO_3$. Available from Hach Chemical Co., PO Box 907, Ames, IA 50010; Betz Laboratories, Inc., 4636 Somerton Rd, Trevose, PA 19047; or equiv.

10.B05 ***Determination***

Pipet 20 ml prepd sample, **10.001**, into 250 ml erlenmeyer. Add 100 ml H_2O, 3 ml 5*N* NaOH, and 0.5 ml calcein indicator soln. Swirl to mix. Titr. with EDTA soln at 1 drop/sec, using overhead light and black background until yellow-green fluorescence is replaced by orange-brown color. mg Ca/L = ml EDTA std soln × 20.

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DRUGS

Infrared Identification of Maleic Acid in Pharmaceutical Maleate Salts

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Difficulties are encountered in the USP melting point identification test for maleic acid extracted from chlorpheniramine maleate. A general procedure is described for the isolation and infrared identification of the maleic acid moiety of pharmaceutical salts from drug substances or dosage forms. This procedure permits the concurrent infrared identification of the amine moiety.

The identification tests for chlorpheniramine maleate in USP XV–XIX (1) include an identity test for the maleic acid moiety in which the maleic acid is isolated and its melting point is determined. This test for maleic acid has definite limitations. Because of the tendency of that acid to form maleic anhydride, the rate of heating may affect the observed melting point. The spread in published melting points attests to its unsuitability as a standard. That listed in the USP XIX is 128–133°C (1); other published values range from 130°C (2) to 139–140°C (3).

The infrared (IR) spectrum of the maleic acid isolated as described in the chlorpheniramine maleate monograph differs in several areas from that prepared from reagent grade maleic acid. These differences in the spectra are seen at about 3500 and 1105 cm^{-1} (Fig. 1). Therefore, material isolated by this procedure would not be suitable for use in an IR identification test. Differences can also be noted among the IR spectra of maleic acid found in published compilations (4–6).

A simple procedure has now been developed for isolating maleic acid from its pharmaceutical salts; IR spectra are reproducible, identical with that of reagent grade material, and thus suitable for purposes of identification. The method is readily applicable not only to the drug substances, but also to solid dosage forms. Because of the presence of other anions in such liquid preparations as cough sirups, e.g., citrate, the identification in such products would be of little value.

Experimental

Drug Substance

Transfer ca 30 mg amine maleate to separatory funnel containing 2 ml water. Add 5 ml $CHCl_3$ and 3 ml ca 0.1*N* NaOH, shake thoroughly, and then

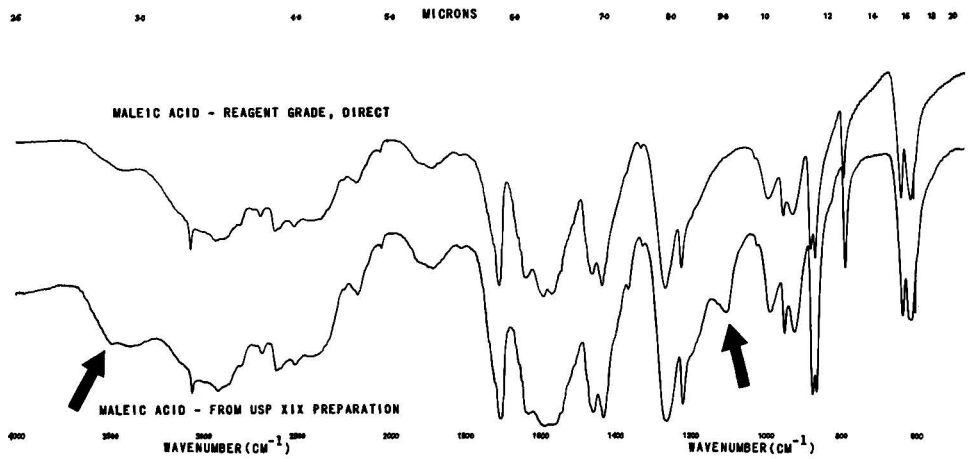

FIG. 1—IR spectra of maleic acid: reagent grade material and material isolated according to USP method.

draw off $CHCl_3$ extract (this can be used for identification of base). Acidify aqueous solution with 1 ml concentrated HCl, and extract with 50 ml ether. Filter ether extract through paper into beaker and concentrate on steam bath to ca 10 ml. Add 10 ml benzene and evaporate to dryness. Add 5 ml benzene to residue and repeat evaporation. Intimately mix 1 mg residue with 200 mg previously dried potassium bromide. Form transparent disk by pressing mixture in die to 25,000 psi under vacuum. Scan IR spectrum.

Tablets

Transfer quantity of ground tablets equivalent to ca 40 mg drug substance to 25 ml Erlenmeyer flask. Add 5 ml methanol and heat to boiling on steam bath. Add 25 ml $CHCl_3$, shake thoroughly, and filter into separatory funnel. Extract with 10 ml ca 0.1*N* NaOH. Let layers separate. Both layers may remain quite emulsified and incompletely separated; if phase separation is slow, reshake with additional 10-15 ml $CHCl_3$ to accelerate separation. Draw off lower layer (reserve for base identification). Without regard to cloudiness of upper layer or incompleteness of withdrawal of lower layer, acidify aqueous layer with 1 ml concentrated HCl and extract with two 25 ml portions of $CHCl_3$. Discard $CHCl_3$ extracts. Extract aqueous layer with 50 ml ether and continue as under *Drug Substance,* beginning with "Filter ether extract . . .".

Identification of Parent Amine

Filter $CHCl_3$ extract of alkaline solution, which was previously set aside. Evaporate on steam bath to ca 10 ml, add 10 ml benzene, and evaporate to dryness. Dissolve residue in ca 0.5 ml $CHCl_3$ and transfer dropwise to 20 mm diameter NaCl plate under IR heat lamp until no odor of $CHCl_3$ can be detected. (*Caution:* Wear contrast safety goggles and carry out this procedure in hood.) Cover residue with second salt plate and scan IR spectrum.

Results and Discussion

The procedure has been applied to chlorpheniramine maleate, carbinoxamine maleate, dimethindene maleate, pyrilamine maleate, thiethylperazine maleate, and prochlorperazine maleate drug substances, and to chlorpheniramine maleate (4 mg), carbinoxamine maleate (4 mg), coated thiethylperazine maleate (10 mg), and coated prochlorperazine maleate (5 mg) tablets. In each case the spectra of both maleic acid and the parent amine were identical with those of authentic materials.

Relatively clean extracts are obtained from tablet material because of the absence of water. Because some of the salts, e.g., chlorpheniramine maleate and pyrilamine maleate, are readily soluble in chloroform, this solvent alone without the methanol can be used for tablets of these compounds. However, in describing a general procedure, chloroform cannot be specified as the solvent because other salts, e.g., thiethylperazine maleate, are not chloroform-soluble and because some formulations may entrap the soluble salts within chloroform-insoluble matrices. The initial methanol treatment was effective in permitting the extraction in every case.

The sodium hydroxide extraction of the methanol-chloroform solution will remove not only the maleic acid moiety of the drug but also stearic acid used in the tablet formulation. The latter is extracted by chloroform from the acidified solution, while maleic acid, because of its low distribution constant, remains in solution in the aqueous layer and is subsequently extracted with ether.

The benzene which is added to the extract during the evaporation step effects the azeotropic removal of water which condenses during the evaporation. Thus the extracts need not be heated in a drying oven.

The occurrence of thiethylperazine as both the malate and the maleate is a potential source of confusion. The method as described was applied to both of these salts and the spectra of malic acid and of maleic acid, respectively, were obtained from the appropriate fractions.

Vigorous grinding of maleic acid may cause a reversal in the relative intensities of the doublet at 864 and 876 cm^{-1} (Fig. 2). If the spectrum

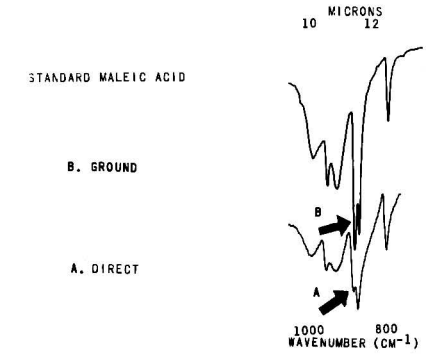

FIG. 2—IR spectra of maleic acid; A, direct; B, after grinding.

of the maleic acid under examination differs from that of the standard at only this region, regrinding and subsequent reconstitution of the disks will eliminate this difference.

The test may not be practical for low dosage drugs. Ergonovine maleate tablets, for example, contain only 0.2 mg of the drug in each tablet. A sample of 200 tablets would be required to provide the 40 mg drug substance specified in the test. While it would be possible to decrease this to some extent, an inordinate number of tablets would still be required.

In summary, a procedure is described for isolating maleic acid from pharmaceutical salts in a form which gives reproducible IR spectra free of extraneous absorption peaks. The IR spectra so obtained are suitable for identification purposes. The procedure provides for the concurrent IR identification of the base.

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Received September 25, 1975.

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Abstracts of papers or reports to be presented at the 90th Annual Meeting of the AOAC, Oct. 18–21, 1976, MUST be submitted on standard, preprinted forms. The forms are available, on request, from the AOAC.

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In addition to the abstract, *six* complete copies (including abstract) of all Associate Referee reports (1 copy for each member of the Subcommittee, the Statistical Consultant, and the Editorial Office) and three complete copies of all contributed papers must be submitted to the AOAC office. The manuscripts should be double-spaced throughout. Unless otherwise specified, all manuscripts received will be considered for publication in *JAOAC,* pending satisfactory review. Each Associate Referee should also send one copy of his report to his General Referee.

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Spectrophotometric Analysis of Alkaloids with Picrolonic Acid

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The spectroscopic characteristics of the interaction of alkaloids with picrolonic acid were studied. In solvents of low and intermediate polarity, the presence of alkaloids caused a red shift of the 322 nm band of nonionized picrolonic acid to 355 nm, corresponding to the anionic resonance band. There was also a considerable increase in absorptivity, which was dependent on both the basicity (pKa) and molar concentration of the alkaloid present. Arylamines, aromatic *N*-heterocycles, and alkaloids lacking an aliphatic amine moiety did not show observable shifts. The interaction was developed into a spectrophotometric assay for the following alkaloids in pharmaceutical preparations: atropine, ephedrine, codeine, emetine, quinine, and strychnine. The method is sensitive to 2 μg alkaloid/ml, with an accuracy of ±1.5% and a standard deviation of ±1.05–1.31%.

Picrolonic acid is a well known microcrystalloscopic reagent for alkaloids and other basic nitrogenous compounds (1, 2). It is frequently used for characterization by crystalline form and melting point, and for gravimetric quantitation of alkaloids via the formation of crystalline salts of constant composition and high melting points (1–3).

In spite of the wealth of literature concerning the formation of the picrolonates for the purpose of identification of many alkaloids, synthetic amines, and bivalent metals as well as their quantitation (1–7), no detailed spectral study of the interaction of picrolonic acid with alkaloids is available.

Among the various polynitro organic reagents for alkaloids, picrolonic acid is not as widely employed as picric acid, which is by far the most popular, although by no means the most selective, of these reagents. In fact, picric acid is a poorly selective reagent. Not only does it give crystalline salts with amines but it also yields crystalline addition compounds, mostly of the charge-transfer type, with a variety of organic compounds such as aromatic hydrocarbons (1, 8), aryl ethers (9), and proteins (3). The charge-transfer hydrocarbon picrates should not be confused with the amine picrates, which are substituted ammonium picrate salts formed by proton transfer.

On the other hand, picrolonic acid is a more selective reagent. Although it interacts with some divalent cations such as the alkaline earth metals, it is generally regarded (1, 3) to be specific to basic nitrogenous compounds, particularly the alkaloids.

In view of the above considerations, this spectral study of the interaction of picrolonic acid with alkaloids was conducted and developed into a spectrophotometric assay for many alkaloids. The spectral behavior of picrolonic acid in various solvents and at different pH values, which was hitherto unreported, is also included.

Experimental

Reagents and Apparatus

(a) *Chemicals.*—Picrolonic acid was obtained commercially (Prolabo, Paris, France). All other chemicals used were analytical reagent or pharmaceutical grade obtained from various manufacturers and were used as working standards. The aliphatic and aromatic amines were purified by redistillation shortly before use. Solvents used were spectrograde or rendered so by the proper treatment (10).

(b) *Picrolonic acid solution.*—Two concentrations were used: $1.5 \times 10^{-5}M$ solution in ethylene dichloride (4 μg/ml) for qualitative screening; $1 \times 10^{-4}M$ solution in ethylene dichloride (26.5 μg/ml) for quantitative assay.

(c) *Buffer.*—Adjust 0.2*M* dibasic potassium phosphate, against glass and calomel electrodes, to pH 9.5 with 0.5*N* NaOH solution.

(d) *Standard solutions.*—Dissolve calculated amount of available alkaloidal salt in water and dilute solution quantitatively and stepwise to 1.0 mg base/ml.

(e) *Spectrophotometer.*—Ultraviolet (UV) and visible range (Spektromom 203, Mom, Budapest, Hungary).

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Received September 11, 1975.

Procedure

Pipet 1 ml standard solution into 30 ml separatory funnel. Add 5 ml buffer and extract with two 4 ml portions of ethylene dichloride, passing separated organic layers through 2 g anhydrous Na_2SO_4 suitably supported in small funnel. Dilute filtrate to 10 ml with ethylene dichloride.

Pipet 1 ml ethylene dichloride solution into dry 5 ml volumetric flask, add 1 ml picrolonic acid solution ($1 \times 10^{-4}M$), and dilute to volume with ethylene dichloride. Read absorbance of solution at 362 nm against blank treated concurrently.

Application to Pharmaceutical Preparations

The following commercial preparations were subjected to the analytical procedure:

(*1*) Atropine sulfate injection (Chemical Industries Development Laboratories, Talbia, Giza, Egypt): contains 1 mg salt/1 ml ampoule.

(*2*) Coramine-ephedrine drops (Swiss-Pharma, Amirya, Cairo, Egypt): contains 250 mg niketha-mide and 15 mg ephedrine hydrochloride/ml.

(*3*) Codinal tablets (Chemical Industries Development Laboratories): contains 15 mg codeine phosphate and 10 mg phenobarbital/tablet.

(*4*) Emetine hydrochloride injection (Kahira Pharmaceutical Co., Shobra, Cairo, Egypt): contains 30 mg salt/1 ml ampoule.

(*5*) Quinine sulfate tablets (Misr Co. for Pharmaceuticals, Zytun, Cairo, Egypt): contains 150 mg quinine sulfate/tablet.

(*6*) Strychnine sulfate injection (El-Nile Co. for Pharmaceuticals, Amirya, Cairo, Egypt): contains 1 mg salt/1 ml ampoule.

(a) *Liquid preparations.* — Transfer aliquot equivalent to 1 mg alkaloidal salt, or measured contents of single dose container of injection, into 5 ml buffer in 30 ml separatory funnel and continue as described above.

(b) *Tablets.*—Place equivalent of 1 tablet from composite of 10 powdered tablets in 5 ml buffer in 30 ml separatory funnel and proceed as described above, making necessary dilution before adding picrolonic acid so sample contains 5–25 μg alkaloidal base/ml.

Results and Discussion

Spectral Characteristics of Picrolonic Acid.— It was essential to examine the electronic absorption spectrum of picrolonic acid in various solvents prior to the study of any spectral changes which may result upon interaction with alkaloids. No explicit reference to the ultraviolet spectrum of picrolonic acid could be found in the available literature which included collections and catalogs (11, 12) as well as more specialized reference works (13, 14).

The acid exhibited a major absorption band, the position and intensity of which varied with variation of solvent polarity and pH as outlined in Table 1.

The more red shifted and intensified bands in polar solvents most probably correspond to the enolized, more conjugated form of the acid. However, previous reports indicate, with few exceptions, the predominancy of enol forms of analogous compounds in *non*polar solvents (13–16). Consequently, other factors must be considered to explain the spectrum of picrolonic acid.

By analogy to other similar heterocyclic nuclei, such as the hydroxypyridines, the contribution of zwitterionic tautomers must be considered (13, 15, 17). Thus polar solvents such as water and alcohols will permit an intramolecular proton transfer, hence favoring the conjugated zwitterion. This explains the red shift and intensified absorption in these solvents. Less polar solvents such as benzene and chloroform will not allow such facile proton transfer; thus the neutral keto form is favored. It may be reasonably assumed that a protonated keto form exists in acidulated water, with a slightly red shifted maximum, rather than the neutral form. The observed shift occurred either because of increased polarity of 0.2*N* HCl or because of the position of the added proton (13, 15).

The anion may be formed in aqueous and alcoholic solvents by the addition of alkali. It has the same maximum as the zwitterion, with some increased absorptivity, which suggests the same basic chromophore.

Preliminary Study with Amine Classes.—In order to define the interacting moiety in the complex alkaloidal molecules which interact

Table 1. Peak position and intensity of picrolonic acid in various solvent media

Solvent	λ_{max}, nm	ϵ_{max}[a]
Benzene	322	17,800
Chloroform	322	19,100
Ethylene dichloride	322	19,700
Methanol or ethanol	350	22,700
Water, pH 7	350	20,200
0.2*N* HCl	330	18,200
0.2*N* NaOH	350	23,050
0.1*N* ethanolic KOH	350	24,000

[a] Average of 3 determinations.

with picrolonic acid, it was necessary to examine first the spectra of systems of picrolonic acid with simpler amines representing the various classes.

Scanning of aliphatic and alicyclic amines such as propylamine, diethylamine, piperidine, triethylamine, and *N*-methylpiperidine revealed a red shift of the 322 nm band of the neutral keto form of picrolonic acid to 355 nm with considerable increase in band intensity. A typical case, exemplified by diethylamine, is shown in Fig. 1.

The new band may be assigned to the picrolonate anion formed by the action of the basic amine. The additional 5 nm red shift in peak position is probably due to an increase in ionic radius of the complementary cation in the case of the organic base; this is in line with previous reports on analogous systems (15, 18, 19).

The intensity of the anionic band depends on both the basicity (pKa) and molar concentration of the amine present, although the peak position was unchanged.

With arylamines, such as aniline and *N*-methylaniline, no spectral changes were observed, while with mixed amines, e.g., *N*,*N*-diethylaniline, only a minor shift was observed, again confirming the dependency on the pKa (Fig. 2).

With aromatic *N*-heterocycles such as pyridine and quinoline, no distinct shift could be observed. However, there was a minor disturbance of the picrolonic acid band with large concentration of the base (Fig. 3).

Alkaloid Systems.—Similar red shifts of the major band of picrolonic acid were observed on interacting the acid dissolved in ethylene dichloride with a number of selected alkaloids, namely, ephedrine, atropine, sparteine, codeine, quinine, strychnine, emetine, and nicotine. The typical case of ephedrine is illustrated in Fig. 4. All these alkaloids have pKa values of 8 or above, and contain some sort of an aliphatic or alicyclic amine moiety. With papaverine, however, which lacks such a moiety and with a pKa of 6.4, only the minor disturbance previously observed with aromatic *N*-heterocycles occurred.

It may be inferred, therefore, that for the amine to interact spectroscopically with picrolonic acid in nonpolar solvents it should be sufficiently basic with a pKa in the neighborhood of 8 or above.

The above conclusion is supported by the good correlation, outlined in Table 2, between the pKa value of the base and observed absorbance of the resulting picrolonate anion caused by $1 \times 10^{-5} M$ base. Regression analysis of this

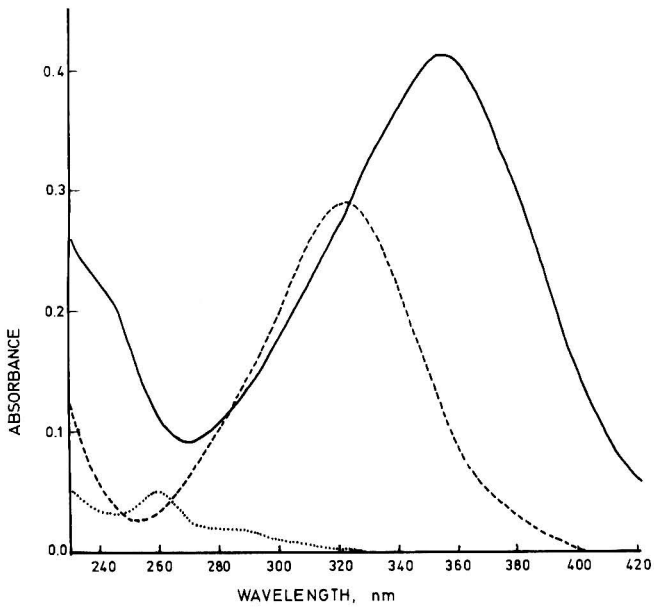

FIG. 1—Absorption spectra of the interaction of picrolonic acid (4 μg/ml) with diethylamine (7 μg/ml) in ethylene dichloride: — reaction product; – – – picrolonic acid; and ... diethylamine.

