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MICROBIOLOGICAL METHODS

Culture Method for Detection of *Salmonella* in Dried Active Yeast: Collaborative Study

PAUL L. POELMA, AIDA ROMERO, CLYDE R. WILSON, and WALLACE H. ANDREWS
Division of Microbiology, Food and Drug Administration, Washington, DC 20204

A method for detecting *Salmonella* in dried active yeast was subjected to collaborative study. This method employs trypticase soy broth as the pre-enrichment medium, a sample-to-broth ratio of 1:10, and subsequent transfers to lauryl sulfate tryptose broth and tetrathionate before streaking onto selective agars. Each collaborating analyst received ten 25 g samples of dried active yeast. Duplicate 25 g samples were each inoculated with *Salmonella oranienburg* at a low level (28 cells) and a high level (107 cells). Similarly, duplicate 25 g samples were each inoculated with *S. senftenberg* at a low level (30 cells) and a high level (114 cells). The remaining 2 of 10 samples were not inoculated. Results from 12 of 13 collaborators were evaluated. Only 2 (8.2%) of the 24 low level *S. oranienburg* samples were reported incorrectly as negative. Twelve of the analysts detected *S. senftenberg* at both levels and *S. oranienburg* at the high level in the inoculated samples. Results from 12 collaborators used in the final evaluation show that 117 of 119 (98.3%) collaborative determinations are in agreement. The official final action method for the detection and identification of *Salmonella*, 46.013–46.026, has been revised official first action to include applicability to dried active yeast.

An improved method for the isolation of *Salmonella* from dried active yeast was developed by Wilson *et al.* (1). The initial method used by the Food and Drug Administration specified pre-enrichment in 1% tryptone broth, a sample-to-broth ratio of 1:5, and subsequent transfers to selenite cystine (SC) broth and tetrathionate (TT) broth before streaking onto selective agars. The improved method employs trypticase soy (TS) broth as the pre-enrichment medium, a sample-to-broth ratio of 1:10, and subsequent transfers to lauryl sulfate tryptose (LST) broth and tetrathionate broth before streaking onto selective agars. This improved method gave significantly higher most probable number (MPN) counts with 5 of 5 *Salmonella* serotypes, when comparative studies were conducted to evaluate the improved method.

Collaborative Study

The test samples were prepared from large amounts of commercial dried active yeast with an aerobic plate count of 22,000/g and with a viable yeast count of 1.3×10^{10} cells/g, using the standard plate counting procedure with wort agar. The yeast powder was pooled in a large container, thoroughly mixed, and dispensed in 25 g portions into individual containers. The samples were prepared by adding *Salmonella* test microorganisms to the yeast powder samples as described in Table 1. No other microorganisms were added to the powder because it contained enough non-*Salmonella* organisms including *Citrobacter*, a microorganism that resembles *Salmonella* and has often been confused with it. Each collaborating analyst received ten 25 g samples of dried active yeast.

Table 1. Description of collaborative samples[a] of dried active yeast

Sample	Microorganisms added	Level added/25 g
A, B	*S. oranienburg*	28
C, D	*S. oranienburg*	107
E, F	*S. senftenberg*	30
G, H	*S. senftenberg*	114
I, J	none	—

[a] Duplicate 25 g positive and negative samples were prepared.

These particular serotypes of *Salmonella* were selected for study because they are representative of the serotypes isolated from commercial dried active yeast. *S. senftenberg* was a strain recovered from a batch of positive commercial yeast powder.

The yeast samples were stored at 5–8°C until they were sent to the collaborators. Before samples were shipped, one set of 10 samples was analyzed; the results confirmed the presence of test microorganisms in the samples as prepared according to Table 1. Each sample was assigned a different sample number selected from a standard statistical table of random numbers (Rand Corp.) (2). Each of the 13 collaborating analysts received an identical set of 10 samples with final instructions for the collaborative method study. Analysts were requested to store samples at 5–8°C until analysis. Analysts were requested to start their examination on the same day and to report results, suggestions, and comments within 1 month.

METHOD

Media and Reagents

All media and reagents used in this study were prepared as described in *Official Methods of Analysis* (3), **46.013–46.026**, with the following additions:

46.015 ***Detection***

(c) *Dried active yeast.*—Aseptically weigh **25 g** sample into sterile, empty, wide-mouth, screw cap pt (**500** ml) jar. Add **225** ml sterile trypticase (tryptic) soy broth, **46.036(f)** (omit **10%** NaCl), and let yeast form smooth suspension. Determine pH with test paper, **46.014(1)**. Adjust pH, if necessary, to **6.8±0.2** with sterile **1*N*** NaOH or HCl, **46.014(c)** or **(d)**, capping jar securely and mixing well before determining final pH. (If pH is adjusted before yeast is evenly suspended, final pH will be less than desired.) Incubate **24±2** hr at **35°C**.

For active dried yeast, substitute lauryl sulfate tryptose broth, **43.036(b)**, for selenite cystine broth, **46.013(b)(*1*)** or **(*2*)**.

Results and Recommendations

Analytical results were reported by each of the 13 collaborators. Results of the positive and negative samples reported by the collaborators and the control collaborator are presented in Table 2. The control collaborative results were obtained by the authors by analyzing a set of samples during the time period specified for the study. Besides confirming the viability of the *Salmonella* serotypes added to the collaborative samples (Table 1), the results showed that 2 samples not inoculated with *Salmonella* were *Salmonella*-negative. The participating analysts were asked to report the results for each sample as either "*Salmonella*-negative" or "*Salmonella*-positive."

Results from 10 of the 13 collaborators (Table 2) agree in that each reported the 8 positive samples as *Salmonella*-positive and the 2 negative samples as *Salmonella*-negative. One analyst (Collaborator 2) reported that a sample was lost during examination. With the exception of the one sample that was lost, all analysts reported the negative samples as *Salmonella*-negative.

Results from one analyst (Collaborator 4) were negative for the 8 *Salmonella*-positive samples. This analyst stated that a *Salmonella* control culture failed to grow when it was inoculated into tetrathionate broth used in the collaborative analysis. On the basis of this medium deficiency, the data from the analysis of this set of 10 samples were excluded from further evaluation.

Using the improved method, 12 analysts detected *S. senftenberg* at each of the 2 inoculation levels (30 and 114 cells/25 g) in the 24 positive samples. Similarly, *S. oranienburg* was detected in 24 positive samples at the high level of inoculation (107 cells/25 g) by the 12 analysts. At the low level of inoculation (28 cells/25 g), however, *S. oranienburg* was not detected by 2 of the analysts.

Results from 12 collaborators used in the final evaluation indicate that the majority (117 of 119 or 98.3%) of the collaborative determinations are in agreement, and the method does detect *Salmonella* from positive dried active yeast at the level of *Salmonella* contamination (about 1/g) employed in this study. Statistical analysis indicates that there is no significant difference between the 12 collaborators in the proportion of correct responses.

It is recommended that the official final action method for the detection of *Salmonella*, **46.013–**

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

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

Table 2. Collaborative results for culture method of detecting *Salmonella* in dried active yeast samples[a]

	S. oranienburg				*S. senftenberg*							
	Low level		High level		Low level		High level		Negative		Total	
Coll.	A	B	C	D	E	F	G	H	I	J	Pos.	Neg.
1	+	+	+	+	+	+	+	+	−	−	8	2
2	+	+	+	+	+	+	+	+	−	−	8	2
3	+	−	+	+	+	+	+	+	[b]	−	7	2
4[c]	−	−	−	−	−	−	−	−	−	−	0	10
5	+	+	+	+	+	+	+	+	−	−	8	2
6	+	+	+	+	+	+	+	+	−	−	8	2
7	+	+	+	+	+	+	+	+	−	−	8	2
8	+	+	+	+	+	+	+	+	−	−	8	2
9	+	+	+	+	+	+	+	+	−	−	8	2
10	+	+	+	+	+	+	+	+	−	−	8	2
11	+	+	+	+	+	+	+	+	−	−	8	2
12	+	+	+	+	+	+	+	+	−	−	8	2
13	−	+	+	+	+	+	+	+	−	−	7	3
Control	+	+	+	+	+	+	+	+	−	−	8	2

[a] Duplicate 25 g samples prepared; + = positive; − = negative.
[b] Sample lost.
[c] Results were excluded from analysis; see text.

46.026, be revised to include applicability to dried active yeast by using pre-enrichment in trypticase soy broth with a sample-to-broth ratio of 1:10 and subsequent transfers to lauryl sulfate tryptose broth and tetrathionate broth before streaking onto selective agars.

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TOXICOLOGICAL TESTS

Biological Parameters and the Acute LD_{50} Test

FREDERICK SPERLING and JOSEPH L. McLAUGHLIN*
Department of Pharmacology, Howard University College of Medicine, Washington, DC 20059
** Division of Biological Sciences, Consumer Products Safety Commission, Washington, DC 20207*

The acute LD_{50} is an inadequate index of toxicity because the catastrophe of death obscures the mechanisms and sites of action. Thus, nonlethal toxicity is not evaluated, and the LD_{50} is not extrapolated to the possible effects of low level exposures for long periods of time. Collaborative studies should be undertaken which would develop methods to reasonably permit such evaluation and extrapolation.

The ED_{50} is the median effective dose. It is the dose which will produce a specific effect or response in one-half of a defined population of a given strain or species of living organism. The LD_{50} is simply a special case of the ED_{50} in which the response, or end point, is death. The acute ED_{50} or LD_{50} refers to the effects from a single dose or exposure.

Over the years, the acute LD_{50} has assumed special importance. The numerical value has come to be accepted as an index of toxicity; the lower the numerical value, the greater the degree of toxicity, and degrees of toxicity were thus classified by Gleason *et al.* (1) and Hodge and Sterner (2). These classifications were then adopted by government agencies and incorporated in the regulations of the Federal Insecticide, Fungicide and Rodenticide Act and the Hazardous Substances Act. They have also been accepted implicitly and explicitly in the various amendments and regulations of the Food, Drug and Cosmetics Act. These classifications ignore other parameters associated with the quantitation of mortality, for instance, whether large or small dose increments are required to produce equivalent increments of mortality, or whether deaths occur rapidly or slowly. Small dose increments which produce large effect increments present problems different from those in which large dose increments are required to produce equally large effects. Early or delayed deaths reflect mechanisms or sites of action and may have forensic importance as well. These important parameters are not considered in the LD_{50} evaluations mentioned later.

The LD_{50} does not include evaluation of morbidity or nonlethal effects. Liver and kidney damage, convulsions or unconsciousness, induced abortion or fetal resorption, embryonic malformations, cancer, etc., are not factors in the calculation of the LD_{50}, acute or nonacute, or in the standard classifications of toxicity based on this parameter. Consequently, the acute LD_{50} is only marginally informative, toxicologically inadequate, and misleading. It does have forensic value. It is important in projecting acceptable short-term levels of exposure for selected groups of individuals, such as in a catastrophe, in industrial exposures, or in accidental poisonings or suicides. It has little prognosticating value for nonlethal toxicity resulting from single or multiple low-level exposures such as may be encountered industrially and environmentally.

Much effort has gone into establishing the reliability and reproducibility of the numerical value of the acute LD_{50} and its extrapolation to lower values. Weil and his colleagues (3) repeated the acute LD_{50} values of a series of compounds for 12 consecutive years. Standard operating conditions were somewhat varied during this period. The acute LD_{50} numerical values of the 26 chemicals tested were practically the same throughout nevertheless. However, variation in the strain of animals used had a significant influence on the numerical value of the acute LD_{50}. Other parameters of toxicity were not considered. Griffith (4) and Weil and Wright (5) conducted collaborative interlaboratory acute LD_{50} tests. The results from the various laboratories, for each compound, were generally distributed normally. The differences between extremes varied by 2 to 3 times, which were small differences, biologically. Continuing the effort, Haley *et al.* (6) concluded that it was possible to accurately extrapolate acute LD_{50} data to

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values as low as $LD_{0.1}$. Weil (7; private communication, 1976) contended that even at the median level, the LD_{50} was inaccurate. But none of these efforts was concerned with the evaluation of nonlethal toxicity parameters and few with other parameters associated with mortality. Extreme precision notwithstanding, it cannot be shown that the LD_0, that dose which predictably will not be fatal, is equivalent to the ED_{100}, that dose which will produce nonlethal toxicity in every instance. Mortality frequencies at the lowest level simply cannot define actual toxicity.

Fühner (8) showed that many parameters which measured nonlethal toxicity were unrelated to mortality. An examination of the data reported by Smyth *et al.* (9), Weil *et al.* (3), and Scholz (10) also shows this. In each instance, significant toxicity was found at doses as much as 1000 times below the acute LD_{50} level. Boyd (11) showed that 2 different death rates from lethal doses of aspirin in rats reflected 2 separate mechanisms of action. Others (12, 13) showed that death rate observations may need to be prolonged for many days or even weeks. Obviously, toxicity needs to be defined in terms of morbidity as well as mortality and by parameters associated with mortality.

In a thorough study of acute LD_{50}, Boyd and co-workers (11, 14–20) showed that, besides the rate of death, survivor recovery rates based on food consumption and body weight changes were significant parameters for the evaluation of toxicity. Important information was gleaned also from autopsies of animals which died during the testing and of survivors at the end of the test. Boyd and co-workers (11, 14–20), Hayes (21, 22), and many others have shown that clinical observations which included gastrointestinal effects such as diarrhea or constipation, respiratory rates, and behavioral effects such as tremors, convulsions, lethargy, or unconsciousness are important parameters which should be evaluated.

Among the most important characteristics of living organisms are variability and change. Even clones show these. Standard strains do not remain standard for very long. In the past 25 years the lifespan of standard laboratory strains of rodents has doubled. Many of their hormonal and enzymic characteristics have become modified during that time. Even standard cultures of relatively simple organisms such as bacteria require periodic re-evaluation because of changing biochemical and enzymic parameters. Since standard animals tend to become "unstandardized," it is important to define test animals according to basic characteristics rather than to attempt to standardize any animal strain or species.

Standardization of acute toxicity must currently consider not only a numerical value for the LD_{50}, but the mechanism of action and the nature of nonlethal toxicity and clues for the prognostication of low-level, repetitive, long-term effects. The acute LD_{50}, regardless of refinements of methodology, is insufficient. Further attention to this parameter will have little value. Standardization should take into consideration biological realities. The animal species should be characterized, not standardized, by its sex, maturity (age and weight), state of health, and strain (biochemical characteristics). Ambience should be concerned with avoiding stresses associated with extremes. Collaborative studies should be undertaken to develop clinical and pathological parameters which will be most informative regarding mechanism of action, development of nonlethal toxicity, and prognostication of low-level, long-term effects. The single dose LD_{50} was not a good indicator or prognosticator. A toxicity profile should be developed which will be indicative of the toxicity potential of any chemical compound, whether used industrially or domestically, or produced as an environmental contaminant or therapeutic agent.

The toxicological community is invited to contribute its expertise to study these suggestions as applied to the acute LD_{50}. The AOAC referees are working with various government agencies and organizations such as the National Academy of Sciences, National Research Council. Various individuals and groups have already agreed to help with the acute LD_{50} investigations by the AOAC referees.

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PESTICIDE FORMULATIONS

Estimation of Milligram and Submilligram Amounts of Busan Fungicide

VERNON L. MILLER, ELIZABETH CSONKA, and CHARLES J. GOULD
Departments of Agricultural Chemistry and Plant Pathology, Washington State University, Western Washington Research and Extension Center, Puyallup, WA 98371

A procedure is described for the estimation of Busan 72 ((2-thiocyanomethylthio)benzothiazole) in the range of 0.01–1.0 mg/ml. An aqueous suspension of the material is heated with dilute sodium hydroxide, cooled, and treated with copper ion. The insoluble reaction product is filtered, and excess copper is determined by using a pyridine-thiocyanate procedure. Analysis may be completed by either spectrophotometry or comparison with described artificial standards.

Aqueous suspensions of Busan 72 (2-(thiocyanomethylthio)benzothiazole) of up to 1000 ppm are used to control *Fusarium* and *Penicillium* spp. that cause rot in plant materials, including Iris, Tulipa, and Narcissus bulbs grown in western Washington, and to treat seed, leather, and lumber (1). Since the bulb treatment involves contact of the fungicide with bulbs, soil, tanks, metals, etc., a rapid procedure is needed as a tankside test to assure that the recommended level of fungicide is maintained during bulb treatment.

Busan has several absorption peaks in the ultraviolet (UV) spectrum. The highest is at 280 nm. However, when the absorption of the calculated amount of Busan in a formulation is compared with that of pure material, results are 15–25% high. Thus, measurement of a peak in the UV spectrum is unsuitable for analytical purposes. Procedures reported by the manufacturer for the concentrate and for residues in paper pulp, based on the work of Pohloudek-Fabini *et al.* (2), were not satisfactory for levels of material in bulb-treating tanks. The procedure described is based on alkaline hydrolysis, reaction of a copper salt with the hydrolysate, and estimation of excess copper by a pyridine-thiocyanate reaction (3). The procedure is satisfactory in the range of 0.01–1.0 mg Busan/ml.

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METHOD

Reagents

(a) *Hydroxylamine solution.*—Dissolve 2.0 g ACS hydroxylamine HCl in water.

(b) *Alkaline copper solution.*—Dissolve 1.774 g ACS $CuCl_2 \cdot 2H_2O$ in small amount of water in 200 ml volumetric flask, add 50 g practical grade triethanolamine, dilute to volume with water, and mix.

(c) *Pyridine-thiocyanate solution.*—Dissolve 10 g ACS NaSCN in ca 40 ml water; add 20 ml ACS pyridine, followed by 17 ml 6*N* HCl. Dilute to 100 ml with water and mix.

(d) *Sealed simulated standards.*—For direct use as tankside test, combine several colored solutions to make artificial standards (Table 1). Seal standards, each containing 9.5 ml of the described solutions, in 150 × 18 mm od test tubes.

Procedure

Thoroughly mix suspension in tank to be tested and remove ca 100 ml sample in 250 ml bottle. Mix subsample gently to avoid foaming and quickly remove 10.0 ml with serological pipet from suspensions containing 0.1–1.0 mg/ml. Transfer sample to 50 ml centrifuge tube (Kimax 45192, or equivalent). Add 0.50 ml 1*N* NaOH, swirl to mix, and place in boiling water bath 10 min. Remove from bath and place in beaker of cold water $\leq$10 min. When cool, add 0.50 ml hydroxylamine solution, followed by 2.00 ml alkaline copper solution. Swirl to mix after each addition. Add 0.1 ml acetone, cover mouth of tube with plastic film (Parafilm), and mix by inversion. Filter through Whatman No. 5 paper. Filtrate must be clear. Transfer 3.00 ml filtrate to small separatory funnel and add 2.0 ml pyridine thiocyanate solution and 10.0 ml $CHCl_3$. Shake 1 min. Filter $CHCl_3$ phase through plug of cotton in stem of separatory funnel and measure absorbance at 450 nm. Compare absorbance with calibration curve made with known amounts of

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Table 1. Preparation of simulated artificial standards[a]

Reagent	Busan equiv. of std, mg				
	10.0	8.0	6.0	4.0	2.0
50.0% acetic acid, ml	9.22	7.76	5.96	5.14	3.89
4.0% $CuCl_2 \cdot 2H_2O$, ml	0.00	0.15	0.30	0.30	0.34
16.5% $NiCl_2 \cdot 6H_2O$, ml	0.41	0.75	1.16	1.73	2.37
0.1% sodium fluorescein, ml	0.36	1.35	2.58	2.82	3.41

[a] All solutions were prepared in 50% acetic acid.

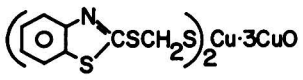
pure Busan treated similarly. Alternatively, compare $CHCl_3$ solution with artificial color standards as tankside test.

For samples containing 10–100 μg/ml, procedure is the same except use 0.1 ml reagent (a), 0.20 ml reagent (b), and 0.02 ml acetone. Measure absorbance at 410 nm. There are no simulated standards for lower concentration procedure.

Results and Discussion

Pulido (4) reported that Busan is prepared in an aromatic hydrocarbon solvent. However, the inclusion of toluene or xylene had no effect on the UV spectrum of Busan above 280 nm when the solvent used in the UV measurement was chloroform or ethanol. The inclusion of acetone or methyl ethyl ketone in amounts possible in the formulation increased the UV absorption in either solvent sufficiently to account for the 15–25% higher values observed.

The procedure depends on the combination of a Busan degradation product with copper ion in the ratio of 2:1. The precipitate formed when hydroxylamine, copper ion, and acetone were added to the alkaline hydrolysis product of Busan contained 35.2% copper and 4.33% nitrogen. Organic thiocyanates are reported to react with sodium hydroxide to yield the sodium salt of the substituted thiol and sodium cyanate (5). The alkaline copper is believed to react with the sodium salt of thiomethylthiobenzothiazole to yield Compound I. Compound I refluxed with 6*N* HCl or HBr yielded an insoluble yellow compound stable to prolonged boiling. The analysis of the compound and solution for copper indicated the compound was the copper salt of thiomethylthiobenzothiazole and the solution contained the 3 CuO groups from I. Calculated values for



Compound I

$C_{16}H_{12}CuN_2S_6$ are 13.1% copper and 5.74% nitrogen; observed values were 13.0 and 5.92%, respectively.

Hydroxylamine solution must be added prior to alkaline copper solution to ensure formation of a filterable precipitate. The exact role of hydroxylamine is not clear. Other aldehyde reagents such as sodium sulfite and semicarbazide hydrochloride were unsatisfactory. Hydrazine sulfate was better, but inferior to hydroxylamine hydrochloride. Since the copper is slowly reduced by hydroxylamine, acetone was added to combine with the excess, presumably to form inert acetone oxime. Undue delay in the completion of the analyses after addition of sodium hydroxide must be avoided.

The reagents for preparation of the artificial standards are given in Table 1. An organic dye was used, since the solubility of uranyl acetate was too low to give the necessary yellow color. Also, 50% acetic acid gave a colored solution more like chloroform in appearance than the previously prepared, similar aqueous artificial standards for thiabendazole and benomyl (6, 7). The artificial standards were calibrated against formulated Busan. Pure 2-(thiocyanomethylthio)-benzothiazole was used as a primary standard for spectrophotometry. The solubility of pure Busan in aqueous medium was very low. Pure material was weighed into the reaction tube, the correct volumes of water and 1*N* NaOH were added, and the reaction medium was stirred vigorously with a magnetic stirrer during heating. Absorbance was measured at the less sensitive 450 nm wavelength with larger amounts of Busan (0.1–1.0 mg/ml) to keep the readings on scale. With smaller amounts of Busan (0.01–0.1 mg/ml), absorbance was read at 410 nm, the most sensitive wavelength.

The effect of compounds that may be present or accumulate in treating tanks is given in Table 2. Many materials do not interfere at the level of 100 mg. Glucose interferes by reduction of

Table 2. Tolerance level of several compounds in the proposed procedure

Material	Amt tolerated, mg
$CaCl_2 \cdot 6H_2O$	100
$MgCl_2 \cdot 6H_2O$	25
$NaNO_3$	100
$K_2C_2O_4$	100
$Na_3PO_4 \cdot 12H_2O$	100
KF	100
$Na_2B_4O_7 \cdot 10H_2O$	50
Glucose	25
Sucrose	50
Sodium maleate	100
Na_2CO_3	100
Na_2SO_4	100
NaCl	100
$CH_3COONa \cdot 3H_2O$	100
$NaHCO_3$	50
Sodium gluconate	100
Sodium citrate	100
Sodium potassium tartrate	100

copper. Also, any appreciable pH change must be avoided.

Accuracy of the procedure and effect of interferences that may come from conditions of bulb treatment are given in Table 3. Busan concentration may be estimated ±50 ppm against the artificial standards; this is generally sufficient for a tankside test. Standard deviation of the data from spectrophotometric measurements as given in Table 3 is 26 ppm. Only Iris bulb husk seemed to have an adverse effect on the concentration of Busan on standing 48 hr.

Acknowledgment

The authors are grateful to M. L. Pulido of Buckman Laboratories, Inc., Memphis, TN for pure Busan standard and manuscript review.