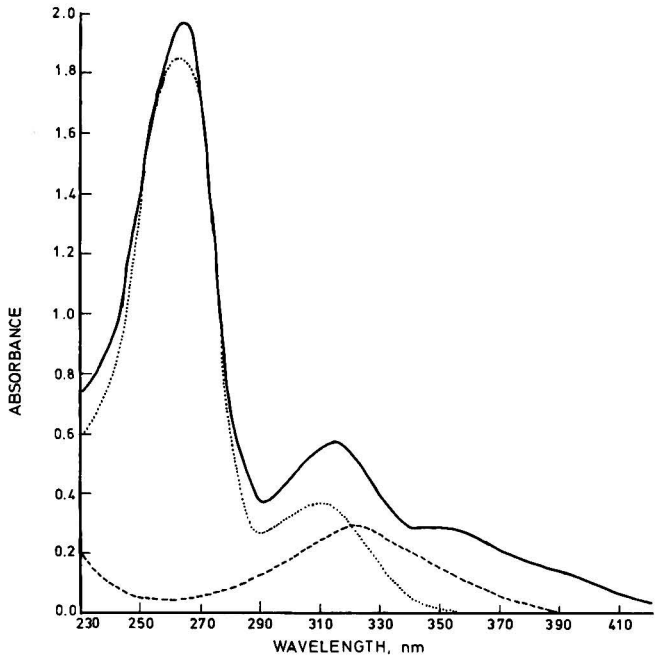

FIG. 2—Absorption spectra of the interaction of picrolonic acid (4 μg/ml) with *N,N*-diethylaniline (20 μg/ml) in ethylene dichloride: — reaction product; – – – picrolonic acid; and ... *N,N*-diethylaniline.

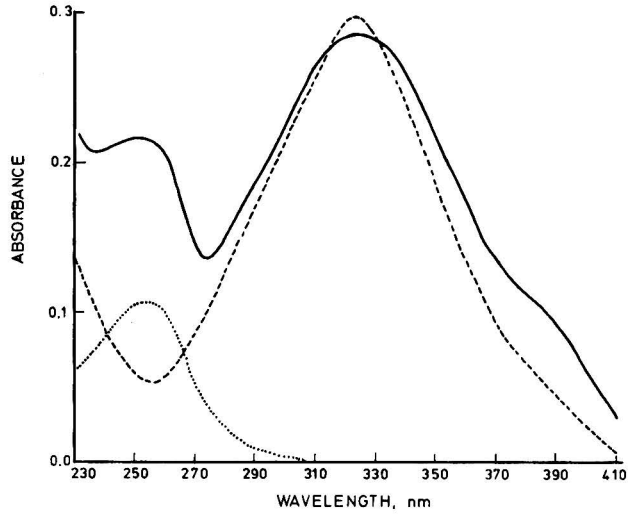

FIG. 3—Absorption spectra of the interaction of picrolonic acid (4 μg/ml) with pyridine (80 μg/ml) in ethylene dichloride: — reaction product; – – – picrolonic acid; and ... pyridine.

correlation, by the method of least squares, afforded the following correlation parameters: correlation coefficient, 0.915; correlation constant, 0.08; and relative standard deviation, 0.04 (Fig. 5).

Quantitative Analysis.—Other factors being equal (concentration of picrolonic acid, pKa of base, etc.), the intensity of the 355 nm anionic band depended on the concentration of the added alkaloid; this permitted the development of the interaction into a sensitive spectroscopic assay because of the high molar absorptivity of the

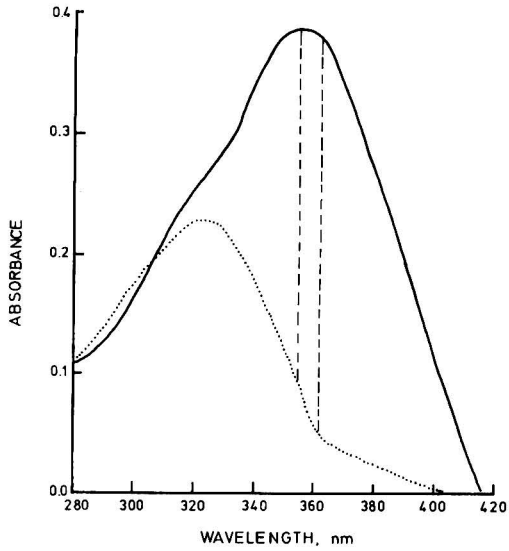

FIG. 4—Absorption spectra of the interaction of picrolonic acid (4 μg/ml) with ephedrine (1.6 μg/ml) in benzene: — reaction product; and ... picrolonic acid.

Table 2. Correlation between pKa of base and absorbance of picrolonate anion at 355 nm

Amine	Symbol in Fig. 5	pKa[a]	A_{355} of $1 \times 10^{-5}M$ soln[b]
Aniline	An	4.63	0.00
N-Methylaniline	NM	4.84	0.00
Quinoline	Ql	4.90	0.003
Pyridine	Py	5.25	0.009
Papaverine	Pa	6.40	0.031[c]
N,N-Diethylaniline	NN	6.61	0.089
Nicotine	Nc	8.02	0.210
Codeine	Cd	8.21	0.235
Emetine	Em	8.23	0.225
Strychnine	St	8.26	0.252
Brucine	Bc	8.28	0.272
Quinine	Qn	8.52	0.275
Atropine	At	9.65	0.340
Ephedrine	Ep	10.14	0.364
Diethylamine	De	10.49	0.411
Propylamine	Pr	10.71	0.415
Triethylamine	Tr	11.01	0.440
Piperidine	Pi	11.12	0.420

[a] Data from ref. 12 at 25°C.
[b] Average of 2 determinations, using $5 \times 10^{-5}M$ picrolonic acid.
[c] Shoulder.

picrolonate anion. Although ethylene dichloride was the better solvent for qualitative screening because of its low cutoff point, both benzene and chloroform are equally useful for quantitative work. The relatively long wavelength maximum of the anion permitted the use of solvents with high cutoff up to 300 nm. Furthermore, there was minimum overlap with original alkaloid bands, since most common alkaloids scarcely absorb above 310 nm (2, 3).

In constructing calibration curves with a blank of picrolonic acid, a wavelength of 362 nm afforded maximum differential between the picrolonate anion and nonionized keto form (Fig. 4) better than the 355 nm wavelength. At 362 nm and with $1 \times 10^{-4}M$ picrolonic acid, plots obeyed Beer's law in all cases in the general concentration range of 2–20 μg alkaloid base/ml.

Application to Dosage Forms.—The applicability of the method to commercial dosage forms was checked by analyzing 6 different, relatively simple preparations representing originally low (atropine, ephedrine, and codeine) and high UV-absorbing (quinine, strychnine, and emetine) alkaloids. The results which are shown in Table 3 confirm the suitability of the proposed method

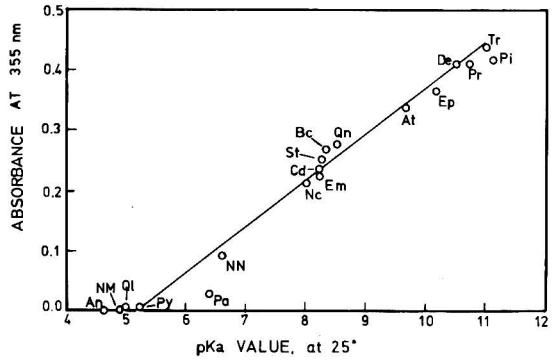

FIG. 5—Correlation of pKa of amine and intensity of anionic band of picrolonic acid at 355 nm in ethylene dichloride: See Table 2 for abbreviation symbols of individual alkaloids.

Table 3. Analysis of commercial alkaloid preparations

Preparation[a]	Alkaloidal salt content, mg/unit				
	Label claim	Found[b]	Added	Recovered,[b] total	Std dev., %
Atropine sulfate injection	1.0	0.98	1.0	2.01	1.24
Coramine-ephedrine drops	15.0	15.11	15.0	29.85	1.05
Codinal tablets	15.0	14.35	15.0	29.50	1.17
Emetine hydrochloride injection	30.0	28.85	30.0	58.65	1.31
Quinine sulfate tablets	150.0	151.55	50.0	201.61	1.23
Strychnine sulfate injection	1.0	1.05	2.0	3.10	1.06

[a] See text for detailed composition.
[b] Average of 5 determinations.

to analyze commercial preparations without interference from common excipients of tablets and formulations, or from non-basic ingredients such as nikethamide and phenobarbital. The former compound is neutral and did not interact spectroscopically with picrolonic acid, while the latter acidic compound was eliminated in the course of the alkaline partition of the procedure.

With more complex formulations and preparations containing alkaloid mixtures, a suitable method of separation should be employed (2, 3). In such cases recovery will be further limited by the skill and accuracy of the analyst.

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Spectrophotometric Determination of Allylisothiocyanate in Mustard Seed Oil

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Allylisothiocyanate is determined spectrophotometrically after reaction with 2,3-dichloro-1,4-naphthoquinone. For pure samples, the color intensity is proportional to allylisothiocyanate content in the range 0.8–3.0 mg/ml reaction mixture. A modified procedure is used to estimate allylisothiocyanate content of mustard seed oil. The reaction is linear for allylisothiocyanate concentrations in the range 40–240 μg/ml reaction mixture. Two mustard seed oil samples contained 0.995 ± 0.020 and 0.981 ± 0.019% allylisothiocyanate.

Allylisothiocyanate is a constituent of mustard seed oil (1). While mustard seed oil is used as a fungicide (2), germicide (3), and stabilizer for vitamin A and D preparations (4), allylisothiocyanate is used in therapy as an emetic and rubefacient (5). The various methods reported for the estimation of allylisothiocyanate include volumetric (6–8), gravimetric (9), spectrophotometric (10), and gas chromatographic (11) procedures. In the Pharmacopoeia of India assay (12), allylisothiocyanate is steam distilled from the mustard seed oil and estimated by Volhard's method which is time consuming and tedious.

Recently, isothiocyanates have been detected by the color reaction with 2,3-dichloro-1,4-naphthoquinone (13). We used this reagent to estimate allylisothiocyanate content in pure samples and expressed oil of mustard seed. Various reaction conditions of the test are standardized. Allylisothiocyanate is allowed to react with sulfanilamide to form the corresponding thiourea which is then allowed to react with 2,3-dichloro-1,4-naphthoquinone in the presence of ammonia. The procedure does not involve preliminary distillation of allylisothiocyanate. The results compare favorably with those obtained by the Indian assay (12).

METHOD

Reagents and Apparatus

Store all solutions and reagents at 20°C.

(a) *Allylisothiocyanate.*—(E. Merck). 2.122% w/v in freshly distilled $CHCl_3$.

(b) *2,3-Dichloro-1,4-naphthoquinone.* — 0.026% w/v in ethanol.

(c) *Ethanolic ammonia.*—Pass dry ammonia into absolute ethanol (Pharmacopoeia of India) until its weight increases ca 20%. Dilute resulting solution with absolute ethanol to obtain 10% w/v ammonia solution.

(d) *Sulfanilamide solution.*—1% w/v sulfanilamide (British Pharmaceutical Codex) in ethanol.

(e) *2,3-Dichloro-1,4-naphthoquinone and N^2-allyl-N^1-(p-sulfonamidophenyl)-thiourea.* — Synthesized by known methods (14, 15).

(f) *Mustard seed oil.*—Pharmacopoeia of India.

(g) *Spectrophotometer.*—Spectronic 20 (Bausch & Lomb) with 4 matched 10 ml cells with 1.0 cm light path.

Preparation of Standard Curve

Procedure A.—Pipet 1 ml allylisothiocyanate standard solution into 10 ml flask. Add 3 ml ethanolic ammonia. Immerse flask in 0°C ice bath. After 25 min, quantitatively transfer contents to 25 ml volumetric flask with aid of absolute ethanol. Add 11 ml 2,3-dichloro-1,4-naphthoquinone reagent and dilute to volume with absolute ethanol. Let mixture stand 10 min at room temperature. Measure absorbance at 520 nm against blank prepared simultaneously.

Procedure B.—Pipet 0.05 ml allylisothiocyanate standard solution into 25 ml flask. Add 5 ml sulfanilamide solution. Immerse flask in water bath at 60°C. After 90 min, cool solution to room temperature. Add 5 ml ethanolic ammonia. Immerse flask in 0°C ice bath. After 30 min quantitatively transfer contents to 25 ml volumetric flask. Adjust to final volume with 2,3-dichloro-1,4-naphthoquinone reagent solution. Let stand 15 min at room temperature. Measure absorbance at 540 nm against blank prepared simultaneously.

Preparation of Samples

Transfer 300 mg mustard seed oil to 25 ml flask with aid of 5 ml ethanol and continue as in *Preparation of Standard Curve, Procedure B,* beginning with "Add 5 ml sulfanilamide solution . . .". Determine amount of allylisothiocyanate by referring to standard curve.

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Results and Discussion

Adding 2,3-dichloro-1,4-naphthoquinone regent to the mixture of allylisothiocyanate and ammonia gives a purple solution. The maximum color intensity was obtained in the presence of 3 ml ammonia in Procedure A and 5 ml ammonia in Procedure B (Fig. 1a and 1b).

Maximum absorbance was obtained in the presence of 11 and 14 ml reagent solution in Procedures A and B, respectively (Fig. 2a and 2b).

The effect of temperature was studied at 0 and 24°C in Procedure A and 24 and 60°C in Procedure B. The highest absorbance was obtained for the allylisothiocyanate-ammonia mixture held 25 min at 0°C for Procedure A, and for the allylisothiocyanate-sulfanilamide mixture warmed 90 min at 60°C for Procedure B (Fig. 3a and 3b).

Color intensity was at a maximum in the final reaction mixture after 10 and 15 min in Procedures A and B, respectively (Fig. 4a and 4b). On further standing, the color intensity decreased somewhat.

Maximum absorbance was obtained in Procedure B for allylisothiocyanate reacted with 5 ml 1% w/v sulfanilamide solution (Fig. 5).

Under the experimental conditions of Procedure A, the color intensity was proportional to the amount of allylisothiocyanate in the range 0.8–3.0 mg/ml reaction mixture, while the Lam-

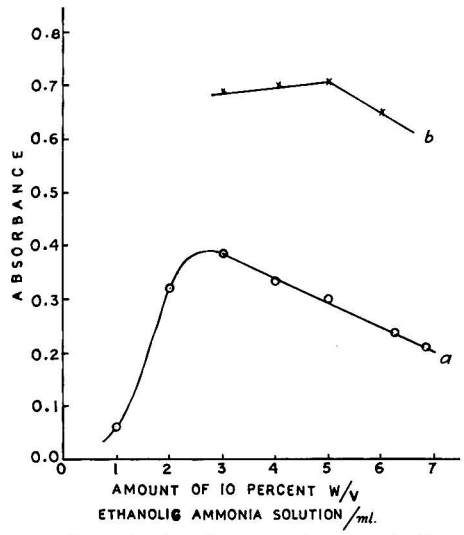

FIG. 1—Effect of ethanolic ammonia concentration on sensitivity: a, Procedure A; b, Procedure B.

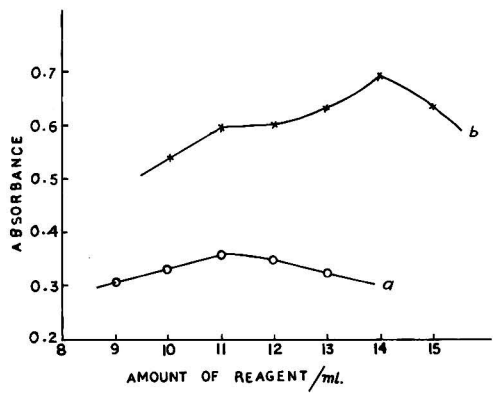

FIG. 2—Effect of 2,3-dichloro-1,4-naphthoquinone concentration on sensitivity: a, Procedure A; b, Procedure B.

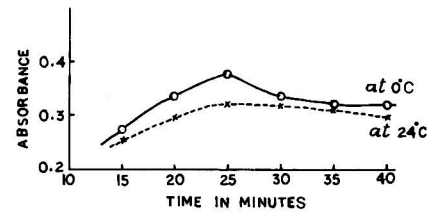

FIG. 3a—Effect of temperature on sensitivity (Procedure A).

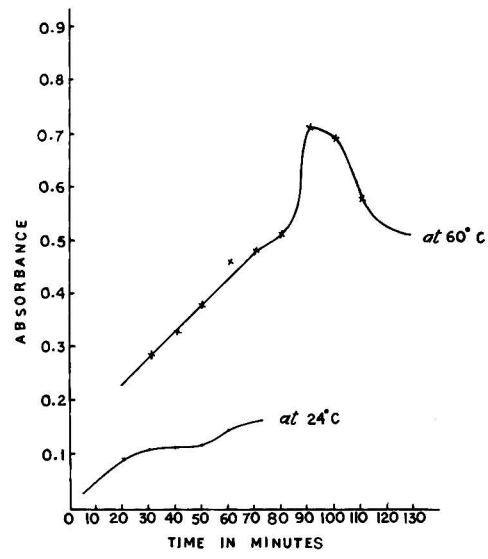

FIG. 3b—Effect of temperature on sensitivity (Procedure B).

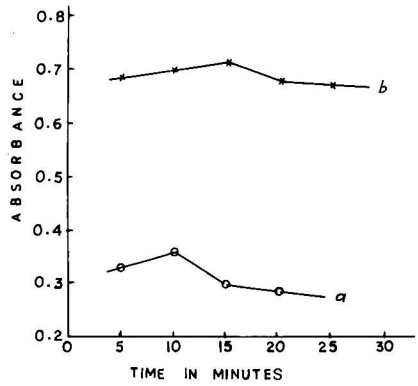

FIG. 4—Effect of time of reaction on sensitivity: a, Procedure A; b, Procedure B.

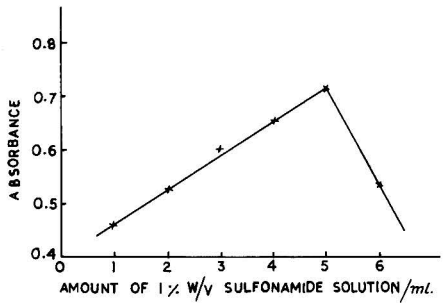

FIG. 5—Effect of sulfanilamide concentration on sensitivity (Procedure B).

bert-Beer law was obeyed in the range of 40–240 μg/ml reaction mixture for the conditions described under Procedure B.

The isothiocyanates form thiourethanes with absolute ethanol (16), so stock solutions of allylisothiocyanate were prepared in freshly distilled chloroform. However, attempts to use chloroform as the reaction medium gave markedly low color intensity. Therefore, ethanol was used as a reaction medium in the present work.

Ammonia reacts with allylisothiocyanate to give the corresponding allylthiourea in ethanolic medium (17). The thiourea thus formed reacts with 2,3-dichloro-1,4-naphthoquinone in the presence of ammonia to give a purple solution (13). However, the color intensity of the test is too low to assay mustard seed oil (by Procedure A) which contains only about a 1% w/v allylisothiocyanate. In addition, the reaction mixture separated into 2 layers in the presence of such a large quantity of oil.

It has been reported that arylthioureas are more sensitive to the test than alkylthioureas (13). Therefore, various aromatic amines such as aniline, *p*-aminobenzoic acid, and sulfonamides were allowed to react with allylisothiocyanate to form the corresponding thioureas. Sulfanilamide was the most suitable amine, because it does not react with the reagent (18). The N^2-allyl-N^1-(*p*-sulfonamidophenyl)-thiourea gives a purple product with maximum absorbance at 540 nm under Procedure B conditions.

The proposed method is applicable to the analysis of pure samples of allylisothiocyanate (Table 1). The modified method (Procedure B) is used to assay mustard seed oil (Table 2). The results are in good agreement with those obtained by the steam distillation method (12). Recoveries of known amounts of allylisothiocyanate added to mustard seed oil ranged between 97 and 102.5% (Table 3). Commonly

Table 1. Recovery (%) of allylisothiocyanate by the iodimetric and spectrophotometric methods[a]

Sample	Iodimetric method (19)	Spectrophotometric method	
		Procedure A	Procedure B
A	99.99±0.412	100.04±0.700[a]	99.62±0.500[a]
B	99.52±0.420	99.60±0.690	99.50±0.510

[a] The standard deviation is based on 10 experiments.

Table 2. Determination of allylisothiocyanate (% found) in mustard seed oil by silver nitrate and spectrophotometric methods

Sample	Silver nitrate method (12)	Spectrophotometric method, Procedure B
A	0.922±0.014[a]	0.995±0.020[a]
B	0.983±0.015	0.981±0.019

[a] The standard deviation is based on 7 experiments.

Table 3. Recovery of added standard allylisothiocyanate in mustard seed oil by spectrophotometric assay (Procedure B)

Analysis	Composite sample wt, mg	Found, mg	Rec.,[a] mg	Added, mg	Rec., %
1	201.1	3.135	1.155	1.100	101.4
2	201.1	3.135	1.155	1.100	101.4
3	202.2	4.125	2.145	2.200	97.0
4	202.2	4.235	2.255	2.200	102.5
5	203.3	5.225	3.245	3.300	98.3
6	203.3	5.280	3.300	3.300	100.0
7	204.4	6.490	4.510	4.400	102.4
8	204.4	6.435	4.455	4.400	101.2

[a] Recovery = mg found − mg assay value (in ethanol) before fortification. Assay value for each analysis = 1.980 mg.

found adulterants (12)—arachis, sesame, and cottonseed oil—do not seem to interfere in the estimation of allylisothiocyanate in mustard seed oil.

Acknowledgment

The authors wish to express their sincere thanks to college authorities for the facilities to carry out this work.

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PESTICIDE FORMULATIONS

High-Speed Liquid Chromatographic Determination of Phenothiazine in Commercial Pesticide Formulations

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Phenothiazine (thiodiphenylamine) in commercial pesticide formulations is analyzed by high-speed liquid chromatography and ultraviolet absorption detection. Phenothiazine is extracted from the formulation with methanol and injected into the liquid chromatograph. Methanol-water (1+1) is the mobile phase. The amount of phenothiazine is calculated from peak height ratios. The liquid chromatographic procedure is much faster than the infrared or colorimetric method and yields values in close agreement with both of these methods.