Table 3. Busan fungicide concentrations (ppm) in suspensions determined before and 48 hr after addition of contaminants

Before addn			After addn		
Wt	Absorbance	Visual comp.	Absorbance	Visual comp.	Contaminant/200 ml suspension[a]
639	650	600	650	650	5 g iris bulb
1005	1050	1050	995	1000	2 g soil
954	950	950	915	950	1 g peat soil
900	945	950	960	950	2 g leather
744	760	750	710	750	2 g wood chips
864	855	900	730	750	0.5 g iris husk
954	950	900	920	900	brass
591	600	600	610	600	aluminum
870	900	850	860	850	lead
519	550	500	530	550	iron
1023	1000	1000	995	1000	stainless steel
600	550	600	570	600	galvanized iron
897	900	850	885	850	copper
525	510	550	465	500	2 g concrete

[a] Metals were 2 pieces, 2 × 1 cm, polished to bright metal.

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High-Performance and Gas-Liquid Chromatography of Methyl Parathion Formulations

EDWIN R. JACKSON
Mississippi State Chemical Laboratory, Box CR, Mississippi State, MS 39762

Methyl parathion formulations (a synthetic sample, a technical material, and 6 emulsifiable concentrates) were analyzed by high-performance liquid chromatography (HPLC), gas-liquid chromatography (GLC), and infrared (IR) spectroscopy. The HPLC method used in ultraviolet detector and a Permaphase ODS column with a methanol-water mobile phase. The GLC method used an SE-30/OV-210 column and flame ionization detection. The IR spectrum was recorded at 8.16 μm. A synthetic sample formulated to contain 45.5% methyl parathion (analytical grade) was analyzed 8 times by each method. HPLC yielded an average value of 45.4%, GLC 45.9%, and IR 45.7%. Analyses of variance of this repeatability study showed that the differences between the GLC mean and both the theoretical value and the HPLC mean were small, but significant. Technical methyl parathion and 6 emulsifiable concentrates were analyzed in duplicate by each method. Variance calculations for each set of duplicates by each method indicate a high probability of difference in the methods. High results from the IR analysis are probably due to impurities in the technical material because analysis of the synthetic sample was satisfactory. The HPLC and GLC methods appear to be precise and will be studied collaboratively.

The insecticide methyl parathion (*O,O*-dimethyl *O*-*p*-nitrophenyl phosphorothioate) is used in large quantities for insect control on cotton and other plants. In this country, most methyl parathion is formulated alone or as a mixture with parathion, toxaphene, endrin, malathion, or EPN.

This insecticide has been available for over 15 years but the AOAC does not have a method for its determination in formulations. Analytical methods for parathion (1–8) were adapted to the determination of methyl parathion and are still in use. Infrared (IR) spectroscopy (9) is widely used, and many laboratories employ in-house gas-liquid chromatographic (GLC) methods for formulations. Hayes (10) has established the feasibility of using high-performance liquid chromatography (HPLC) to analyze methyl parathion and there is considerable interest in this approach. We examined the various approaches to the analysis of methyl parathion so a method could be recommended for AOAC collaborative study.

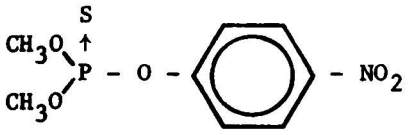
Technical methyl parathion used in preparing various formulations is usually guaranteed to contain 80% methyl parathion. Impurities in the technical product include *p*-nitrophenol (PNP), *O*-methyl *O,O*-bis(*p*-nitrophenyl) phosphorothioate (dinitro), and *O,S*-dimethyl *O*-*p*-nitrophenyl phosphorothioate (*S*-isomer) (11). Many formulations are emulsifiable concentrates in petroleum solvents, and these added components can cause problems in an analytical determination. For this work, a sample of the dinitro compound was obtained from Kerr-McGee Chemical Corp., Hamilton, MS, and the *S*-isomer was prepared in this laboratory (12).

One of the parathion methods being used in the analysis of methyl parathion is based on the reduction and diazotization of the nitro group. Another involves alkaline hydrolysis to yield the salt of PNP which is determined colorimetrically. Free PNP can be removed, but the dinitro compound and the *S*-isomer cause either of these methods to yield high results for methyl parathion.

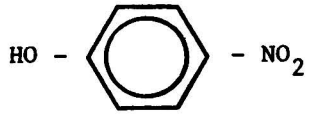
An examination of the IR spectra of the dinitro compound and the *S*-isomer indicates that these impurities would interfere to some extent at 8.16 μm. The spectra are very similar and no methyl parathion absorption is completely free of interference from these 2 impurities.

Both HPLC and GLC should be less susceptible to interferences. This is especially true for those formulations containing other pesticides in addition to methyl parathion. Laboratories presently using the GLC technique are using primarily nonpolar or slightly polar stationary phases (report from P. R. Caudill (1974), Chairman of Check Sample Committee, Association of American Pesticide Control Officials). The best

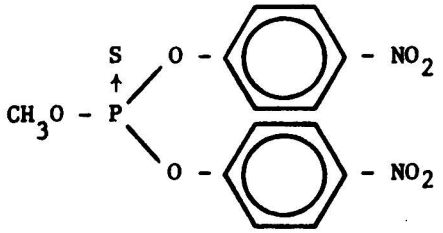
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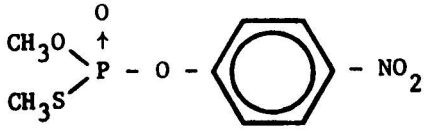
O,O-Dimethyl *O*-*p*-nitrophenyl phosphorothioate



p-Nitrophenol (PNP)



O-Methyl *O,O*-bis(*p*-nitrophenyl) phosphorothioate (dinitro compound)



O,s-Dimethyl *O*-*p*-nitrophenyl phosphorothioate (*S*-isomer)

stationary phase seems to be one that gives baseline separation of methyl parathion in the presence of the largest number of mixed formulations and that gives acceptable quantitative results. The column must be able to separate PNP, the dinitro compound, methyl parathion, and the *S*-isomer from other components as well as an internal standard.

A possible drawback to the GLC approach is the question of instability of methyl parathion at elevated temperatures. Methyl parathion may be converted to the *S*-isomer by prolonged heating at 125°C and this could be the source of some of the problems associated with the GLC determination. It is the experience of the author that low column and injector temperatures are desirable, but the design and condition of the GLC system are also important. An all-glass system should be used; on-column injection has given better results in this laboratory than has a system with a separate glass-lined injector. However, a dirty inlet from the injection of a large number of emulsifiable concentrates promotes the formation of *S*-isomer.

The HPLC method with ultraviolet (UV) detection lacks the versatility possible in the GLC approach because of wide differences in UV absorbance of the various pesticides that are commonly mixed with methyl parathion.

A synthetic emulsifiable concentrate, a technical material, and 6 commercial emulsifiable concentrates were analyzed by HPLC, GLC, and the published IR method (9) to establish that GLC and HPLC can be used for assaying methyl parathion in formulations.

High-Performance Liquid Chromatography

Apparatus and Reagents

(a) *High-performance liquid chromatograph with recorder.*—Model ALC-202 liquid chromatograph with fixed 254 nm UV detector and 6000 psi constant flow solvent delivery system (Waters Associates, Inc., Milford, MA 01757, or equivalent). Operating conditions: flow rate 1.2 ml/min; ambient temperature; range 0.32 absorbance unit full scale on 10 mv recorder; injection volume 10 μl with sample loop; operating pressure ca 1600 psi.

(b) *HPLC column.*—1 m × ¼″ drilled stainless steel tube (2.1 mm id) equipped with ¼ × 1/16″ low dead volume end fittings with 5 μm stainless frit in exit fitting (Alltech Associates, 202 Campus Dr, Arlington Heights, IL 60004). Pack tube with Permaphase ODS (Dupont Instruments, 1500 S Shamrock, Monrovia, CA 91016) according to method of Snyder and Kirkland (13). (Prepacked column may be purchased from Dupont Instruments.)

(c) *Methanol.*—ACS, redistilled from all-glass system.

(d) *HPLC eluting solvents.*—Prepare methanol-water (40+60) solution and thoroughly de-gas by stirring vigorously 5 min under moderate vacuum.

(e) *Methyl parathion stock solution.*—Weigh 220 mg analytical grade methyl parathion (Stauffer Chemical Co., Westport, CT 06880) into 100 ml

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glass-stoppered volumetric flask and dilute to volume with methanol.

(f) *Internal standard stock solution.*—Weigh 5.5 g reagent grade naphthalene into 500 ml glass-stoppered volumetric flask and dilute to volume with methanol.

(g) *Working standard.*—Transfer 25 ml aliquot of methyl parathion stock solution to 100 ml glass-stoppered volumetric flask, add 10 ml internal standard solution, and dilute to volume with methanol.

Preparation of Sample

(a) *Technical methyl parathion.*—Weigh 340 mg sample into 50 ml glass-stoppered volumetric flask and dilute to volume with methanol. Transfer 10 ml aliquot to 100 ml glass-stoppered volumetric flask, add 10 ml internal standard solution, and dilute to volume with methanol.

(b) *Emulsifiable concentrates.*—Weigh sample containing 55 mg methyl parathion into 100 ml glass-stoppered volumetric flask, add 10 ml internal standard solution, and dilute to volume with methanol.

Determination

Inject 10 μl aliquots of working standard until peak height ratio of methyl parathion to naphthalene varies ≤1%. Alternatively, inject standard and sample to give duplicate injections of each sample. Peak height response should be 60–80% full scale. Calculate peak height ratio of methyl parathion to naphthalene for each injection. Average the 2 sample and 3 adjacent standard peak height ratios.

$$\text{Methyl parathion, } \% = (R \times W' \times P)/(R' \times W)$$

where R and R' = average peak height ratios for sample and standard, respectively; W' = mg methyl parathion in working standard; W = mg sample in final 100 ml volumetric flask; and P = purity (%) of methyl parathion analytical standard.

Gas-Liquid Chromatography

Apparatus and Reagents

(a) *Gas chromatograph.*—With flame ionization detector, recorder, and provisions for on-column injection. GLC conditions: temperatures (°C)—inlet 200, column oven 180, detector 250; nitrogen carrier gas flow set to give retention time of ca 4 min for methyl parathion; attenuation set to give 60–80% full scale deflection for peaks.

(b) *Column.*—4′ × 4 mm id Pyrex column packed with 1.5% SE-30/1.5% OV-210 on 80–100 mesh Gas-Chrom Q. Condition ≥24 hr at 250°C.

(c) *Internal standard solution.*—Dissolve 5 g 2,2-bis-(*p*-chlorophenyl)-1,1-dichloroethylene (*p,p′*-DDE) (Aldrich Chemical Co., 940 N St. Paul Ave, Milwaukee, WI 53233, No. 12,389-7) in carbon disulfide, and dilute to 1 L.

(d) *Working standard solution.*—Weigh 125 mg analytical grade methyl parathion into 50 ml glass-stoppered Erlenmeyer flask, pipet 25 ml *p,p′*-DDE solution into flask, and swirl to dissolve.

Determination

Weigh technical methyl parathion or methyl parathion emulsifiable concentrate containing 125 mg pure material into 50 ml glass-stoppered Erlenmeyer flask. Pipet 25 ml *p,p′*-DDE internal standard solution into flask; swirl to dissolve.

Inject 1.5 μl aliquots of working standard solution until peak height ratios of methyl parathion to *p,p′*-DDE vary ≤1% on successive injections. Alternatively, inject 1.5 μl aliquots of working standard and sample to give duplicate injections of each sample. Calculate ratio of peak height of methyl parathion to *p,p′*-DDE for each injection. Average peak height ratios and calculate per cent methyl parathion as in high-performance liquid chromatography.

Results and Discussion

Bondapak C_{18}/Corasil, Bondapak Phenyl/Corasil, μ-Bondapak/C_{18} (all from Waters Associates, Inc.), and Permaphase ODS HPLC columns were tested for elution characteristics of methyl parathion, using (*a*) isopropanol-water, (*b*) acetonitrile-water, and (*c*) methanol-water mobile phases. Methyl parathion eluted from all of these chemically bonded stationary phases, but only μ-Bondapak/C_{18} and Permaphase ODS gave satisfactory separation from the impurities in a typical formulation. The methanol-water mobile system produced a more desirable elution profile than did acetonitrile-water and required a lower pressure than did the isopropanol-water system. The μ-Bondapak/C_{18} column appeared to be the column of choice, but it developed an extremely high back pressure during the study and was not available for the completion of this work. A liquid-solid system using the Waters μ-Porasil column and a methylene chloride-hexane mobile phase was also tested. This proved to be a highly efficient column, but it did not elute all components of the technical formulation under the conditions required to elute methyl parathion. A system of this type might be more difficult to maintain because small amounts of water would deactivate the adsorbant.

The Permaphase ODS column used was packed

in this laboratory and fitted with low dead volume end fittings constructed from standard Swagelok unions. Retention volume of methyl parathion on this column under stated conditions is 3.8 ml; Fig. 1 is a chromatogram of a typical 4 lb methyl parathion/gal. formulation. Volume retention ratios of other compounds of interest relative to methyl parathion are: *S*-isomer 0.42, PNP 0.44, parathion 2.5, naphthalene (internal standard) 2.6, the dinitro compound 7.3, and EPN 10.5. The system is capable of handling formulations of methyl parathion alone as well as mixtures with parathion. The UV absorbance of malathion at 254 nm is considerably lower than that of methyl parathion, while endrin shows practically no absorbance at this wavelength.

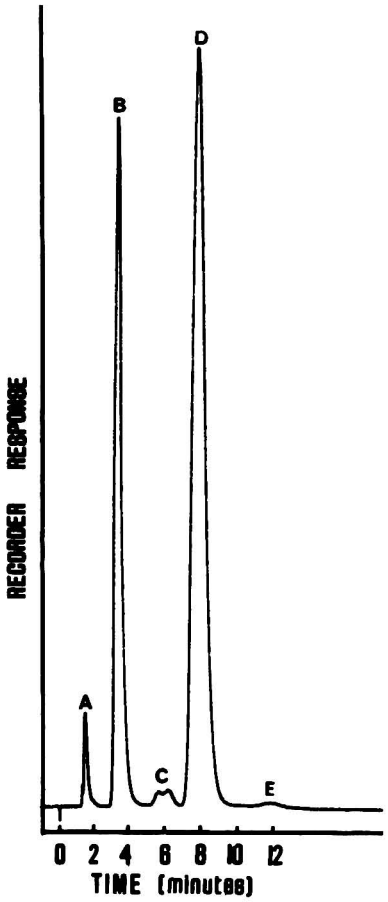


FIG. 1—High-performance liquid chromatogram of A, PNP and S-isomer; B, methyl parathion (5.5 μg); C and E, unknown; D, naphthalene (internal standard) (11 μg).

Linearity of this system was checked by chromatographing solutions of methyl parathion varying in concentration from 0.1 to 1.0 mg/ml while holding the concentration of naphthalene internal standard in these solutions constant at 1.1 mg/ml. Peak height ratios plotted against methyl parathion concentrations gave a straight line passing through the origin.

Investigation of GLC stationary phases showed that SE-30, DC-550, DC-710, OV-17, OV-25, OV-210, and OV-225 were unable to effect all separations that might be encountered in mixed methyl parathion formulations. The mixed SE-30/OV-210 column is able to handle most of these separations, but sizeable changes in column temperature and carrier flow are required for some of them. When methyl parathion is eluted at 4 min on this column, the relative retention ratios of other possible components of mixtures are: PNP 0.26, malathion 1.13, parathion 1.36, *p,p'*-DDE 1.62, *S*-isomer 1.87, endrin 2.02, and EPN 6.24. The dinitro compound does not chromatograph. Methyl parathion in a mixture with toxaphene can be analyzed by GLC, using a phosphorus-sensitive detector.

A synthetic emulsifiable concentrate was prepared in the laboratory according to a commercial formula except that analytical grade methyl parathion was used instead of the technical product. Table 1 lists results of 8 analyses of this synthetic sample by each method. At a 95% confidence level, only the GLC mean is significantly different from the theoretical value. This is explained in part by an abnormally low standard deviation for the GLC determinations.

Table 1. Results of 8 assays of methyl parathion (%) in a synthetic formulation[a] by HPLC, GLC, and IR methods

Method	Methyl parathion	Av.	Std dev.	Coeff. of var.
HPLC	45.4, 44.9, 45.8, 45.8, 45.0, 45.4, 45.2, 45.9	45.4	0.40	0.89
GLC	45.8, 45.9, 46.1, 45.8, 45.9, 46.0, 45.8, 45.6	45.9	0.15	0.33
IR	46.2, 46.2, 46.4, 44.6, 44.9, 45.6, 46.0, 45.5	45.7	0.65	1.4

[a] Theoretical value = 45.5% methyl parathion prepared from recrystallized methyl parathion, emulsifiers, and petroleum solvent.

Table 2. Analysis of methyl parathion (MP) in commercial formulations by HPLC, GLC, and IR methods

	HPLC			GLC			IR		
Sample	MP, %	Av.	Std dev.	MP, %	Av.	Std dev.	MP, %	Av.	Std dev.
1	43.0	43.1	0.14	43.2	43.5	0.35	44.5	44.7	0.22
	43.2			43.7			44.9		
2	41.5	41.6	0.14	42.7	43.0	0.35	43.0	43.5	0.69
	41.7			43.2			43.9		
3	39.5	39.6	0.21	41.1	41.0	0.07	42.2	42.7	0.71
	39.8			41.0			43.2		
4	41.7	41.4	0.42	41.9	42.2	0.42	42.2	42.0	0.35
	41.1			42.5			41.7		
5	42.7	42.9	0.28	42.6	42.8	0.28	43.4	43.6	0.30
	43.1			43.0			43.8		
6	43.5	43.8	0.35	44.2	44.3	0.14	45.5	45.3	0.30
	44.0			44.4			45.1		
Mean		42.1	0.28[a]		42.8	0.30[a]		43.6	0.46[a]
7[b]	77.7	77.1	0.85	76.7	77.0	0.42	78.0	78.2	0.23
	76.5			77.3			78.3		

[a] These standard deviations were calculated from the combined variances of duplicate assays on 6 different samples.
[b] Technical methyl parathion.

There is also a statistically significant difference between means obtained by the HPLC and GLC methods.

The repeatability of peak height ratios (methyl parathion/internal standard) for standard injections was evaluated for both HPLC and GLC. The ratios for the 17 HPLC standards injected during the synthetic sample determinations showed a standard deviation of 0.006, a mean of 0.923, and a coefficient of variation of 0.62%. For the GLC standard injections, the standard deviation was 0.008, the mean 0.989, and the coefficient of variation 0.79%.

The 6 commercial samples, 4 lb/gal. emulsifiable concentrates from 3 different formulators, were analyzed in duplicate by each method. Results are given in Table 2 along with the results from one technical methyl parathion sample. Means by each method were calculated. Student's *t*-test showed a significant difference between any 2 of the means at the 95% confidence level. IR results on the commercial samples were higher than those obtained by either of the other methods; this indicates that the bulk of IR interference may come from technical material. Results of the analysis of one technical methyl parathion sample support this.

Both the HPLC and GLC methods are precise and it is the opinion of the author that no conclusions should be drawn on superiority of either from such limited data. The Associate Referee recommends that both the HPLC and GLC methods be evaluated by a collaborative study.

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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 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**, 379.

Gas-Liquid Chromatography and Infrared Spectroscopy of Resmethrin in Nonaqueous Aerosols

MARK W. LAW, WARREN R. BONTOYAN, and JACK B. LOOKER
TSD-Chemical and Biological Investigations, Environmental Protection Agency, Beltsville, MD 20705

Two rapid and precise methods have been developed for the determination of resmethrin in nonaqueous aerosol formulations. The propellents are evaporated, the residue is dissolved in carbon disulfide, and the resulting solution is analyzed by gas-liquid chromatography or infrared spectroscopy. The accuracies of the methods were confirmed by a collaborative study.

Resmethrin (5 - benzyl - 3 - furylmethyl - *dl* - *cis-trans*-chrysanthemate) is a synthetic insecticidal pyrethroid, effective against houseflies, mosquitos, and cockroaches, and with low toxicity to mammals. Murano *et al.* (1) adapted methods for allethrin and phthalthrin to the determination of resmethrin in technical formulations and found gas-liquid chromatography (GLC) to be the best. Heath (2) reported a modified GLC method to determine resmethrin in dusts and petroleum distillates.

An effort to develop an interim specification analysis for the U.S. military brought to our attention the fact that no rapid specific method exists for determining resmethrin in aerosols. The search for a rapid and accurate method led to the development of simple determinations by a specific infrared (IR) and less specific GLC method.

Determination of resmethrin concentrations in carbon disulfide solution was linear over the range 1–12 mg/ml for both IR and GLC analyses. We chose a working concentration of 6 mg/ml to give optimum results by IR, using a 0.2 mm sodium chloride cell. This permits one solution to be used for both IR and GLC analyses.

Methanol also is a suitable solvent for the GLC determinations, and the same solution can be used for analysis by high-pressure liquid chromatography.

METHOD

Apparatus and Reagents

(a) *Gas chromatograph.*—With thermal conductivity detector and 4′ × ¼″ stainless steel column, packed with 10% SP-2100 on 80–100 mesh Chromosorb 750. Operating conditions: helium carrier gas flow 50 ml/min, injector 290°C, column 260°C, detector 270°C, attenuation 2×. Retention time of resmethrin is 4.5 min.

(b) *Infrared spectrophotometer.*—Double beam ratio recording, with matched 0.2 mm NaCl or KBr cells.

(c) *Resmethrin standard solution.* — (S. B. Penick & Co., New York, NY). Accurately weigh 150 mg into 25 ml volumetric flask and dilute to volume with ACS CS_2. Add small amount of anhydrous Na_2SO_4.

Preparation of Sample

Weigh full aerosol can to nearest 0.1 g and record as gross weight. Freeze overnight in freezer or 2 hr in Dry Ice box. Punch holes in top of can to release low boiling gases; then cut out top of can. Let can come to room temperature and then remove higher boiling propellents on ca 30°C water bath. Wipe can dry with lint-free towel and cool to room temperature. Reweigh can and top. Quantitatively transfer residue to 50 ml volumetric flask by dissolving and rinsing with CS_2. Dilute to volume with CS_2. Weigh can and top for tare and calculate per cent nonvolatiles. Take aliquot of solution and dilute to ca 6 mg nonvolatile materials/ml CS_2.

Gas-Liquid Chromatography

Inject 15 μl standard in duplicate. Then inject each sample at least twice. Multiple injections of same volume should agree ±1%. Measure peak areas for sample and standard and use average of these areas for calculations.

Infrared Spectroscopy

With CS_2 in reference cell, and using optimum quantitative analytical settings for IR instrument being used, scan both standard and sample from 1800 to 1600 cm^{-1} (5.6 to 6.25 μm). Determine absorbance of standard and sample by using peak at 1720 cm^{-1} (5.82 μm) and baseline from 1765 to 1660 cm^{-1} (5.67 to 6.02 μm).

Calculations

$$\text{Nonvolatiles, \%} = \frac{\text{(wt nonvolatiles/}}{\text{net wt contents)}} \times 100$$

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Table 1. Statistical analysis of results for collaborative study of resmethrin formulations

	1.2% Resmethrin formulation		2.0% Resmethrin formulation	
Coll.	GLC	IR	GLC	IR
1	—	1.20	2.14	2.09
2	1.28	—	2.08	—
3	1.26	1.24	2.18 2.16	—
4	1.35	1.30	2.08 2.02	2.07
5	1.25 1.22	1.32 1.30	2.16 2.18	2.23 2.12
6	1.26 1.31	1.27 1.26	2.01 1.98	2.14
7	1.36	1.28	2.14	2.13
Mean	1.29	1.27	2.10	2.13
Std dev. for repeatability	0.028	0.014	0.026	0.077
reproducibility	0.051	0.040	0.081	0.077
Coeff. of var. for repeatability	2.19	1.10	1.24	3.62
reproducibility	3.98	3.15	3.86	3.62

$$\text{Resmethrin, \%} = (A \times C' \times \text{purity of std} \times \text{\% nonvolatiles})/(A' \times C)$$

where A and A' = peak areas for GLC or absorbances for IR for sample and standard, respectively; and C and C' = concentrations (mg/ml) of sample and standard, respectively.

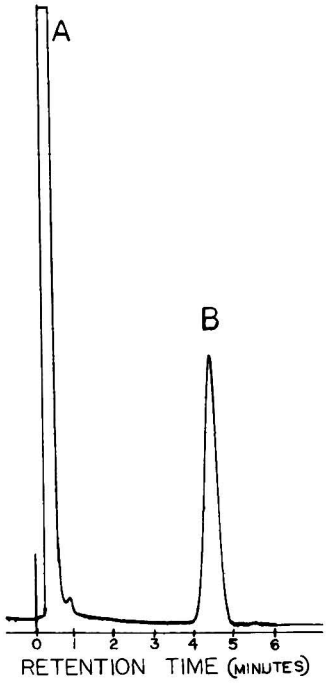


FIG. 1—GLC chromatogram of typical resmethrin sample: A, solvent; B, resmethrin. The standard gives an identical chromatogram.

Results and Discussion

The methods were subjected to a brief collaborative study, limited by number of samples available and number of collaborators possessing proper equipment. Samples containing 1.2 and 2% resmethrin were prepared by the S. B. Penick Co. and distributed to 7 collaborators for analysis. The data in Table 1 show good repeatability (within-collaborator variability) and good reproducibility (between-collaborator variability) for both methods tested.

Figure 1 shows a gas chromatogram of a typical resmethrin sample injection. Standard resmethrin gives an identical chromatogram.

Figure 2 shows IR scans of resmethrin standards before and after collection from a gas chromatograph. The most noticeable change appears in the region around 800 cm^{-1} (12.5 μm). The linearity of response and the precision of the method indicate that the thermal change can be considered to be quantitative. This procedure of trapping the eluate for IR analysis should be routinely used in GLC method development. It not only determines thermal decomposition, but also eliminates any doubt as to whether any other material present elutes at the same time, which would give high results. The primary reason suspected for slightly lower precision in the GLC method is the different injection techniques practiced by different analysts. To eliminate this problem we are developing an internal standard GLC method. We are also investigating high-pressure liquid chromatography as an alternative to GLC, since this would eliminate thermal factors.

Recommendations

Although the statistical analysis indicates that the IR and GLC methods are highly accurate, the AOAC requirements of at least 30 analyses

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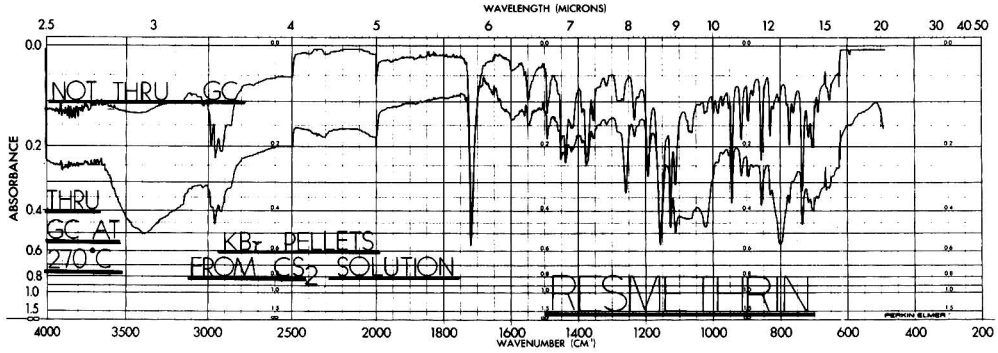


FIG. 2—Infrared scan of resmethrin standard before and after passage through a gas chromatograph.

for official first action were not met. Therefore, the Associate Referee recommends a larger collaborative study for the IR and GLC methods. Further study is recommended for GLC and high-pressure liquid chromatographic methods, using internal standards. Methods to determine resmethrin in water-based aerosol formulations for household use should also be studied.

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Collaborative Study of the High-Pressure Liquid Chromatographic Analysis of Picloram-2,4-D Formulations

NORMAN E. SKELLY, ROBERT J. RUSSELL, and DEBORAH F. PORTER
Analytical Laboratories, The Dow Chemical Co., Midland, MI 48640

A high-pressure liquid chromatographic method for the analysis of picloram-2,4-D formulations has been studied by 11 collaborators. The method requires no sample preparation other than weighing and dilution. Salicylic acid is used as the internal standard. The compounds are separated on DuPont Zipax SAX (strong anion exchange resin) with 0.01*M* sodium tetraborate and 0.002*M* sodium perchlorate as the mobile phase, and measured by ultraviolet spectroscopy at 280 nm. Coefficients of variation for the 6 samples studied ranged from 1.35 to 3.54% for picloram (4-amino-3,5,6-trichloropicolinic acid) and 0.83 to 2.30% for 2,4-D (2,4-dichlorophenoxyacetic acid). Comments from the collaborators were generally favorable. The method has been adopted as official first action for the determination of both picloram and 2,4-D in picloram-2,4-D formulations.

A number of herbicide formulations (Table 1) containing the active ingredient picloram (4-amino-3,5,6-trichloropicolinic acid) together with various chlorophenoxyacetic acids are marketed by The Dow Chemical Co. under the registered trademark Tordon®. An infrared method (Dow Analytical Method No. 87120a, Oct. 21, 1968) developed at the time these products were introduced gives satisfactory results, but requires extraction, solvent evaporation, and finally solvent addition before infrared measurement. Mueller (1) studied a method which required formation of the methyl ester followed by a partition chromatographic separation and ultraviolet (UV) measurement. A recent gas-liquid chromatographic (GLC) method (2) for the chlorophenoxy acids has been introduced; however, it also requires sample manipulation and derivatization before analysis.

Table 1. Composition and sample weights for the analysis of picloram-containing herbicides

Formulation	Sample wt, g	Picloram acid equiv., %	2,4-D acid equiv., %
Tordon 10K	1.0	10.0	
Tordon 22K	0.5	21.5	
Tordon 101	1.6	5.7–6.2	21.2–22.7
Tordon 101R	3.2	3.0	11.2
Tordon 212	0.8	10.1	20.2
Tordon 472	0.9	2.0	32.3

A high-pressure liquid chromatographic (HPLC) method for the analysis of picloram-2,4-D (2,4-dichlorophenoxyacetic acid) formulations has been studied collaboratively. The procedure requires no sample manipulation other than weighing and dilution. The compounds are separated by using an isocratic mobile phase and the eluate is monitored by a UV detector at 280 nm. Calculations are based on peak height measurements for the sample and internal standard.

Collaborative Study

Six samples of a picloram-2,4-D formulation were sent to 11 collaborators. The formulation contained water, 2-propanol, and the active ingredients as their triisopropanolamine salts. The collaborators were also supplied with standards of the active ingredients, the internal standard (salicylic acid), and a packed high-pressure liquid chromatographic column. They were instructed to make duplicate injections from 2 separate weighings and to average the results. All 11 collaborators responded to this request.

METHOD

Picloram (4-Amino-3,5,6-Trichloropicolinic acid) and 2,4-D (2,4-Dichlorophenoxyacetic Acid)

High Pressure Liquid Chromatographic Method
Official First Action

6.B09 ***Apparatus***

(a) *Liquid chromatograph.*—MiniPump® Model 396-31 (available from Milton Roy Co., 5000 Park St N, PO Box 12169, St. Petersburg, FL 33733), or equiv.; Altex Model 151, 280 nm UV detector (available from Altex Scientific, Inc., 1450 Sixth St, Berkeley, CA 94710), or equiv.; and Valco No. FSV-6-HPax injection valve (available from Valco Instruments, PO Box 19032, Houston, TX 77024), or equiv. Alternatively, septum injection head may be used; however, stop-flow injection is recommended.

Operating conditions: eluant flow rate, 0.7 ml/min (ca 1000 psi); detector sensitivity, 0.08 *A* unit full scale; temp., ambient, but within ±2.5°.

(b) *Liquid chromatographic column.*—No. 316 stainless steel, 1000 × 2.1 mm id, with Varian No. 96-000075-00 reducing union (⅛″ × 1/16″) contg 2 μm frit (regular reducing union packed with glass wool may be used instead) packed with DuPont No. 820960005 Zipax® SAX (strong anion exchange) resin. Preclean column with few ml each of $CHCl_3$, acetone, and MeOH, and vac.-dry. Pack in small increments over 40 min period while tapping column on hard surface.

6.B10 *Reagents*

(a) *Eluants.*—Prep. sep. solns of 0.01*M* $Na_2B_4O_7 \cdot 10H_2O$ (3.8 g/L) and 0.002*M* $NaClO_4 \cdot H_2O$ (0.28 g/L) in previously boiled and cooled deionized H_2O.

(b) *Salicylic acid internal std soln.*—Accurately weigh ca 3.6 g USP Ref. Std Salicylic Acid into 1 L vol. flask, dil. to vol. with 0.05*N* NaOH in isopropanol-H_2O (1 + 1), and mix.

(c) *Picloram-2,4-D std soln.*—(4 mg picloram + 12 mg 2,4-D + 3.6 mg salicylic acid)/ml. Accurately weigh ca 100 mg picloram ref. std, 99+% pure (Dow Chemical Co.), and ca 300 mg 2,4-D ref. std, 99+% pure (Dow Chemical Co.), into glass vial, pipet in 25 ml salicyclic acid internal std soln, and shake to dissolve.

6.B11 *Preparation of Sample*

Accurately weigh ca 1.6 g sample into ca 10 dram glass vial, pipet in 25 ml salicyclic acid internal std soln, and shake to dissolve.

6.B12 *Determination*

Inject 2 μl picloram-2,4-D std soln onto column and adjust attenuation to give largest possible on-scale peaks. Repeat injections until peak ht ratios of herbicide:internal std vary ≤1% for successive injections. Without changing conditions, inject 2 μl aliquots sample soln until peak ht ratios vary ≤1%. Average last 2 peak ht ratios for picloram and 2,4-D and calc. % herbicide.

$$\% \text{ Herbicide} = (R_x/R_s) \times (W_s/W_x) \times P,$$

where R_x and R_s = av. peak ht ratios of each herbicide to the internal std for sample and std, resp.; W_s = mg herbicide in std; W_x = mg sample; and P = % purity of std.

Discussion

The linearity of the UV detector response was determined over a 10-fold concentration range at 0.08 absorbance unit full scale. A plot of peak height vs. concentration for picloram, 2,4-D, and the internal standard was linear.

Liquid chromatograms of a typical Tordon 101 herbicide formulation together with a standard mixture are given in Fig. 1. The small peak appearing before picloram is an impurity in the technical product.

To analyze formulations other than Tordon 101, the weights of the analytical standards should approximate those of the active ingredients in the formulation.

The various chlorophenoxy acids do not interfere with the picloram peak. Retention times (min) are as follows: picloram 4.0; 2,6-D 4.0; salicylic acid 7.8; 2-(2-methyl-4-chlorophenoxy) propionic acid 10.7; 2-methyl-4-chlorophenoxyacetic acid 12.0; 2,4,6-trichlorophenoxyacetic acid 12.6; 2-(2,4-dichlorophenoxy)propionic acid 12.9; 2,4-D 14.3; silvex 33.1; 4-(2-methyl-4-chlorophenoxy)butyric acid 36.9; 2,4,5-trichlorophenoxyacetic acid 38.5; 4-(2,4-dichlorophenoxy)butyric acid 46.8. The impurity 2,6-D, generally present in 2,4-D at 1–6%, has the same retention time as picloram; however, at this concentration and because of its low UV sensitivity at 280 nm, interference is minimal.

It should also be noted that not all the chlorophenoxy acids are separated from each other. Generally a formulation will contain only one chlorophenoxy acid together with picloram. If it is suspected that the formulation contains more than one chlorophenoxy acid, infrared examination will be required.

The response factor for picloram relative to the internal standard salicylic acid was studied as a function of temperature. It varied by 0.3%/°C, based on peak height measurement. Therefore, if the ambient temperature changes by 5°C during an analysis, temperature control is necessary.

Although there are references to degradation of coated ion exchangers, column life ranged from 3 to 6 months with mobile phase being pumped continuously for that period of time. The mobile phase was recycled into the reservoir, and changed monthly. The column was cleaned at this time with more concentrated perchlorate solution.

The sample medium should be basic to prevent esterification of the salicylic acid. When

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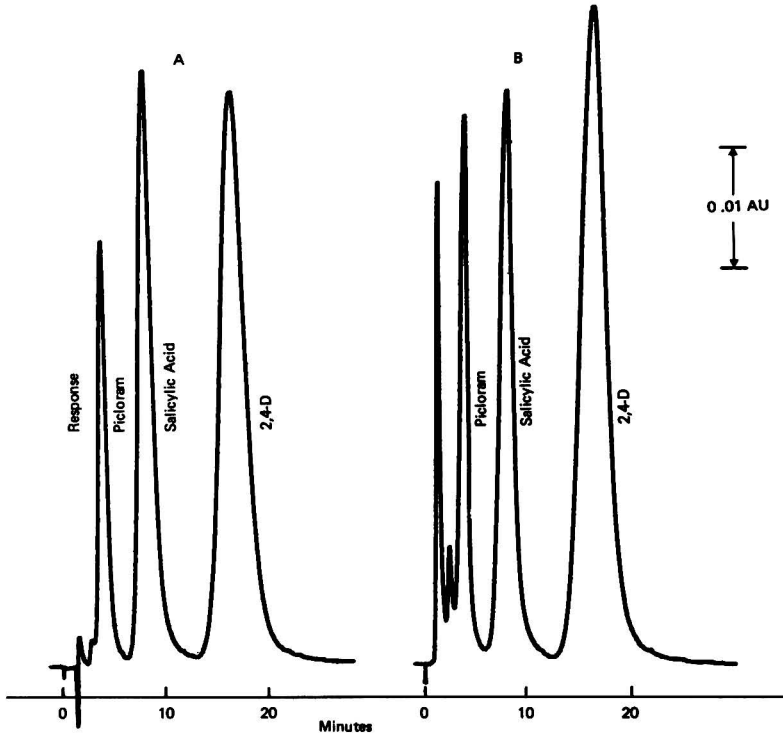


FIG. 1—High-pressure liquid chromatogram of standard and sample picloram-2,4-D formulation.

neutral 2-propanol was used as a solvent, formulations containing small amounts of acidic additives showed a slow reduction in salicylic acid with time which was attributed to ester formation.

The method has been used successfully for the analysis of Dow Chemical DMA-4 and DMA-6 herbicide formulations which nominally contain 40 and 60% 2,4-D, respectively.

Results

The results from the 11 collaborators are given in Table 2. Comments by the collaborators were generally favorable. Four collaborators suggested adding the internal standard by volume rather than by weight. This change has been incorporated into the method. However, it is imperative that the internal standard be added to the analytical standard and sample at the same time to preclude errors caused by temperature change.

The liquid chromatographic column used by Collaborator 3 deteriorated following single determinations of the samples. The column was returned and, indeed, did show poor resolution. No explanation could be offered for this.

Collaborator 2 recommended temperature control on the column. Based on the temperature study described earlier, temperature control is recommended if the ambient temperature varies >5°C.

The ranked results are given in Table 3. Collaborator 11 exceeded the approximate 5% 2-tail limits of 15–57 (3) for both picloram and 2,4-D. Therefore, these results were excluded from standard deviation calculations (Table 2) on the basis of systematic error. Also, one value from Collaborator 2 was not used. There were a few additional erratic values; however, they were generally within 2 standard deviations, and therefore were not discarded.

The results of Collaborator 1 were slightly out of the range of the 5% 2-tail limits for 2,4-D. Examination of the original data indicated that the second set of duplicate determinations for

Table 2. Collaborative results for high-pressure liquid chromatographic analysis of picloram-2,4-D formulations (% acid equivalent)[a]

	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6	
Coll.	Pic.	2,4-D	Pic.	2,4-D	Pic.	2,4-D	Pic.	2,4-D	Pic.	2,4-D	Pic.	2,4-D
1	6.31	20.10	6.39	19.73	6.30	19.82	6.12	19.86	6.37	19.48	6.42	19.55
2	5.35[b]	19.45	6.36	19.90	6.54	20.45	6.09	20.32	6.55	20.93	6.31	19.94
3	6.16	19.77	6.79	20.05	6.95	19.50	6.56	20.17	6.57	19.77	6.55	18.44
4	6.18	19.92	6.28	20.02	6.20	20.02	6.00	20.05	6.33	19.68	6.42	19.94
5	6.35	20.4	6.39	20.1	6.40	20.1	6.20	20.1	6.35	19.8	6.40	19.77
6	6.27	20.33	6.30	20.15	6.18	20.19	6.06	20.43	6.33	19.95	6.35	19.78
7	6.39	20.11	6.42	20.27	6.48	20.32	6.29	20.30	6.48	19.73	6.39	20.00
8	6.30	20.22	6.33	20.30	6.30	20.27	6.04	20.06	6.36	19.94	6.28	19.54
9	6.41	20.37	6.45	20.05	6.35	20.11	6.16	20.22	6.40	19.77	6.42	19.96
10	6.31	20.16	6.32	19.94	6.25	19.80	6.26	20.04	6.37	19.57	6.21	19.42
11[c]	6.61	21.70	6.54	21.51	6.53	21.45	6.56	21.04	6.66	21.62	6.55	21.21
Mean	6.30	20.08	6.40	20.05	6.39	20.06	6.18	20.16	6.41	19.86	6.38	19.63
Std dev.	0.085	0.30	0.15	0.17	0.23	0.28	0.16	0.17	0.090	0.40	0.094	0.47
Coeff. of var., %	1.35	1.48	2.28	0.85	3.54	1.42	2.65	0.83	1.40	2.03	1.47	2.30

[a] *See* Table 1 and text for a description of samples.
[b] Outline excluded by Dixon test in the statistical calculations.
[c] Data excluded because of systematic error; *see* text and Table 3.

Table 3. Ranked results for high-pressure liquid chromatographic analysis of picloram-2,4-D formulations

	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6		Coll. score	
Coll.	Pic.	2,4-D	Pic.	2,4-D	Pic.	2,4-D	Pic.	2,4-D	Pic.	2,4-D	Pic.	2,4-D	Pic.	2,4-D
1	5.5	8	5.5	11	7.5	9	7	11	6.5	11	4	8	36	58
2	11	11	7	10	2	2	8	3	3	2	9	4.5	40	32.5
3	10	10	1	6.5	1	11	1.5	6	2	6.5	1.5	11	17	51
4	9	9	11	8	10	8	11	9	10.5	9	4	4.5	55.5	47.5
5	4	2	5.5	5	5	7	5	7	9	5	6	7	34.5	33
6	8	4	10	4	11	5	9	2	10.5	3	8	6	56.5	24
7	3	7	4	3	4	3	3	4	4	8	7	2	25	27
8	7	5	8	2	7.5	4	10	8	8	4	10	9	50.5	32
9	2	3	3	6.5	6	6	6	5	5	6.5	4	3	26	30
10	5.5	6	9	9	9	10	4	10	6.5	10	11	10	45	55
11	1	1	2	1	3	1	1.5	1	1	1	1.5	1	10	6

2,4-D was noticeably lower than the first. Relative to the mean value for 2,4-D, the results did not deviate excessively and therefore were retained in this study.

Recommendation

Because of the variety of liquid chromatographic instrumentation used, the variation in aliquot injected (some collaborators had a 20-μl injection valve on their apparatus and therefore made 10-fold dilutions prior to analysis), and adjustments in eluant strength to obtain an optimum chromatogram, the method has, in effect, been put through a ruggedness test. One part of the method not checked is packing of the column. However, prepacked columns are available from the manufacturer.

Based on the results of this study is it recommended that the method be adopted as official first action.

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A. A. Carlstrom and J. R. Updyke, Chevron Chemical Co., Richmond, VA

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee A and was adopted by the Association. *See* (1976) *JAOAC* **59**, 379.

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

R. H. Collier and R. K. Gard, Purdue University, West Lafayette, IN

E. W. Day, Jr., Eli Lilly and Co., Greenfield, IN

D. L. Frasch, State Chemical Laboratory, Vermillion, SD

L. R. Kallenbach, Gulf Oil Chemicals Co., Merriman, KS

W. H. McDermott, Union Carbide Corp., South Charleston, WV

J. P. Minyard, Jr., Mississippi State Chemist Laboratory, Mississippi State, MS

L. Mujica, Dow Chemical Co., Mexico City, Mexico

A. E. Rash, State Laboratories Department, Bismark, ND

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ATTENTION

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.

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.

DEADLINE for receipt of abstracts and papers—August 13, 1976.

Collaborative Study of the Determination of Color in White Wines Dust and Powder Formulations

WILLIAM H. McDERMOTT
Research and Development Department, Union Carbide Corp., PO Box 8361, South Charleston, WV 25303

Some 10% and lower guarantee carbaryl dusts cannot be quantitatively extracted by shaking with chloroform as specified in the official first action infrared method for carbaryl formulations, 6.293–6.295. The official method has been compared in a collaborative study with an alternative method in which a simple rotator has replaced the wrist-action shaking machine and a solution of 10% (v/v) methanol in chloroform has replaced chloroform. The results indicate the alternative method is superior to the official method when applied to difficult-to-extract formulations and equal to it for all other formulations. The mixed solvent-rotator alternative method has been adopted as official first action to replace the present method for the carbaryl analysis of carbaryl formulations.

Several years ago it was brought to the attention of the General Referee on Pesticide Formulations and the Associate Referee on Carbaryl that not all carbaryl formulations can be assayed by the official first action AOAC infrared method for carbaryl formulations, **6.293–6.295** (1).

The essence of the problem is that carbaryl cannot be quantitatively extracted from some formulations by the simple chloroform extraction specified in the method. To date all complaints have dealt with formulations with assay guarantees of $\leqslant$10%. An investigation of this problem was initiated in 1972, and the preliminary results were reported at the 86th Annual Meeting of the AOAC (2).

A modification of the infrared method in which 10% (v/v) methanol in chloroform replaced chloroform and in which a simple rotator replaced the wrist-action shaker was developed and reported at the 88th Annual Meeting of the AOAC (3).

A repeatability study was made in which 5 formulations were assayed on 10 different days, using both the mixed solvent and chloroform. All samples were extracted in the simple rotator. The coefficients of variation ranged from 0.51 to 0.90% for the mixed solvent, and from 0.75 to 1.16% for chloroform. In no formulations were the results with chloroform higher than those with the mixed solvent. In 3 of the 5 formulations, the results with the mixed solvent were significantly higher than the results with chloroform.

Several samples found deficient in State laboratories by the official method were assayed by the official infrared method, the modified infrared method, and a colorimetric method (4). Results of these assays demonstrated that both the modified infrared method and the colorimetric method achieved quantitative recovery of the carbaryl in all formulations studied, and the official method did not.

As a result of this work, the Associate Referee recommended that the modified infrared method be collaboratively studied, and this recommendation was approved by Subcommittee A and accepted by the Association. This report concerns the results of the collaborative study.

Collaborative Study

Each collaborator received a copy of the method, an analytical grade sample of carbaryl, and 4 carbaryl formulation samples. Collaborators were asked to follow the method as closely as possible and to report any deviations. They were asked to prepare all equipment and apparatus, make a practice run on the carbaryl 50W (wettable powder) formulation if they felt it necessary, and then assay each sample once using each solvent, remembering to read the absorbance of the chloroform extract at both 5.75 and 8.94 μm. In addition, the inhibitor content of the chloroform used was requested.

A major difficulty in this investigation has been finding samples representative of the extraction problem in sufficient quantity to use in analytical experiments. For the collaborative study, special formulation samples were prepared. Small quantities of 2 difficult-to-extract

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5% carbaryl dust formulations were obtained and blended. This blend is Sample 1 in the collaborative study. Sample 2 was prepared by blending a random 10% carbaryl dust with 10% carbaryl dust which had been partially decomposed in an accelerated storage stability test. Sample 3 was a random 50% carbaryl wettable powder formulation. Sample 4 was prepared by blending a random 80% carbaryl sprayable powder formulation and an 80% sprayable powder formulation partially decomposed in an accelerated storage stability test. It was hoped that all the extraction difficulties possible in dust and powder formulations would be encountered in these samples.

METHOD

Carbaryl (1-Naphthyl Methylcarbamate) Official First Action

(*Caution: See* **51.018, 51.040, 51.041,** and **51.056.**)

6.B01 ***Apparatus***

(a) *Centrifuge.*—Clinical model, 8 place, or equiv.