Introduced in 1935, phenothiazine (thiodiphenylamine) is one of the oldest pesticides known. It has a low toxicity to mammals and is used mainly as an anthelmintic wormer fed in salt or mineral supplements to control horn and face flies in cattle (1). A powdered form mixed with trichlorfon and piperazine is used as a wormer for horses. Its oxidation products also have a slight fungicidal effect (2).

We have 2 current analytical methods in our laboratory for the analysis of phenothiazine. The infrared (IR) method (3) requires a 3–4 hr extraction, while the AOAC colorimetric method (4) requires approximately 1 hr to complete. For this reason we investigated high-speed liquid chromatography (HPLC) and found it to provide a rapid and accurate method of analysis for phenothiazine in commercial pesticide formulations.

METHOD

Apparatus and Reagents

(a) *Liquid chromatograph.*—Varian 8500 with recorder and variable ultraviolet (UV) detector. Operating conditions: room temperature, pressure 70 atm, flow rate 70 ml/hr, methanol-water (1+1) mobile phase, wavelength 254 nm, chart speed 10″/hr, mev 10, absorbance range 0–1.0.

(b) *Liquid chromatographic column.*—Vydac reverse phase column, 50 cm × ¼″ od × 2.0 mm id, octadecyl silicone chemically bonded packed column (Applied Science Laboratories, Inc., PO Box 440, State College, PA 16801).

(c) *Methanol.*—Spectrographic grade (Burdick and Jackson Laboratories Inc., Muskegon, MI 49442).

(d) *Phenothiazine standard.*—(Available from West Chemical Products, Inc., Long Island City, NY 11101.) Weigh ca 30 mg recrystallized phenothiazine (from 10% solution in toluene) into 50 ml volumetric flask and dilute to volume with methanol. Prepare fresh daily.

(e) *o-Phenylphenol internal standard solution.*—Dissolve 300 mg *o*-phenylphenol (Dow Chemical Co., Midland, MI 48640) in 500 ml methanol.

Preparation of Sample

Weigh portion containing ca 0.6 mg phenothiazine/ml into 150 ml separatory funnel. Add 20.0 ml internal standard solution and shake 15–20 min. Pour methanol extract into centrifuge tube and centrifuge.

Table 1. Per cent phenothiazine found in 4 formulations, 6 replicate determinations

Detn	Formulation 1 (0.90%) supplement	Formulation 2 (2.0%) molasses block	Formulation 3 (40%) pellets	Formulation 4 (3.5%) medicated feed
1	0.821	1.83	40.23	3.70
2	0.825	1.81	40.72	3.85
3	0.813	1.74	41.40	3.68
4	0.834	1.88	41.31	3.65
5	0.836	1.76	41.34	3.79
6	0.824	1.86	40.96	3.73
Av.	0.824	1.81	40.99	3.73
Std dev.	0.003	0.05	0.45	0.07
Coeff. of var., %	0.37	2.76	1.10	1.87

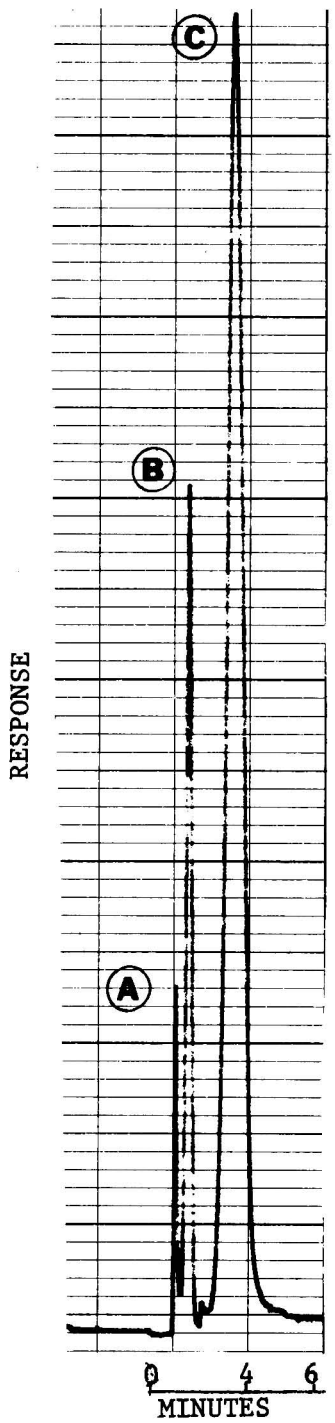

FIG. 1—Typical formulation chromatogram showing: A, unidentified peak; B, o-phenylphenol internal standard; and C, phenothiazine.

Determination

Using stop flow technique, inject 4 μl methanol solution into liquid chromatograph operating at conditions described. Calculate peak height ratio for sample and standard and determine per cent phenothiazine.

$$\text{Phenothiazine, } \% = (R'/R) \times (W'/W) \times (V/V') \times P$$

where R' and R = peak height ratios for standard and sample, respectively; W' and W = weights (g) of standard and sample used, respectively; V' and V = volume (ml) of standard and sample solutions, respectively; and P = purity (%) of standard.

Results and Discussion

Peak height was proportional to concentration of phenothiazine in the range 0.1–1.0 mg/ml. Nine consecutive injections of 4 μl standard samples indicated a ±1% reproducibility of peak height ratios, with a coefficient of variation of 0.35. Figure 1 is a chromatogram obtained for a 4 μl injection of a formulation sample.

Four commercial formulations containing phenothiazine were analyzed. Formulations 1, 2, and 4 were collected by Environmental Protection Agency inspectors during their visits to commercial distributors. Formulation 3 was obtained from the Environmental Protection Agency laboratory in Beltsville, MD. Phenothiazine concentrations in these samples ranged from 0.81 to 41.4%. The results presented in Table 1 show the precision of the method for 6 replicate analyses.

Formulation 4 was analyzed by IR and the AOAC UV method. Analytical results are given in Table 2. The HPLC method compares favorably with these 2 spectrophotometric procedures. Agreement between the IR and the UV

Table 2. Per cent phenothiazine found in 3.5% medicated feed formulation, 3 analytical methods, 6 replicate determinations

Sample	IR	AOAC-UV	HPLC
1	3.67	3.54	3.70
2	3.63	3.65	3.85
3	3.67	3.50	3.68
4	3.52	3.82	3.65
5	3.61	3.71	3.79
6	3.59	3.64	3.73
Av.	3.61	3.64	3.73
Std dev.	0.05	0.11	0.07
Coeff. of var., %	1.39	3.02	1.87

methods when compared with the HPLC method tends to confirm that phenothiazine is the only UV absorber eluted from the formulation solution at the measured retention time.

The HPLC method described for the analysis of phenothiazine formulations is simple, rapid, and accurate. It can easily be applied to include all commercial phenothiazine formulations that we receive in our laboratory.

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Chemical Composition of Technical Chlordane

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Technical chlordane, a multicomponent organochlorine insecticide, was separated into 26 peaks by temperature-programmed gas chromatography, using a 3% OV-17 column. Peaks were identified by using combined gas chromatography-mass spectrometry (GLC-MS) and, where individual standards were available, by comparing retention times on various columns. Technical chlordane constituents were also investigated by GLC-MS, using a Dexsil 300 column; 19–20 peaks were observed. Quantitation studies indicated differences in the per cent contributions of the constituents, particularly for heptachlor and *trans*-chlordane, in 2 different technical chlordane reference standards. For the same reference standard higher results were obtained by using ^{63}Ni electron capture detection than by using flame ionization detection. By using a combination of 4 GLC columns to overcome peak overlap, we determined that the 11 known constitutents of technical chlordane constituted approximately 40%.

Technical chlordane is an organochlorine insecticide comprising a mixture of chlorinated hydrocarbons. The earliest publications related to its chemical composition are those of Vogelbach (1) and March (2). Vogelbach isolated 7 crystalline compounds by column chromatography. These were 3 heptachloro, 3 octachloro, and 1 nonachloro constituents, while March (2), using the same technique, identified heptachlor, 2 isomers of chlordane, and small amounts of unreacted chlordene.

Gas-liquid chromatography (GLC) has also been used to separate the multicomponents of technical chlordane. The gas chromatogram of standard reference grade material showed at least 11 components separated on a 4.5% DC-11/0.5% Epon 1001 column. These were identified by Polen (1966, Velsicol Chemical Corp., Chicago, IL) by letters A to K (Fig. 1) in information submitted to the IUPAC Commission on Terminal Residues, Geneva, Switzerland, in 1966. Of these 11 labeled peaks the structural identities of only 5 constituents are known with certainty, i.e., chlordene, heptachlor, *trans*-chlordane, *cis*-chlordane, and *trans*-nonachlor. In 1969, Saha and Lee (3), using a 5% SE-30 column, labeled 14 peaks (Fig. 2) and made assignments based on retention times and mass spectral and occasionally infrared characteristics. Both these gas chromatograms were obtained using relatively nonpolar liquid phases. Minor variations in the chromatograms of technical chlordane are exhibited on mixed-phase columns. Recently, Harris (4) separated 21 component peaks of technical chlordane and from the analysis of a typical formulation found only 8.59 and 10.53% *cis*- and *trans*-chlordane, respectively.

The main components of technical chlordane are the isomers of chlordane, i.e., *cis*- and *trans*-chlordane. The estimated percentages of these 2 components given by the manufacturer are 19± 3 and 24±2%, respectively, i.e., approximately 43% (5). These figures are the "normalized" responses of *cis*- and *trans*-chlordane when the total GLC response of standard technical chlordane is equated to 100%. This numerical characterization of technical chlordane was designed to establish the constancy of composition of dif-

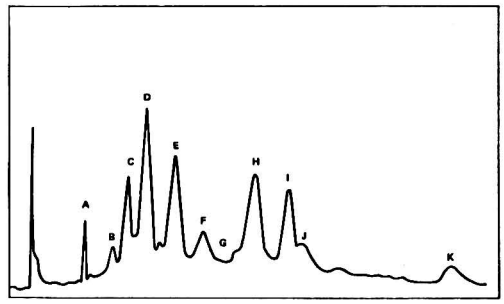

FIG. 1—Gas-liquid chromatogram of standard technical chlordane on a column of 4.5% DC-11/0.5% Epon 1001 on Chromosorb W (ABS) at 175°C with flame ionization detection. Reprinted with permission from *J. Agric. Food Chem.*, American Chemical Society, Washington, DC.

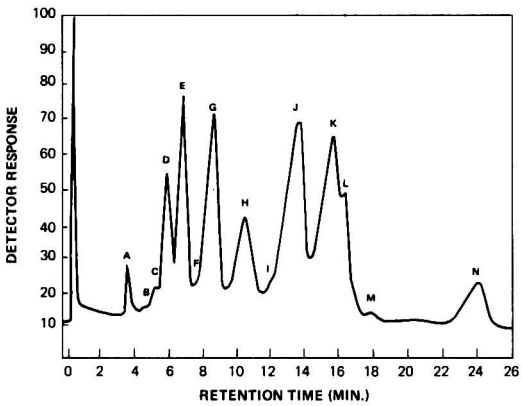

FIG. 2—Chromatogram of technical chlordane, using a column of 5% SE-30 on Chromosorb W at 180°C with thermal conductivity detection (3). Reprinted with permission from *Bull. Environ. Contam. Toxicol.*, Springer-Verlag, New York.

ferent batches and not as an indication of the precise analyses of technical chlordane.

In an effort to quantitate and ascribe structures to the remaining major constituents of technical chlordane, the present work was carried out by using GLC-mass spectrometry (MS) and synthesis techniques.

Experimental

GLC analyses were performed on a Pye 104 gas chromatograph fitted with ^{63}Ni (pulsed) and flame ionization detectors. GLC-MS analyses were performed on a Pye 104 gas chromatograph interfaced with a DuPont 21-490B mass spectrometer by means of a jet separator. The effluent from the column was split to the flame ionization detector and the mass spectrometer, where the total ion current was recorded. The parent ion (M‡) referred to in Tables 1 and 2 is the ^{35}Cl isotope.

Table 1. Gas chromatographic peak identification of technical chlordane constituents

3% OV-17 at 165°C, Fig. 3		5% SE-30 at 180°C, Fig. 2		4.5% DC-11/0.5% Epon 1001 at 175°C, Fig. 1	
Peak	Assignment	Peak	Assignment	Peak	Assignment
A, B	isomers of monochlorinated derivatives of tetrachlorochlordene	A	not identified	A	adduct of pentachlorocyclopentadiene and cyclopentadiene
C	chlordene	B	chlordene	B	chlordene (isomer)
D	chlordene isomer	C	not identified		
E	monochlorinated derivative of pentachlorochlordene	D	monochlorinated derivative of pentachlorochlordene	C	chlorinated derivative of peak A adduct
F	heptachlor (single compound peak)	E	heptachlor + chlordane isomer, mp 168–170°C	D	heptachlor + chlordene isomer
G	M‡ 336				
H	—	F	chlordane isomer	—	
I	M‡ 370 (heptachloro derivative)				
J	γ-chlordene	G	heptachlor isomer + chlordane analog, mp 135–137°C	E	γ- and β-chlordene (2:1 ratio)
K	mixture of 3 compounds				
L	M‡ 406	H	chlordane isomer (no m/e 270)	F	chlordane analog
M	β-chlordene				
N	M‡ 406 (+ 440 impurity)	I	not identified	G	chlordane analog
O	*trans*-chlordane	J	*trans*-(γ)-chlordane	H	*trans*-(γ)-chlordane
P	chlordane isomer M‡ 406	—		—	
Q	*cis*-chlordane	K	*cis*-(α)-chlordane	I	*cis*-(α)-chlordane
R	M‡ 454, an epoxide	L	chlordane isomer + nonachlor	J	*trans*-(δ)-nonachlor
S	*trans*-nonachlor	M	chlordane isomer	—	
T	chlordane isomer + heptachloro epoxide				
U	same as peak T	—		—	
V	decachloro compound				
W	*cis*-nonachlor				
X	compound (peak) K	N	nonachlor	K	dichlorinated α-chlordene (peak K)

Table 2. Comparison of retention time data and GLC-MS assignments on Dexsil 300

Fig. 4	Retention times of stds[a]	Fig. 5	GLC-MS of tech. chlordane[b]
A, B, C, D	—[c]	—	
E	compound C	A	M‡ 336 (m/e 236 + 100) compound C
F	heptachlor	B	heptachlor
G	α-chlordene	C	M‡ 336, α-chlordene
H	—	D	M‡ 370 (m/e 270 + 100), heptachloro isomer
I	mixture of β- and γ-chlordene	E/F	M‡ 336, mixture of γ- and β-chlordene
J	—	G	mixture, contains MW 404
K	—	H	M‡ 406 (m/e 235 + 100)
L	—	I	M‡ 336
M	*trans*-chlordane	J	M‡ 370 (m/e 235 + 135)
—	*trans*-nonachlor	K	M‡ 406 (+ small 440), mixture of *trans*-nonachlor and *trans*-chlordane
N	*cis*-chlordane	L	*cis*-chlordane
—		M	M‡ 454, nonachloro epoxide ($C_{18}H_3Cl_9O$)
O	—	N	M‡ 474, decachloro compound
P	—	O	M‡ 406
Q	*cis*-nonachlor	P	*cis*-nonachlor
R	compound K	Q	compound K
		R	M‡ 440 and 406, mixture
		S	M‡ 440 and 454, mixture
		T	M‡ 440
		U	M‡ 406

[a] 5% Dexsil 300 on Chromosorb W (AW) at 200°C (Fig. 4).
[b] 6′ × ⅛″ Dexsil 300 column coupled to DuPont 21-490B mass spectrometer interfaced with DuPont 21-09L MS data system (Fig. 5).
[c] No standards available.

The all-glass columns employed were:

(a) *Column I.*—3% OV-17 on 100–120 mesh Gas-Chrom Q for both GLC-MS and per cent composition analysis (Fig. 3). Before use the column was extensively conditioned as described previously (6). For both types of analysis the column was maintained at 165°C until after the appearance of *cis*-chlordane, and then programmed at 6°C/min for 20 min more (Fig. 3) to 285°C.

(b) *Column II.*—5% Dexsil 300 on 80–100 mesh acid-washed Chromosorb W. For peak comparison with standards the column was monitored at 200°C (Fig. 4). For GLC-MS work, the column was started at 165°C followed by programming at 6°C/min (Fig. 5).

(c) *Column III.*—4% OV-101/6% OV-210 on 80–100 mesh Chromosorb W operated at 185°C for per cent composition analysis only.

(d) *Column IV.*—3% SE-30 on 80–100 mesh Gas-Chrom Q operated at 195°C for per cent composition analysis only.

Analytical standards of compound C, α-, β-, γ-chlordene, and compound K were supplied by the Velsicol Chemical Corp., Chicago, IL.

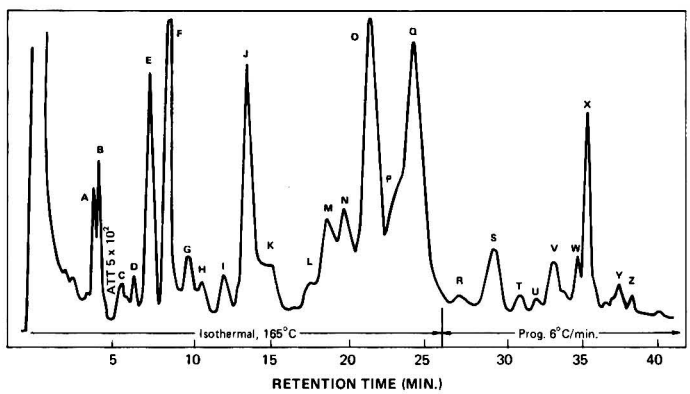

FIG. 3—Chromatogram of standard technical chlordane, using a column of 3% OV-17 on Gas-Chrom Q, at temperatures shown, with flame ionization detection.

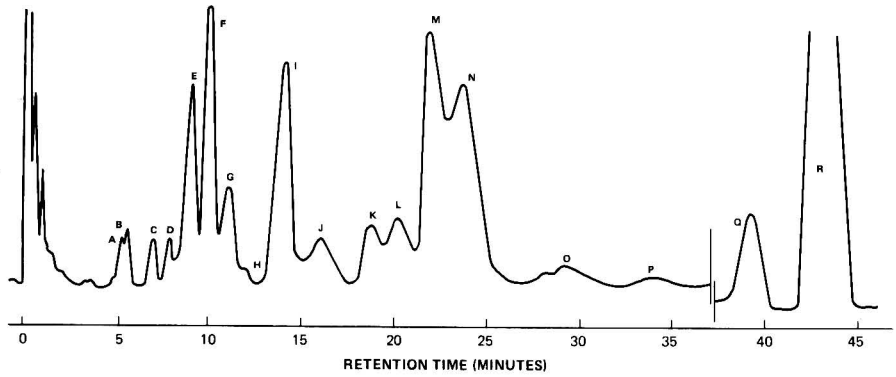

FIG. 4—Chromatogram of standard technical chlordane, on a column of 5% Dexsil 300 on Chromosorb W (AW) at 200°C with flame ionization detection.

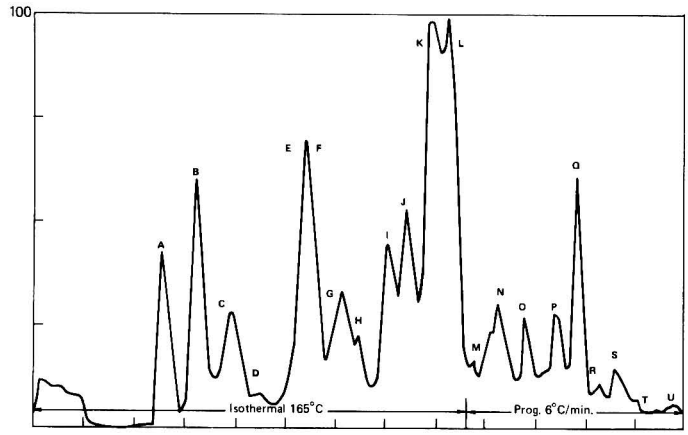

FIG. 5—Chromatogram obtained from the GLC-MS computer printout for standard technical chlordane on 6′ × ⅛″ Dexsil 300 column.

Results and Discussion

A number of column substrates were investigated to determine which gave the best separation of the individual components. A 3% OV-17 column and temperature programming after appearance of the main *cis*- and *trans*-chlordane constituents separated 26 peaks (Fig. 3), 9 of which appeared after *cis*-chlordane (Fig. 3, peak Q). From the mass spectra of these 26 peaks, tentative identities were assigned and, where possible, cross-correlated with the findings of Polen (Fig. 1) and Saha and Lee (Fig. 2). As expected, the identities of chlordene, heptachlor, *cis*- and *trans*-chlordane, and *trans*-nonachlor were in agreement (Table 1). However, the probable identities of some of the other major and minor constituents were different. Polen's (Fig. 1) peak A was postulated as possessing structure 1 (Fig. 6); however, separation on the OV-17 column resolved this early eluting peak into 2 components. Each component (peaks A and B, Fig. 3) had a molecular weight of 302 and mass spectral fragmentation patterns consistent with stereoisomeric forms of structure II (Fig. 6). Retro-diene fragments at m/e 202 and 100 indicated the 4:1 distribution of the chlorine atoms in the 2 cyclopentadiene ring systems.