(b) *Hypodermic syringe.*—1 ml, glass barrel with rubber-tipped plastic plunger (1 ml B-D Glaspak Tuberculin disposable syringe supplied by Becton, Dickinson, and Co., Rutherford, NJ 07070, is suitable). Disposable syringe may be used repeatedly. Wash with H_2O and acetone or MeOH, air-dry, and lubricate rubber plunger tip with silicone stopcock grease.

(c) *Infrared spectrophotometer.*—Perkin-Elmer Corp., Model 337, or equiv. Operator must adapt conditions to his instrument.

(d) *Rotator.*—Tube type, BBL, or equiv.

(e) *Shaking machine.*—Wrist-action shaker (Burrell Corp., 2223 Fifth Ave, Pittsburgh, PA 15219, or equiv.).

(f) *Tubes.*—Culture tubes, borosilicate glass, 16 × 150 mm with screw caps and Teflon liners (Corning Glass Works No. 9826, or equiv.).

6.B02 ***Reagents***

(a) *Methanol-chloroform soln.*—10% (v/v) MeOH in $CHCl_3$.

(b) *Carbaryl std solns.*—*(1) 8 mg/ml.*—Transfer 0.12±0.01 g carbaryl (anal. grade, available from Union Carbide Corp., Agricultural Products and Services), weighed to nearest 0.1 mg, to culture tube. Pipet 15 ml MeOH-$CHCl_3$ soln into tube, cap securely, and rotate or shake mech. 30 min. *(2) 2.5 mg/ml.*—Transfer 0.25±0.01 g carbaryl, weighed to nearest 0.1 mg, to 250 ml g-s erlenmeyer. Pipet 100 ml $CHCl_3$ into flask, stopper, and swirl to dissolve.

6.B03 ***Preparation of Sample***

(a) *Carbaryl dust and powder formulations.*—Transfer weighed sample (≤2.4 g) contg 0.12±0.01 g carbaryl to culture tube. Pipet 15 ml MeOH-$CHCl_3$ soln into tube and cap securely. Rotate or shake mech. 30 min and centrf. 10 min.

(b) *Liquid suspensions.*—Following steps must be performed in order described, as any deviation can cause erroneous results due to faulty sample transfer and incomplete extn: Place ca 20 g Na_2SO_4 in 250 ml g-s erlenmeyer. Pipet 100 ml $CHCl_3$ into flask. Vigorously shake sample bottle. Draw appropriate vol. sample into hypodermic syringe without needle. Use ca 0.5 ml sample for carbaryl 4 lb/gal. and ca 1.0 ml for carbaryl 2 lb/gal. Wipe outside of syringe with paper towel and weigh syringe and contents to nearest 0.1 mg. Add sample to erlenmeyer by slowly depressing syringe plunger. Do not let syringe or sample touch sides of flask. Sample must drop into $CHCl_3$. Reweigh syringe and calc. sample wt by difference. Stopper flask and shake vigorously 30 min on mech. shaker.

6.B04 ***Determination***

(a) *Carbaryl dust and powder formulations.*—Using matched 0.2 mm NaCl cells, scan sample soln against MeOH-$CHCl_3$ soln from 5.2 to 6.0 μm (1900–1600 cm^{-1}). Repeat scan with std soln. Measure A of carbaryl peak at 5.75 μm (1740 cm^{-1}), using A at 5.40 μm (1850 cm^{-1}) as 0 point. A = ca 0.4 for both std and sample.

$$\% \text{ Carbaryl by wt} = (A \times B' \times P)/(A' \times B),$$

where A and A' = absorbance of sample and std, resp., at 5.75 μm; B and B' = mg sample and mg std/ml, resp.; and P = % purity of carbaryl std.

(b) *Liquid suspensions.*—Proceed as in (a), except use matched 0.5 mm NaCl cells and scan sample soln against $CHCl_3$.

Results and Recommendations

Eleven collaborators reported results in this study. The raw data from the study are recorded in Tables 1–4. Collaborators 1, 2, 5, 6, 7, and 11 used rotators of some kind for the extraction step. Collaborators 3 and 8 used wrist-action shakers. Collaborator 9 used a reciprocating shaker. Collaborator 4 and 10 did not report any deviation, so it may be assumed they used rotators. The data show no dependence upon the

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 (except he recommended measurement of carbaryl at both 5.75 and 8.94 μm) and by Subcommittee A and were adopted by the Association. *See* (1976) *JAOAC* **59**, 379–380.

Table 1. Collaborative results for the analyses of 5% carbaryl dust[a] (% carbaryl by wt)

	Mixed solvent	Chloroform	
Coll.	5.75 μm	5.75 μm	8.94 μm
1	5.42	3.74	3.95
2	5.53	3.90	3.93
3	5.31	5.01	5.12
4	5.35	3.70	3.87
5	5.47	4.00	3.99
6	5.61	4.90	5.00
7	5.44	3.79	3.94
8	5.17	3.62	3.62
9	5.24	3.14	3.30
10	5.39	3.70	3.45
11	5.27	3.68	3.93
$\bar{X}$	5.38	3.93	4.01
s^2	0.0170	0.306	0.322
s	0.130	0.55	0.57
CV	2.42	14.08	14.16
sp	0.402	0.560	
$\bar{X}_1 - \bar{X}_2$	1.45	0.08	
t	8.459	0.335	
t (99) tabulated	2.845	2.845	

[a] $\bar{X}$ = average, s^2 = variance, s = standard deviation, CV = coefficient of variation, sp = pooled standard deviation, and t = t-test.

Table 2. Collaborative results for the analyses of 10% carbaryl dust[a] (% carbaryl by wt)

	Mixed solvent	Chloroform	
Coll.	5.75 μm	5.75 μm	8.94 μm
1	9.99	9.86	9.78
2	10.06	9.90	9.89
3	9.90	9.89	10.06
4	9.65	9.77	9.39
5	9.98	9.70	9.68
6	10.67[b]	10.10	10.18
7	9.87	9.91	9.61
8	9.35	9.66	10.07
9	10.19	9.67	9.72
10	9.88	9.93	10.10
11	9.74	9.76	9.74
$\bar{X}$	9.86	9.83	9.84
s^2	0.0557	0.0179	0.0596
s	0.236	0.134	0.244
CV	2.39	1.36	2.48
sp	0.189	0.197	
$\bar{X}_1 - \bar{X}_2$	0.03	0.01	
t	0.363	0.119	
t (99) tabulated	2.861	2.845	

[a] *See* footnote, Table 1.
[b] Rejected on the basis of the Dixon test at the 95% confidence level.

Table 3. Collaborative results for the analyses of 50% carbaryl wettable powder[a] (% carbaryl by wt)

	Mixed solvent	Chloroform	
Coll.	5.75 μm	5.75 μm	8.94 μm
1	50.44	51.24	49.57
2	52.39[b]	50.51	51.43
3	50.07	50.64	50.86
4	49.83	50.79	48.49
5	50.28	50.83	49.62
6	51.30	51.30	51.30
7	50.30	49.93	50.01
8	49.77	48.30[b]	51.07
9	50.82	50.56	50.41
10	50.70	50.10	50.70
11	50.13	50.12	50.21
$\bar{X}$	50.36	50.60	50.33
s^2	0.221	0.214	0.771
s	0.47	0.46	0.88
CV	0.93	0.91	1.75
sp	0.467	0.712	
$\bar{X}_1 - \bar{X}_2$	0.24	0.27	
t	1.149	1.219	
t (99) tabulated	2.879	2.861	

[a] *See* footnote, Table 1.
[b] Rejected on the basis of the Dixon test at the 95% confidence level.

Table 4. Collaborative results for the analyses of 80% carbaryl sprayable powder[a] (% carbaryl by wt)

	Mixed solvent	Chloroform	
Coll.	5.75 μm	5.75 μm	8.94 μm
1	80.07	79.40	80.75
2	80.50	81.13	82.68
3	79.92	80.92	81.01
4	79.77	79.64	80.62
5	80.18	78.39	79.55
6	79.30	79.20	82.60
7	78.80	78.25	81.52
8	77.37[b]	78.00	84.43
9	80.75	79.73	80.90
10	79.30	79.70	81.40
11	79.33	79.89	83.03
$\bar{X}$	79.79	79.48	81.68
s^2	0.372	1.01	1.89
s	0.61	1.00	1.38
CV	0.76	1.26	1.68
sp	0.841	1.20	
$\bar{X}_1 - \bar{X}_2$	0.31	2.20	
t	0.843	4.300	
t (99) tabulated	2.879	2.845	

[a] *See* footnote, Table 1.
[b] Rejected on the basis of the Dixon test at the 95% confidence level.

method of agitation. Collaborators 1, 2, and 11 used chloroform with an ethanol inhibitor content of 0.75%. Collaborator 4 used chloroform with an ethanol content of 1.0%. In the other cases the inhibitor content was not known or redistilled chloroform was used. The data show no dependence on the inhibitor content of the chloroform.

Collaborator 2 commented that he did not approve of transferring the extract directly from a centrifuge tube to an infrared cell without filtering the extract to prevent introducing solid particles into the cell. Collaborator 4 commented that infrared cells are always damaged by methanol and he could, therefore, not recommend the method. He prefers acetone extraction followed by evaporation and solution of the residue in chloroform.

The data in Tables 1–4 were statistically evaluated in the same manner as the data generated in the repeatability study reported at the 88th Annual Meeting (3). The coefficient of variation for the mixed solvent modification for the 5% carbaryl dust formulation (Table 1) is an acceptable 2.42%. In the repeatability study, the coefficient of variation was 0.68%. The difference is that expected between a repeatability and a reproducibility study. The coefficients of variation for the 2 alternatives of the official method are 14.08 and 14.16%. These are unacceptably high, and the probable cause is the large variation caused by minor differences in technique when the extraction is incomplete. The *t*-tests demonstrate that both alternatives in the official method yield the same assay, while the assay is significantly higher by the mixed solvent modification. When the Dixon test for outliers (5) was applied to the data from the mixed solvent extraction, no results were found to be outliers. In the chloroform extraction data, 2 assays were found to be outliers but these assays were not eliminated from the statistical calculations because the standard deviations in these series were so large that elimination of 2 results would be meaningless.

The data for the 10% carbaryl dust formulation (Table 2) show that the coefficient of variation for the mixed solvent modification is an acceptable 2.39%. One assay from this series was found to be an outlier by the Dixon test, and it was eliminated from the statistical study. The coefficients of variation for the 2 alternatives of the official method were 1.36 and 2.48%, both acceptable for assay methods of analysis. The *t*-tests demonstrate that all 3 alternatives yield the same assay.

The data for the 50% wettable powder formulation are presented in Table 3. One of the assays from each of the 5.75 μm series was eliminated on the basis of the Dixon test. The coefficients of variation of 0.93, 0.91, and 1.75% for the 3 alternatives are very good for infrared reproducibility studies, and the *t*-tests indicate that the 3 alternatives yield the same assay. The quality of these data is doubly significant because this is the only commercial formulation used in this study that was not blended or diluted in the laboratory.

The data for the 80% carbaryl sprayable powder formulation are presented in Table 4. One of the assays from the mixed solvent modification was rejected on the basis of the Dixon test. The coefficients of variation of 0.76, 1.26, and 1.68% for the 3 modifications are well within the 2% expected of an infrared method in a collaborative study. The *t*-test demonstrates that the assays obtained at 5.75 μm are the same with either solvent. The *t*-test applied to the 2 alternatives of the official method shows that the assays calculated from the 8.94 μm absorbance are statistically higher than those calculated from the 5.75 μm absorbance. This phenomenon was evident in the repeatability study also. Because the 8.94 μm peak is a measure of the C—O—C bond, the most likely cause of this difference is the presence of a polyoxyethylene-polyoxypropylene block copolymer surfactant in the carbaryl 80% sprayable formulation.

The objection of Collaborator 2 to transferring the supernatant solution to an infrared cell for fear of introducing solid particles into the cell is a legitimate objection, but it is the opinion of the Associate Referee that this manipulation can be accomplished by a technician trained in the proper techniques of handling salt plate infrared cells with minimum risk to the cells. The technique of filtering a solvent as volatile as chloroform is also accompanied by risks because evaporation of the solvent will lead to high results and the cooling of the filter paper and apparatus can cause water condensation and thus water contamination even in controlled humidity.

The objection of Collaborator 4 to the use of methanol in infrared cells is also legitimate, but the Associate Referee has not been able to detect any damage to sodium chloride infrared cells caused by 10% (v/v) methanol in chloroform over a period of several years.

Overall, the data generated in this collaborative study prove that the mixed solvent modification and the use of the 5.75 μm absorbance peak are superior to the other modifications in

the determination of carbaryl in difficult-to-extract dust formulations and in 80% sprayable formulations, and this combination is equal to the other modifications in the assay of all other powder and dust formulations. Therefore, the Associate Referee recommends that the official first action method for carbaryl in formulations be rewritten to include the mixed solvent modification and to eliminate the chloroform modification and the 8.94 μm modification in the assay of dust and powder formulations.

Because no difference in assays attributable to tube rotator or wrist-action shaker was discernible from these data, it is recommended that both methods of agitation be included in the official method.

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E. R. Winterle, Florida Department of Agriculture and Consumer Services, Tallahassee, FL

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Collaborative Study of a Gas-Liquid Chromatographic Method for the Determination of Simazine

ARTHUR H. HOFBERG, JR., LEE C. HEINRICHS, and GENE A. GENTRY
Ciba-Geigy Corp., PO Box 11422, Greensboro, NC 27409

A gas-liquid chromatographic method for the determination of simazine in a wettable powder formulation containing about 80% active ingredient was collaboratively studied, using a matched pair scheme. The samples were dissolved in dimethylformamide containing dioctyl phthalate as an internal standard, chromatographed on Carbowax 20M, and detected by using a flame ionization detector. Analysis of the 2 samples, based on peak height measurements, showed the following results: 1.5% overall coefficient of variation, 1.03% coefficient of variation for random error, and 0.23% systematic error. Sample A was an 80% powder formulation; Sample B was a 5% dilution of Sample A by the addition of inert material. The coefficients of variation were 1.20% for Sample A and 1.54% for Sample B. The larger variation in Sample B most likely resulted from nonuniform mixing during the dilution of Sample A. Since all collaborators used the same reference standard, the variation found in Sample A is more indicative of the variability of the method. The method has been adopted as official first action.

Gas-liquid chromatographic (GLC) methods involving the extraction of a pesticide from formulated products with a solvent containing an internal standard, followed by peak height measurement, were previously collaboratively studied with atrazine, prometryn, prometon, ametryn, propazine, and terbutryn (1–4) and have been adopted as official methods by the AOAC. The purpose of the present study was to determine whether the GLC procedure used for these compounds could be applied to formulations containing simazine (2-chloro-4,6-bis(ethylamino)-*s*-triazine).

The test for closely matched pairs described by Youden (5) was followed. Each collaborator was sent the technical herbicide to be used as a standard, technical dioctyl phthalate for use as an internal standard, a formulation (Sample A), and a dilution of Sample A with inert ingredients (Sample B) for each study. The samples were dissolved in dimethylformamide containing dioctyl phthalate. The extracted pesticide was chromatographed on a column containing 3% Carbowax 20M on 80–100 mesh Gas-Chrom Q and detected by flame ionization. Peak height ratios (simazine to dioctyl phthalate) of the unknown samples were compared with the corresponding peak height ratios of the standard solution containing a known amount of simazine.

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METHOD

Standard Solutions

(a) *Dioctyl phthalate internal std soln.*—Weigh 3.0±0.1 g tech. dioctyl phthalate, dissolve in ca 200 ml DMF, and dil. to 1 L with DMF. (*Caution: See* **51.053.**) Std should be >98% pure and contain no impurities eluting at retention time of simazine.

(b) *Pesticide std soln.*—Accurately weigh 150 mg tech. simazine of known purity (available from Ciba-Geigy Corp., PO Box 11422, Greensboro, NC 27409) into 4 oz bottle with Al-lined screw cap. Pipet in 50 ml internal std soln (a), and shake mech. to dissolve.

Preparation of Sample

Accurately weigh ca 190 mg simazine powder formulation into 4 oz bottle with Al-lined screw cap. Pipet in same vol. internal std soln used for prepn of std soln and shake mech. 30 min. Let insol. materials settle, or centrf. portion of ext to obtain clear soln.

Gas Chromatography

Use instrument equipped with flame ionization detector and 6′ × 4 mm id glass column packed with 3% Carbowax 20M (Applied Science Laboratories, Inc.) on 80–100 mesh Gas-Chrom Q. Condition 24 hr at 240° with N or He at ca 40 ml/min. Column should have ≥1500 theoretical plates (*see* **6.155(a)**).

Operate at the following conditions: temps—inlet, 250°; column, 210±5°; detector 250°; N or He carrier gas, 80–100 ml/min; air and H flows as recommended for the detector; attenuation varied so that peak hts of simazine and internal std are 60–80% full scale. Retention times: simazine 6–8 min, dioctyl phthalate 10–14 min.

Determination

Inject 2 μl aliquots std soln until peak ht ratio of simazine:internal std varies $\leq 1\%$ for successive injections. Then make duplicate injections of sample followed by duplicate injections of std. Peak ht ratios of stds must be with $\pm 1\%$ of first accepted std values or repeat series of injections. Repeat for addnl samples.

Calculations

Calc. peak ht ratios for duplicate std injections both preceding and following sample. Average the 4 values (R'). Calc. and average peak ht ratios of the 2 samples (R).

Simazine, $\% = (R/W) \times (W' \times P/R')$, where W and W' = mg sample and std, resp.; and $P = \%$ purity of std.

Results and Discussion

Collaborators were requested to perform only one determination on each sample, as described in the method. The raw data and scans returned by the collaborators were rechecked. Recalculations showed some differences caused by deviation from the requested calculation procedure, use of too few decimal points, or minor mathematical errors. However, the data were used as submitted by the collaborators, since the magnitudes of these errors were less than 1% relative.

Table 1 shows the results for the matched pair study of simazine for 35 collaborators. Two points deviate significantly from the main pattern. These points represent results from Collaborators 14 and 28 and may be rejected after application of the Dixon test (6). The standard deviation of the estimation for the random error, S_r (0.96%), does not change with the rejection of these results, but the standard deviation for the systematic error, S_b, decreases from 0.51 to 0.12%. The overall standard deviation, S_d, is reduced from 1.97 to 1.18 with this omission.

Average values after rejection of results from Collaborators 14 and 28 were $81.47 \pm 0.72\%$ for Sample A and $78.32 \pm 0.87\%$ for Sample B. The overall coefficient of variation was 1.5%, coefficient of variation for the random error, 1.03%, and systematic error, 0.23%. These values compare favorably with those found by a standard wet chemical titration procedure (7): Sample A was found to contain 81.9% simazine and Sample B, 77.9%.

The coefficient of variation for Sample A by the method studied was 1.20%, while that of Sample B was 1.54%. Since Sample B was produced by diluting a portion of Sample A with clay in a blender, it is most likely that the larger variation in results is due to nonuniform mixing. It is felt that, since each collaborator used the same reference standard with the same assigned purity, the variability of the method is more accurately measured by Sample A.

Evaluation of all previous studies by these authors shows that the coefficient of variation for Sample B was significantly greater than for Sample A for all dilutions of powdered formulations. Offering additional support to this hypothesis is the fact that no significant differences

Table 1. Collaborative results for the determination of simazine (%) in matched pair samples

Coll.	Sample A	Sample B	Diff.	Total
1	81.45	76.63	4.82	158.08
2	81.82	78.31	3.51	160.13
3	82.85	78.31	4.54	161.16
4	80.18	77.50	2.68	157.68
5	80.70	78.98	1.72	159.68
6	81.04	77.82	3.22	158.86
7	80.39	80.04	0.35	160.43
8	82.11	78.21	3.90	160.32
9	81.28	76.96	4.32	158.24
10	83.26	80.89	2.35	164.15
11	83.03	77.34	5.69	160.37
12	81.02	78.87	2.15	159.89
13	82.24	78.10	4.14	160.34
14[a]	(78.41)	(73.67)	(4.74)	(152.08)
15	80.97	77.62	3.35	158.59
16	81.66	79.17	2.49	160.83
17	81.51	78.70	2.81	160.21
18	78.74	79.16	−0.42	157.90
19	81.43	77.62	3.81	159.05
20	81.88	77.67	4.21	159.55
21	82.02	80.83	1.19	162.85
22	81.38	78.15	3.23	159.53
23	80.70	77.53	3.17	158.23
24	83.38	78.58	4.80	161.96
25	82.04	82.15	−0.11	164.19
26	81.30	77.77	3.53	159.07
27	81.26	77.63	3.63	158.89
28[a]	(76.46)	(72.15)	(4.31)	(148.61)
29	79.80	78.02	1.78	157.82
30	81.78	77.86	3.92	159.64
31	80.00	77.08	2.92	157.08
32	81.46	78.43	3.03	159.89
33	81.98	78.10	3.88	160.08
34	81.89	77.11	4.78	159.00
35	81.87	77.42	4.45	159.29
Av.	81.24	78.01	3.23	159.25
S_d	1.37	1.74		1.97
S_r			0.96	
S_b			0.51	
Excluding Colls. 14 and 28:				
Av.	81.47	78.32	3.15	159.79
S_d	0.98	1.21		1.18
S_r			0.95	
S_b			0.12	

[a] Rejection with 95% significance level for Dixon test.

in coefficients of variation existed for Samples A and B when liquid formulations were studied.

Recommendation

The method as presented is rugged, accurate, and within the accepted limits of AOAC methods. It is recommended that the gas-liquid chromatographic method for the determination of simazine in formulations be adopted as official first action.

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H. D. Antles, Washington Department of Agriculture, Yakima, WA

J. B. Audino, California Department of Agriculture, Sacramento, CA

W. Black, Illinois Department of Agriculture, Springfield, IL

L. J. Burton, Agriculture Canada, Calgary, Alberta, Canada

M. J. Byrne, Environmental Protection Agency, Denver, CO

D. L. Campbell, Canadian Agricultural Plant Products, Ottawa, Ontario, Canada

A. A. Carlstrom, Chevron Chemical Co., Richmond, CA

R. H. Collier, Purdue University, West Lafayette, IN

J. E. Forrette, Velsicol Chemical Corp., Chicago, IL

I. R. Garcia Faure, Laboratorios Regionales, Madrid, Spain

R. P. Gideon, Ciba-Geigy Corp., McIntosh, AL

C. R. Guertal, Ohio Department of Agricultural Laboratories, Reynoldsville, OH

M. D. Guthrey, Alabama Department of Agricultural Industries, Auburn, AL

H. Hammond, North Dakota State Laboratories Department, Bismarck, ND

E. F. Hawkins, Allied Chemical Co., Baltimore, MD

F. A. Janecka, New Mexico Department of Agriculture, Las Cruces, NM

F. A. Jennings, Virginia Department of Agriculture, Richmond, VA

L. Kerr, Regina Research Station, Regina, Saskatchewan, Canada

J. E. Launer, Oregon Department of Agriculture, Salem, OR

J. J. Maher, Ciba-Geigy Corp., Greensboro, NC

V. Mattinen, State Institute of Agricultural Chemistry, Helsinki, Finland

W. M. McCaskill, Clemson University, Clemson, SC

W. H. McDermott, Union Carbide Corp., South Charleston, WV

J. Meier, Ciba-Geigy Corp., Basle, Switzerland

J. P. Minyard, Jr., Mississippi State Chemical Laboratory, State College, MS

G. Muth, Environmental Protection Agency, San Francisco, CA

G. Owens, Ciba-Geigy Corp., St. Gabriel, LA

H. H. Polvsen, Kemikaliekontrollen, Lyngby, Denmark

H. Sauer, Sandoz AG, Basle, Switzerland

D. C. VandeLinde, West Virginia Department of Agriculture, South Charleston, WV

G. P. Vann, Mobil Chemical Co., Mt. Pleasant, TN

W. C. Ware, Arkansas State Plant Board, Little Rock, AR

G. R. Winstead, III, North Carolina Department of Agriculture, Raleigh, NC

E. R. Winterle, Florida Department of Agriculture, Tallahassee, FL

H. K. Wipf, Socar AG, Dubendorf, Switzerland

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The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee A and was adopted by the Association. *See* (1976) *JAOAC* **59**, 380. The method has been incorporated into **6.372–6.376** (revision, 1975), "Changes in Methods" (1976) *JAOAC* **59**, 458.