On OV-17, peaks C and D were assigned as chlordene isomers, corresponding to previous findings of peak B of Polen and Saha and Lee as chlordene (Table 1). Livăr *et al.* (7) had previously shown the existence of 2 chlordene isomers (structures III and IV, Fig. 6) in heptachlor containing unreacted chlordene. The chlor-

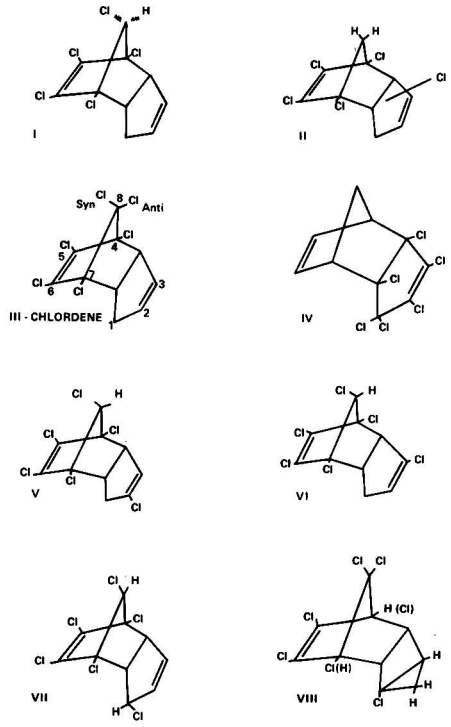

FIG. 6—Structure of chlordene and various related compounds.

dene with structure III predominates and compound IV is readily isomerized to III at temperatures above 160°C.

In agreement with previous work, peak E (Fig. 3) was a monochlorinated derivative of pentachlorochlordene. This constituent is normally referred to as compound C (5). The retention time was compared with those of the $CrCl_2$ mono-dechlorinated products of 2- and 3-chlorochlordene (Fig. 6, V and VI, respectively) (8) and was different from peak E. Therefore, its structure was tentatively postulated as VII (Fig. 6). Compound VII was synthesized from 1-hydroxychlordene in 2 steps, first by reaction with $CrCl_2$ to remove the *anti*-chlorine atom at position 8 and then chlorination of the OH with $SOCl_2$-pyridine. Although VII and peak E had identical retention times on a 4% OV-101/6% OV-210 column, more conclusive proof of identity was required. As this work was in progress small quantities of compound C, isolated from technical chlordane, became available so that nuclear magnetic resonance (NMR) analysis was possible. Comparison of the NMR and melting points indicated that compound C did not possess the structure depicted by VII. The NMR spectrum of compound C exhibited no resonance peaks in the olefinic or *anti*-C_6 proton regions. All 6 proton signals for peak E appeared in the region 6.3–7.6 τ. The IR spectrum showed the ClC=CCl grouping at 1600 cm^{-1}, while the mass spectrum gave retro-diene fragments at m/e 236 and 100. From such spectral and synthetic data it is postulated that compound C has a structure consistent with VIII (Fig. 6). Although other isomeric structures can be postulated, placing the H at position 4 follows from NMR comparisons with the endosulfan series of compounds containing 5 chlorine atoms on the bicyclo [2.2.1] hexene ring (9).

Retention times and mass spectral characteristics agreed well for heptachlor in all 3 studies. On OV-17 heptachlor was monitored as a single peak. Polen (1966, Velsicol Chemical Corp., Chicago, IL) indicated that the peak he designated as peak D (Fig. 1) contained heptachlor as well as a chlordene isomer; this was most probably α-chlordene, which on OV-17 appears as peak G. Saha and Lee (3) assigned their peak G as comprising a heptachlor isomer plus a chlordane analog (mp 135–137°C), while Polen's corresponding peak E was attributed to a mixture of the γ- and β-isomers of chlordene present in a 2:1 ratio (Table 1). In the present study, peak J (Fig. 3) was identical to authentic γ-chlordene, while β-chlordene appeared slightly later as peak M.

α-, β-, and γ-chlordene are empirical isomers of chlordene formed during the rearrangement of chlordene heated in the presence of organic peroxides (10–12). A mixture containing primarily the β- and γ-isomers was introduced in commercial form (Bandane®) in 1967 as an experimental crab grass herbicide by the Velsicol Chemical Corp. The structures of the α-, β-, and γ-chlordene isomers have been elucidated only recently (13) and are shown in Fig. 7. α-Chlordene can add Cl_2 to give an empirical isomer of chlordane ($C_{10}H_6Cl_8$) which is identical to Polen's peak K of technical chlordane.

On the OV-17 column, peak I (Fig. 3) proved to be a heptachlor isomer whose mass spectrum gave retro-diene fragments m/e 236 and 100. This constituent was tentatively identified as 3-chlorochlordene. Peaks K and N were mixtures, while L was an octachloro compound. No struc-

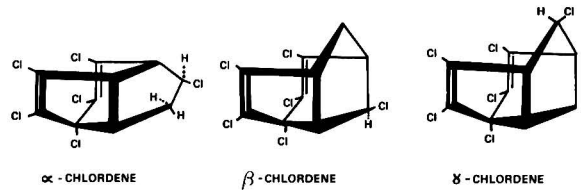

FIG. 7—Structures of the isomeric α-, β-, and γ-chlordenes.

tural assignments were made for these peaks. Peaks O and Q are the *trans*- and *cis*-isomers of chlordane, respectively, and peak P is a chlordane ($C_{10}H_6Cl_3$) isomer. Although *trans*-nonachlor is well defined on a temperature-programmed OV-17 column, it appears as a shoulder peak following *cis*-chlordane on an SE-30 or OV-1 column.

Nine peaks were observed after *cis*-chlordane on the temperature-programmed OV-17 column. Three of these peaks, i.e., peaks R, T, and U, exhibited fragmentation patterns, which indicated the presence of epoxy compounds. The fragmentation patterns obtained for these peaks were in accord with the findings of Damico *et al.* (14) for dieldrin and heptachlor epoxide. In addition to a strong M − 35 ion, an M − 35 + 28 ion, corresponding to M − (Cl + CO), indicated the presence of an epoxide group. In the absence of an epoxide group the M − 35 ion had a low intensity. This may be an indication that reference grade technical chlordane (6) changes with time due to air oxidation and, therefore, has a definite shelf-life. Such changes, if pronounced, would affect its usefulness as an analytical reference standard unless certain precautions are taken.

The mass spectra indicated that peaks S and W were both nonachloro compounds; peak S had the same retention time as *trans*-nonachlor, and W had the same retention time as the *cis*-isomer. Peak V exhibited the mass spectrum of a decachloro compound, while peak X was identical with the product of chlorination obtained from α-chlordene and described by Polen as compound K, i.e., peak K, Fig. 1.

The identities of the technical chlordane constituents were also investigated, using a Dexsil 300 column (Figs. 4 and 5) and the same mass spectrometer as that used in the OV-17 experiment. Assignments based on retention time data of individual standards and the GLC-MS study are compared in Table 2. In this instance, 19–20 peaks were obtained; the retention times of 10 of these agreed with those of the 10 available standards. Again, β- and γ-chlordene appeared as a single peak as previously found with the SE-30 and OV-101/OV-210 columns. Also no separate *trans*-nonachlor peak was observed because its retention time coincided with the *cis*- and *trans*-chlordane peaks. *Cis*-nonachlor, compound K, and a decachloro peak were readily discernible on the Dexsil column. Indications of epoxy compounds were also obtained in the later-eluting peaks.

Table 3 gives the per cent composition of technical chlordane, calculated from 11 individual standards. All 11 standards could not be used with the OV-17 column because peak overlap and lack of temperature programming (for peaks Q through X) precluded quantitative calculation of these later-eluting peaks. To determine these constituents, a 3% SE-30 column was used. Two technical chlordane reference standards, dated 1967 and 1974, were analyzed using this SE-30 column. The major differences were in the percentages of heptachlor and *trans*-chlordane. Heptachlor accounted for only 4.4% on the OV-17 column where it had been shown by GLC-MS to constitute a single constituent peak, while 5.71 and 4.99% were calculated from peaks on the SE-30 column. A similar situation occurs with *trans*-chlordane. The precision data for heptachlor and *trans*-chlordane on the SE-30 column were 1.8 and 3.2%, respectively. This indicates that these minor differences were in the composition of the samples and not due to variation of the analytical results.

For comparison, the per cent composition calculated from peaks on an OV-1 column, which has the same resolution characteristics as the SE-30 column, was substantially higher for all 6 constituents detected by an electron capture detector. The 6.4, 10.3, and 9.8% figures for

Table 3. Approximate composition of technical chlordane (TC)

Constituent	OV-17[a] Fig. 3 peak	OV-17[a] TC1, %	SE-30[a] TC1,[b] %	SE-30[a] TC2,[b] %	OV-1[c] TC1, %
Chlordene	C	0.60	0.19	0.05	—
Compound C	E	3.37	2.70	3.31	4.2
Heptachlor	F	4.40	5.71	4.99	6.4
α-Chlordene	G	1.10	—	—	—
γ-Chlordene	J	6.00	6.9	6.3	8.5
β-Chlordene	M	2.8			
trans-(γ)-Chlordane	O	7.93	9.60	8.90	10.3
cis-(α)-Chlordane	Q	—	8.01	7.08	9.8
trans-(δ)-Nonachlor	S	—	1.55	3.25	6.3
cis-(β)-Nonachlor	W	—	3.58	3.97	—
Compound K	X	—			

[a] Flame ionization GLC was used for quantitation. All values are averages of 3 replicates.
[b] Two technical chlordane standards were analyzed on the 3% SE-30 column at 185°C. TC1 = American Association of Pesticide Control Officials technical chlordane standard, 1967; TC2 = Velsicol technical chlordane standard, April 1974.
[c] A 3% OV-1 column at 180°C with ^{63}Ni detection was used for quantitation.

heptachlor and *trans*- and *cis*-chlordane compare with 8.46, 10.53, and 8.5% respectively, found by Harris (5) with an electron capture detector (the column was not specified). The values 6.9, 6.3, and 8.5% for combined γ- and β-chlordene (they appeared as a single peak) on SE-30 and OV-1 were calculated by using a 1:2 mixed standard of β- and γ-isomers (6). Similarly, the peak comprising *cis*-nonachlor and compound K was obtained by using a 3:2 mixed standard. We calculated this ratio by analysis of the individual components on a mixed OV-101/OV-210 column which yielded baseline separation. Using ^{63}Ni detection, *cis*-nonachlor and compound K were calculated at 1.7 and 1.0%, respectively. Therefore, from Table 3 it can be calculated, using the flame ionization detector results singly or in combination, that the 11 known constituents of technical chlordane constitute only 37.85–40.50%. In a technical sample, compounds having different chlorine contents but the same carbon content can co-chromatograph. Since the electron capture detector is more sensitive to changes in chlorine content than in carbon content, the response to the technical sample compared with the standard will be enhanced.

Acknowledgments

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Ultraviolet and Infrared Analysis of Rotenone: Effect of Other Rotenoids

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Biases evidenced by past collaborative trials of ultraviolet (UV) and infrared (IR) methods for rotenone can be caused by interference from other rotenoids that occur in extracts of cubé and derris. In the UV analysis, sumatrol, rotenolone, and deguelin cause positive errors of decreasing magnitude; elliptone, toxicarol, tephrosin, dehydrodeguelin, and dehydrorotenone produce negative biases of increasing magnitude. In the IR analysis, error depends on rotenoid concentration relative to rotenone concentration. At equal proportions, bias is positive with toxicarol and deguelin and negative with other rotenoids. The baseline version of the IR method has an inherent positive bias of approximately 8% but is superior to the base point version because of greater insensitivity to deguelin. The official first action UV method for the determination of rotenone in derris and cubé powder, 6.162–6.163, has been deleted, and the IR method, 6.164–6.165, has been revised to exclude derris products.

Two new procedures for the analysis of rotenone in plant extracts are reasonably rapid and apparently accurate: One is based on gas-liquid chromatography (GLC) (1), the other on high-performance liquid chromatography (HPLC) (2). These methods should be evaluated for possible adoption as official AOAC methods for rotenone. The problem is which of the existing AOAC methods should be used for comparison.

At present there are 3 official AOAC methods for the determination of rotenone in cubé and derris powder: an official final action method based on crystallization (3), and 2 official first action methods based on ultraviolet (UV) spectroscopy (4) and infrared (IR) spectroscopy (5). Comparison of these 3 methods in repeated cooperative trials (6–11) has disclosed inexplicable and occasionally large biases among them. Before attempting to compare either the GLC or the HPLC method with any of the existing official methods in cooperative trials, the cause of these biases should be determined so that results may be interpreted correctly.

Variable results between the UV and IR methods apparently are due to materials in cubé extracts (derris has not been tested) that interfere with absorption measurements by either method or both. The most likely suspects are compounds of closely related structure known to occur in these extracts. However, the effect of other rotenoids has never been determined for the UV and IR methods. This study supplies that needed information. Without considering non-rotenoid interfering materials, the data offer a reasonable explanation for past discrepancies which will facilitate future evaluations of new assay procedures.

Experimental

With the exception of 6aβ,12aβ-rotenolone, all rotenoids were characterized earlier at the Northern Regional Research Laboratory (12). Rotenolone was synthesized by the method of Crombie and Godin (13), which gave white crystals (mp, 113–118°C; IR peaks, 1670, 1615, 1520, 1460, 1360, 1335, and 1300 cm^{-1}; UV peaks, 237 and 293 nm). All rotenoids were analyzed via GLC (1) and found to be >95% pure (i.e., free from other rotenoids). Only sumatrol contained a detectable amount of rotenone, which had no significant effect on the results reported.

UV measurements were made with a Beckman Model DK-2A recording spectrophotometer set at a chart scale of 10 nm/cm and a speed of 36 nm/min. The rotenoid concentration was 10 μg/ml in absolute ethanol. Each rotenoid solution was assayed separately by the official method for rotenone (4).

IR measurements were made with a Perkin-Elmer Model 337 grating spectrophotometer at a scanning speed of approximately 120 cm^{-1}/min with 0.1 mm sodium chloride cells. Chloroform solutions containing 5 mg rotenone/ml and 0, 2.5, 5, 7.5, or 10 mg of the other rotenoids/ml were assayed according to the official method (5).

Results and Discussion

Rotenoids in Plant Extracts

In fresh cubé root extracts, the only rotenoids present are rotenone and deguelin, together with

small amounts of their primary oxidation products (6aβ,12aβ-rotenolone and tephrosin). Most likely the latter preexist in plants (*see* discussion in ref. 14), but also they form readily by air oxidation in solution (15). In limited experience with cubé at this laboratory, deguelin concentrations have ranged from approximately 50 to 100% that of rotenone (1, 16). Generally, the tephrosin concentration has been from 5 to 10% that of deguelin; presumably, the ratio of rotenolone to rotenone is similar. In old extracts or stored commercial formulations, the parent rotenoids may exist almost entirely as their oxidation products (1): rotenolone, tephrosin, 6a,12a-dehydrorotenone, 6a,12a-dehydrodeguelin, and smaller amounts of further degradation products (15).

Derris root extracts are more complex chemically than cubé extracts. Commercial derris root may be either *Derris elliptica, D. malaccensis,* or a mixture of the 2. *D. elliptica* contains rotenone, deguelin, and elliptone. Six samples examined at this laboratory (1) contained from 51 to 123% as much deguelin and from 19 to 27% as much elliptone as rotenone. *D. malaccensis* contains sumatrol, α-toxicarol, and malaccol, which are 11-hydroxy derivatives of rotenone, deguelin, and elliptone, in addition to the parent compounds. One variety (*Sarawakensis*) contains the 11-hydroxy rotenoids, but little or no rotenone (17). In fresh extracts, any of the above 6 rotenoids may be present, together with small amounts of their corresponding 12a-hydroxy derivatives. Old extracts and stored commercial products may be expected to contain larger amounts of the latter compounds, together with appreciable amounts of the 6a,12a-dehydroderivatives and other oxidation products.

All cubé rotenoids mentioned were included in this study. Of the additional rotenoids found in derris, elliptone, sumatrol, and toxicarol were available; malaccol and oxidation products of these 4 rotenoids were not.

Effect of Rotenoids on Ultraviolet Method

Each rotenoid was measured individually by the standard 3-wavelength method. The apparent amount of rotenone given by other rotenoids compared with the result for an equal concentration (by weight) of rotenone ((rotenoid result × 100)/rotenone result) is as follows: sumatrol 210, 6aβ,12aβ-rotenolone 105, deguelin 9, elliptone −13, α-toxicarol −18, tephrosin −31, 6a,12a-dehydrodeguelin −230, 6a,12a-dehydrorotenone −235. So, the UV method is susceptible to interference. The high value for sumatrol makes the technique unsuitable for derris. Even for cubé the method is unreliable because of large biases caused by rotenolone, dehydrodeguelin, and dehydrorotenone. The small amounts of these compounds (especially rotenolone) in fresh extracts increase on storage. Moreover, there is no way of telling from the UV spectrum when these compounds may be present in appreciable quantities.

In past trials, rotenone results for cubé extracts were 9% higher on the average by UV analysis than by crystallization (Table 1). Considering our results, such bias can be explained as the influence of deguelin and rotenolone in extracts, although possible bias in the crystallization method and interference in the UV method by other extract components are not ruled out.

Effect of Rotenoids on Infrared Method

Unlike the UV technique for rotenone, it is not feasible to obtain IR spectra of individual pure compounds and predict results with their mixtures: The IR peaks are sharp and narrow and the exact position of the interfering peak

Table 1. Results of past trials comparing UV or IR methods with the official AOAC crystallization method[a]

No. of samples	No. of colls.	Ref.	Average bias,[b] % UV	IR Base point (8)	IR Baseline (5)
Fresh Cubé Extracts					
2	15	(6)	13	—	—
3	15	(7)	6	—	—
3	5	(8)	5	7	2
2	1	(9)	0	−1	−8
5	13	(11)	11	—	4
Commercial Products or Resins[c]					
15	1	(18)	12	—	—
2	15	(7)	10	—	—
2	1	(8)	−1	26	9

[a] Results of the 1963 trial (10) with all 3 methods were omitted because of large, unexplained differences between laboratories.

[b] Compared with official AOAC crystallization method (3).

[c] "Resins" means dried plant extracts.

is critical in determining whether it affects either the rotenone peak height at 7.65 μm or the baseline points at 7.57 or 7.75 μm. For this reason, we tested the effect of contaminants with solutions of rotenone containing 0, 50, 100, 150, or 200% of other rotenoids. Results were calculated by both the original base point method of Knoerlein (8) and the present baseline method of Samuel (5, 19) (Figs. 1 and 2).

The 2 IR versions are in general similar. Both show excessive interference from toxicarol; hence they are unsuitable for analyzing derris extracts. Compared with the base point method, the baseline method gives high results with rotenone alone, less positive bias of rotenone results with equal or greater amounts of deguelin, and more of a negative bias with the remaining rotenoids.

The positive bias with rotenone alone in the baseline version is a result of measuring the

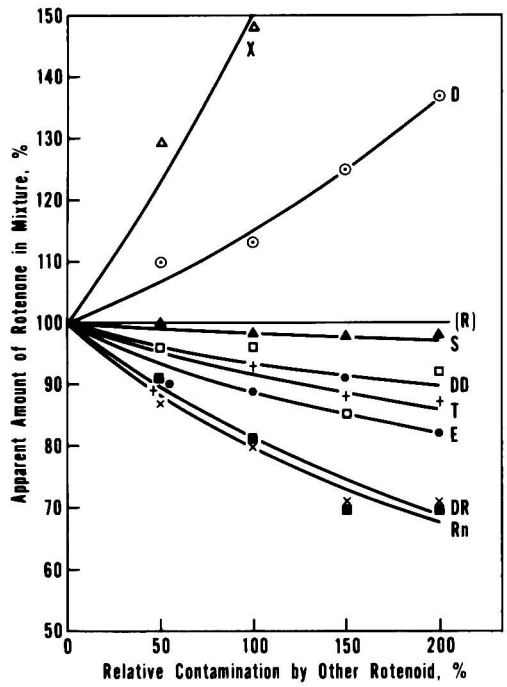

FIG. 1—Effect of other rotenoids on the analysis of rotenone by the base point IR method (8).

Curves represent rotenone (5 mg/ml chloroform) together with varying proportions (0, 2.5, 5, 7.5, and 10 mg/ml) of α-toxicarol (X, △), deguelin (D, ⊙), sumatrol (S, ▲), 6a,12a-dehydrodeguelin (DD, □), tephrosin (T, +), elliptone (E, ●), 6a,12a-dehydrorotenone (DR, ■), and 6aβ,12aβ-rotenolone (Rn, x). The horizontal line at 100% represents rotenone (R) alone.

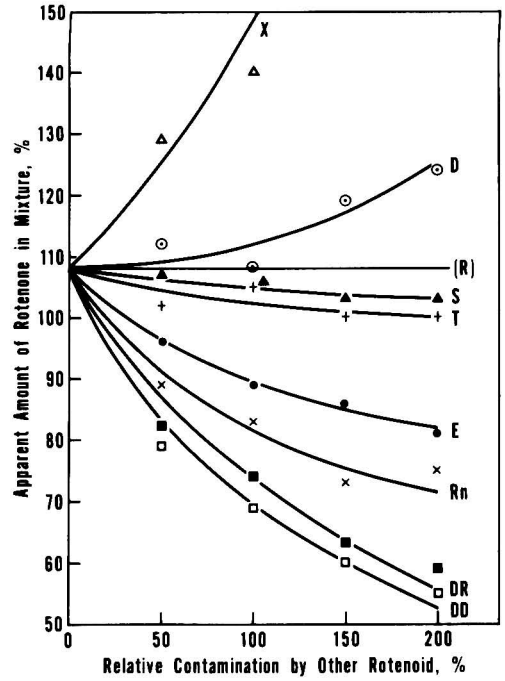

FIG. 2—Effect of other rotenoids on the analysis of rotenone by the baseline IR method (5). Abbreviations and symbols same as in Fig. 1. Horizontal line at 108% represents rotenone alone (see text for explanation).

standard peak height as the distance from the peak at 7.65 μm to the base point at 7.57 μm, and measuring the sample peak height as the distance from the peak at 7.65 μm to the baseline between 7.57 and 7.75 μm. Since absorbance is less at 7.75 μm than at 7.57 μm with pure rotenone (cf. Fig. 1, part 5, in ref. 8), the peak height is greater if measured to the baseline (as in the sample) than if measured to the base point (as in the standard). In the base point method of Knoerlein (8), of course, both sample and standard peaks are measured the same way.

Other rotenoids which do not absorb in the 7.57–7.75 μm region would not affect either method, but all rotenoids tested showed at least some absorption in this critical region. Those absorbing at 7.65 μm increased the apparent rotenone concentration the same amount in each version. Those absorbing at the base point at 7.57 μm decreased the apparent rotenone concentration more for the base point method than for the baseline version, which uses roughly the

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average of absorption at 7.57 and 7.75 μm. Those absorbing at 7.75 μm decreased the apparent rotenone concentration only in the baseline method. Those absorbing at more than 1 of these 3 critical wavelengths gave intermediate effects.

It is obvious from this study that the IR method for rotenone is sensitive to the presence of other rotenoids, although less so than the UV method. The most important effect is due to deguelin which is always present in cubé or other rotenoid plant extractives at significant levels (50–200% that of rotenone). Fortunately, the other rotenoids in cubé extracts produce an opposite bias; hence the errors tend to cancel each other. Moreover, from a practical standpoint the errors are in the "right" direction; deguelin causes rotenone to be overestimated, but is itself a good insecticide (20). Also, rotenone and deguelin oxidation products cause negative errors so that the rotenone content of stored samples or formulated products will not be overestimated because of them (these compounds are of little value as insecticides). Most important, however, is the possibility that the presence of interfering rotenoids could be estimated from other portions of the IR spectrum and suitable corrections could be made. This possibility will be explored in a future report.

Although this IR study does not rule out nonrotenoid interfering compounds in cubé extracts, there is no direct evidence for their presence. The results in past collaborative trials have been reasonably close to those of the crystallization method (Table 1). The extremely high biases reported by Rund (9) for 2 commercial samples of cubé resin may have been the result of unusually high deguelin-to-rotenone ratios (i.e., 150–200:100). The higher biases he found by the base point compared with those found by the baseline modification are consistent with this hypothesis.

Now that the major sources of error are better understood, the AOAC IR method should be satisfactory for evaluating the newer GLC and HPLC methods: the 2 chromatographic methods can be used to quantitatively determine the amounts of other rotenoids in the extracts and suitable corrections can be made for the bias that they produce in the IR method. This should be better than using the AOAC crystallization method as a reference because it is cumbersome and could easily be as biased.

Recommendations

It is recommended—

(*1*) That the UV method for rotenone be deleted.

(*2*) That the IR method for rotenone be deleted for derris products, but be temporarily retained in official first action status for cubé analysis, pending possible improvement and further testing.

(*3*) That more reliable methods be sought for the analysis of rotenone and other rotenoids to replace or supplement the IR method for rotenone.

Acknowledgment

I thank W. F. Kwolek, Biometrician, North Central Region, Agricultural Research Service, U.S. Department of Agriculture, stationed at the Northern Laboratory, for statistically evaluating the results of previous collaborative trials by others.

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This report of the Associate Referee was presented at the 89th Annual Meeting of the AOAC, Oct. 13–16, 1975, at Washington, DC.

The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee A and were adopted by the Association. *See* (1976) *JAOAC* **59**, 380.

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The mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

Gas-Liquid Chromatographic Determination of Pentachloronitrobenzene in Pesticide Formulations

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A gas-liquid chromatographic method has been developed to determine pentachloronitrobenzene (PCNB) in pesticide formulations including dusts, powders, granules, liquids, and fertilizers. Captan, disulfoton, and Terrazole do not interfere. Samples are extracted with chloroform, and an aliquot is mixed with an equal volume of internal standard solution containing *o*-terphenyl. PCNB is chromatographed on a 5% SE-30 column and quantitated by peak height ratios. The method has been subjected to a ruggedness test which indicates little sensitivity to changes in extraction and chromatographic conditions.

Pentachloronitrobenzene (PCNB, Terrachlor) is a chlorinated hydrocarbon developed by Olin Mathieson Chemical Co. in 1950 and used as a soil fungicide and seed disinfectant. Commercial dust, powder, liquid, granular, and fertilizer formulations which contain PCNB alone or in combination with disulfoton, captan, or Terrazole are available. The PCNB guarantees in these formulations range from 0.5 to 75%. Therefore, an analytical method for PCNB must be applicable to a variety of formulations.

There is no official AOAC method of analysis for PCNB. A gas-liquid chromatographic (GLC) method has been reported for PCNB in fertilizers (1). This paper presents a similar method, employing a different internal standard and a different GLC column.

METHOD

Apparatus and Reagents

(a) *Gas chromatograph with recorder.*—With flame ionization detector and 6′ × ¼″ od (4 mm id) glass column packed with 5% SE-30 on 80–100 mesh Chromosorb W (dimethylchlorosilane-treated) (Analabs). Condition newly packed column 24 hr at 285°C with low nitrogen flow. Operating conditions: temperatures (°C)—column 175 (isothermal), detector 250, injector 200; adjust nitrogen carrier gas to give desired elution profile (*see* Fig. 1); adjust hydrogen and air as recommended for detector by manufacturer.

(b) *PCNB standard solution.*—2.0 mg/ml $CHCl_3$. Weigh 0.2 g PCNB into 100 ml volumetric flask and dilute to volume with $CHCl_3$.

(c) *Internal standard solution.*—0.8 mg/ml $CHCl_3$. Weigh 0.2 g *o*-terphenyl (Eastman Kodak Co.) into 250 ml volumetric flask and dilute to volume with $CHCl_3$.

(d) *Mixed standard solution.*—1.0 mg PCNB + 0.4 mg *o*-terphenyl/ml. Pipet 25 ml each of PCNB and internal standard solutions into vial and mix.

Preparation of Sample

(a) *Solid formulations.*—Grind 100 g well mixed fertilizer or grindable granules to pass 1 mm sieve. Weigh portion of well mixed, ground sample equivalent to 0.2 g PCNB into 250 ml glass-stoppered Erlenmeyer flask. Add 100 ml $CHCl_3$, stopper, and shake 2 hr on rotary shaker. Let insoluble matter settle.

(b) *Dusts, powders, and nongrindable granules.*—Weigh portion of well mixed sample equivalent to 0.2 g PCNB into 250 ml glass-stoppered Erlenmeyer flask and proceed with extraction as for solids.

(c) *Liquids.*—Weigh portion of well mixed sample equivalent to 0.2 g PCNB into 100 ml volumetric flask and dilute to volume with $CHCl_3$. Mix.

(d) *Solution for analysis.*—Pipet 10 ml sample extract and 10 ml internal standard into vial, cap, and mix.

Determination and Calculation

Inject 4 μl aliquots of mixed standard solution until variation in peak height ratios of PCNB to *o*-terphenyl is ca 1%. Adjust GLC operating conditions to give peak heights ca 60–80% full scale. Inject mixed standard, inject sample twice, and then repeat injection of mixed standard. Calculate peak height ratios (PCNB:*o*-terphenyl) for the 2 mixed standard and sample injections. Average peak height ratios and calculate per cent PCNB:

$$\text{PCNB, } \% = (R/R') \times (W'/W) \times P$$

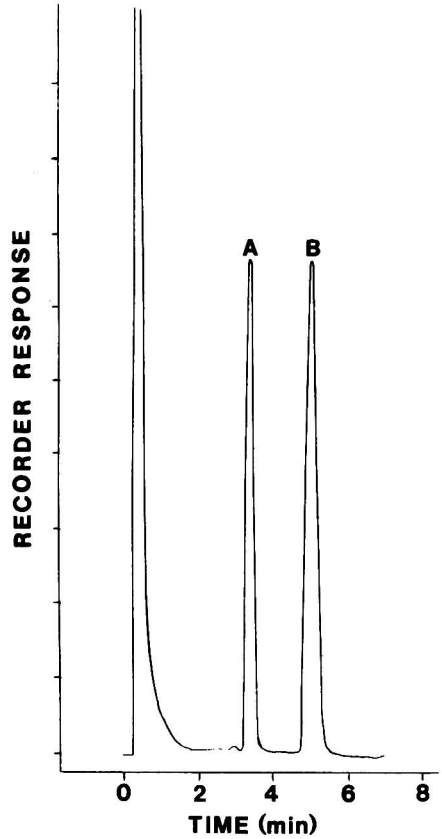

FIG. 1—Gas chromatogram of 4 μl mixed standard solution: A, o-terphenyl; B, PCNB.

where R′ and R = average peak height ratios for mixed standard and sample, respectively; W' = g PCNB/100 ml standard solution (0.2 g for method described); W = g sample extracted for analysis; and P = per cent purity of standard. This equation applies only if the procedure described is followed.

Results and Recommendation

From 1 to 6 μl mixed standard was injected to determine the effect of injection volume on the peak height ratio of PCNB to *o*-terphenyl. Response of the flame ionization detector to these 2 compounds was linear over the range tested: 1–6 μg for PCNB and 0.4–2.4 μg for *o*-terphenyl. The average peak height ratio was 0.987

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee A and was accepted by the Association. *See* (1976) *JAOAC* **59**, 380.

Table 1. Analysis of commercial samples for PCNB

Analysis	A, 10%	B, 30%	C, 24%	D, 20%	E, 75%	F,23.2%	G, 10%	H, 10%	I, 2.25%	J, 2.25%
1	10.40	30.29	26.72	21.41	73.98	28.27	9.33	9.28	1.43	3.97
2	10.35	30.54	26.83	21.26	73.91	28.37	9.36	9.51	1.44	3.81
3	10.25	30.63	26.61	20.94	74.41	28.53	9.37	9.23	1.45	3.86
4	10.28	30.19	26.68	21.11	73.54	28.17	9.34	9.50	1.43	3.87
Mean	10.32	30.41	26.71	21.18	73.96	28.34	9.35	9.38	1.44	3.88
Std dev.	0.07	0.21	0.09	0.20	0.36	0.15	0.02	0.15	0.01	0.07
Coeff. of var., %	0.68	0.69	0.34	0.94	0.49	0.53	0.21	1.6	0.69	1.8
Sample descr.[a]	nongrindable granule with 0.75% disulfoton	seed protectant with 30% captan (WP)	liquid EC	seed dust with 5% Terrazole	WP	liquid EC with 5.8% Terrazole	dust with 10% captan	dust	fertilizer	fertilizer

[a] WP = wettable powder; EC = emulsifiable concentrate.

(range 0.971–1.001) with a standard deviation of 0.009.

We selected 4 μl as a convenient injection volume and used it throughout the remainder of this study. Twelve consecutive injections of 4 μl mixed standard gave an average ratio of 0.983 with a standard deviation of 0.002, indicating excellent reproducibility for this injection volume. We did not compare peak height vs. peak area measurements for quantitation; however, it is doubtful that even peak area determinations by electronic integration would significantly improve the precision obtained using peak heights (2). Figure 1 illustrates the chromatographic profile obtained for a 4 μl injection of the mixed standard.

In a previous report on the determination of PCNB, aldrin was used as an internal standard (1). The use of a second pesticide as an internal standard was not desired; several non-pesticidal candidates were explored and *o*-terphenyl was selected (3). Also, a different GLC liquid phase than that previously used (11%, OV-17 + QF-1) was selected to avoid the use of a mixed phase and to employ a more commonly used material. The liquid phase chosen, SE-30, has recently been classified as one of the most used and preferred liquid phases (4).

Eight commercial pesticide formulations and 2 commercial fertilizers containing PCNB were analyzed by the method described herein (*see* Table 1). Good precision was obtained in the analysis of all samples. The other pesticides, captan, disulfoton, and Terrazole, in the samples did not interfere with the analysis for PCNB. In addition, the results obtained are in reasonable agreement with the product label claims. Recovery was not determined for the formulations tested in this investigation; however, a previous report indicates recovery of PCNB from fertilizers is complete, using the same extraction procedure (1). Analysis of Samples E and H by a total chloride method (5) gave results of 73.4 and 9.5%, respectively, which agree well with the levels of PCNB found by GLC. Therefore, recovery does not appear to be a problem.

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Table 2. Effects of varying conditions on PCNB analysis

Condition	Value	PCNB, %	Diff.
Removal of	settle	74.75	
suspended matter	filtration	75.95	−1.2
Injection	4.0	75.02	
volume, μl	3.5	75.68	−0.66
Detector	250	75.13	
temperature, °C	240	75.57	−0.44
Column	175	75.20	
temperature, °C	180	75.51	−0.31
Extract used, ml	10	75.32	
	15	75.37	−0.05
Extraction time, hr	2.0	75.39	
	1.5	75.32	+0.07
Injector	200	75.53	
temperature, °C	195	75.18	+0.35

The GLC method for PCNB has been subjected to a ruggedness test (6) to determine the effects of changing chromatographic and extraction conditions. A 75% PCNB wettable powder was analyzed under varying experimental conditions (Table 2). None of the conditions chosen for study showed sensitivity to slight changes.

The Associate Referee recommends that the GLC method for the determination of PCNB in pesticide formulations be subjected to a collaborative study.

Acknowledgments

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Collaborative Study of a Method for the Analysis of Metoxuron and Its Formulations

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The method collaboratively studied is based on 3 steps: free interfering amines are removed by acid extraction; dimethylamine is liberated by alkaline hydrolysis; and the liberated amine is distilled into an absorbing solution and continuously titrated. Interfering byproducts are separately determined by a thin layer chromatographic method. Two samples of metoxuron, the technical active ingredient, and an 80% wettable powder, were analyzed by 18 collaborators from Europe, the United States, and South America; results were satisfactory with an overall coefficient of variation of about 0.8%. Only one outlier was noticed. The method was adopted in 1974 as a full CIPAC method. The method was adopted in 1975 as an official first action AOAC method.

Metoxuron is the ISO (International Standards Organization) name for 3-(3-chloro-4-methoxyphenyl)-1,1-dimethylurea; the molecular weight of this compound is 228.67 and the empirical formula is $C_{10}H_{13}ClN_2O_2$. Metoxuron is used in many countries, but not at present in the United States, as a selective post-emergence herbicide on winter wheat and barley, as well as on carrots. It is effective against many graminaceae and the main annual dicotyledons. Metoxuron is commercially available as an 80% wettable powder.

In 1973, a CIPAC (Collaborative International Pesticides Analytical Council) collaborative study was proposed in CIPAC information sheet No. 39. The study was initiated by the PAC (Pesticides Analysis Advisory Committee)-Switzerland to test one analytical method for metoxuron preparations as well as for chlorotoluron (3 - (3 - chloro - 4 - methylphenyl) - 1,1 - dimethylurea) preparations.

The method involves alkaline hydrolysis, distillation, and titration of the liberated dimethylamine. Interfering byproducts are determined by a semiquantitative thin layer chromatographic (TLC) method. Eighteen laboratories participated in this study. They received directions for the method, guidelines, standards, and sample preparations.

The metoxuron part of this study is described here; the chlorotoluron work is published separately (1).

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Collaborative Study

The collaborative study was intended to check the performance of the method for the quantitative determination of metoxuron content in the following samples: metoxuron technical active ingredient and Dosanex 80 WP (80% wettable powder). Each collaborator also received one sample each of metoxuron technical active ingredient and an 80% wettable powder formulation for practice.

The following instructions were given to the collaborators:

Collaborators should use the practice samples to become familiar with the procedures. Once the analyst is well acquainted with the methods, 3 titrimetric determinations should be made on each sample. Only one TLC determination need be carried out for each sample. All values should be reported on the attached lists and *all* individual modifications of the methods should be noted.

METHOD

Chlorotoluron [3-(3-Chloro-4-methylphenyl)-1, 1-dimethylurea] or Metoxuron (Dosanex®) [3-(3-Chloro-4-methoxyphenyl)-1,1-dimethylurea]

Official First Action

CIPAC-AOAC Method

6.B05 ***Principle***

Pesticide is extd from formulations with CH_2Cl_2, free amines are removed with acid, and ext is hydrolyzed by alkali to Me_2NH which is distd and titrd. Related byproducts, 3-(3-chloro-4-methylphenyl)-1-methylurea (I), 3-(4-methylphenyl)-1,1-dimethylurea (II) (from

chlorotoluron) and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (III), 3-(3-chloro-4-hydroxyphenyl)-1,1-dimethylurea (IV), and 3-(4-methoxyphenyl)-1,1-dimethylurea (V) (from metoxuron), which may interfere, are detd by semiquant. TLC. Two other byproducts, 1,3-bis(3-chloro-4-methylphenyl) urea (VI) and 1,3-bis(3-chloro-4-methoxyphenyl) urea (VII), do not interfere with chlorotoluron and metoxuron detns, resp.

6.B06 *Preparation of Sample*

(a) *Technical formulation.*—Accurately weigh ca 3 g sample and transfer, using 100 ml CH_2Cl_2, into separator, dissolve, and add 50 ml 1*N* HCl.

(b) *80% Wettable powder.*—Accurately weigh ca 3.5–4.0 g sample into 200 ml beaker. Add 100 ml CH_2Cl_2 and stir mag. 5 min. Filter thru fritted glass crucible, and rinse beaker and crucible with portions of CH_2Cl_2 to total vol. of ca 200 ml. Use only slight vac. to prevent crystn of pesticide on walls of crucible. Transfer quant. to 500 ml separator, and add 50 ml 1*N* HCl.

6.B07 *Determination*

Vigorously shake mixt. 1 min and drain lower org. layer into second separator. Add 25 ml 1*N* HCl, shake 30 sec, and drain lower layer into 500 ml r-b flask. Wash the 2 acid layers successively with same 100 ml portion CH_2Cl_2 and drain lower layer into the 500 r-b flask. Discard acid.

Vac.-evap. CH_2Cl_2 in rotary evaporator to dryness at max. of 40°. Add 100 ml propylene glycol, 40 g KOH, and some boiling stones to residue. Immediately connect flask securely to distn app. (Fig. **6:B1**) whose joints are lubricated with thin film of silicone grease. Place end of condenser delivery tube (≥10 mm id) in 400 ml beaker below level of absorbing soln of 0.2 g H_3BO_3 and 1 ml mixed indicator soln (40 mg methylene blue and 60 mg Me red dissolved in 100 ml alcohol) in 150 ml H_2O. (To enhance end point, use 150 ml MeOH (2+1).)

Gently warm flask until all particles dissolve; then boil 10 min or until propylene glycol distils into condenser. Titr. distd Me_2NH continuously with stdzd 1*N* HCl, **50.011–50.017**. Complete distn by carefully adding H_2O dropwise from dropping funnel at rate of 1 drop/sec. Continue titrn until end point persists 2 min (*V* ml). Perform blank detn (*B* ml) with each series.

$$\% \text{ Pesticide} = [(V - B) \times N \times F/\text{g sample}] - \% \text{ byproducts (from 6.B08)},$$

where $F = 21.27$ for chlorotoluron or 22.87 for metoxuron, and N = normality of stdzd HCl.

6.B08 *Determination of Byproducts*

(a) *For chlorotoluron.*—Dissolve 100 mg each of byproducts I and II (**6.B05**) (available from Ciba-Geigy Ltd, Analytical Department, CH-4002, Basel, Switzerland) together in tetrahydrofuran and dil. to 50 ml in vol. flask. Dil. aliquots of 1, 2, 3, 4, and 5 ml to 20 ml with tetrahydrofuran, equiv. to 0.2, 0.4, 0.6, 0.8, and 1.0%, resp., of each byproduct.

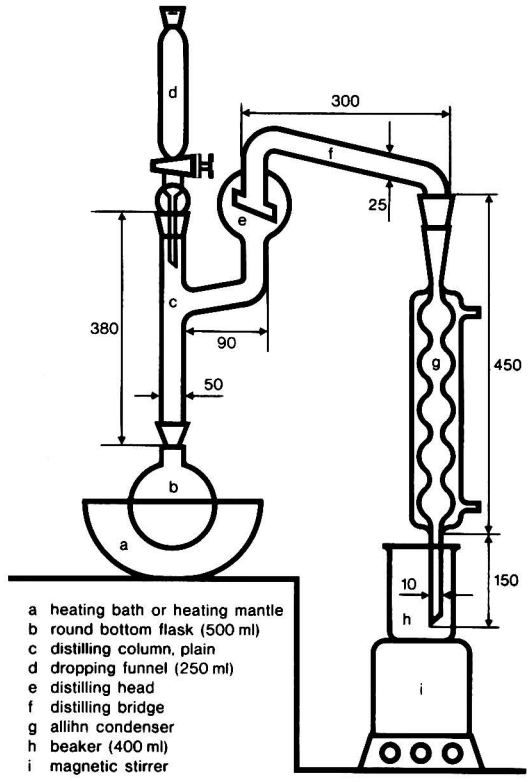

FIG. 6:B1—Distillation apparatus (all dimensions in mm)

Dissolve 1.0 g sample in tetrahydrofuran, and dil. to 20 ml with same solv.