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

FOODS AND FEEDS

Determination of Sodium in Alkali-Treated Cereal Straws, Lye-Peel Wastes, and Brine-Frozen Foods with an Ion Selective Electrode Compared with Atomic Absorption Spectrometry

GERALDINE E. SECOR, GARY M. McDONALD, and ROLLAND M. McCREADY
Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Berkeley, CA 94710

A simple procedure is described for determining sodium in sodium hydroxide-treated rice straw and rye grass, lye-peeled potato wastes, and brine-frozen vegetables. Samples containing ≤4% sodium were extracted with 0.05*M* ammonium carbonate and the extracts were analyzed directly by using a sodium ion selective electrode over the range 10^{-4} to 10^{-2}*M* sodium ion. Comparable analyses of the same extracts by atomic absorption spectrometry agreed to within 10%. Recovery of sodium added to control rice straw was 102%.

Research on the processing of rice straw, rye grass, and other agricultural cellulosic byproducts to increase their digestibility for ruminant feed is now extensive. The development of ruminant feed from these waste products promises to provide an economically and ecologically attractive alternative to present high-cost feed grains and hay, and to waste disposal methods such as open-field burning. Alkali treatment (1) increases the cellulose digestibility of the straw and improves its feed value. Ground straw is treated with about 4% by weight of sodium hydroxide applied as a concentrated solution (30–50%) and the treated material is compacted. The sodium content of the feed product must be known for chemical control of the dosage of sodium hydroxide on the treated straw. A sodium ion selective electrode procedure previously used for the determination of sodium ion in pulp mill liquors (2) combined with a portable, direct-reading specific ion meter was investigated. This paper reports the sodium content of sodium hydroxide-treated rice straw and rye grass determined by a sodium ion selective electrode procedure and compares the results with those obtained by atomic absorption spectrometry (AAS). Sodium analyses by both methods are included for lye-peeled potato wastes and brine-frozen vegetables where the residual sodium content is important for ruminant feed, waste disposal, and food processing.

METHODS

Apparatus

(a) *Electrode equipment.*—Sodium ion selective electrode (No. 94–11A); single-junction reference electrode (No. 90–01), filled with lithium trichloroacetate-lithium chloride filling solution (No. 90–00–19); specific ion meter (Model 404), all available from Orion Research Inc., 11 Blackstone St, Cambridge, MA 02139.

(b) *Atomic absorption spectrophotometer.* — Perkin-Elmer Model 303, equipped with 3-slot Boling burner head.

Reagents

Reagents were analytical grade unless otherwise stated, and redistilled (in glass) water was used in reagents and sample solutions. Plastic ware used was aliphatic polyolefin type.

(a) *Ionic strength adjustor (ISA).* — 0.05 and 0.1*M* ammonium carbonate. Dissolve 8.3 g (for 0.05*M*) and 16.6 g (for 0.1*M*) reagent grade ammonium carbonate (mixture of ammonium hydrogen carbonate and ammonium carbonate, assumed formula weight 157 and 95% purity), respectively, in water. Dilute each solution to 1 L with water and store in 1 L plastic, screw-capped bottles.

(b) *Sodium stock standard solution.* — 0.1*M* NaCl. Dissolve 1.4611 g NaCl (recrystallized from water, dried 1 hr at 550°C) in water and dilute with water to 250 ml in Pyrex volumetric flask.

(c) *Sodium working standard solutions.*—Prepare fresh daily in Pyrex volumetric flasks in sodium ion concentration range of sample solutions to be measured in 0.05*M* ISA. For 0.001, 0.005, and 0.01*M* standard solutions, pipet 1, 5, and 10 ml 0.1*M* stock NaCl solution into separate

Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

100 ml volumetric flasks, add 50 ml 0.1*M* ISA to each, and dilute to volume with water. Dilute 1 ml 0.1*M* NaCl and 100 ml 0.1*M* ISA to 200 ml with water for 0.0005*M* standard. Dilute 20 ml 0.0005*M* NaCl to 100 ml with 0.05*M* ISA for 0.0001*M* standard.

(d) *Ashing reagents.*—Concentrated H_2SO_4 in redistilled 95% ethanol (10+90 (v/v)) for ashing, and aqueous 6*N* HCl for dissolving metallic ions in ash residue.

Preparation and Extraction of Sample

(a) *Sample extraction procedure.* — Accurately weigh 5 g alkali-treated rice straw or rye grass into Waring Blendor. Add 500 ml 0.05*M* ISA and blend 5 min at low speed. Pour mixture into 600 ml plastic beaker, cover top of beaker with plastic film (i.e., Saran Wrap), and secure with rubber band. After 10–30 min, stir solution, and filter rapidly through double thickness of cheese cloth. Discard first 50 ml filtrate and collect remainder in 500 ml plastic Erlenmeyer flask. For measurement of sodium ion with ion selective electrode, pipet 25 or 50 ml filtered extract into 250 ml plastic beaker and bring total volume to 100 ml with 75 or 50 ml 0.05*M* ISA.

(b) *Alternative sample extraction procedure.*— Accurately weigh 5 g alkali-treated rice straw or rye grass into 600 ml plastic beaker. Add 500 ml 0.05*M* ISA. Stir thoroughly 5 min with stainless steel tablespoon. Cover top of beaker with plastic film, secure with rubber band, and let stand $\geq$10 min. For measurement of sodium ion with ion selective electrode, use tablespoon to hold back bulk of straw while decanting and measure exactly 25 or 50 ml extract in graduated cylinder. Transfer to 250 ml plastic beaker, using 75 or 50 ml 0.05*M* ISA to rinse cylinder, and dilute to 100 ml with 0.05*M* ISA.

(c) *Sample preparation, ashing.* — Accurately weigh 5 g alkali-treated rice straw or rye grass into 100 × 40 mm platinum dish. Wet sample with 10 ml ethanolic H_2SO_4 reagent and char 30 min on steam bath. Heat ca 30 min on infrared heater (Fisher Duplex Infra-Rediator, 115 v ac/dc, Model 11–504–10) until sample ceases smoking. Ash 2–3 hr in 550°C muffle furnace (ashes are light gray). Cool in desiccator. Wet ash with 5 ml 6*N* HCl and transfer with water to 500 ml Pyrex volumetric flask. Dilute to volume with water. Mix well and let ash settle. Pipet 25 or 50 ml ash solution into 100 ml Pyrex volumetric flask. Add 50 ml 0.1*M* ISA from graduated cylinder, dilute to volume with water, and mix. Pour into 250 ml plastic beaker for ion selective electrode measurement.

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Note: The 25 or 50 ml aliquots of sample solution used in above procedures for electrode measurement of sodium ion in straw and grass samples, when diluted to 100 ml, will have sodium ion concentration within range of standard solutions used for calibration (10^{-4} to 10^{-2}*M* sodium ion).

Sodium Ion Selective Electrode Measurements

Ionic strength adjustor method (2, 3) was used with direct readout of sodium ion concentration. Determine sodium ion in standard and sample solutions under same conditions: Use 100 ml solution, 10^{-4} to 10^{-2}*M* sodium ion in 0.05*M* ISA, in 250 ml plastic beaker containing 1½ × 5/16″ Teflon stirring bar. pH of this mixture is ca 9 and is controlled by ISA. Place beaker on magnetic stirrer with mat of thermal insulating material between stirrer and beaker to minimize any temperature change in solution from motor heat during stirring. Insert electrodes to ca one-half depth of solution. Magnetically stir solution 5 min at moderate, constant rate to equilibrate system. Read sodium ion concentration in solution directly on upper logarithmic scale of specific ion meter. Between solutions, rinse electrodes with water from plastic wash bottle and blot dry with low sodium, nonabrasive, absorbent paper tissue (i.e., Kimwipes Type 900-S, Kimberly-Clark Corp.).

Calibrate (3) initially with 2 standard solutions with sodium ion range (an even power of 10) that brackets concentration of sodium in sample solutions. Recheck calibration with third standard solution with sodium concentration in middle of range. If calibration reading exceeds ±2%, repeat calibration before reading sample solutions.

Atomic Absorption Spectrometry

Use original 0.05*M* ISA extract of sample or 0.06*N* HCl solution of ashed sample for AAS determination as was used for ion selective electrode measurement. Solutions should be decanted or filtered to remove particulate matter. Analyze blanks of 0.05*M* ISA and 0.06*N* HCl.

Use air-acetylene oxidizing flame. For high concentrations of sodium, as in experiments reported here, set atomic absorption spectrometer at ultraviolet range, wavelength 330 nm and slit 4 (1.4 nm). Sodium concentration should be ca 20–400 μg/ml. (*Note:* For low concentrations of sodium (0.1–10 μg/ml), set instrument at visible range and 295 (589 nm) (4).)

Results and Discussion

Theoretically, AAS measures the total sodium in solution and the sodium ion selective electrode measures the sodium ionic activity. The activity

of the sodium ion/concentration of the sodium ion = activity coefficient, which varies mainly with the total ionic strength of the solution. By adding a noninterfering ionic strength adjustor to both standard and unknown solutions, the ionic strength is controlled (2, 3) and the electrode can be calibrated to measure sodium ion concentration. The 0.05*M* ammonium carbonate acts as an ionic strength and pH adjustor (2) for the ion selective electrode procedure, and extracts (ion exchange) the sodium ion from the samples.

The accuracy of the method, including the measurement, extraction, and ashing procedures, was determined by the recovery (Table 1) of added amounts of sodium ion. Aliquots of 1*M* NaOH (standardized against 0.5*N* HCl) were added to 5 g samples of control rice straw at the levels of sodium found in the processed straw. The treated samples were placed in a 70°C forced draft air oven and dried 3 hr. They were analyzed as described for extracted and ashed samples. The same aliquots of 1*M* NaOH were diluted to 500 ml in 0.05*M* ISA and measured by the electrode and AAS procedures. The recovery (Table 1) of sodium by the sodium ion selective electrode method averaged 104% for direct measurement in 0.05*M* ISA, 102% for the extracted rice straw, and 100% for the ashed rice straw. The electrode results were within 10% of the AAS results.

Table 2 shows the results of the sodium analyses by the sodium ion selective electrode and AAS procedures for control rice straw, and NaOH-treated rice straw and rye grass at 2 levels of sodium. The electrode results were within 10% of the AAS results. The precision was determined by analyzing 10 extracted (Waring Blendor) and 12 ashed preparations of NaOH-treated rice straw Sample A. Sample A was a typical pilot plant sample, ½–1″ in straw lengths, not compacted and not reground before sodium analysis. The results are listed in Table 2 and are essentially the same for the electrode and AAS methods.

Table 1. Recovery of added sodium as measured by sodium ion selective electrode and atomic absorption spectroscopy (AAS)[a]

Sodium added to	Calcd[b,c]	Electrode Found[b]	Electrode Rec., %	AAS Found[b]	AAS Rec., %
0.05*M* Ammonium carbonate (ISA)	1.82	1.85	102	1.85	102
	4.09	4.24	104	4.18	102
		4.29	105	—	—
Control, untreated rice straw:					
Extracted	1.82	1.87	103	1.86	102
		1.86	102	1.87	103
Extracted	4.09	4.11	100	4.31	105
		4.15	101	4.40	108
Ashed	1.82	1.84	101	1.87	103
Ashed	4.09	4.00	98	4.39	107

[a] Results represent single analyses.
[b] Corrected for sodium found in control, untreated rice straw (Table 2).
[c] Sodium concentration expressed in g/10 L 0.05*M* ISA, or % in rice straw.

The agreement in the sodium values for the extracted and ashed samples (Tables 1 and 2) indicates that there was no significant interference from organic compounds or from the rice silica. Potassium affects the response of the sodium ion selective electrode if the ratio of potassium to sodium ion exceeds 100:1 (3, 5). The amount of potassium ion found in the rice straw relative to the sodium was less than 5:1 in these experiments, too low to interfere.

Our analyses indicate the sodium ion selective electrode can be used successfully to determine sodium in lye-peeled potato wastes (6) and brine-frozen vegetables (7). Aqueous samples from each process, 3–5 g in 100 ml 0.05*M* ISA, were analyzed for sodium by the electrode and AAS procedures. The lye-peeled potato waste samples were also analyzed by the ashed sample procedure. Sample 1 was wash water and Sample 2 was a thickened sludge of peel waste from the lye-peeled potatoes. The sodium content of Sample 1 is particularly critical because it determines the amount of land area needed for its disposal. The sodium results (Table 3) for Samples 1 and 2 indicate that all the sodium present was measured in the ISA extracts and that there was no interference from the starch or sugar content at the concentrations present (<0.04% (w/v) starch and <0.003% (w/v) total sugar). The brine-frozen vegetable samples (7) were cooked, homogenized, and filtered, and the filtrates were analyzed for sodium and chloride. The sodium content found agrees well with the sodium calculated from the chloride measured by coulometric titration. The per cent sodium (cooked, drained, vegetable weight) calculated from the per cent chloride, and found by sodium ion selective electrode and AAS, was 1.02, 1.04, and 1.02 for carrots; 0.51, 0.53, and 0.54

Table 2. Sodium analysis of rice straw and rye grass by sodium ion selective electrode and atomic absorption spectroscopy (AAS)

Sample	No. of detns	Electrode		AAS	
		Na found, av. %	Range	Na found, av. %	Range
Control, untreated rice straw:					
Extracted	3	0.07	0.06–0.08	0.07	0.06–0.08
Ashed[a]	2	0.06	0.05–0.07	0.06	0.06–0.06
Sample A, NaOH-treated rice straw:					
Extracted	10	1.81 (±0.15)[b]	1.57–2.07	1.87 (±0.14)[b]	1.65–2.14
Ashed[a]	12	1.65 (±0.25)[b]	1.33–2.20	1.70 (±0.25)[b]	1.44–2.25
Sample B, NaOH-treated rice straw:					
Extracted	3	4.21	4.14–4.25	4.22	4.20–4.25
Ashed	3	4.11	4.05–4.14	4.18	4.15–4.20
Sample C, NaOH-treated rye grass:					
Extracted	2	1.64	1.52–1.75	1.57	1.52–1.61
Ashed	1	1.50	—	1.52	—
Sample D, NaOH-treated rye grass:					
Extracted	2	3.17	3.17–3.17	3.19	3.17–3.20
Ashed	1	3.08	—	3.32	—

[a] Potassium 0.5%.
[b] Standard deviation.

Table 3. Sodium analyses (3 for each sample) of lye-peeled potato wastes, wet weight, by sodium ion selective electrode and atomic absorption spectroscopy (AAS)

Waste sample	Electrode		AAS	
	Na found, av. %	Range	Na found, av. %	Range
Sample 1, wash:[a]				
Extracted	0.04	0.04–0.04	0.04	0.04–0.04
Ashed	0.04	0.04–0.04	0.05	0.04–0.05
Sample 2, peel:[b]				
Extracted	0.66	0.65–0.66	0.65	0.64–0.66
Ashed	0.66	0.65–0.66	0.68	0.68–0.68

[a] Starch 0.63%; total sugar 0.09%.
[b] Starch 2.54%; total sugar 0.14%.

for snow peas; and 0.27, 0.27, and 0.27 for string beans. The ratios of potassium to sodium ion were less than 1, too low to interfere with the electrode determination of sodium.

The sodium results for the samples in Tables 1–3 show that these samples do not have to be ashed before analysis by either the electrode or AAS method. If a large number of samples are to be analyzed, the electrode method is preferred because particulate matter in the ISA extracts does not have to be removed if it does not prevent uniform mixing and stirring during the sodium ion measurement. A particle-free solution is necessary for the AAS method. Thus the electrode procedure saves filtration time and glassware and eliminates a possible source of sodium contamination from filter paper (8).

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PRESERVATIVES AND ARTIFICIAL SWEETENERS

Rapid Screening Procedure for the Determination of Preservatives in Ground Beef: Sulfites, Benzoates, Sorbates, and Ascorbates

ALBERT B. KARASZ,[1] JOHN J. MAXSTADT,[2] JUERGEN REHER, and FRANK DECOCCO
New York State Food Laboratory, Department of Agriculture and Markets, Albany, NY 12235

A procedure is described for the examination of large numbers of ground beef samples for the 4 most commonly used preservatives. A single sample solution is prepared and aliquots are taken for the various tests. Sulfite is determined colorimetrically with acid-bleached *p*-rosaniline. Ascorbic acid is titrated with 2,6-dichloroindophenol. Benzoic and sorbic acids are measured by ultraviolet absorption. Negative samples are rapidly sorted out in this procedure and positive samples are completed for quantitative determination of the preservative. Recoveries are 95% for sodium sulfite, 103% for sodium benzoate, 90% for potassium sorbate, and 81% expected recovery for sodium ascorbate. The limit of detection is 0.005% for potassium sorbate, and 0.01% for the 3 other preservatives.

Federal and State regulations allow no preservatives in ground beef. Nevertheless, the addition of sulfites and ascorbates to ground beef to preserve the red color has been a fairly common trade practice in New York State. Benzoates and sorbates are also used as antimicrobial preservatives that may make the product appear fresher than it is. Ground beef is generally prepared in retail stores, and since in New York State there are an estimated 15,000 such establishments, an effective surveillance program calls for the examination of a very large number of samples, which, in turn, requires a rapid test for the presence of preservatives in ground beef.

In the procedure we developed for this purpose, time is saved initially by preparing a single sample solution and taking aliquots for the various determinations. Sulfites are determined by an adaptation of the *p*-rosaniline colorimetric method which is much faster than the quantitative Monier-Williams distillation method. No comparison is made with the malachite green test for sulfite in meat (1) because it is a qualitative test used principally as a field test in New York State. The *p*-rosaniline test is specific for sulfites and has been adopted as official final action for dried fruit by the AOAC (2). Ascorbic acid is titrated with 2,6-dichloroindophenol, an official final action method for many foods, but not for meat (3). Benzoic and sorbic acids are extracted from an aliquot of the sample solution with petroleum ether and determined by their absorption at 225 and 250 nm, respectively. Similar procedures for the determination of benzoic and sorbic acids in other food products have been reported previously by this laboratory (4, 5).

These tests can be used to rapidly screen out the samples that contain no preservatives. For example, if 1.0 ml indophenol solution turns the sample aliquot pink, no added ascorbic acid is present. Similarly, sulfites are absent if purple does not develop with the *p*-rosaniline reagents. The absence of benzoic and sorbic acids is promptly noted if there is no absorbance of the petroleum ether extract at 225 and 250 nm.

If the presence of any of the preservatives is indicated, the test is continued for a quantitative estimation. Any amount found constitutes a violation, since there is no tolerance for preservatives in ground beef.

The sulfites, benzoates, and sorbates are not completely extracted by this method. However, the quantities of preservatives present in the unknown samples can be estimated satisfactorily if standards are processed in the same manner as samples. The results are expressed in terms of the sodium salt for sulfite, benzoate, and ascorbate and as the potassium salt for sorbate, since generally preservatives are added to the ground beef as these salts.

[1] Retired.
[2] To whom correspondence and inquiries should be addressed.

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The sample solution is prepared by adding 80 ml liquid to 30 g meat, which contains about 20 ml water. (The average moisture content of meat is 66%.) The total volume of the sample solution therefore is assumed to be 100 ml. An aliquot of this solution is titrated with indophenol that has been standardized with pure ascorbic acid. Sulfites, benzoates, and sorbates are quantitated by reference to standard absorption vs. concentration plots that need to be prepared only once, initially.

METHOD

Reagents

(a) *Extracting solution.*—Dissolve 200 g HPO_3 and 100 ml H_3PO_4 in ca 700 ml water and dilute to 1 L. Store in refrigerator and prepare fresh every 2 weeks.

(b) *2,6-Dichloroindophenol solution.*—25 mg/100 ml. Prepare and standardize as in 43.052(c).

(c) *Ascorbic acid solution.*—1 mg/ml. Dissolve 100 mg USP Ascorbic Acid in 100 ml extracting solution (a). Prepare solution just before use.

(d) *Sodium benzoate solution.*—3 mg/ml. Dissolve 300 mg sodium benzoate in 100 ml extracting solution (a).

(e) *Potassium sorbate solution.*—1.5 mg/ml. Dissolve 150 ml potassium sorbate in 100 ml extracting solution (a).

(f) *Ultraviolet reference solution.*—Mix 30 ml solution (a) with 50 ml ethanol and 20 ml water.

(g) *Acid-bleached p-rosaniline solution.*—Place 100 mg *p*-rosaniline HCl (certified) and 200 ml water in 1 L volumetric flask. Add 160 ml HCl (1+1) and dilute to volume. Let stand 12 hr. Dilute 20 ml of this solution to 100 ml with water.

(h) *Sodium tetrachloromercurate.*—Place 23.4 g NaCl and 54.3 g $HgCl_2$ in 2 L volumetric flask. Dissolve in ca 1900 ml water and dilute to volume.

(i) *Formaldehyde solution.* — 15 mg/100 ml. Dilute 2 ml formalin to 100 ml with water. Dilute 2 ml of this solution to 100 ml with water.

(j) *Sodium sulfite solution.*—3 mg/ml. Dissolve 300 mg sodium sulfite in 100 ml extracting solution (a).

Preparation of Sample Solution

Weigh 30 g ground beef into 200 ml beaker. Add, a little at a time, 30 ml extracting solution and blend into meat. Add 50 ml ethanol, stir well, and let stand 10 min, but not longer. Decant solution through moistened 15 cm folded paper (S&S 560, or equivalent). Keep most of the solids out of filter. Discard first few ml filtrate. Take aliquot for the sulfite test from first 10 ml collected.

Determination of Sulfites

Pipet 5 ml *p*-rosaniline solution and 2 ml mercurate reagent into 200 mm test tube. Add 2 ml solution followed by 10 ml formaldehyde solution. Mix and let stand 30 min. If purple develops, measure absorbance at 550 nm against reagent blank solution in which 2 ml water has been substituted for sample solution. Find corresponding per cent sodium sulfite in sample from standard plot.

Preparation of Standard Plot for Sulfites

Weigh 30 g portions of pure ground beef into each of 6 beakers. Add to these, respectively, 1, 2, 3, 4, 5, and 6 ml sulfite solution, followed by enough extracting solution to total 30 ml added liquid. To each add 50 ml ethanol, mix, and filter to obtain sulfite standard solutions. Take 2 ml aliquots for color development as described in sulfite determination. Plot absorbance found at 550 nm against sodium sulfite concentration in meat in range between 0.01 and 0.06%.

Determination of Benzoates and Sorbates

Pipet 5 ml sample solution into 50 ml glass-stoppered cylinder, add petroleum ether to 50 ml mark, and shake 1 min. Similarly, shake 5 ml ultraviolet reference solution with 45 ml petroleum ether to obtain reference extract. If sample extract indicates absorbance at 225 or 250 nm, scan against reference extract between 300 and 200 nm and check for characteristic benzoic or sorbic acid absorption curve. If benzoic acid is identified by curve, measure absorbance at 225 nm and find corresponding per cent sodium benzoate in sample from standard plot. Similarly, if sorbic acid is identified, measure absorbance at 250 nm and find corresponding per cent potassium sorbate in sample from standard sorbate plot.

Preparation of Standard Plot for Benzoates and Sorbates

Weigh 30 g portions of pure ground beef into each of 12 beakers. Add 1, 2, 3, 4, 5, and 6 ml benzoate solution to first 6 beakers, and same volumes of sorbate solution to second 6 beakers. To each add enough extracting solution to total 30 ml added liquid, followed by 50 ml ethanol. Mix, and filter to obtain standard benzoate and sorbate solutions. Take 5 ml aliquots and proceed as with benzoate and sorbate determinations. Plot absorbances found against concentrations in meat in range 0.01–0.06% for sodium benzoate and 0.005–0.030% for potassium sorbate.

Determination of Ascorbates

Add 1 ml indophenol solution to 25 ml solution. If dye turns pink and remains so for 10 sec, no added ascorbic acid is present. If dye is decolorized, add indophenol from buret to pink end point. Add to buret reading the 1 ml dye used for limit test.

Ascorbic acid, mg/100 g = (ml titer × mg ascorbic acid × 100 ml × 100 g)/(ml dye × 25 ml × 30 g)

To simplify and convert to sodium ascorbate:

Sodium ascorbate, mg/100 = (ml titer × mg ascorbic acid × 15)/ml dye

Results and Recommendation

Ground beef known to be free of preservatives was used to prepare the samples for recovery studies. Preservatives, in the quantity most likely to be encountered in retail samples, were added to 30 g portions of meat. Replicate determinations were made for each preservative, each replicate representing an individual fortified sample. The results of the analysis of these samples are shown in Table 1.