Spot 5 μl each of sample and std solns on 20 × 20 cm glass plates precoated with 0.25 mm layer thickness of silica gel 60 F-254 (No. 5715, E. Merck, Darmstadt, Germany, or equiv.), and develop by ascending technic in tank, presatd 30 min with developing solv. $CHCl_3$-EtOAc (4+1), without filter paper linings, for ca 70 min (13 cm migration). Expose plate to 254 nm UV light and compare spots of samples with those of stds to est. concn of byproducts. Approx R_f values: chlorotoluron, 0.50; byproduct I, 0.25; byproduct II, 0.35; and byproduct VI (does not interfere), 0.82.

(b) *For metoxuron.*—Proceed as in (a), except use 100 mg each of byproducts III, IV, and V (available from Sandoz Ltd, Agrochemical Division, Research, CH-4002, Basel, Switzerland). Approx. R_f values: metoxuron, 0.25; byproduct III, 0.34; byproduct IV, 0.08; byproduct V, 0.13; and byproduct VII (does not interfere), 0.46.

Results and Discussion

All 18 collaborators completed the study and returned the results as indicated in Table 1. Collaborators 1, 3, 11, and 16 did not carry out the desired number of analyses. Results from Collaborator 3 were not included in the statistical evaluation. No major difficulties were reported. The results of one collaborator had to be corrected because he used the wrong molecular weight. Only one result was rejected as an outlier by the Nalimov test (2) and was not included in the statistical evaluations.

Table 1. Collaborative results (%) of the determination of metoxuron technical and wettable powder formulation[a]

Coll.	Technical	Wettable powder
1	99.28	81.16
	98.52	81.53
2	98.6	80.0
	98.6	79.9
	98.4	80.2
3	98.12	79.82
4	97.8	80.2
	97.8	79.5
	97.4	79.5
5	98.0	79.9
	98.0	80.3
	98.4	79.9
6	99.8	79.8
	99.5	80.3
	99.2	79.8
7	99.8	87.15[b]
	100.6	80.3
	101.7	80.8
8	99.2	80.2
	99.1	80.3
	99.2	80.3
9	98.98	79.92
	98.50	80.28
	99.07	80.25
10	99.3	80.3
	99.3	80.3
	99.4	80.0
11	98.5	79.3
	98.8	79.3
	98.6	
12	99.4	80.7
	99.6	80.7
	99.1	80.3
13	99.9	80.5
	100.6	80.6
	100.3	80.2
14	98.7	80.1
	99.9	79.2
	99.1	80.4
15	98.8	79.9
	99.0	80.0
	99.3	80.5
16	96.9	79.5
	98.4	79.4
17	97.79	78.19
	97.79	80.00
	97.45	79.98
18	98.1	79.5
	98.3	79.8
	97.7	79.2

[a] Each value represents one determination.
[b] Rejected as outlier.

Table 2 summarizes the per cent mean values of metoxuron found in the alkaline hydrolysis procedure and the corresponding per cent values of byproducts found by the TLC determination. Table 3 indicates averages, ranges, and standard deviations. The overall range was 96.9–101.7% for metoxuron technical and 78.2–81.5% for metoxuron wettable powder. The mean values ranged from 97.7 to 100.7% for metoxuron technical and from 79.1 to 81.3% for metoxuron wettable powder. The overall coefficient of variation was about 0.8%. The byproducts were found within a range from <0.4 to 0.9% (in metoxuron technical) and <0.2 to 0.8% (in metoxuron wettable powder) with a standard deviation of 0.15%.

The results of the analysis of variance are shown in Table 4. Small, yet statistically significant, differences in the mean values are observed between laboratories. This is a well known fact, however, with methods using very precise techniques such as the titrimetric technique. The figures of variance within collaborators are small (0.15 and 0.17). Therefore, small but inevitable differences as, for instance, in the skill

Table 2. Collaborative results (%) of the determination of the active ingredients and byproducts III, IV, and V in metoxuron technical and wettable powder formulation[a]

	Technical		Wettable powder	
Coll.	Active ingred.	III, IV, & V	Active ingred.	III, IV, & V
1	98.9	0.8	81.3	0.4
2	98.5	0.9	80.0	0.5
3	98.1	0.6	79.8	0.5
4	97.7	0.8	79.7	0.8
5	98.1	0.5	80.0	0.7
6[b]	99.5	0.8	80.0	0.6
7	100.7	0.6	80.5	0.4
8	99.2	0.7	80.3	0.5
9	98.9	0.8	80.1	0.7
10	99.3	0.8	80.2	0.4
11	98.6	0.7	79.3	0.6
12	99.4	0.9	80.6	0.5
13	100.3	0.7	80.4	0.5
14	99.2	0.8	79.9	0.6
15	99.0	0.5	80.1	0.5
16	97.7	0.7	79.5	0.7
17	97.7	0.5	79.1	0.4
18	98.0	<0.4	79.5	<0.2

[a] The values represent 3 determinations of the active ingredient (except for Collaborators 1, 3, 11, and 16) and one determination of the byproducts.
[b] Collaborator 6 is the senior author (HHS).

Table 3. Averages, ranges, and standard deviations for the collaborative results of the determination of metoxuron technical and wettable powder formulation

Statistics	Technical		Wettable powder	
	Active ingred.	III, IV, & V	Active ingred.	III, IV, & V
No. of coll.	18	18	18	18
No. of results	50	18	49	18
Range coll. av.	97.7–100.7	<0.4–0.9	79.1–81.3	<0.2–0.8
Overall range	96.9–101.7		78.2–81.5	
Overall av.	98.8	0.69	80.0	0.52
Std dev. between coll.	0.86	0.15	0.52	0.15
Overall std dev.	0.91		0.55	
Coeff. of var. between coll.	0.87	—	0.65	—
Overall coeff. of var.	0.93		0.69	

Table 4. Analysis of variance[a] of the determination of metoxuron technical and wettable powder formulation

Sample	Source	Variance	Degrees of freedom	F_{found}	$F_{tabulated}$ (95%)
Metoxuron technical	between coll.	2.19	16	12.9	1.97
	within coll.	0.17	32		
	total	—	48		
Metoxuron wettable powder	between coll.	0.61	16	4.07	1.99
	within coll.	0.15	30		
	total	—	46		

[a] Excluding results of Collaborator 3.

of operators, equipment, and reagents between different laboratories can result in the mathematically drawn conclusion of a systematic error. However, the range of mean values and single results as well as the overall coefficient of variation is satisfactory and comparable to earlier collaborative studies (3, 4).

Collaborator's Comments

The proposed method did not cause any major difficulties. Only minor modifications in equipment and reagents were used or suggested. Most of these involved the distillation step.

The collaborators' remarks are summarized below; some of them have been incorporated in the analytical method.

One collaborator recommended that a lower normality of hydrochloric acid be used in the extraction. Another investigator simplified the method for determining the formulated product by omitting the extraction step; although this modification does not lead to significant deviations when samples of high purity are analyzed, it cannot be adopted for the general method. In the evaporation of methylene chloride, one collaborator preferred to distill a portion of the solvent under nitrogen before transferring it to the rotary evaporator. The amount of potassium hydroxide required for hydrolysis was questioned. However, according to tests by one collaborator, the amount specified in the method is necessary for complete hydrolysis. One laboratory favored ethylene glycol rather than propylene glycol. One participant did not obtain a sharp end point in the titration.

In the TLC method, some investigators observed that the sample spots were more extended than standard spots. This impaired the evaluation to some extent, but it still allowed satisfactory results to be obtained. The sensitivity of detection may be enhanced by using the chlorine gas-potassium iodide-starch reaction.

Recommendation

The results prove that the tested method is rugged, precise, and reliable. The method has been adopted as a full CIPAC method at the 19th Annual Meeting, June 9–12, 1975, at Oeiras, Portugal.

The methods for the analysis of metoxuron and chlorotoluron were studied separately. They have been editorially combined for publication in "Changes in Methods" (1976) *JAOAC* **59**, 455–456 and in this paper.

On the basis of the work reported in this paper, the General Referee on Pesticide Formulations I and Subcommittee A approved the method and it was adopted as an official first action CIPAC-AOAC method at the 89th Annual Meeting of the AOAC, Oct. 13–16, 1975, at Washington, DC.

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Collaborative Study of a Method for the Analysis of Chlorotoluron and Its Formulations

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Chlorotoluron technical and its 80% wettable powder were analyzed by 19 participants in a collaborative study. The analytical method used involves acid extraction to remove interfering free amines, followed by alkaline hydrolysis of the extraction residue, distillation, and titration of the liberated dimethylamine. Related by-products which may interfere are determined by semiquantitative thin layer chromatography. Results obtained from 19 government and industrial collaborators showed good repeatability within laboratories. Good agreement was also achieved between laboratories. An overall coefficient of variation of about 0.8% was obtained. In 1974 the analytical method was accepted as a full CIPAC method. In 1975, the method was adopted as an official first action AOAC method.

A variety of methods are available for the assay of urea herbicides in technical material and formulations, including titrimetric, spectrophotometric, gas-liquid chromatographic, and high-pressure liquid chromatographic procedures.

The present study was initiated by PAC (Pesticides Analysis Advisory Committee)-Switzerland to evaluate the precision and reliability of the widely used and time-proven titrimetric method for chlorotoluron (3-(3-chloro-4-methylphenyl)-1,1-dimethylurea). This involves alkaline hydrolysis, followed by distillation and titration of the split-off aliphatic amine. The required degree of specificity was achieved by (a) removing free amines by extraction prior to hydrolysis and (b) determining interfering related compounds by thin layer chromatography (TLC).

Collaborative Study

One sample each of chlorotoluron technical and 80% wettable powder formulation, together with standards and detailed guidelines, were sent to 20 collaborators in Europe, the United States (at the present time chlorotoluron is not being used in the United States), and South America. A parallel series of tests of the same analytical method was carried out with metoxuron (3-(3-chloro-4-methoxyphenyl)-1,1-dimethylurea) (1).

All collaborators were asked to perform the wet chemical analysis in triplicate; only a single TLC determination was required. They were also asked to submit the raw data.

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METHOD

Chlorotoluron [3-(3-Chloro-4-methylphenyl)-1,1-dimethylurea] or Metoxuron (Dosanex®) [3-(3-Chloro-4-methoxyphenyl)-1,1-dimethylurea]

Official First Action

CIPAC-AOAC Method

6.B05 ***Principle***

Pesticide is extd from formulations with CH_2Cl_2, free amines are removed with acid, and ext is hydrolyzed by alkali to Me_2NH which is distd and titrd. Related byproducts, 3-(3-chloro-4-methylphenyl)-1-methylurea (I), 3-(4-methylphenyl)-1,1-dimethylurea (II) (from chlorotoluron) and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (III), 3-(3-chloro-4-hydroxyphenyl)-1,1-dimethylurea (IV), and 3-(4-methoxyphenyl)-1,1-dimethylurea (V) (from metoxuron), which may interfere, are detd by semiquant. TLC. Two other byproducts, 1,3-bis(3-chloro-4-methylphenyl) urea (VI) and 1,3-bis(3-chloro-4-methoxyphenyl) urea (VII), do not interfere with chlorotoluron and metoxuron detns, resp.

6.B06 ***Preparation of Sample***

(a) *Technical formulation.*—Accurately weigh ca 3 g sample and transfer, using 100 ml CH_2Cl_2, into separator, dissolve, and add 50 ml 1*N* HCl.

(b) *80% Wettable powder.*—Accurately weigh ca 3.5–4.0 g sample into 200 ml beaker. Add 100 ml CH_2Cl_2 and stir mag. 5 min. Filter thru fritted glass crucible, and rinse beaker and crucible with portions of CH_2Cl_2 to total vol. of ca 200 ml. Use only slight vac. to prevent crystn of pesticide on walls of crucible. Transfer quant. to 500 ml separator, and add 50 ml 1*N* HCl.

6.B07 ***Determination***

Vigorously shake mixt. 1 min and drain lower org. layer into second separator. Add 25 ml 1*N* HCl, shake 30 sec, and drain lower layer into 500 ml r-b flask. Wash the 2 acid layers successively with same 100 ml portion CH_2Cl_2 and drain lower layer into the 500 r-b flask. Discard acid.

Vac.-evap. CH_2Cl_2 in rotary evaporator to dryness at max. of 40°. Add 100 ml propylene glycol, 40 g KOH, and some boiling stones to residue. Immediately connect flask securely to distn app. (Fig. **6:B1**) whose joints are lubricated with thin film of silicone grease. Place end of condenser delivery tube (≥10 mm id) in 400 ml beaker below level of absorbing soln of 0.2 g H_3BO_3 and 1 ml mixed indicator soln (40 mg methylene blue and 60 mg Me red dissolved in 100 ml alcohol) in 150 ml H_2O. (To enhance end point, use 150 ml MeOH (2+1).)

Gently warm flask until all particles dissolve; then boil 10 min or until propylene glycol distils into condenser. Titr. distd Me_2NH continuously with stdzd 1*N* HCl, **50.011–50.017**. Complete distn by carefully adding H_2O dropwise from dropping funnel at rate of 1 drop/sec. Continue titrn until end point persists 2 min (*V* ml). Perform blank detn (*B* ml) with each series.

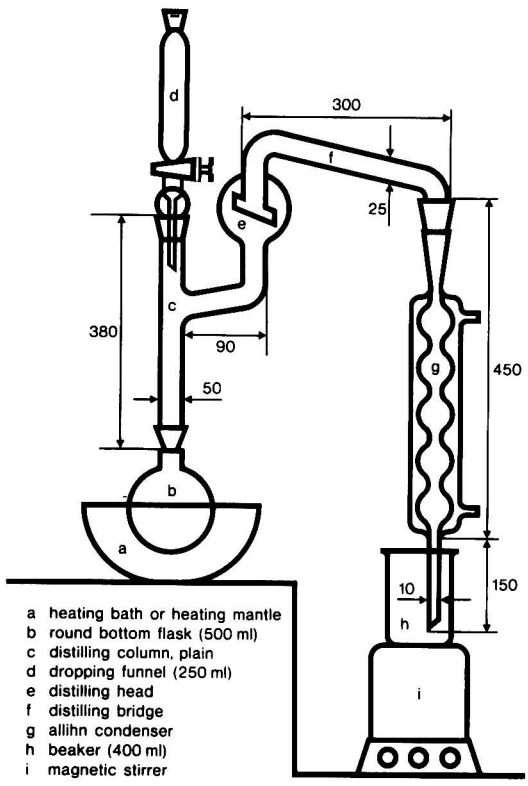

FIG. 6:B1—Distillation apparatus (all dimensions in mm)

% Pesticide = [(*V* − *B*) × *N* × *F*/g sample] − % byproducts (from **6.B08**),

where *F* = 21.27 for chlorotoluron or 22.87 for metoxuron, and *N* = normality of stdzd HCl.

6.B08 ***Determination of Byproducts***

(a) *For chlorotoluron.*—Dissolve 100 mg each of byproducts I and II (**6.B05**) (available from Ciba-Geigy Ltd, Analytical Department, CH-4002, Basel, Switzerland) together in tetrahydrofuran and dil. to 50 ml in vol. flask. Dil. aliquots of 1, 2, 3, 4, and 5 ml to 20 ml with tetrahydrofuran, equiv. to 0.2, 0.4, 0.6, 0.8, and 1.0%, resp., of each byproduct.

Dissolve 1.0 g sample in tetrahydrofuran, and dil. to 20 ml with same solv.

Spot 5 μl each of sample and std solns on 20 × 20 cm glass plates precoated with 0.25 mm layer thickness of silica gel 60 F-254 (No. 5715, E. Merck, Darmstadt, Germany, or equiv.), and develop by ascending technic in tank, presatd 30 min with developing solv. $CHCl_3$-EtOAc (4+1), without filter paper linings, for ca 70 min (13 cm migration). Expose plate to 254 nm UV light and compare spots of samples with those of stds to est. concn of byproducts. Approx R_f values: chlorotoluron, 0.50; byproduct I, 0.25; byproduct II, 0.35; and byproduct VI (does not interfere), 0.82.

(b) *For metoxuron.*—Proceed as in (a), except use 100 mg each of byproducts III, IV, and V (available from Sandoz Ltd, Agrochemical Division, Research, CH-4002, Basel, Switzerland). Approx. R_f values: metoxuron, 0.25; byproduct III, 0.34; byproduct IV, 0.08; byproduct V, 0.13; and byproduct VII (does not interfere), 0.46.

Results and Discussion

Results were received from 19 of the 20 collaborators (Table 1). Not all of the participants had performed the desired number of analyses, but all of the results were used for statistical evaluation.

We reviewed the raw data and some results were recalculated. No result was rejected as an outlier by the Nalimov test (2). Some values were just rejectable at the 95% level of the test, but all were acceptable at the 99% level.

Table 2 contains averages and absolute and relative standard deviations as well as the ranges. The analysis of variance is given in Table 3.

The statistical treatment reveals 3 important facts. (*1*) The reproducibility of the method is

The methods for the analysis of metoxuron and chlorotoluron were studied separately. They have been editorially combined for publication in "Changes in Methods" (1976) *JAOAC* **59**, 455–456 and in this paper.

Table 1. Collaborative results (%) of the determination of chlorotoluron technical, wettable powder formulation, and byproducts

	Chlorotoluron		Byproducts (ΣI + II)	
Coll.	Technical	Wettable powder	Technical	Wettable powder
1	99.87	80.76	—	0.3
	99.91	80.95		
2	99.3	80.8	0.1	0.4
	99.6	80.8		
	99.4	80.5		
3	99.34	81.48	0.2	0.6
		81.21		
4	99.7	81.3		
	100.5	81.3	0.4	0.6
	100.1	81.6		
5	99.8	81.4		
	99.8	81.5	0.3	0.8
	99.7	81.3		
6	97.9	81.2		
	99.7	81.5	0.1	0.3
	98.9	81.5		
7	100.5	81.2		
	100.1	77.9	0.2	0.6
	99.4	80.1		
8	101.4	81.9		
	101.4	81.5	0.1	0.4
	101.4	81.4		
9	98.70	80.96		
	99.07	80.90	0.4	0.6
	99.26	80.98		
10	100.0	81.5		
	100.1	81.5	—	0.4
	100.0	81.5		
11	99.0	80.8		
	98.9	81.0	—	0.2
	99.3	81.1		
12	99.9	81.5		
	99.9	81.2	0.2	0.3
	99.7	81.1		
13	99.6	81.7		
	100.6	81.4	0.2	0.3
	99.7	81.6		
14	100.6	81.0		
	100.4	81.1	0.2	0.6
	100.2	81.2		
15	99.7	82.1		
	100.2	81.7	0.2	0.2
	99.5	81.3		
16	97.6	79.4	<0.2	0.4
	99.1	80.4		
17	98.12	80.41		
	97.46	80.41	0.15	0.2
	98.25	80.61		
18	99.3	81.2		
	99.3	80.7	<0.2	<0.2
	99.0	80.7		
19	99.37	81.23		
	98.95	80.77	—	—
	99.50	79.34		
	98.82	81.68		

very satisfactory, with an overall coefficient of variation of about 0.8%. (*2*) Small, yet significant, differences in the mean values are observed between laboratories; however, the range of mean values is satisfactory and in line with other collaborative studies (3, 4). (*3*) The TLC values show good reproducibility, with an absolute standard deviation of about 0.1%.

The use of the amine evolution method did not lead to any major difficulties. Only minor modifications referring to equipment and reagents, particularly at the distillation step, were adopted or recommended by the participants.

The collaborators' remarks are summarized below; some of them have been incorporated into the analytical method.

One collaborator suggested that a hydrochloric acid of lower normality be used in the extraction. Another participant simplified the method for formulations by omitting the extraction step; although this modification does not lead to significant deviations in the case of samples of high purity, it cannot be adopted for the general method. In the evaporation of methylene chloride, a collaborator distilled part of the solvent under nitrogen before transferring it to the rotary evaporator. The amount of potassium hydroxide required for hydrolysis was questioned; according to our own studies, however, the amount specified is necessary for complete hydrolysis. In the distillation step, nitrogen was recommended as the vehicle; one laboratory preferred ethylene glycol instead of propylene glycol. One collaborator did not obtain a sharp end point in the titration.

Some co-workers observed that the TLC sample spots were more diffuse than the reference spots. This impaired the evaluation somewhat, but satisfactory results were still obtained. The sensitivity of detection may be enhanced by using the chlorine gas-potassium iodide-starch reaction.

Conclusion

As the results reveal, the analytical methods tested are rugged, accurate, and within the accepted limit of AOAC methods. A small, yet statistically significant bias between laboratories has often been noted in collaborative studies. This may arise from inevitable differences in equipment, reagents, and skill of the operators. However, the overall standard deviation, calculated from all results, appears to be satisfactory.

On the basis of work reported in this paper, the General Referee on Pesticide Formulations I and Subcommittee A approved the method and it was adopted as an official first action CIPAC-AOAC method at the 89th Annual Meeting of the AOAC, Oct. 13–16, 1975, at Washington, DC.

Table 2. Averages, ranges, and standard deviations for the collaborative results of the determination of chlorotoluron technical and wettable powder formulation

Parameter	Technical	Wettable powder	Byproducts (ΣI + II)	
			Technical	Wettable powder
No. of coll.	19	19	19	19
No. of results	54	55		
Range, coll. av.	97.9–101.4	79.9–81.7	0–0.4	0–0.8
Overall range	97.5–101.4	77.9–82.1		
Overall av.	99.6	81.0	0.2	0.4
Std dev. between coll.	0.77	0.55	0.12	0.20
Overall std dev.	0.83	0.68		
Coeff. of var. between coll.	0.78	0.68	—	—
Overall coeff. of var.	0.83	0.84		

Table 3. Analysis of variance of the determination of chlorotoluron technical and wettable powder formulation

Sample	Source	Variance	Degrees of freedom	F_{found}	$F_{tabulated}$ (95%)
Chlorotoluron technical	between coll.	1.71	18	10.5	1.97
	within coll.	0.16	35		
	total	—	53		
Chlorotoluron wettable powder	between coll.	0.82	18	2.86	1.94
	within coll.	0.29	36		
	total	—	54		

Only slight modifications to the method have been brought to our attention.

The analytical method was accepted as a full CIPAC method at the 19th Annual Meeting, June 9–12, 1975, at Oeiras, Portugal.

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TECHNICAL COMMUNICATIONS

Comparison of Antibiotic-Amended Potato Dextrose Agar and Acidified Potato Dextrose Agar as Growth Substrates for Fungi

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Fifteen fungal species, all isolated from foods, were compared for their growth abilities on potato dextrose agar acidified to pH 3.5, and on nonacidified potato dextrose agar amended with 40 ppm chlortetracycline hydrochloride. Comparisons were made at 16, 21, 26, 32, and 37°C. Of the 15 species, only *Penicillium expansum* exhibited better growth on the acidified medium than on the nonacidified antibiotic medium, while 9 species grew better on the nonacidified antibiotic medium. Five species grew equally well on either medium.

A number of methods presently utilized to determine yeast and mold counts in foods usually involve the use of an agar medium acidified to a pH of 3.5 with a given organic acid (tartaric, lactic, etc.). The medium most commonly utilized is potato dextrose agar. The use of low pH agar substrate for determining yeast and mold counts in foods was first proposed in 1931 by White and Hood (1) who reported that undesired bacteria were selectively inhibited while yeasts and molds were not. However, recent reports (2, 3) indicate that higher yeast and mold counts may be obtained if the pH of the agar substrate is not adjusted to 3.5. Bacterial inhibition may be accomplished by the addition of antibiotics to the substrate. Preliminary investigations in our laboratory have indicated, likewise, that nonacidified, antibiotic-amended agar media result in higher total yeast and mold counts of the same food sample than do acidified agar media.

In view of the above reports (2, 3), plus our own preliminary findings, the following study was conducted to determine if the lower counts on acidified agar media were due, at least in part, to delayed or restricted growth abilities on a low pH substrate of a number of yeast and mold species commonly isolated from foods. Fifteen species, all isolated from foods and including 9 species known to produce mycotoxins, were examined for their ability to grow on potato dextrose agar acidified to pH 3.5 with tartaric acid (PDAT) vs. their ability to grow on nonacidified potato dextrose agar amended with 40 ppm chlortetracycline hydrochloride (PDAC). The initial pH of PDAC was 5.6. Growth ability and rate of growth by each species on each substrate was based on colony diameter, measured in mm, attained after 5 days. The individual species were single-spot inoculated onto the agar surface of plastic petri dishes, each containing ca 25 ml agar medium. For each species, comparisons and measurements were made at 5 temperatures: 16, 21, 26, 32, and 37°C. Duplicate plates of each medium were incubated at each temperature.

Table 1 lists the species investigated and the results obtained. The data show that growth was established in 5 days by all 15 species on both media at 26, 21, and, except for *Aspergillus versicolor* on PDAT, at 16°C. Not all of the 15 species showed growth in 5 days at 37 and 32°C. The data indicate, however, that fungal growth at these higher temperatures is more apt to occur on the antibiotic-amended substrate (PDAC) than on the acidified substrate (PDAT).

With regard to rate of growth on the 2 agar media, the data in Table 1 suggest that the 15 species can be subdivided into 3 categories. Category 1, PDAT favored over PDAC: According to the data, only *Penicillium expansum* grew better on PDAT than on PDAC. Interestingly, this species is the primary agent for apple rot, and the pH of most apple varieties ranges from 2.9 to 3.3. Category 2, PDAC favored over PDAT: The following 9 species, including 6 of the 9 mycotoxin producers, favored PDAC over PDAT, *Aspergillus flavus, A. ochraceus, A. versicolor, Geotrichum candidum, Penicillium brevi-compactum, P. cyclopium, P. urticae, P. viridicatum,* and *Rhodotorula aurantiaca.* Category 3, no difference: Five species grew equally well on both substrates: *Aspergillus niger, Candida albicans, Penicillium citrinum, P. rubrum,* and *Saccharomyces cerevisiae.*

This study thus indicates that the lower total yeast and mold counts obtained from foods by others (2, 3) and by ourselves on acidified agar media vs. nonacidified, antibiotic-amended agar media are due, at least in part, to more restricted growth rates of a number of fungal species common to foods, including several mycotoxin producers, on the acidified substrate. This study fur-

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Table 1. Effect of temperature and type of agar substrate on 5-day growth of 15 food-contaminating fungal species

Species	Agar substrate[a]	Growth in mm at 37°C	32°C	26°C	21°C	16°C
Aspergillus flavus	PDAC	55	65	37	27	8
	PDAT	40	42	30	20	4
A. niger	PDAC	67	80	46	32	9
	PDAT	65	72	46	30	9
A. ochraceus	PDAC	8	16	22	15	6
	PDAT	3	8	12	9	3
A. versicolor	PDAC	—[b]	4	9	7	3
	PDAT	—	—	5	3	—
Candida albicans	PDAC	6	6	5	4	2
	PDAT	6	6	4	3	2
Geotrichum candidum	PDAC	6	29	33	29	19
	PDAT	—	7	12	10	8
Penicillium brevi-compactum	PDAC	—	2	16	15	10
	PDAT	—	—	12	12	8
P. citrinum	PDAC	7	9	15	14	7
	PDAT	6	8	15	13	6
P. cyclopium	PDAC	—	2	21	20	15
	PDAT	—	—	14	12	11
P. expansum	PDAC	—	2	20	19	14
	PDAT	—	4	23	22	16
P. rubrum	PDAC	13	21	22	18	7
	PDAT	13	20	22	17	7
P. urticae	PDAC	3	10	21	17	12
	PDAT	2	5	16	12	8
P. viridicatum	PDAC	—	2	17	17	12
	PDAT	—	—	14	13	8
Rhodotorula aurantiaca	PDAC	7	7	7	7	6
	PDAT	2	5	5	5	4
Saccharomyces cerevisiae	PDAC	5	6	5	4	4
	PDAT	5	6	5	4	4

[a] PDAC, potato dextrose agar amended with 40 ppm chlortetracycline hydrochloride; PDAT, potato dextrose agar acidified to pH 3.5 with tartaric acid.
[b] —, no visible growth observed in 5 days.

ther indicates that the determination of total yeast and mold counts of foods should be carried out in the 21–26°C temperature range, regardless of the pH of the agar substrate.

Recommendation

It is recommended that study be continued.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee F and was accepted by the Association. *See* (1976) *JAOAC* **59**, 397.

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(2) Koburger, J. A. (1970) *J. Milk Food Technol.* **33**, 433–434
(3) Ladiges, W. C., Foster, J. F., & Jorgensen, J. J., III (1974) *J. Milk Food Technol.* **37**, 302–304

This report of the Associate Referee, P. B. Mislivec, was presented at the 89th Annual Meeting of the AOAC, Oct. 13–16, 1975, at Washington, DC.

Derivatization Procedure for Identification of Aflatoxin M_1 on a Thin Layer Chromatogram

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A commodity extract containing presumptive aflatoxin M_1 is placed on an origin spot of a thin layer chromatographic plate and overspotted with trifluoroacetic acid. The mixture is held in the dark 30 min at ambient temperature and then 30 min at 55°C. The plate is developed with $CHCl_3$-acetone-2-propanol (85+10+7). The R_f values of reacted and unreacted aflatoxin M_1 are compared with authentic M_1 similarly treated for identification. The lowest concentration that has been identified is 0.1 μg/kg.

A method for confirmation of identity of aflatoxin M_1 has been adopted as official first action by the AOAC (1, 2). The method is based on the formation of the acetate and the hemiacetal derivatives of presumptive aflatoxin M_1 in a clean extract obtained either by preparatory thin layer chromatography (TLC) or cellulose column partition chromatography, and comparison of the TLC characteristics of the presumptive reaction products with the reaction products from authentic aflatoxin M_1. Although the method specifies 30 ng aflatoxin M_1 in the extract, a test can be successfully performed with as little as 1 ng aflatoxin M_1 (3).

A method based on the trifluoroacetic acid (TFA)-catalyzed derivatization of presumptive aflatoxin B_1 in a TLC origin spot of commodity extract has been adopted as official first action by the AOAC (3, 4). Because of the simplicity of the method and the presence in aflatoxin M_1 of the same furan unsaturation in aflatoxin B_1, the method was applied to aflatoxin M_1, but no obvious reaction products were found. However, by altering the reaction conditions, aflatoxin M_1 does react in the presence of TFA on a TLC origin spot to give a blue fluorescent derivative that can be used for confirmation of aflatoxin M_1 identity. The product of this acid-catalyzed reaction on the TLC plate is not, however, the same product obtained by the same reaction in a test tube. Several attempts were made to isolate and identify this derivative, but it was too unstable after removal from the TLC plate (R. D. Stubblefield, 1975, U.S. Department of Agriculture, Peoria IL, personal communication).

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Experimental

Apply sample extract containing ca 1–2 ng presumptive aflatoxin M_1 in $CHCl_3$ to TLC origin spot, and overspot with 1 μl TFA. Repeat procedure with authentic aflatoxin M_1, and with sample extract and authentic M_1 in common origin spot.

Hold TLC plate 30 min in dark at ambient temperature and additional 30 min at 55°C for complete reaction and removal of TFA. Develop with $CHCl_3$-acetone-2-propanol (85+10+7).

Let solvent evaporate at room temperature and examine plate under longwave ultraviolet light for blue fluorescent spots. R_f values of M_1 derivative and unreacted M_1 are ca 0.24 and 0.67, respectively, on Adsorbosil-1 spread 0.5 mm thick.

Discussion

To test the degree of conversion of M_1 to derivative, pure aflatoxin M_1 was applied to a TLC plate at 0.5, 1.0, and 2.0 ng and allowed to react with TFA as described above. After plate development the amount of unreacted aflatoxin M_1 was determined by fluorescence densitometry with conditions previously described (5), taking precautions against spot fading (6). The average amounts of unreacted aflatoxin M_1 from 4 spots at each level were 0.1, 0.12, and 0.38 ng. The unreacted aflatoxin M_1 was 12–20% of the original compound with an overall average of 17%.

At levels of contamination (0.1–0.2 μg/kg) where interferences may be present on the TLC plate, it is necessary to separate the M_1 derivative from these interferences, using the 2-dimensional TLC spotting technique of Yin *et al.* (7): After spotting, develop plate in first direction with $CHCl_3$-acetone-2-propanol (90+10+1) in unequilibrated, unlined chamber. Add 1 μl TFA to spot on plate expected to contain presumptive aflatoxin M_1 as judged by aflatoxin M_1 in reference channel and appearance of developed extract spot; also add 1 μl TFA to authentic aflatoxin M_1 in second dimension reference channel. Use reaction conditions outlined above, including chromatogram development and inspection.

This procedure has been successfully applied to a variety of cheeses, fluid milk, and nonfat dried milk solids.

Acknowledgments

The author is grateful to Robert D. Stubblefield for the aflatoxin M_1 standards and Leonard Stoloff for his advice and helpful suggestions.

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(2) *Official Methods of Analysis* (1975) 12th Ed., AOAC, Washington, DC, secs. **26.079–26.083**
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(5) *Official Methods of Analysis* (1975) 12th Ed., AOAC, Washington, DC, sec. **26.055**
(6) Nesheim, S. (1971) *JAOAC* **54**, 1444–1445
(7) Yin, L., Campbell, A. D., & Stoloff, L. (1971) *JAOAC* **54**, 102–105

CORRECTIONS

JAOAC **57**, 53–59 (1974), "Collaborative Study of the Gas Chromatographic Determination of Six Thiocarbamate Herbicides in Formulations," by J. E. Barney II, p. 56, Table 3, S_d value for Sutan, Formulation E

Change "0.277" to "2.77." Corrected

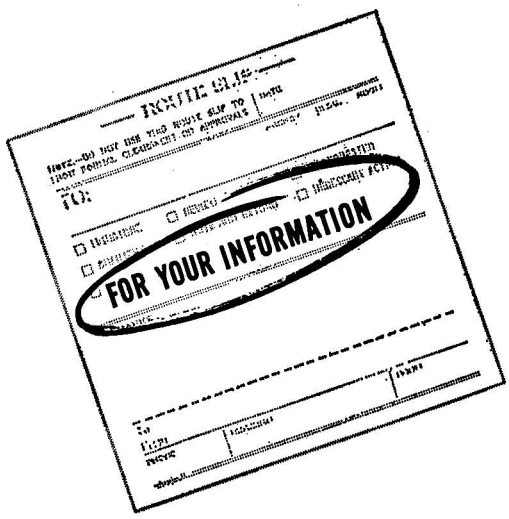
JAOAC **58**, 50–57 (1975), "The Purity of Sulfanilic Acid," by D. M. Marmion, pp. 51–52, captions for Figs. 2–4

Change to read: ". . . 2, orthanilic acid, concentration 10%, amplitude 25; 3, metanilic acid, concentration 10%, amplitude 25; 4, sulfanilic acid, concentration 10%, amplitude 40." Corrected

JAOAC **58**, 500–506 (1975), "Screening Method for the Detection of Aflatoxins in Mixed Feeds and Other Agricultural Commodities with Subsequent Confirmation and Quantitative Measurement of Aflatoxins in Positive Samples," by T. R. Romer, p. 501, left column

Change parenthetical statement to read "(Applicable to detection of $\geq$10 ppb total aflatoxins ($B_1 + B_2 + G_1 + G_2$) in white and yellow corn, peanut and cottonseed meals, peanuts, peanut butter, and pistachio nuts, and $\geq$15 ppb total aflatoxins in mixed feeds)."

V.P.
3.Feb.1977



NBS . . . More Accurate Measurements for Industrial Pollutants

The Commerce Department's National Bureau of Standards (NBS) has developed a new series of Standard Reference Materials (SRMs) designed to provide more accurate measurements of pollutants that are potential health hazards to industrial workers. The new SRMs are the outgrowth of a program, jointly sponsored by the National Institute of Occupational Safety and Health (NIOSH) and NBS, to develop reference materials for industrial hygiene analysis and for monitoring the workplace atmosphere.

The 3 members of the series now available from the NBS Office of Standard Reference Materials are: Freeze-Dried Urine Certified for Fluorine, Beryllium on Filter Media, and Metals on Filter Media.

Freeze-Dried Urine Certified for Fluorine (SRM 2671) consists of 2 freeze-dried samples of human urine containing low and elevated levels of fluoride when reconstituted with water. The standard, which will be useful for industrial hygiene analysis in both aluminum reduction plants and the fertilizer industry, is issued as a set of 2 bottles containing the freeze-dried material for *in situ* reconstitution.

Beryllium on Filter Media (SRM 2675), useful for monitoring the workplace atmosphere in the specialty ceramics and refractory materials industries, consists of carefully reproduced quantities of salts of beryllium deposited upon membrane filters. With both Beryllium and Metals on Filter Media, 3 concentrations of metal salts are deposited upon the membrane to simulate the amount collected under typical workplace conditions when atmospheric concentrations are either near, equal to, or well above the threshold level of detection.

Metals on Filter Media (SRM 2676) consists of quantities of lead, cadmium, zinc, and manganese deposited upon the membrane filter. This standard is useful for monitoring the workplace atmosphere of metal working companies, foundries, paint shops, and plating and welding operations. Each unit of SRM 2675 and 2676 contains a set of 3 membrane filters.

The cost of each SRM is $80 per unit. They may be ordered from the Office of Standard References Materials, Room B311, Chemistry Building, National Bureau of Standards, Washington, DC 20234.

Interim Official First Action Methods

This category of official first action methods was established at the 1972 Annual Meeting to permit methods to be given status or recognition between annual meetings. The requirements and procedural details were described in the July 1973 issue of *JAOAC* 56, p. 1031.

The following 2 methods have been adopted as interim official methods since the 1975 Annual Meeting of the AOAC: gas-liquid chromatographic method for the determination of non-elution materials (polymers and oxidation products) in heated vegetable oils, submitted by Walter E. Waltking, Best Foods Research Center, CPC International Inc., Union, NJ 07083, published in (1975) *JAOAC* 58, 898–901; and a flameless atomic absorption method for the determination of mercury in water, submitted by Theodore O. Meiggs, Environmental Protection Agency, Denver, CO 80225, Method D 3223-32, American Society for Testing and Materials, Philadelphia, PA. Copies of these methods are available from the AOAC office, Box 540, Benjamin Franklin Station, Washington, DC 20044, at a nominal charge. The Association will vote on full acceptance of these interim actions at the 1976 Annual Meeting; if the interim actions are approved, these methods will appear in "Changes in Methods" (1977) *JAOAC*, March issue.

Adoption of Methods

The Association acted on a total of 48 new and revised methods during the 89th Annual Meeting, Oct. 13–16, 1975, as Washington, DC. The sources and numbers of methods adopted are: Food and Drug Administration 17, U.S. Department of Agriculture 6, other U.S. government agencies 4, State regulatory agencies 1, industry 17, and universities and institutes 3.

New or revised methods were adopted in the following topics: Feeds 3; Pesticide Formulations

6; Drugs 4; Dairy Products 3; Fish and Other Marine Products 2; Food Additives 1; Meat and Meat Products 2; Microchemical Methods 1; Mycotoxins 1; Alcoholic Beverages 4; Cereal Foods 1; Fruits and Fruit Products 2; Vitamins and Other Nutrients 4; Pesticide Residues 2; Metals and Other Elements 1; Extraneous Materials 4; Microbiological Methods 3; Drugs in Feeds 2; Drug and Feed Additives in Animal Tissues 1; Forensic Sciences 1.

Enzyme Nomenclature

Enzyme Nomenclature: Recommendations (1972) of the International Union of Pure and Applied Chemistry and the International Union of Biochemistry. Supplement 1: Corrections and Additions (1975) was published in Volume 429 of *Biochimica et Biophysica Acta*, pp. 1–45, appearing in March 1976. Reprints may be purchased from: Elsevier Scientific Publishing Co., PO Box 1345, Amsterdam, The Netherlands. Prices (including postage) are: Dfl 15.00 each ($5.63) for 1–9 copies; Dfl 14.00 each ($5.26) for 10–49; Dfl 12.00 each ($4.51) for 50–99; Dfl 11.00 each ($4.13) for 100 or more.

Census Suggestion Box

The Census Bureau in the United States is now planning for the 1980 census. The full content of the basic census questionnaire must be determined by spring of 1977 so that final preparatory steps can be accomplished successfully. Although there are many constraints on the census in terms of what and how much information can be collected and tabulated, the Bureau is trying to obtain and review the recommendations of as wide a range of users and potential users of decennial census data as possible. Therefore, AOAC members who have any suggestions, questions, or comments on the 1980 census are invited to direct them to: Director, Bureau of the Census, U.S. Department of Commerce, Washington, DC 20233.

Summer Programs

The Massachusetts Institute of Technology (MIT) will offer a 2-week elementary course in Design and Analysis of Scientific Experiments, June 14–25, 1976. The course will be taught by Professors Harold Freeman and Paul Berger. MIT will also hold a short course in Enzymes and Their Use in Analysis and Clinical Diagnosis, July 12–16, 1976. The objective of the program is to develop the participants' abilities to use enzymes as analytical reagents and to measure enzyme activities in biological materials. For further information on both courses contact: Director of the Summer Session, Room E19-356, MIT, Cambridge, MA 02139.

Microscopy Conference

The McCrone Research Institute of Chicago will sponsor the INTER/MICRO 76, The International Conference on Microscopy, June 28–July 1, 1976 at the McCormick Inn, Chicago, IL. The McCrone Institute will also sponsor a variety of 3–5 day microscopy courses throughout the year in Chicago and other locations. For a catalog and schedule of the courses, and program and exhibitor information on the conference, write: Walter C. McCrone, McCrone Research Institute, 2508 S. Michigan Ave, Chicago, IL 60616.

Nuclear Science Abstracting Service

An international nuclear science abstracting service is now available in INIS ATOMINDEX. ATOMINDEX incorporates into its data base the service formerly provided by Nuclear Science Abstracts, the U.S. Energy Research and Development Administration (ERDA) periodical which is being discontinued. The index will cover all nuclear-related information generated by ERDA and other U. S. sources.

ATOMINDEX has been produced since 1970 by International Nuclear Information System (INIS), a project of International Atomic Energy Agency (IAEA). It pinpoints information necessary to identify, locate, assess, and obtain all items recorded in the System. These items include books, technical reports, journal articles, and conference papers as well as patents, standards, and theses. Each twice-monthly issue of ATOMINDEX is fully indexed, and multivolume Cumulative Indexes are published semiannually. Subjects covered in ATOMINDEX include: life sciences; health, safety and the environment; engineering and technology; isotope and radiation applications; physical sciences; chemistry, materials, and earth sciences; other aspects of nuclear energy—economics, law, documentation, safeguards, inspection.

INIS ATOMINDEX is available from Unipub, Box 433, Murray Hill Station, New York, NY 10016. Rates for 1976 are: (a) 24 issues + 2 cumulative indexes, $150.00; (b) 24 issues only, $110.00; (c) 2 cumulative indexes only, $40.00. A sample copy of ATOMINDEX is available on request.

Air Sampling Methods Book Available

The second edition of *Methods of Air Sampling and Analysis* is now available from the American Public Health Association. It was produced by an Intersociety Committee from 13 professional associations (APCA, ACS, ACGIH, AIHA, AIChE, APHA, APWA, ASCE, ASME, AOAC, HPS, ISA, SAE). Major topics in the new edition include:

General Precautions and Techniques/Methods for Ambient Air Sampling and Analysis; Recommended Factors and Conversion Limits for Analysis of Air Pollutants; Industrial Hygiene, Air and Biological Sampling Methods in the Work Place.