Recoveries of added sulfite, benzoate, and sorbate were quite satisfactory. Ascorbate recovery of 73% was rather low at the 0.0276 g/100 g level, possibly attributable to some loss due to oxidation by oxyhemoglobin and oxymyoglobin in the meat. Additional tests with higher fortifications gave better recoveries: 85% ascorbate was recovered in trials when 0.040 g/100 g and 0.053 g/100 g samples were prepared, so that recovery of about 81% may be expected over the probable range. As much as 0.062 g sodium ascorbate/g is permitted in cured meats, although none is allowed in ground beef.

In the sulfite determination there is a tendency to haze formation. This will be negligible, however, if the instructions for the preparation of the sample solution and the taking of the aliquot are closely followed. The possible effect of haze on absorption at 550 nm can be checked by noting the absorbance at 720 nm, which should be minimal.

We have found no interferences in the procedure for benzoates and sorbates. Pure meat shows little UV absorption, as seen in Fig. 1, which also shows the absorption curves for the extracts of the same ground beef fortified with sodium benzoate and potassium sorbate. These curves are easily identified. Benzoic and sorbic acids together in the same solution yield an absorption curve of a composite shape, but the maxima are still distinct. In such an event, the preservatives can still be estimated. At the concentrations considered, benzoic acid has little absorption at 250 nm, so the absorption at this wavelength can be used to compute sorbic acid. On the other hand, sorbic acid does have significant absorption at 225 nm, which is 38% of its absorption at 250 nm. Making a correction for this, the absorption at 225 nm due to benzoic acid is $A_{225} - 0.38 \times A_{250}$. In the course of several years of application of this method, how-

Table 1. Recovery of added preservatives

Statistic	Na sulfite	Na benzoate	K sorbate	Ascorbic acid
Added, g/100 g	0.020	0.040	0.020	0.0276
Av. recd, g/100 g	0.019	0.0412	0.018	0.0201
Rec., %	95	103	90	73
Range	0.004	0.020	0.007	0.0055
Replicates	8	10	10	11
Std dev.	0.0013	0.008	0.002	0.0019
Coeff. of var., %	6.8	19.4	11.1	9.6

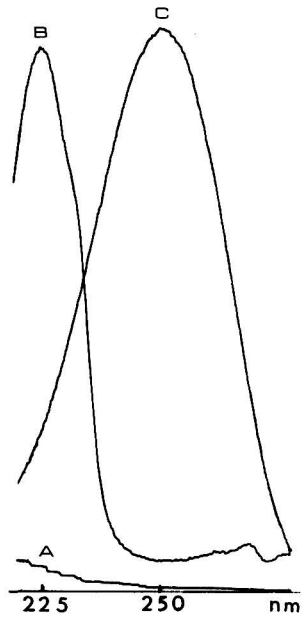


FIG. 1—Absorption curves of ground beef with and without added preservatives: A, unfortified ground beef; B, the same ground beef fortified with 0.06% sodium benzoate; C, the same ground beef fortified with 0.025% potassium sorbate.

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**, 289–290.

ever, we have not found benzoic and sorbic acids in ground beef simultaneously. The sensitivity of the method is 0.005% for potassium sorbate while sodium sulfite, sodium benzoate, and sodium ascorbate can be determined at the 0.01% level.

It is recommended that the method described be subjected to collaborative study.

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

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REMINDER . . .

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ALCOHOLIC BEVERAGES

Determination of the Congeners Produced from Ethanol During Whisky Maturation

GEORGE H. REAZIN, SIDNEY BALDWIN, HARRY S. SCALES, HAROLD W. WASHINGTON, and ARTHUR A. ANDREASEN
Joseph E. Seagram & Sons, Inc., Louisville, KY 40201

A study was made to determine which congeners are derived from ethanol during maturation of whisky. Radioactive ethanol-1-^{14}C was added to a barrel of new bourbon whisky and the radioactivity of the major individual congeners was determined during the maturation period. During 56 months of maturation, ethanol was transformed into acetic acid, acetaldehyde, ethyl acetate, and ethanol lignin as indicated by their radioactivity. No radioactivity was found in the solids, fusel oil alcohols, or the aromatic aldehydes derived from ethanol lignin. The chemistry of maturation is discussed.

It is well known that the chemical and physical properties of alcoholic distillates change during storage in a charred oak barrel. Extensive chemical analyses of whisky aged for different periods have established those congeners that increase during maturation (1–5). These data suggest that congeners are derived during maturation in the following ways: (*1*) reactions of and between ethanol and congeners of the high wine, (*2*) interaction of ethanol and the barrel wood, and (*3*) extraction of congeners from the char and wood of the barrel. For example, esters, aldehydes, and volatile acids are believed to be produced by reactions involving the ethanol of the high wine. Ethanol lignin is thought to be produced by an interaction of ethanol with the barrel wood, while fixed acids, tannins, furfural, and other complex organic substances probably are the result of extraction from the barrel wood and char. Color may be derived by a combination of extraction and reaction. However, in most cases the evidence cited for these reactions has been obtained by indirect means. The use of ethanol labeled with radioactive carbon provides a system whereby one of these reactions can be directly monitored. Since the chemical properties of radioactive ethanol are the same as those of nonradioactive alcohol, any congener derived from ethanol will be both radioactive and nonradioactive. Those congeners that increase during maturation, but are not radioactive, are not derived from ethanol.

This paper summarizes data obtained by isolating the individual congeners in treated whisky during maturation and determining their radioactivity to demonstrate which of the congeners are derived from ethanol.

Experimental

Warehousing Conditions

A new, charred white oak barrel was filled and placed in a non-air-conditioned storage area of the research and development building under government supervision. Customary precautions in the use of radioactive materials were observed.

After the barrel was filled with bourbon high wine, 5 mCi radioactive ethanol-1-^{14}C was added. The total volume of radioactive ethanol was less than 0.1 ml in ca 50 gal. high wine. The radioactive ethanol was obtained from New England Nuclear Corp., Boston, MA. The contents were then mixed by using a motorized stirrer with a long shaft that extended through the bung hole. After ca 30 min of mixing equilibrium was reached, the 0 time sample was siphoned from the barrel, and the bung was inserted. Samples were removed at 6-month intervals for chemical and radioactivity analyses. The experiment was terminated after 56 months of maturation.

The temperature of the storage area was not controlled; however, temperature was measured over a 2-year period. The average temperature for April through September was 84°F and October through March, 73°F. Sampling periods were April and October.

Analytical Procedures

Representative samples were obtained by mixing the contents of the barrel for 10 min, removing a sample for color determination, and repeating this process until the same Klett colorimeter readings were obtained on at least 2 consecutive samples. About 1.5 L material was then siphoned from the

barrel for analysis. Proof, pH, and color intensity were determined. AOAC methods were used to determine fusel oil, acids (fixed and volatile), aldehydes, and esters (6). The concentrations of individual higher alcohols of fusel oil were measured by gas-liquid chromatography (GLC) (7). Methods for separation of individual congeners are as follows:

(a) *Acids.*—Place 200 ml aged whisky, 200 ml water, and 5 ml 10% sodium bicarbonate in Kjeldahl flask. Distill ca 190 ml into 200 ml volumetric flask and dilute to volume. Ethanol, esters, aldehydes, and fusel oil alcohols are in distillate, while acids, solids, and color remain in residue. Process 800 ml of sample in this manner, so that 2 portions of 200 ml can be used for duplicate aldehyde analyses and 2 portions of 200 ml can be used for duplicate ester and fusel oil determinations.

Combine and divide residue into 2 portions for duplicate determinations of acids. Add 2–3 pellets of NaOH to make each solution basic. Evaporate residue solutions on steam bath to reduce volume and place each concentrated solution in 25 ml volumetric flask for steam distillation.

Acidify concentrated solutions and steam distill (8) to remove volatile acids. Collect 300–400 ml, add excess NaOH, and evaporate to dryness. Dissolve dried acids in small volume of water, add 1–2 drops of strong HCl to remove red color of phenolphthalein added when samples were initially treated, and dilute to 10 ml with water. Determine radioactivity of 0.5 ml aliquot of acidified portion, using Tri-Carb scintillation spectrometer (7).

Evaporate 50 ml of original undistilled whisky sample to dryness on steam bath. Redissolve this material in 100° proof spirits and place aliquots on metal planchets for radioactivity determinations, using a Chicago Nuclear methane flow proportional counter. This radioactivity will represent radioactive carbon present in fixed acids and other nonvolatile residues of whisky sample.

(b) *Aldehydes.* — Aldehydes are precipitated from duplicate 200 ml Kjeldahl distillates obtained in first step of acid fractionation. Initially, place distillate in 500 ml round-bottom flask and chill in ice bath. Add 2,4-dinitrophenylhydrazine solution (2.5 g/L 2*N* HCl) on molar basis so ≥ 5 times more reagent is present than aldehyde. Stopper flask and remove from ice bath after ca 1 hr. Hydrazone settles to bottom of flask. After 3–4 days, collect precipitate on filter paper. Wash with 10 times original volume with water to remove any radioactive ethanol contamination. Dissolve precipitate on filter paper with excess chloroform. Dry chloroform solution over anhydrous Na_2SO_4 and evaporate to 10 ml. Place 0.5 ml aliquots on metal planchets, dry, and determine the radioactivity present, using proportional counter.

(c) *Ester and fusel oil.*—Fractionate remaining duplicate 200 ml distillates obtained in isolation of acids into esters, ethanol, and fusel oil by distillation in Podbielniak fractionation still (7). This fractionation recovers 90–100% of the radioactivity. Since some ethanol is present in ester fraction, further purify ester material with preparative GLC techniques (7). Trap GLC fractions in a cold finger submerged in −60°C bath.

Separate fusel oil components by GLC (7). Determine radioactivity present in each alcohol with Tri-Carb scintillation spectrometer.

(d) *Ethanol lignin.*—Concentrate 500 ml whisky in rotary film evaporator (30 mm mercury) to ca 150 ml at 35–45°C (9). Collect ethanol lignin residue by method previously reported (9), dry in tared dish, weigh, and calculate amount of material present. Place ca 10 mg of this material into 10 ml graduated test tube and dissolve sample in 0.2 ml acetone and 0.3 ml water. Dilute to 1 ml with acetone-water mixture and dilute to 2 ml with water. Place 0.5 ml aliquots on metal planchets, dry, and determine radioactivity with proportional counter.

Results

Color intensity, pH, and major congener values are summarized in Table 1. These congener data, in general, show the same trends in congener development previously reported (1–5) and indicate that, although the amounts of some of the congeners may be higher than in normally warehoused whisky, the maturation relationships of the experimental radioactive whisky are normal.

Radioactivity results for the different congener groups are described below:

Ethanol

Table 2 shows the total radioactivity present at each sampling interval over the entire maturation period. Based on the addition of 5 mCi of radioactive ethanol to the original 402 lb of 110° proof high wine, 25.3 μCi/PL (PL = 100 L at 100° proof) should be present. The data agree reasonably well with this value. The total radioactivity would be expected to change due to evaporation losses and other causes, but has not been analyzed critically.

Of the total radioactivity present, virtually all of it is due to ethanol. At zero time, 0.1% of the total radioactivity was found in the congener

Table 1. Chemical analysis of whisky during maturation

Age, months	Proof	Color[a]	pH	Congeners, g/100 PL[b]					
				Acids		Aldehydes	Esters	Fusel oil	Ethanol lignin
				Fixed	Volatile				
0	110.0	0	—	0.26	2.12	0.7	6.87	207	0
6	112.0	147	—	4.10	25.63	3.37	17.68	226	13.6
12	110.0	167	—	5.44	36.15	2.67	21.55	250	27.1
18	111.9	195	—	10.51	41.52	5.15	27.44	228	25.3
24	112.6	215	4.3	12.98	45.03	4.96	33.15	240	41.9
30	111.5	251	4.1	10.95	56.72	5.87	38.22	243	35.7
42	119.0	248	4.0	14.36	49.66	8.25	51.83	249	33.9
56	125.0	270	—	—	—	9.23	57.20	266	—

[a] Klett units (No. 54 filter) at barrel proof.
[b] g/100 L at 100° proof.

Table 2. Radioactivity analysis of whisky during maturation

Age, months	Total in whisky, μCi/PL	Congener radioactivity, nCi/PL[a]					
		Acids		Aldehydes	Ester	Fusel oil	Ethanol lignin
		Fixed	Volatile				
0	25.514	0	0	0	0	0	0
6	27.393	3.77	4.17	0.29	8.75	5.07	0.110
12	23.302	2.41	5.10	1.71	14.65	6.12	0.225
18	21.857	4.79	9.70	19.10	20.09	2.18	0.201
24	21.741	2.85	6.83	12.73	39.45	1.08	0.514
30	20.490	—	7.16	—	56.27	2.84	0.350
42	22.965	—	4.95	27.07	54.37	—	0.407
56	23.235	—	—	32.66	57.52	1.18	—

[a] nCi ^{14}C/L at 100° proof.

fractions. This radioactivity represents impurities present in the radioactive alcohol added. After 56 months of maturation, about 0.4% of the radioactivity was associated with the congeners studied. Corrected for impurities, about 0.3% of the ethanol originally present was transformed into congeners during maturation.

Acids

The radioactivity of the volatile and fixed acids is shown in Table 2. In general, volatile acids increased in concentration and the radioactivity also increased during aging. The deviations, particularly at 18 and 42 months, probably are due to experimental error. Since the volatile acids were radioactive and the radioactivity showed a general increase, volatile acids must have been produced from ethanol.

The volatile acid fraction was then investigated to determine which of the individual acids was radioactive. The volatile acid fraction from a 24-month maturation sample was evaporated to 0.5 ml. An equal volume of 5% sulfuric acid was added and 250 μl was injected into a gas chromatograph equipped with a 0.24″ × 15′ column packed with 5% FFAP on Chromosorb G (7).

As shown in Table 3, 86% of the volatile acid radioactivity was present in acetic acid, which accounted for 97% of the total volatile acids. Relatively high radioactivity was found in the isobutyric, *n*-butyric, and isovaleric acid fractions in relation to their low concentration in the whisky. Formation of these acids from ethanol is not explained. Their radioactivity may originate from contamination in the ^{14}C-ethanol or from tailing of radioactive materials in the GLC column. However, the data indicate that ethanol is transformed into acetic acid during maturation.

The fixed acids also increased and contained radioactivity (Table 2). The amount of radioactivity increased during the first months only. Since the radioactivity was not determined on a purified fraction, but on whisky evaporated to dryness, the radioactivity is probably due to nonvolatile materials other than fixed acids. Although radioactive ethanol lignin is present, its

Table 3. Radioactivity of individual components of volatile acid fraction[a]

Peak	Per cent of total volatile acid	Radioactivity[b] nCi[c]	%	Compound
1	—	0.01	2.3	pre-acetate unknowns[d]
2	97.2	0.37	86.0	acetic acid
3	0.1	0.00	0	propionic acid
4	0.6	0.02	4.6	isobutyric acid
5	1.3	0.01	2.3	*n*-butyric acid
6	0.8	0.02	4.6	isovaleric acid
7	—	0.00	0	post-acids[e]
Total	100	0.43	99.8	

[a] Sample obtained from 24-month aged whisky.
[b] Fractionation by gas-liquid chromatography.
[c] Total injection was 0.33 nCi.
[d] Fraction collected prior to acetic acid peak.
[e] Fraction collected after last acid peak.

total activity is too low (*see* below) to account for the amount of radioactivity found.

Aldehydes

Tables 1 and 2 show that as the aldehydes increase during maturation so does the radioactivity. The deviations are probably due to technical problems with the aldehyde hydrazone derivative procedure. That is, in forming the derivatives, some of the radioactive ethanol could have been converted to aldehyde, causing an abnormally high radioactivity level; also, in the extensive washing of the precipitate to remove ^{14}C-ethanol, some of the hydrazone could have been lost. However, this would apply generally to all samples. It is significant that the radioactivity of the aldehyde fraction increased as maturation time increased. Therefore, aldehyde is formed from ethanol during maturation.

Determinations also were made to establish the individual aldehydes present and their radioactivity. The aldehyde hydrazone from the 24-month whisky sample was dissolved in chloroform. Paper chromatograms of this material were made by placing drops of the chloroform solution uniformly on a line 3.5″ from the top of a 7 × 17″ sheet of Whatman No. 4 filter paper, across the width of the sheet. The sheet was then placed in a glass chromatographic tank and developed descendingly with cyclohexane-dimethylformamide (300+50). The 5 bands of dinitrophenylhydrazones were cut from the original chromatogram and the material was dissolved in chloroform. The radioactivity of these solutions was determined with the proportional counter.

The various bands were identified by comparison with a chromatogram of a mixture of known aldehyde hydrazones. The results of this study are summarized in Table 4 and indicate that at least 79% of the radioactivity present was associated with acetaldehyde. Two unknown bands were found which probably represent materials other than simple aldehydes, i.e., diacetyl and glyoxal. Some radioactivity was found in these unknowns. Since these bands were between the origin and the acetaldehyde, and the band nearest the acetaldehyde had a higher radioactivity level than that nearest the origin, some tailing of the acetaldehyde may be indicated. Therefore, the radioactivity in these unknown bands may represent acetaldehyde contamination. These data indicate that acetaldehyde is formed from ethanol during maturation.

Esters

As shown in Table 2, the ester fraction was radioactive. Only ethyl acetate was detected. Therefore, the data indicate that ethanol is transformed into ethyl acetate during the maturation process.

Fusel Oil

There was a very low level of radioactivity in the fusel oil fraction (Table 2) except for unexplained higher levels at 6 and 12 months.

When the concentrations of the individual higher alcohols present at different maturation ages were compared (Table 5) no significant differences were observed in isobutyl, *d*-amyl, and isoamyl alcohols. The concentration of *n*-propyl alcohol increased during the first 18 months of maturation, after which it remained relatively constant. This higher alcohol gave only a slight color when measured by the

Table 4. Radioactivity of individual components of aldehyde fraction[a]

Band	Radioactivity[b] nCi	%	Compound
1	0.015	1.0	origin
2	0.102	7.0	unknown
3	0.179	12.4	unknown
4	1.142	79.4	acetaldehyde
5	0.000	0	propionaldehyde
6	0.001	0	isobutyraldehyde
Total	1.439	99.8	

[a] Sample obtained from 24-month aged whisky.
[b] Fractionation by paper chromatography.

Table 5. Change in radioactivity of higher alcohols during maturation

Age, months	*n*-Propyl		Isobutyl		*d*-Amyl		Isoamyl	
	C[a]	R[b]	C	R	C	R	C	R
0	9.35	0	64.50	0	47.4	0	113.0	0
6	9.72	3.33	64.50	0.94	44.7	0.63	103.0	0.17
12	12.08	2.63	76.70	0.01	41.6	1.82	111.4	1.66
18	17.80	0	76.0	0	49.9	1.49	117.1	0.69
24	18.67	0	82.5	0	45.2	0.39	119.2	0.69
30	20.58	0	68.8	0	57.6	1.39	155.2	1.30
56	16.94	0	63.8	0	48.3	0.89	118.2	0.23

[a] Concentration in g/100 PL.
[b] Radioactivity in nCi/PL.

Komorowsky colorimetric reaction, which was used for the values in Table 1.

Only traces of radioactive carbon, if any, were found in the individual higher alcohols. These traces are assumed to be due to tailing of the ethanol in the GLC fractionation of the fusel oil. This is supported by the fact that when only the radioactive ethanol fraction, isolated from the Podbielniak fractionation, is subjected to GLC fractionation, radioactivity is found in the various fractions even though ethanol is not detected. Therefore, the data of Tables 2 and 5 have been corrected by the amount of radioactivity contamination found at zero time.

Since there was no increase in the concentration of the isobutyl, *d*-amyl, and isoamyl alcohols, and there was no significant radioactivity, these congeners can be considered not to be formed from ethanol during maturation. Although there was an increase in *n*-propyl alcohol, no significant radioactivity was found. If *n*-propyl alcohol is formed during maturation, it may originate from the wood of the barrel, but not from the ethanol.

Ethanol Lignin and Aromatic Aldehydes

The amount of ethanol lignin isolated from the whisky did not increase significantly after the first 2 years of maturation (Table 1). It was radioactive (Table 3) and the activity paralleled the concentration.

Aromatic aldehydes in the 24-month sample were isolated and separated by paper chromatography (9). However, when they were tested individually for radioactivity, none was found. It would appear that the aromatic aldehydes are not derived from the ethanol moiety.

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

Discussion

As previously stated, congener development during maturation is generally attributed to reactions of and between high wine components, interaction between the barrel wood and high wine components, and extractions from the barrel wood. The data of this report show that all 3 systems occur.

Reactions of High Wine Components

The data indicate that ethanol can undergo an oxidation reaction to produce acetaldehyde, which is in turn oxidized to acetic acid. These reactions are illustrated by Equations 1 and 2, with the asterisk indicating the position of the radioactive carbon.

$$2\ CH_3C^*H_2OH + O_2 \longrightarrow 2\ CH_3C^*HO + 2\ H_2O \quad (1)$$

$$2\ CH_3C^*HO + O_2 \longrightarrow 2\ CH_3C^*OOH \quad (2)$$

The amount of acetaldehyde found is small, since it is easily oxidized to acetic acid. Thus, the amount of acetaldehyde present at any sampling period will be the difference in the reaction rates of Equations 1 and 2.

By determining the amount of radioactive carbon per mg carbon present (specific activity) in the congeners and comparing this value with the specific activity of the ethanol at zero time, a measure of the proportion of a congener that is produced from ethanol can be obtained. For example, if all the congener found is produced from ethanol, it should have the same specific activity as the ethanol. Table 5 shows the specific activities of the ethanol, acetaldehyde, acetic acid, and ethyl acetate.

The calculated specific activity of the ethanol, based on the amount of radioactivity added and the proof of the high wine, was 0.11 nCi/mg

carbon. The specific activities of the ethanol in the various samples taken during maturation agree, within experimental error, with this value. This indicates no ethanol was formed during maturation, as would be expected.

The specific activity of the acetic acid was lower than that of the ethanol. The ratio of specific activities between the ethanol and acetic acid is roughly 3:1, indicating that more acetic acid is being formed from other sources, e.g., extracted from the barrel, than is being produced from ethanol. These data agree with those of Tables 1 and 2, which show a greater increase in the g/100 PL of acetic acid than in radioactivity.

The specific activity data for acetaldehyde are not valid inasmuch as the activities from the older samples were much larger than those obtained for ethanol. This high value is explained by the probability that some of the ethanol was oxidized to acetaldehyde during dinitrophenylhydrazone formation in the aldehyde isolation phase of the study. As a result, abnormally high specific activity values would be obtained when the calculations are based on the aldehyde content of the original whisky. This error would be expected to remain more or less constant throughout the experiment. Although the validity of the quantitation procedure is in question, the acetaldehyde and its total radioactivity increased in such a manner during aging that the specific activity remained more or less constant after 18 months. These data indicate that acetaldehyde is formed from ethanol during maturation, but it would be speculative to conclude that acetaldehyde is formed from sources other than ethanol, e.g., during the early stages of maturation.

Equation 3 illustrates the formation of ethyl acetate.

$$CH_3C^*H_2OH + CH_3COOH \longrightarrow CH_3C^*H_2OOCCH_3 + H_2O \quad (3)$$

This reaction shows the esterification of acetic acid with radioactive ethanol to form radioactive ethyl acetate. As maturation proceeds, acetic acid is formed from the oxidation of ethanol and will react to produce ethyl acetate according to Equation 4.

$$CH_3C^*H_2OH + CH_3C^*OOH \longrightarrow CH_3C^*H_2OOC^*CH_3 + H_2O \quad (4)$$

Therefore, radioactive ethyl acetate can contain radioactive carbon in both the ethanol and acetic acid moieties of the molecule. If Reaction 3 were the only system operating, the specific activity of the ethyl acetate would be half that of the ethanol, or 0.05 nCi/mg carbon, because of dilution by cold carbon from the acetic acid. However, if only Reaction 4 occurred, the specific activity would be the same as that of ethanol. Since the specific activity of the acetic acid was about one-third that of ethanol, the ethyl acetate specific activity should be less than that of ethanol, but perhaps greater than one-half of the ethanol specific activity.