The book can be ordered at a special prepublication price of $28.00 plus $0.50 handling charge per order until May 31, 1976. Regular price is $35.00. Order from American Public Health Association, 1015 18th St, NW, Washington, DC 20036.

9th ICC Congress to Meet in Vienna

The 9th Congress of the International Association of Cereal Chemistry (ICC) will meet in Vienna, Austria, May 12–14, 1976 at Kongressaal, Bundeswirtschaftskammer, Hoher Markt 3, Vienna 1.

The chairman of the ICC Technical Committee will report to the Congress on results achieved in the Association's Study Groups. Symposia will be held on the following subjects: Protein Analysis—Chairman: V. F. Golenkov, USSR; Protein Structure—Chairman: M. Jankiewicz, Poland; Contribution of Wheat Flour Components to Breadmaking—Chairman: Y. Pomeranz, U.S.A.; Theoretical Bases of Dough Rheology—Chairman: A. H. Bloksma, The Netherlands.

Working languages of the Congress will be English, French, and German, and simultaneous translations of the above presentations will be held. Two other symposia (without simultaneous interpretation) will be: Composite Flours—Chairman: D. de Ruiter, The Netherlands and Moisture Problems in Cereals—Chairman: J. L. Multon, France.

Proceedings of the 8th International ICC Working and Discussion Meetings, containing the full text of all papers presented, is now available at a cost of 200 Austrian Shillings ($11.00 U.S.) including shipping costs. Airmail rate will be charged separately. Orders may be sent to ICC Secretariat General, Schmidgasse 3-7, A-2320 Schwechat, Austria.

Forensic Toxicology Symposium To Be Held in Belgium

The European Symposium of the International Association of Forensic Toxicologists will be held at the State University of Ghent, Belgium, Faculty of Pharmaceutical Sciences, Department of Toxicology, Aug. 27–29, 1976. The language of the symposium will be English. Further details may be obtained from: Department of Toxicology, State University of Ghent, Hospitaalstraat 13, 9000 Ghent, Belgium.

AOAC Publishes EPA Pesticide Formulations Manual

The AOAC has scheduled publication of the *EPA Manual of Chemical Methods for Pesticides and Devices* for July 1, 1976. The manual, compiled by the Environmental Protection Agency (EPA) and State laboratories, will include over 200 currently used methods for analyzing commercial pesticide formulations—covering germicides, fungicides, herbicides, insecticides, and rodenticides —by means of gas-liquid, thin layer, and high-pressure liquid chromatography, infrared, ultraviolet, and atomic absorption spectroscopy, and classical chemical procedures.

The 1000-page, loose-leaf volume, sponsored jointly by the EPA, the Association of American Pesticide Control Officials, and the AOAC, will include such special features as a pesticide formulations bibliography, a cross-reference index, and more than 350 individual infrared spectra of pesticide compounds.

The manual is intended for use as a supplement to the 12th edition (1975) of the AOAC *Official Methods of Analysis*. Since the number of official methods applicable to pesticide formulations is limited, the new EPA manual makes available the analytical procedures presently used by EPA and State laboratories in enforcing State and Federal environmental laws.

The manual is available from the AOAC at a cost of $25.00 per copy plus $2.00 for postage and handling. Orders may be sent to: AOAC, Box 540, Benjamin Franklin Station, Washington, DC 20044.

AOAC Slide Series

A descriptive slide series on the AOAC is now available for presentation to persons and agencies interested in learning more about the Association and taking an active role in its endeavors.

The series, developed and updated by AOAC Executive Secretary Luther Ensminger and Dr. Fred J. Baur, Proctor & Gamble, Cincinnati, OH, will be shown to interested groups by members of the AOAC Executive Committee and executive employees. It consists of 70 slides highlighting information on AOAC activities, membership, and structure.

The slides explain in detail the goals of the Association and depict which agencies, industries, and laboratories use AOAC methods of analysis. The slides also describe the topics researched by AOAC analysts and illustrate the responsibilities and activities of each sector of the membership: Subcommittee members, General Referees, Associate Referees, collaborators, and administrative staff.

Also highlighted are classes of membership, publications of the AOAC, sources of program support, and sources of General and Associate Referees.

The slide series should serve as a valuable informative tool for those scientists and administrators interested in active participation in the Association. It has already been presented before a number of Food and Drug Administration organizational units in Washington, DC and the field, as well as several State agencies.

Arrangements to have the slide series shown can be made by contacting: Kathleen Fominaya, AOAC, PO Box 540, Benjamin Franklin Station, Washington, DC 20044.

NEW PUBLICATIONS

Acceptable Common Names and Chemical Names for the Ingredient Statement on Pesticide Labels. 3rd Ed. U. S. Environmental Protection Agency, Washington, DC 20460, 1975. 181 pp., paperback. Distributed at no charge (obtain from Mrs. Aleda Evans, PM 215, EPA Information Center, Washington, DC 20460).

This publication is an extensive revision of the compilation prepared in June 1972 and includes all the common names in effect as of January 1975. The more than 930 entries comprise common names, chemical names, and trade names.

Methods of Air Sampling and Analysis. 2nd Ed. Morris Katz (Ed.). American Public Health Association, 1015 18th St, NW, Washington, DC 20036, 1976. 900 pp. Price $28.00 plus $0.50 for handling for each order—special prepublication price until May 31, 1976; $35.00 (regular price).

Major topics covered in the expanded new edition include the following: general precautions and techniques/methods for ambient air sampling and analysis—carbon compounds, halogens and halogen compounds, metals, oxidants, inorganic nitrogen compounds, particulates, radioactivity, sulfur compounds; recommended factors and conversion limits for analysis of air pollutants; industrial hygiene; and air and biological sampling methods for the work place. This manual was a cooperative effort of 13 professional associations: Air Pollution Control Association, American Chemical Society, American Conference of Governmental Industrial Hygienists, American Industrial Hygiene Association, American Institute of Chemical Engineers, American Public Health Association, American Public Works Association, American Society of Civil Engineers, American Society of Mechanical Engineers, Association of Official Analytical Chemists, Health Physics Society, Instrument Society of America, Society of Automotive Engineers, with the cooperation of the Environmental Protection Agency and the National Institute of Occupational Safety and Health.

BOOK REVIEWS

Operational Amplifiers in Chemical Instrumentation. Robert Kalvoda. John Wiley & Sons Inc., New York, 1975. 178 pp. Price $23.50.

Operational amplifiers are electronic devices which can be connected into a circuit to perform various mathematical operations, such as addition, subtraction, multiplication, division, differentiation, and integration. This wide versatility provides the chemist with an opportunity to design and construct instrumentation for measuring specific parameters. Dr. Kalvoda of the J. Heyrovsky Institute of Physical Chemistry and Electrochemistry, Czechoslovak Academy of Sciences, describes how the operational amplifier works and how it can be employed in the construction of chemical instrumentation.

The monograph consists of 8 chapters, a lengthy reference list, and a useful subject index. Chapter 1 presents a scheme for a generalized measuring system. Chapters 2–4 introduce the operational amplifier and describe a variety of operational amplifier circuits. Chapters 5 and 6 discuss important parameters of operational amplifiers and types of operational amplifiers. Chapter 7 presents a number of applications of operational amplifiers to chemical instrumentation. Chapter 8 is a detailed discussion of several operational amplifier module kits.

This is the latest in a series of monographs in analytical chemistry, the Ellis Horwood Series, edited by Dr. R. A. Chalmers. This book will be most helpful to the chemist with some electronic training, especially those with some experience in design and construction of instrumentation.

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Photoelectron and Auger Spectroscopy. Thomas A. Carlson. Plenum Press, New York, 1975. xiii + 417 pp. Price $32.50.

This book is undoubtedly the most comprehensive text on electron spectroscopy currently available. The author presents in detail the theoretical and instrumental bases of the 3 most profitable areas of study in the field—x-ray photoelectron spectroscopy, ultraviolet photoelectron spectroscopy, and Auger electron spectroscopy. He also provides a thorough and up-to-date survey of pertinent literature, encompassing over 1000 references. This book is intended primarily for researchers already working in the field of electron spectroscopy, but it will also be useful to others seeking an introduction to the field and a reference book for demonstrating its accomplishments and potential.

Electron spectroscopy for chemical analysis (ESCA) derives its analytical utility from measurements of the binding energies of atomic and molecular orbitals. The usefulness of the data yielded by photoelectron spectroscopy of the outer shells has been compared with that furnished by optical and mass spectroscopy, while the potential analytical utility of photoelectron spectroscopy of the inner shells (PESIS) has been compared with that of Mössbauer and nuclear magnetic resonance spectroscopy. PESIS and particularly Auger spectroscopy have been used to date most often as techniques of surface analysis for impurities, coverage, inhomogeneity (between bulk and surface), radiation damage, and molecular identification. PESIS also has considerable qualitative, quantitative, and diagnostic potential for use in such diverse applications as biological structure determinations, rapid nondestructive elemental analysis of geological samples, environmental studies, quantitative analysis of polymers and alloys, radiation studies, and spot testing of industrial processes.

Some specific examples cited in the book should be of particular interest to regulatory chemists. ESCA has been used to obtain quantitative estimates of the total protein content of various species of grain by measuring the intensities of the nitrogen and sulfur peaks. In addition, for proteins rich in lysine and arginine, the amine nitrogen has been distinguished from amino nitrogen. Thus, PESIS appears to be a convenient and rapid method for determining the quantity and quality of grain proteins, with potential for use in mass screening operations. PESIS has also been used to measure chemical shifts in the binding energies of arsenic in soil samples spiked with a commercial herbicide containing arsenic, in the form of cacodylic acid, as its active ingredient. Using the soil sample directly, it was possible to estimate the charge state of the arsenic *in situ*. Measurements on a group of narcotics and narcotic antagonists confirmed, both experimentally and theoretically, that the charge density on the nitrogen atom of the protonated species remains nearly invariant for a variety of species, in contrast to earlier speculation based on differences in pharmacological activity.

The foregoing examples are only a few of many cases where photoelectron spectroscopy has been put to work on applied problems. At least several

hundred electron spectrometers have been purchased for industrial and application uses. Commercial instruments with varying operational characteristics are currently available from AEI, DuPont, Hewlett-Packard, McPherson, Perkin-Elmer, Physical Electronics Industries, McCrone-RCI, Vacuum Generators, Inc., and Varian Associates. The author's objective review of the various commercial instruments will be especially helpful to those considering purchase of an electron spectrometer. Price is a drawback, with costs ranging from $40,000 to $150,000; the average is close to the upper end of the price range. However, if the potential ESCA has for structural determination and elemental analysis can be fully realized, this cost would be justified.

ALAN J. SENZEL

AOAC
Washington, DC

Aldehydes—Photometric Analysis. Vols. 1 and 2. E. Sawicki and C. R. Sawicki. Academic Press, London, New York, 1975. xxviii + 283 pp. (Vol. 1); xiv + 344 pp. (Vol. 2). Prices £10.50 (Vol. 1), £10.80 (Vol. 2).

From the title of this series, one would expect to find information on ultraviolet, visible, infrared, fluorometric, and phosphorimetric methods of analysis for aldehydes. The books contain this information and much more. Other analytical techniques which may incorporate photometry, such as paper, thin layer, and high-pressure liquid chromatography, are often included in the discussions.

These volumes comprise an extensive literature survey, organized into 67 alphabetically arranged chapters. Most of the chapters treat individual aldehydes (acetaldehyde through formaldehyde in Vol. 1, 3-formylacrylic acid through vanillin in Vol. 2). Interspersed are general chapters on classes of aldehydes (aliphatic, aromatic, and total aldehydes in Vol. 1, glyoxals, oxybenzaldehydes, peptide and protein aldehydes, and α,β-unsaturated aldehydes in Vol. 2). Most of the chapters contain sections on physical properties, spectral properties (ultraviolet-visible and fluorometric-phosphorimetric characteristics are stressed), measurement techniques, and separation techniques. Measurement techniques include direct photometric measurement of the unreacted aldehydes when feasible, and determination of the aldehydes after reactions to form absorbing or fluorescing species. Separation techniques include those of wet chemistry, as well as manual or instrumental chromatographic methods. The chapters often include directions for purification of critical reagents or solvents and outlines of the more useful or generally applicable methods of aldehyde analysis. Many valuable spectra and tables of data are reproduced from the source literature.

The organization of material and the clarity of writing are excellent. The general chapters give the reader easy access to literature covering properties and methods of analysis for classes of aldehydes, and these chapters are complemented by the more specific discussions of certain individual aldehydes. The latter chapters range in length from one paragraph (e.g., for 3-hydroxypyruvaldehyde) to 36 pages (for formaldehyde). The references are listed at the end of each chapter, and they total about 950 in Vol. 1 and 1180 in Vol. 2. The subject indexes are quite adequate.

Unfortunately, the reader must anticipate a rather high level of error in the reference lists. A spot check of 25 citations against the source literature revealed that 5 of these citations either contained bibliographic errors or were inappropriately cited.

Nonetheless, this set of volumes deserves to appear in any comprehensive scientific library; it will be highly appreciated by chemists engaged in the analysis of samples for aldehydes. The breadth of the material will make these texts useful in many disciplines, including the study of air pollution, the life sciences, pharmaceutical chemistry, and the control of industrial manufacturing processes.

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National Center for Drug Analysis
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Symbols and Abbreviations

kg	kilogram(s)
g	gram(s)
mg	milligram(s)
μg	microgram(s)
ng	nanogram(s)
L	liter(s)
ml	milliliter(s)
μl	microliter(s)
m	meter(s)
cm	centimeter(s)
mm	millimeter(s)
μm	micrometer(s) (*not* micron)
nm	nanometer(s) (*not* millimicron)
amp	ampere(s)
μa	microampere(s)
v	volt(s)
dc	direct current
′	foot (feet)
″	inch(es)
cu in.	cubic inch(es)
gal.	gallon(s)
lb	pound(s)
oz	ounce(s)
ppm	parts per million
ppb	parts per billion
psi	pounds per square inch
sp gr	specific gravity
bp	boiling point
mp	melting point
id	inside diameter
od	outside diameter
hr	hour(s)
min	minute(s)
sec	second(s)
%	per cent
$\overline{\$}$	standard taper
N	normal
M	molar
m*M*	millimolar

(Note: Spectrophotometric nomenclature should follow the rules contained in *Official Methods of Analysis*, "Definitions of Terms and Explanatory Notes.")

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Principles of Regulatory Drug Analysis

By Daniel Banes

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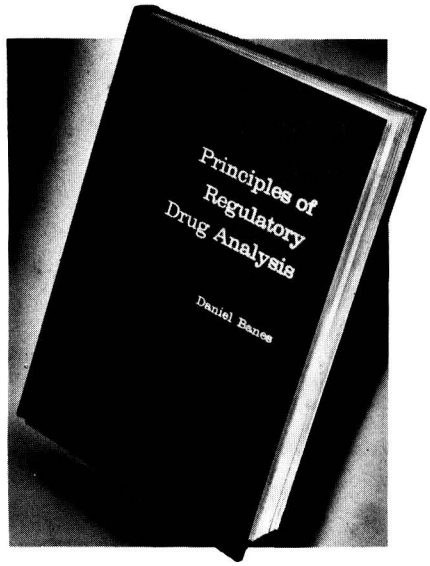

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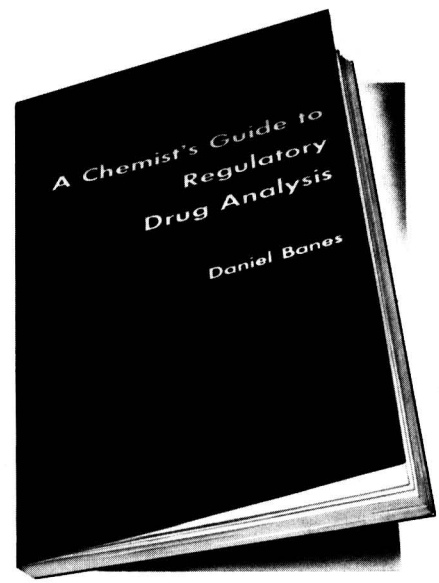

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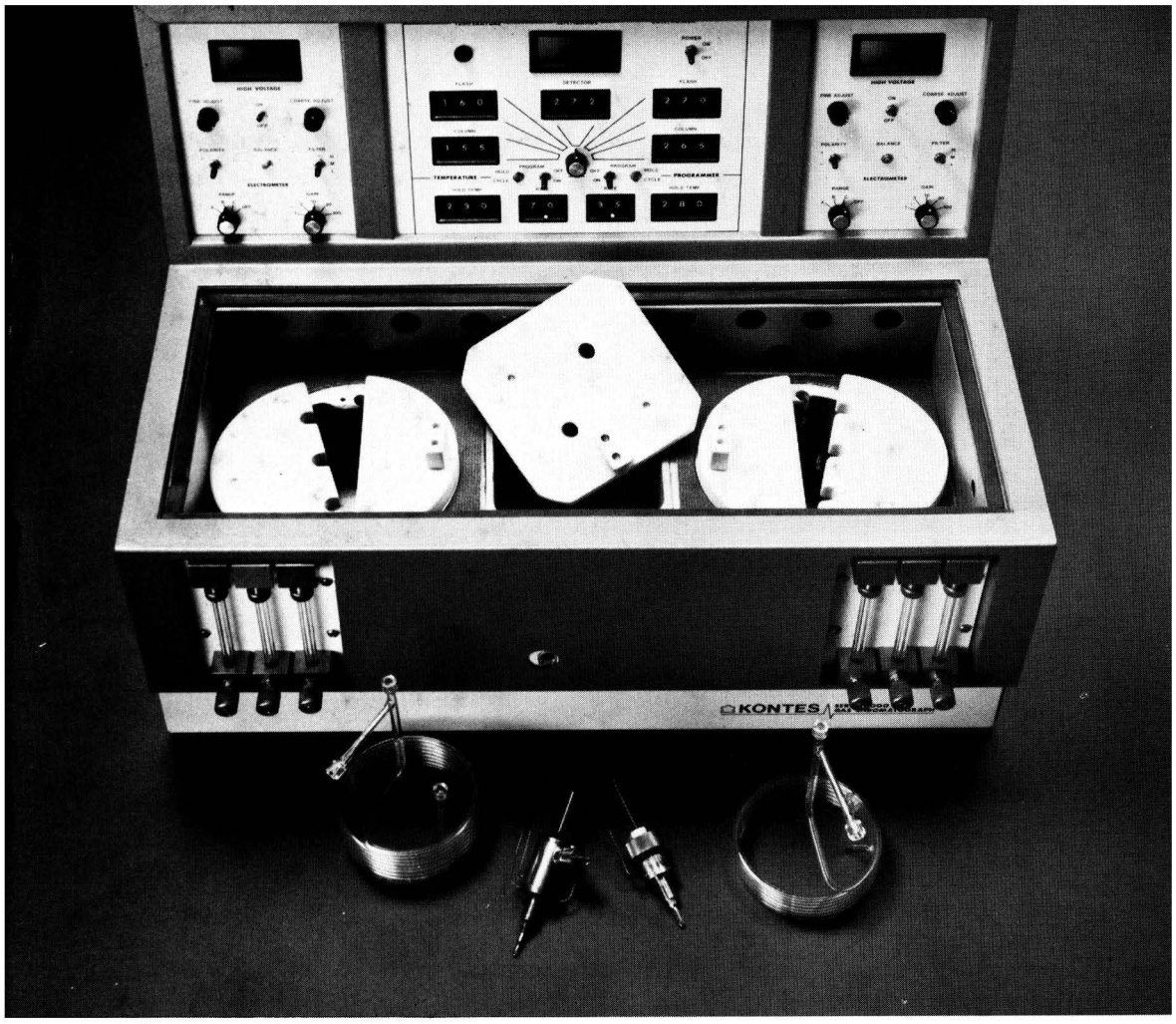

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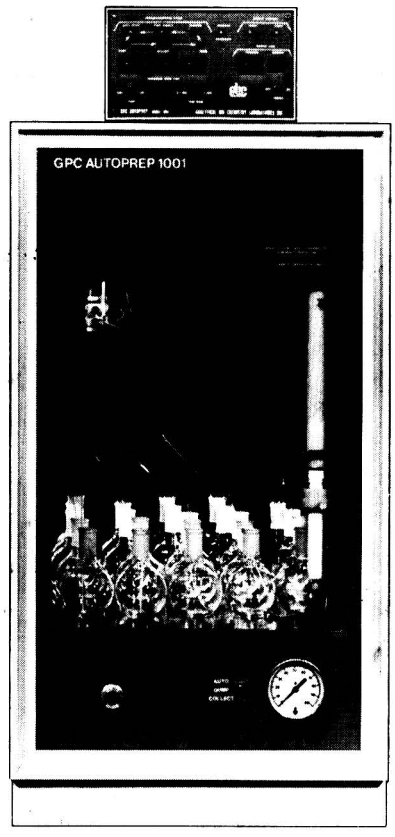

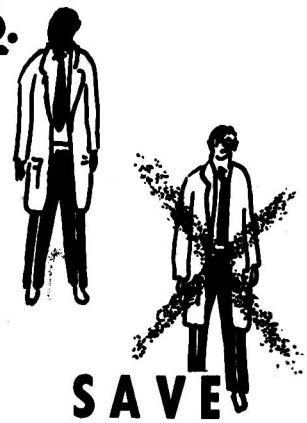

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