The specific activities for ethyl acetate are shown in Table 6. After 2 years of aging, the specific activities were approximately twice that of the ethanol added. This is attributed to a relatively constant experimental error. However, the specific activity values suggest that Reaction 3 operates initially, followed by the introduction of Reaction 4 during the latter part of the maturation period.

It could be expected that, due to the large excess of ethanol to acetic acid, all the acetic acid would be esterified. This does not occur and may be explained by the presence of water, which prevents the equilibrium from shifting completely to ethyl acetate formation. The equilibrium is shown in Equation 5.

$$K_{eq} = (\text{moles ethyl acetate} \times \text{moles water})/(\text{moles acetic acid} \times \text{moles ethanol}) \quad (5)$$

The equilibrium constants for this reaction were

Table 6. Specific activity of ethanol and congeners during maturation

Age, months	Ethanol,[a] nCi/mg C	Acetic acid, nCi/mg C	Acetaldehyde, nCi/mg C	Ethyl acetate, nCi/mg C
0	0.12	0	0	0
6	0.13	0.04	0.02	0.09
12	0.11	0.04	0.12	0.12
18	0.11	0.06	0.68	0.13
24	0.11	0.04	0.47	0.22
30	0.10	0.03	—	0.27
42	0.11	0.03	0.60	0.19
56	0.11	—	0.65	0.18

[a] Specific activity of ethanol-1-^{14}C in barrel initially was calculated as follows (distillate at 110° proof):
g carbon/L = (550 ml ethanol/L × 0.79365 g/ml × 24 g carbon/mole ethanol)/46 g/mole ethanol = 227.743
radioactivity added = 25.3 μCi/L
specific activity = 25.3 μCi/227.743 g carbon/L
= 0.11 nCi/mg carbon.

calculated from the data of Table 1 for the various time periods studied. These values ranged randomly from 1.07 to 1.56 and averaged 1.28. This suggests that the law of mass action primarily governs the formation of ethyl acetate, i.e., esters are formed by a dynamic system influenced by changes in concentration of acetic acid, ethanol, and water. Thus, during maturation a loss of water by evaporation and/or an increase in acetic acid will cause an increase in ethyl acetate formation.

Interaction of Ethanol with Barrel Wood

The radioactivity data obtained for ethanol lignin suggest an interaction of ethanol with barrel lignin. These reactions are shown by Equations 6 and 7.

$$\text{ethanol} + (\text{lignin}_x - \text{wood}) \rightarrow \text{ethanol lignin} + (\text{lignin}_{(x-1)} - \text{wood}) \quad (6)$$

$$\text{ethanol lignin} \xrightarrow{[O]} \text{ethanol} + \text{aromatic aldehydes} \quad (7)$$

The data obtained are consistent with the hypothesis of Baldwin *et al.* (9), and demonstrate another mode of congener formation during aging.

Extraction of Barrel Components

Color substances, tannins, and other compounds are extracted from the wood during maturation (1–5). These materials would not necessarily be expected to become radioactive. However, some radioactivity was found in the solids fraction isolated from the whisky. This can be due to contamination rather than the formation of some radioactive solid, because no change in its radioactivity was observed during maturation and an increase would be expected.

Of the higher alcohols present, only *n*-propyl alcohol increased and was not radioactive. It is conceivable that it could be formed from the decomposition of lignin or tannin constituents of the wood.

Experimental errors appear to be inherent in the conventional analytical techniques used, when applied to tracer work. However, the data presented indicate that during maturation ethanol is transformed to acetic acid, acetaldehyde, and ethyl acetate. Ethanol lignin is also formed by the interaction of ethanol with the lignin present in the wood of the barrel. A study of reactions involving radioactive compounds other than ethanol would be useful in elucidating the changes that occur in whisky during maturation.

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Collaborative Study of the Determination of Color in White Wines

HERB L. WILDENRADT and ARTHUR CAPUTI, JR.
E. & J. Gallo Winery, PO Box 1130, Modesto, CA 95353

A collaborative study was conducted on the determination of color in white wines by trans-reflectometry. The instrument used is designed to give a photometric response approximating the International Commission on Illumination CIE-z tristimulus function and provides transmittance values obtained from a 2.5 cm sample pathlength. Seven collaborators analyzed 13 samples (10 wines and 3 potassium dichromate solutions). Average transmittance values of the samples ranged from 75.34 to 87.56%. The standard deviations obtained in the study varied from 0.20 to 0.67% transmittance. In view of the simplicity and reproducibility of the method, it has been adopted as official first action.

The appearance of a wine is one of its most important characteristics, and color is a significant contributor to that appearance. Since the color of white wines is often an indicator of quality, it is a parameter that is frequently evaluated in the production, processing, and blending of those wines. A simple, accurate, and reproducible method of measuring white wine color is necessary in monitoring and controlling these processes.

The AOAC method in common use (1) is that described for beer, which determines the absorbance of a sample at 430 nm. The deficiencies of this procedure are numerous and have been reviewed at length by Little (2). In order to avoid the problems associated with this method, Little (3) and Little and Simms (4) devised a simple instrument based on the principle of trans-reflectometry. Its photometric response approximates the International Commission on Illumination (Commission Internationale de l'Eclairage) CIE-z tristimulus function; thus it gives results correlating to visual perception. When a sufficient number of these instruments were in use to give valid statistical data, a collaborative study was initiated.

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

Collaborative Study

Seven collaborators participated in this study. They were instructed to standardize their instruments according to the manufacturer's instructions and then to analyze each sample once.

Each collaborator was sent 400 ml potassium chromate standard solution, 400 ml each of 10 different commercially produced wines, and 400 ml each of 3 different potassium dichromate solutions with color values similar to those of white wines.

All samples were distributed to the collaborators in sealed, screw-capped, green glass bottles. The bottles were filled so that a minimum of headspace oxygen was present that could cause oxidative browning of the samples. The wine samples consisted of, and are referred to in this paper as: Sample A, French Colombard; Sample B, Sauvignon Blanc; Sample C, a blended white wine; Sample D, dry sherry; Sample E, a special natural white wine; Sample F, a carbonated apple wine; Sample G, Riesling; Sample H, a special natural white wine; Sample I, a blended white table wine; and Sample J, a blended white table wine. The 3 potassium dichromate solutions, labeled K, L, and M, were to be analyzed as white wine samples. They were, respectively, 0.0400, 0.0500, and 0.0350 g $K_2Cr_2O_7$/L 0.01*N* H_2SO_4.

METHOD

Color in White Wines
Official First Action

11.B01 ***Apparatus and Reagents***

(a) *White wine colorimeter.*—Double beam filter photometer utilizing W incandescent lamp with Corning 5-61 high pass filter, Se photocells, and 1″ path test and ref. cells. Combination of responses of photocell and filter approximates monochromatic peak at 430 nm. Available from Technical Products, 2135 Upper Scenic Dr, PO Box 291, Felton, CA 95018.

(b) *Potassium chromate std soln.*—0.0002059*M*. Dissolve 0.0400 g K_2CrO_4 primary std (J. T. Baker Chemical Co., No. 3058) in 0.05*N* KOH and dil. to 1 L with 0.05*N* KOH.

11.B02 ***Determination***

Let instrument warm up 2 hr. Stdze with K_2CrO_4 soln according to manufacturer's instructions. $\%T$ should be reproducible to $\pm 0.1\%$.

Fill ref. and test cells with H_2O, and place both in colorimeter. Set indicator knob to zero, and null colorimeter by adjusting zero set knob. Remove test cell and replace with zero set cell. Null meter by adjusting indicator. Indicator should read ca 98.5 on duplicate tests. Repeat each hr or after every 10–15 samples.

With zero set cell in place, set indicator to value (ca 98.5) detd above. Null meter with zero set knob. Replace zero set cell with test cell contg wine sample. Null meter by adjusting indicator. Read $\%T$ on indicator.

Results and Recommendations

Table 1 contains the transmittance data obtained in the collaborative study. Sample K sent to Collaborator 5 was broken and therefore was unreported.

The ranked results of the collaborators are given in Table 2, omitting Sample K. The ranked results of Collaborators 3 and 4 are, respectively, lower and higher than the 29–72, 5% 2-tail limits for 12 samples and 7 collaborators. Their colorimeters may have been incorrectly standardized or may have a slight inherent error.

A 2-way analysis of variance (collaborators and the 10 wine samples) (5) was performed on the data (Table 3). A significant difference ($P < 0.01$) was found among collaborators over the 10 wine samples. The standard deviation within samples within a collaborator (precision or repeatability) was estimated from the collaborator × samples interaction. It was 0.318 with a coefficient of variation of 0.4%. The standard deviation among laboratories (reproducibility) was 0.357 with a coefficient of variation of 0.4%. It is evident that the analyses of Samples B and J are the least precise. These 2 wine types are subject to oxidative browning, and although precautions were taken to avoid this problem, variations in transportation and storage environment could have caused slight changes.

The white wine colorimeter drifts at approximately 0.1 transmittance unit/hr and this drift could be a factor influencing the precision and accuracy. Two of the collaborators reported that after completing the wine sample color analysis, they re-analyzed their standard potassium chromate solution and found their colorimeter to indicate 0.3 transmittance unit higher than when standardized prior to the wine sample analysis. It is recommended that the influence of the colorimeter drift be minimized by performing the colorimeter test prior to use, at least once

Table 1. Individual collaborative results (in transmittance units) for the determination of white wine color by trans-reflectometry

	Samples												
Coll.	A	B	C	D	E	F	G	H	I	J	K	L	M
1	84.9	83.0	82.0	84.7	83.3	79.2	87.7	84.7	85.7	80.7	79.6	75.3	81.8
2	85.0	84.1	82.8	84.7	83.4	79.3	87.5	84.7	85.2	80.8	79.7	75.5	81.8
3	85.6	83.3	81.9	84.8	83.5	79.3	87.7	85.1	85.8	81.6	80.0	75.9	82.3
4	84.7	82.8	82.3	84.5	83.0	78.8	87.1	84.1	85.2	80.4	79.1	74.8	81.4
5	85.4	82.6	82.1	85.0	83.7	79.9	87.8	84.7	85.3	81.1	—	75.5	82.1
6	85.2	82.8	81.9	84.4	83.1	78.7	87.6	84.6	85.5	81.4	79.2	74.9	81.5
7	85.2	84.3	82.3	84.6	83.4	79.2	87.5	84.6	85.2	80.8	79.7	75.5	81.9
Av.	85.14	83.27	82.19	84.67	83.34	79.20	87.56	84.64	85.41	80.90	79.55	75.34	81.83
Std dev.	0.30	0.67	0.32	0.20	0.24	0.39	0.23	0.29	0.25	0.49	0.34	0.38	0.31

Table 2. Ranked results in white wine color collaborative study

	Sample												
Coll.	A	B	C	D	E	F	G	H	I	J	L	M	Sums
1	6	4	5	3.5	5	4.5	2.5	3	2	5	5	4.5	50
2	5	2	1	3.5	3.5	2.5	5.5	3	6	7	3	4.5	46.5
3	1	3	6	2	2	2.5	2.5	1	1	1	1	1	24
4	7	5.5	2.5	6	7	6	7	7	6	6	7	7	74
5	2	7	4	1	1	1	1	3	4	3	3	2	32
6	3.5	5.5	6	7	6	7	4	5.5	3	2	6	6	61.5
7	3.5	1	2.5	5	3.5	4.5	5.5	5.5	6	4	3	3	47

Table 3. Analysis of variance for collaborative results for the determination of white wine color

Source of variance	Degrees of freedom	Mean square	Sum of squares
Between colls.	6	2.192	0.3653
Between samples	9	363.9594	40.4399
Colls. × samples	54	5.4566	0.1010

every 10–15 samples, or hourly. The magnitude of all errors encountered is of such a low order that the method appears to have more than adequate precision and interlaboratory reproducibility for quality control and experimental use. It is therefore recommended that the method be adopted as official first action, and that the instrumental values obtained simply be designated transmittance units.

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C. S. Ough, Department of Viticulture and Enology, University of California, Davis, CA

Dennis Ruggles, Division of Mathematics, Food and Drug Administration, Washington, DC, performed the analysis of variance.

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.

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Collaborative Study of the Gas-Liquid Chromatographic Determination of Coumarin (1,2-Benzopyrone) in Wine

RANDOLPH H. DYER and GLENN E. MARTIN
Scientific Services Division, Bureau of Alcohol, Tobacco and Firearms, Department of the Treasury, Washington, DC 20226

The determination of coumarin in wine by using gas-liquid chromatography of a chloroform extract was collaboratively studied. Eleven collaborators analyzed 6 samples: 3 containing 4.0 mg coumarin/L, 2 containing 8.0, and 1 containing 0.0. When the triplicate and duplicate recoveries for the 4.0 and 8.0 levels were averaged and treated singly for the collaborators, there was no statistically significant difference in the accuracy between the 2 levels. However, the accuracy for the 4.0 level was much better than that for the 8.0 level: 100.0 vs. 105.9%. An analysis of the individual values showed no significant differences among collaborator means at the 4.0 level, while there was a significant difference among means at the 8.0 level. All collaborators were within the allowable score limits in the ranking test. The method was adopted as official first action.

In last year's report on coumarin in wine (1), the Associate Referee's results for a gas-liquid chromatographic (GLC) method were presented and a collaborative study was recommended. In addition, modifications of the regulations (2) necessitated a method to quantitate coumarin in wines. Consequently, a collaborative study of the GLC method was initiated and completed.

Collaborative Study

Each of 11 collaborators received 7 samples, the chromatographic packing material, and a small amount of coumarin for the analysis. One sample was a known practice sample containing 5.0 mg coumarin/L in ethanol-water (10+90), and the other 6 samples were commercial wines containing the following amounts (mg/L) of added coumarin: Sample 1, 8.0; Sample 2, 4.0; Sample 3, 4.0; Sample 4, 8.0; Sample 5, 4.0; Sample 6, 0.0.

This report of the Associate Referee, R. H. Dyer, was presented at the 89th Annual Meeting of the AOAC, Oct. 13–16, 1975, at Washington, DC.
Received July 21, 1975.

METHOD

Coumarin (1,2-Benzopyrone)

Gas Chromatographic Method
Official First Action

11.B03 ***Reagents and Apparatus***

(a) *Coumarin std soln.*—0.5 mg/ml alcohol. Accurately weigh 100 mg coumarin (Eastman Kodak No. 79, or equiv.), dissolve in alcohol in 200 ml vol. flask, and dil. to vol.

(b) *Gas chromatograph.*—With flame ionization detector and 6′ (1.8 m) × 2 mm id *glass* column packed with 10% SP-1000 (Carbowax 20 M-TPA) on 100–120 mesh Chromosorb W AW (No. 01-1962, Supelco, Inc., Bellefonte, PA 16823). Operating conditions: He (use gas purifier filter) carrier gas flow rate—30 ml/min; temps (°)—column 180, injector and detector 200. Adjust GLC operating parameters to obtain optimum sepn and efficiency, with coumarin retention time of ca 15 min.

11.B04 ***Determination***

Add 100 ml sample to each of two 250 ml separators. Add 1.0 ml coumarin std soln to one (spiked sample) and 1.0 ml alcohol to other (unspiked sample). Mix thoroly and ext with 20 ml $CHCl_3$, shaking vigorously 2 min. Let layers sep. and drain ca 10–12 ml $CHCl_3$ layer into centrf. tube. Swirl stirring rod in tube to aid in breaking emulsion and freeing $CHCl_3$. Centrf., if necessary, to clarify. Inject 5 μl clear $CHCl_3$ ext onto GLC column. Average 3 values for calcn.

$$\text{mg Coumarin/L} = [U/(S - U)] \times 0.5 \times (1000/100),$$

where U = peak ht of unspiked sample, S = peak ht of spiked sample, and 0.5 = mg coumarin added to 100 ml spiked sample.

Results and Discussion

The individual results of the collaborative study are given in Table 1. The statistical analysis is presented in Table 2. Since the 4.0 and 8.0 mg/L levels had triplicate and duplicate recovery values, respectively, the standard deviations among collaborators are based on the 3 and 2 recovery values, respectively. A 2-factor

Table 1. Individual collaborative results for the GLC determination of coumarin in wine (mg/L)

Coll.	Practice (5.0)	Spl. 2 (4.0)	Spl. 3 (4.0)	Spl. 5 (4.0)	Spl. 1 (8.0)	Spl. 4 (8.0)	Spl. 6 (0.0)
1	4.6	3.9	3.8	3.9	7.9	7.0	<0.2
2	4.5	4.1	3.4	3.8	12.4[a]	14.2[a]	0.0
3	—	3.6	3.8	4.1	8.9	8.5	0.0
4	5.7	4.2	4.7	3.8	8.6	8.6	0.0
5	5.3	3.9	4.1	3.6	9.5	7.9	<0.1
6	5.1	4.4	3.3	4.7	7.8	9.2	0.0
7	5.1	4.0	4.3	3.9	10.8	11.7	neg.
8	5.8	3.7	3.9	3.6	10.0	9.3	<0.4
9	5.5	3.5	3.8	3.9	5.7	8.0	0.0
10	—	4.0	5.7	4.8	6.1	9.2	0.0
11	4.8	4.1	3.9	3.8	7.5	7.3	<0.2

[a] Outlier; not used in statistical calculations.

analysis of variance (collaborators and spiked coumarin levels) was made to compare the collaborator means and the coumarin spiked level means. Although there was no statistically significant difference ($P > 0.05$) in the accuracy (per cent recovery) of the method between the 4.0 and 8.0 levels, the accuracy for the 4.0 level was much better than that for the 8.0 level, 100.0 vs. 105.9%. No significant differences ($P > 0.05$) were found among collaborator means. However, a significant ($P < 0.01$) interaction was found between collaborators and levels.

Separate one-way analyses of the variance were performed on the per cent recoveries for both the 4.0 and 8.0 levels in order to obtain estimates of precision and accuracy of the method at each level. From these analyses no significant differences ($P > 0.10$) were found among collaborator means at the 4.0 level, while significant differences ($P < 0.01$) were found among collaborator means at the 8.0 level. The 8.0 level had a larger within-sample variability (repeatability) than that for the 4.0 level. The variability among collaborators (reproducibility) for the 8.0 level was more than double that for the 4.0 level.

Table 2. Statistical analysis of collaborative results for GLC determination of coumarin in wine

Coll.	Av. rec., % (4.0 mg/L)	Av. rec., % (8.0 mg/L)
1	96.7	93.2
2	94.2	166.2
3	95.8	108.7
4	105.8	107.5
5	96.7	108.8
6	103.3	106.2
7	101.7	140.6
8	93.3	120.6
9	93.3	85.6
10	120.8	95.6
11	98.3	92.5
Statistics for Per Cent Recoveries		
Mean (accuracy)	100.0	105.9
Reproducibility[a]	11.6211	18.4266
Coeff. of var.	11.6	17.4
Repeatability[b]	10.2710	13.0257
Coeff. of var.	10.3	12.3
Statistics for Actual Values		
Mean	4.0	8.5
Reproducibility[a]	0.4648	1.4763
Repeatability[b]	0.4108	1.0424

[a] Standard deviation among collaborators.
[b] Standard deviation within samples.

According to the Youden ranking test (3), all the scores of the collaborators were within the allowable limits of 11–49 as shown in Table 3. In general, the results are quite satisfactory, especially considering the low levels of coumarin present and the simplicity of the method.

During the course of this collaborative study, several important suggestions and modifications were made and were subsequently incorporated in the method. One collaborator suggested that a 5 μl rather than a 10 μl sample injection

Table 3. Ranked results for coumarin collaborative study

	Sample					
Coll.	1	2	3	4	5	Score
1	7	7.5	8	11	5	38.5
2	1	3.5	10	1	8	23.5
3	5	10	8	7	3	33
4	6	2	2	6	8	24
5	4	7.5	4	9	10.5	35
6	8	1	11	4.5	2	26.5
7	2	5.5	3	2	5	17.5
8	3	9	5.5	3	10.5	31
9	11	11	8	8	5	43
10	10	5.5	1	4.5	1	22
11	9	3.5	5.5	10	8	36

should be used. In addition, a gas-purified filter should be placed in line with the carrier gas, since water and oxygen are detrimental to the column packing material. Two collaborators and the Associate Referee found that a stainless steel column should not be substituted for glass because the response for the coumarin decreases and the solvent response increases after only a few injections. It is important to use only glass columns for this determination.

The method is straightforward and requires only a gas chromatograph and basic laboratory supplies; also, the analysis time per sample is relatively short. Although some collaborators reported minor difficulties, most collaborators performed the analyses readily and commented favorably on the method.

Recommendation

The results of this collaborative study indicate that the GLC method is precise and accurate, and it is recommended that the GLC analysis of coumarin in wine be adopted as official first action.

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The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee D and was adopted by the Association. *See* (1976) *JAOAC* **59**, 388.

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FOOD ADDITIVES

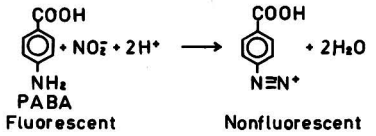
Nitrite in Meat Products Determined by Fluorescence Quenching of *p*-Aminobenzoate Ion

ELIA D. COPPOLA, ALPHONSE F. WICKROSKI, and J. GORDON HANNA
The Connecticut Agricultural Experiment Station, 123 Huntington St, New Haven, CT 06504

A direct fluorometric method is presented for determining nitrites in meat products by means of quenching. The extracted sodium nitrite is consumed in a diazotization reaction with a measured excess of the fluorescent reagent *p*-aminobenzoic acid (PABA) in boiling water and at pH $\leq$1.4. The amount of decrease in fluorescence (quenching) of PABA, in alkaline medium (pH 11.0) with excitation at 265 nm and fluorescence at 339 nm, is directly related to the amount of nitrite present. The slope for the standard curve is 1% decrease in relative fluorescence intensity per 2.76 ppm $NaNO_2$. Results obtained for a series of 20 meat samples analyzed by this fluorometric method are in good agreement with results obtained by a colorimetric method with an average difference of $\pm$6.1 ppm $NaNO_2$.

The use of nitrite as an antibacterial preservative in cured meats is widespread, despite the growing concern over the possibility that nitrite may react with secondary amines and form carcinogenic nitrosamines. We recently reported a fluorometric determination of nitrite in cured meats, involving the use of a controlled excess of sulfanilic acid to react with sodium nitrite, followed by the determination of the remaining sulfanilic acid with fluorescamine (1). The method has the advantage of being free of interferences resulting from cloudy or colored solutions, but it is time-consuming and fluorescamine is a relatively expensive reagent.

The present method uses the quenching of the fluorescent reagent *p*-aminobenzoic acid (PABA) by the nitrite ion as a simple analytical tool to determine nitrite in meat products. In boiling water and at pH $\leq$1.4, nitrites react quantitatively with the amino group of PABA and cause a decrease in the fluorescence (quenching) of the PABA anion. Duggan *et al.* (2) reported that aromatic amines probably lose fluorescence (quenching) because a group capable of donating electrons, such as the amino group, is replaced by an electron-withdrawing group. The diazotization reaction between PABA and the nitrite ion comprises such a substitution because the amino group of PABA is replaced by the electrophilic diazonium ion, as shown:



The decrease in fluorescence is directly related to the amount of nitrite present. The remaining fluorescence is measured in alkaline medium (pH 11.0) with excitation at 265 nm and fluorescence at 339 nm. The meat extract *per se* does not affect the fluorescence of the PABA anion. The proposed method is simpler and more rapid than our previous fluorometric method and offers a useful alternative to the colorimetric method, especially in the analysis of cloudy or colored meat extracts.

METHOD

Apparatus and Reagents

(a) *Fluorescence spectrophotometer.* — Perkin-Elmer Model 203, with excitation wavelength at 265 nm and fluorescence wavelength at 339 nm. Alternatively, a proper filter fluorometer equipped with 265 and 339 nm filters could be used. Because spectra are uncorrected for wavelength dependency of lamp intensity, excitation and fluorescence maxima should be checked with particular spectrofluorometer used.

(b) *pH Meter.*—Model 801, digital (Orion Research, Inc., Cambridge, MA 02139), or equivalent, equipped with combination glass electrode (Beckman No. 39099-E3, or equivalent) and saturated calomel electrode.

(c) *p-Aminobenzoic acid standard solution.*—0.01000*M*. Dissolve 0.1371 g PABA in 100 ml water. Dilute 50-fold to $2.000 \times 10^{-4}M$ PABA.

(d) *Standard sodium nitrite solution.*—0.1000*M*. Dissolve 0.6901 g $NaNO_2$ in 100 ml water. Dilute

100-fold to $1.000 \times 10^{-3}M$ $NaNO_2$. Prepare fresh each week.

(e) *Borate buffer.*—0.20*M*. Adjust 0.20*M* boric acid solution to pH 11.0 with 1.0*M* NaOH and dilute to 1 L.

Preparation of Sample

Weigh 10.0 g finely comminuted and fully mixed meat sample into 250 ml beaker and add ca 60 ml water. Place beaker on steam bath 45 min, stirring occasionally with glass rod. Transfer contents with help of boiling water to 200 ml Kohlrausch volumetric flask and bring water level to slightly below mark. Cool in ice. Add boiling water until all fat is above mark. Pipet additional 4 ml water to compensate for meat volume. Mix, and filter through Whatman No. 40 paper. Use filtrate for $NaNO_2$ determination.

Determination

Pipet 4 ml $2.000 \times 10^{-4}M$ PABA and 2.0 ml 2.4*M* HCl solutions into 100 ml volumetric flask. Add 4 ml meat filtrate and wash flask sides with ca 10 ml water. Place in boiling water 20 min. Cool, add 10 ml borate buffer and 5.569 mequiv. NaOH (25 ml 0.2227*N* NaOH), and dilute to 100 ml. Final pH should be 10.8–11.0.

Fluorometric Procedure—Preparation of Standard Curve

Pipet 4 ml $2.000 \times 10^{-4}M$ PABA and 2.0 ml 2.4*M* HCl into 100 ml volumetric flask. Place in boiling water 20 min. Cool, add 10 ml borate buffer and 5.569 mequiv. NaOH (25 ml 0.2227*N* NaOH), and dilute to 100 ml. Use this $8.000 \times 10^{-6}M$ PABA solution as highest standard.

Pipet 2.0, 4.0, 6.0, and 8.0 ml $8.0 \times 10^{-6}M$ PABA into four 10 ml volumetric flasks. Dilute to volume with water. Prepare blank by adding 2 ml 2.4*M* HCl, 10 ml borate buffer, and 25 ml 0.2227*M* NaOH to 100 ml volumetric flask. Dilute to volume with water. Measure standards within 30 min on fluorescence spectrophotometer at 265 nm excitation wavelength, 339 nm fluorescence wavelength with sensitivity control on 9, selector X-1. Measure relative fluorescence intensity by adjusting highest standard ($8.000 \times 10^{-6}M$ PABA) to 100% and blank to 0%. Plot values of remaining standards. Fluorescence intensity is directly proportional to PABA concentration with practical slope of 1% relative fluorescence per 0.0800 nanomole PABA. Prepare fresh standard each month. After initial standard curve is prepared, omit standard dilutions for successive determinations. *Caution:* Solutions are subject to heating from light source. Place shutter in off position when not reading. Read relative fluorescence intensity values of each sample quickly.

Determination of Sodium Nitrite

Read values of relative fluorescence of meat filtrate and compare with prepared standard curve. Amount of $NaNO_2$ present is inversely proportional to amount of PABA present with slope for standard curve of 1% relative fluorescence per 2.76 ppm $NaNO_2$. Amount of $NaNO_2$ present is obtained by subtracting concentration of PABA found from $8.000 \times 10^{-6}M$ PABA (highest standard) and by using following equation:

$$\text{ppm } NaNO_2 = \text{difference in concentration} \times 69{,}000 \times \text{dilution factor}$$

For instance, if meat sample, prepared as above, gives 80% relative fluorescence reading corresponding to $6.40 \times 10^{-6}M$ PABA or $1.60 \times 10^{-6}M$ $NaNO_2$ with dilution factor of (200 ml/10 g) $\times$ (100/4) = 500, then amount of $NaNO_2$ in ppm originally present in 10 g meat sample is given by $1.60 \times 10^{-6} \times 69{,}000 \times 500 = 55.2$ ppm $NaNO_2$. Alternatively, since decrease in relative fluorescence is 20% and slope is 1%, relative fluorescence per 2.76 ppm = $20 \times 2.76 = 55.2$ ppm $NaNO_2$.

Results and Discussion

Spectral Characteristics

The excitation spectrum (curve A) and the fluorescence spectrum (curve B) for an $8.000 \times 10^{-6}M$ PABA solution are shown in Fig. 1. The excitation spectrum has a maximum at 265 nm and a secondary peak at 299 nm. The fluorescence maximum with the excitation wavelength at 265 nm is at 339 nm. These uncorrected values

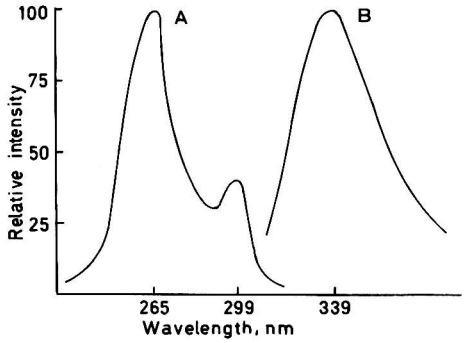


FIG. 1—Excitation (curve A) and fluorescence (curve B) spectra for $8.000 \times 10^{-6}M$ PABA, pH 11.0; sensitivity control 9, selector X-1.

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obtained with a mercury lamp spectrofluorometer are in general agreement with values of 295 nm excitation, 345 nm fluorescence obtained by Duggan *et al.* (2), using a xenon arc lamp. Corrected spectra for the PABA anion were obtained in one run using a Model MPF-4 fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT). The corrected excitation spectrum has a maximum at 278 nm, while the corrected fluorescence spectrum peaks at 330 nm.

pH Dependence

Previous work (2) has shown that the optimum fluorescence maximum for PABA is around pH 11.0. The present work indicates that maximum relative fluorescence is obtained in the pH range 8.5–11.2 where the curve reaches a plateau (98.0±3.0%), as shown in Fig. 2. The fluorescence intensity decreases rapidly for pH values between 8.5 and 3.0 and it is zero below pH 3.0.

For pH values >11.2, the fluorescence intensity also decreases, although less rapidly, and it is 50% of maximum at pH 13.0. The maximum fluorescence intensity in the pH range 8.5–11.2 seems to suggest that the dissociation of PABA to *p*-aminobenzoate anion increases the resonance energy, and thus enhances the fluorescence intensity. On the other hand, the low relative fluorescence for pH values ≤4.50 suggests that the cationic form of PABA decreases the resonance energy, and thus decreases fluorescence.

Determination of Sodium Nitrite

Preliminary work was aimed at achieving quantitative recovery of known amounts of $NaNO_2$ in water. The diazotization reaction between a fixed controlled excess of PABA and $NaNO_2$ is temperature- and time-dependent. In preliminary trials, we used 35 min heating on a steam bath (ca 80°C) because these conditions previously (2) secured a quantitative reaction between sulfanilic acid and a minimum of 5 nanomoles $NaNO_2$. However, with the system PABA-$NaNO_2$ and the above conditions, we obtained only 75–80% recovery of known amounts of nitrite. To quantitatively recover $NaNO_2$, it was necessary to elevate the temperature to 100°C. A recovery of 97.3% (standard deviation ±2.88%) was obtained for 16 known samples of $NaNO_2$ ranging from 1.00×10^{-6} to $1.0 \times 10^{-5}M$ $NaNO_2$ (4–120 nanomoles), when

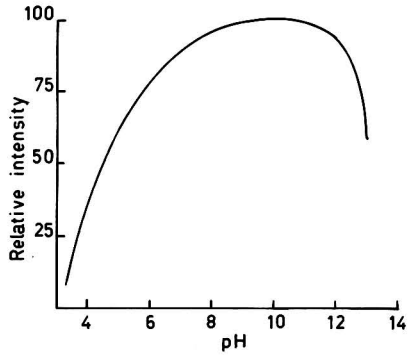


FIG. 2—Plot of relative fluorescence intensity vs. pH for $8.000 \times 10^{-6}M$ PABA; 265 nm excitation wavelength, 339 nm fluorescence wavelength; sensitivity control 9, selector X-1.

the reaction was allowed to proceed for 20 min at 100°C. The lowest amount of 4 nanomoles (4.000 ml $1.00 \times 10^{-6}M$ $NaNO_2$) corresponds to an initial meat sample with 1.38 ppm $NaNO_2$. For samples containing more than 1.38 ppm $NaNO_2$, the time needed for quantitative nitrite recovery is much less. Only 10 min heating time is needed for a 27.6 ppm nitrite sample. Optimum conditions of 20 min heating time in boiling water were used for the rest of the present study.

To assess the validity of our method, a number of cured meat samples were analyzed for sodium nitrite content and the results were compared with the results for the same samples analyzed routinely in our laboratory by the Follet-Ratcliff (3) colorimetric method. For a series of 20 samples, the average deviation between the values obtained by the colorimetric method and those obtained by the fluorometric method was ±6.1 ppm, as shown in Table 1. However, the difference between the 2 methods was unusually high (more than 2.5 the average deviation of 18 remaining values) for only 2 samples (liverwurst 16.8 ppm and frankfurter 14.7 ppm). If we disregard the above 2 values, then the average deviation between the 2 sets of remaining values is ±5.00 ppm, which is slightly better than the average deviation (±5.1 ppm) of our previous method (2).

The present method was initially tested for recoveries of known amounts of sodium nitrite added to cured meat products, all listing sodium nitrite as an ingredient. For 6 determinations,

Table 1. Comparison of sodium nitrite determinations (ppm) in meat products by colorimetric (C) and fluorometric (F) methods

Product	C	F	Diff. (F − C)
Frankfurter	44.0	47.6	+3.60
Abbruzzese sausage	8.0	13.9	+5.90
Kielbasa	50.0	44.9	−5.10
Frankfurter	74.8	74.8	0
Hungarian sausage	28.0	19.7	−8.30
Liverwurst	20.0	3.2	−16.8
Frankfurter	62.0	51.7	−10.3
Frankfurter	70.0	70.8	+0.80
Luncheon sausage	36.0	27.1	−8.90
Kielbasa	64.0	63.5	−0.50
Frankfurter	54.0	39.3	−14.7
Genoa salami	28.0	22.8	−5.20
Frankfurter	58.0	69.3	+11.3
Kielbasa	60.0	64.2	+4.20
Frankfurter	20.0	16.5	−3.50
Luncheon sausage	18.0	27.5	+9.50
Frankfurter	40.0	34.5	−5.50
Frankfurter	0	0	0
Salami	6.0	6.9	+0.90
Frankfurter	42.0	35.7	−6.30
Average			±6.1

the average recovery of 69–200 ppm added sodium nitrite was only 84.7% with a standard deviation of ±5.45%. On the other hand, for 9 determinations, the average recovery of sodium nitrite (range 69–200 ppm) added to meat filtrates was only slightly better, 85.7±5.70%. These low recoveries seem to indicate a reaction between the nitrite added and some meat components with a net loss of free nitrite in the system. The nitrite loss is confirmed by the findings of Fox and Nicholas (4), who reported a substantial loss of nitrite in heated meat slurries. The loss is attributed to the reaction of nitrite with components normally present in cured meats, such as the reductants ascorbate and cysteine and the amino acid histidine.

All samples of cured meats analyzed contained <100 ppm sodium nitrite, which is well below the maximum amount (156 ppm) permitted by Federal regulation. Therefore, for the present study, a standard curve of concentrations ranging from 0 to $8.000 \times 10^{-6}M$ PABA was used because it is equivalent to the pertinent range of 0–276 ppm sodium nitrite. The reagent PABA is a highly fluorescent material and yields a linear standard curve with a minimum of $4.000 \times 10^{-7}M$ PABA. Linearity is observed for concentrations up to $1.000 \times 10^{-5}M$. Above this, self-quenching is responsible for deviations from linearity.

The relative intensity of the PABA fluorescence, as of other fluorescent species (5), is affected by temperature changes, and a decrease is observed because of heat from the light source. We observed a decrease of approximately 30% relative fluorescence intensity when the temperature in the sample cell increased 4°C above room temperature. Therefore, it is very important to avoid unnecessary exposure of the sample to the light source. When cooled to room temperature, the sample will return to the original relative fluorescence intensity reading.

The proposed method is faster and simpler than our previous fluorimetric method and is free of interference resulting from cloudy or colored solutions. Therefore, it offers a valuable alternative to any colorimetric method.

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STATISTICAL TECHNIQUES

Analytical Variation When Proportions of Sampled Units Contain the Active Agent

WILLIAM F. KWOLEK and EIVIND B. LILLEHOJ*
North Central Region, Agricultural Research Service, U.S. Department of Agriculture, Peoria, IL 61604
** Northern Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Peoria, IL 61604*

When a product consisting of discrete units is sampled, the number of units to be selected depends on p, the proportion of units containing an active agent. If the sample is ground, the problem of subsample size is complicated when only a proportion, g, of the subparticles generated from each unit containing the active agent is active. Procedures have been devised for determining sample size, subsample size, and fineness of grind for wide ranges of p and g. The expected precision of the analytical result is influenced by both sampling variation and analytical variation.

When a sample of units (particles, seeds, organs, grains, specimens) is taken, it is often assumed that all units are similar. The sample may be ground and a suitable subsample may be taken for chemical analysis. If the component of interest does not appear in every unit but only in a proportion, p, the sample size may be increased to ensure some positive units in the sample. The aim of a grinding step with subsequent subsampling is to increase the sampling rate and to provide uniformity in the smaller subsample used for the actual analysis. A uniform mixture of subunits ensures that each subunit has an equal chance of being selected for assay. However, it may be that only a certain expected proportion, g, of each positive unit contains the component of interest. Thus when the unit is ground, all subparticles may not be positive but only a certain proportion, pg. Before grinding, a proportion p of the units are positive for the component. After grinding, a proportion pg of the subunits is positive.

The following questions are important when sampling units over a wide range of p and g values:

(*1*) What are the chances that the unit sampled actually contains the material to be assayed?

(*2*) How do changes in particle size after grinding influence the chances that subsamples contain the active agent?

(*3*) How do sample size, grinding, and analytical variation influence precision of the assay?

Background information on sampling is contained in the statistical literature. A recent reference with chemical application in sampling solids is the paper by W. E. Harris and B. Kratochvil (1974) *Anal. Chem.* **46**, 313–315.

Number of Units per Sample

Sampling is common practice when an analyst is faced with a large lot or batch composed of small particles or units, for example, seeds. When a sample is taken, it is assumed that all units have an equal chance of being selected. We may want to estimate the proportion of units possessing a property, the amount of a component per lot, or both. Usually the sample is ground, and a subsample is taken for analysis. When the component to be analyzed does not appear in every unit but only in a proportion, p, sample size has to be set so as to ensure positive units in the sample. As the proportion of positive units, p, decreases, sample size has to be increased to maintain a high probability, T, of including positive units.

The relation was plotted (Fig. 1) between the number of units sampled, n, the true proportion of positives, p, in the lot being sampled, and the percentage, T, of samples that would contain at least one positive. The relation shown in Fig. 1 is:

$$T = 1 - (1 - p)^n \qquad (1)$$

For the Poisson approximation to the binomial set:

$$(1 - p)^n = e^{-np} \qquad (2)$$

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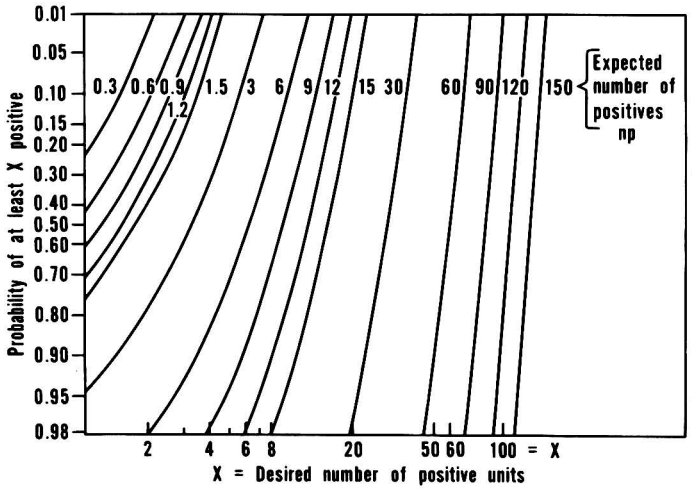


FIG. 1—Relation of sample size *n* to proportion of positives *p* and probability of at least one positive unit out of *n*.

The left-hand side of Equation 2 is the binomial probability of zero positives. The sample size selected is a compromise for values of p, n, and T. For example, n is determined in part by the resources available for handling n units. A value for T is probably most easily fixed. Arbitrarily, we might say that we desire at least one particle in 99% of the samples. Then

$$0.01 = (1 - p)^n \qquad (3)$$

or

$$n = -2/\log\ (1 - p) \cong 4.605/p \qquad (4)$$

and for $p < 0.2$, $\log\ (1 - p) \cong -0.434\ p$.

For a 99% chance of including at least one positive, the expected number of positive particles in the sample is np, or 4.6. To have a high probability (>0.95) of at least one positive unit in a sample, at least $3/p$ units must be taken (Fig. 1).

A more exact statement of the required sample size based on binomial distribution is obtainable from Fig. 2. The abscissa shows the desired number of positive units in a sample of size n. The value for each np contour is given, where p is proportion positive and n is sample size. The ordinate shows the probability of at least X positive units. For example, when $np = 3$, there is a 95% chance of finding at least one positive unit. The chance of having at least 7 positives is less than 0.05. If a 95% chance of at least 10 positives in the sample is desired, np must be about 15. If p were assumed to be 0.01, a sample of 1500 units would be necessary. Note that the np contour of 4.6 would pass through the 0.99 probability for $X = 1$, and for $np = 6.91$ through the 0.999 probability for $X = 1$.

The Poisson and binomial distributions, on which Figs. 1 and 2 are based, are appropriate for sampling from lots where p positive units are randomly distributed. Specification of the underlying distribution may cause considerable disagreement seldom based on adequate information. For units subject to several transfers involving combines, trucks, bins, and elevators, the units are probably well mixed.

Sample Size After Grinding

The grinding or size reduction step, followed by mixing and subsequent subsampling, presumably increases the expected number of positive subunits and ensures that all subunits have an equal chance of being selected. However, the true situation may be that only a certain proportion of each unit contains the active component. When an active unit is ground, all subparticles may not be positive, but only a certain proportion g originating from each positive unit. Thus p is the proportion of intact kernels, for example, containing some active agent, while g is the proportion of positive subparticles after grinding a single positive unit. Various situations

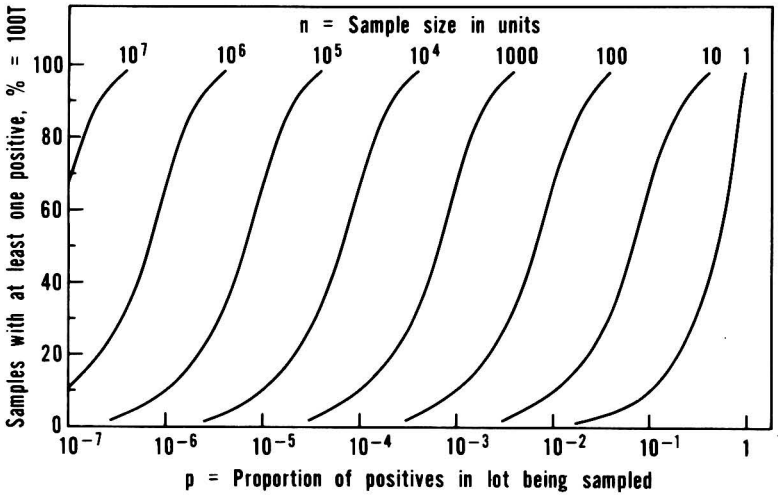


FIG. 2—Probability of X positive units per sample as related to expected number of positives.

arise with respect to p and g. For a method that has been developed for solutions in an analytical laboratory, it is assumed that both p and g equal 1. However, in the analysis of grain samples for protein content, $p = 1$; $g < 0.5$. In the analysis of a sample of seeds for a mycotoxin, $p \ll 0.5$; $g < 0.5$. If p and g are underestimated at the outset, it is likely that the precision of the method will be questioned after it has been put in practice.

As the detection limit of an assay is approached, the problem of variation introduced by the sampling technique dominates. As concentration declines, probably p, g, the concentration per positive unit, or any combination of these is changing.

The problem arising after grinding a sample is illustrated with $np = 5$. When $p = 5 \times 10^{-4}$, the required number of units is $n = 10^4$. The value 5 was selected as providing a 0.99 chance for at least one positive in the primary sample.

If each unit is ground into $m = 100$ subunits, 500 of the total 10^6 subunits would be positive, assuming $g = 1$ and that each positive unit yields 100 positive subunits.

If a subsample of the ground material is taken for analysis and if the sample required is the equivalent of r units (for example, for a 50 g sample of ground corn, $r =$ ca 150, i.e., 0.3 g per kernel), then the subsample equals rm subunits.

If $r = 100$ units is selected, the number of subunits would be $rm = 10^4$ and if $p = 5 \times 10^{-4}$ and $g = 1$, then

$$rmp = 10^4 \times (5 \times 10^{-4}) = 5 \qquad (5)$$

positive subunits would be expected in the analytical subsample. This equation implies that when $g = 1$ there is the same probability of having at least one positive subunit as long as rm, the number of subunits in the analytical sample, is equal to n.

If g is only 0.1, that is, the active agent is contained in only one-tenth of the subunits of each positive unit, then only 10 positive subunits are produced from each positive unit ($m = 100$). The probability of picking a positive subunit is no longer p but instead pg. Thus with $rm = 10^4$, we can no longer expect 5 positive subunits but rather

$$rmpg = (10^4)(5 \times 10^{-4})(0.1) = 0.5 \qquad (6)$$

positive subunits. With $rmpg = 0.5$, the probability of at least one positive subunit is less than 0.01. Now p and g may be considered constant for any sampling of units. Apparently, the only way to increase the expected number of positives in the subsample is to either increase r and take a larger subsample or increase rm by grinding to a smaller subunit size.

The relationships between rm, p, g, n, and the probability of at least one positive subparticle

are of great importance in determining both sample and analytical subsample sizes. There is no guarantee that the number of positives in the primary sample is actually np. Instead, the number of positives follows a binomial distribution with mean np and X equal to the actual number of positive units in a sample of size n.

At the primary sampling stage when n units are taken, the probability of X positive whole units where p is the actual proportion of positive units is assumed to follow the binomial distribution.

$$P(X) = \binom{n}{X} p^X q^{n-X} \quad (7)$$

$$p + q = 1 \quad (8)$$

Assuming the primary sample of n units is then ground and a secondary sample of rm subparticles is taken, the probability of Y positive subunits given X positive units in the primary sample is

$$S(Y|X) = \binom{mr}{Y} (gX/n)^Y (1 - gX/n)^{rm-Y} \quad (9)$$

where g is the proportion of positive subparticles per positive unit. Each unit is ground into m subparticles, and a sample of rm is taken. To determine the probability of at least one positive subparticle, we need to sum $P(X) \cdot S(Y|X)$ for $X \geq 1$, $Y \geq 1$.

The probability of a sample containing at least one positive subparticle is

$$G(Y>0) = \sum_{X=1}^{n} \binom{n}{X} p^X q^{n-X} \sum_{Y=1}^{mr} \binom{mr}{Y} (gX/n)^Y (1 - gX/n)^{mr-Y} \quad (10)$$

$$= \sum_{X=1}^{n} \binom{n}{X} p^X q^{n-X} (1 - (1 - gX/n)^{mr}) \quad (11)$$

$$= 1 - \sum_{X=0}^{n} \binom{n}{X} p^X q^{n-X} (1 - gX/n)^{rm} \quad (12)$$

The value $(1 - gX/n)^{rm} \cong e^{-gXmr/n}$ and, when gX/n is less than 0.1, the discrepancy between these terms is less than 0.005. For more critical ranges in the calculation, $X/n \ll 0.1$. Substituting $e^{-gXmr/n}$ in Equation 12 we find:

$$G(Y>0) = 1 - (1 - p(1 - e^{-mrg/n}))^n \quad (13)$$

We may fix $G(Y>0)$ at some value T and solve for the value of rm required to satisfy T given g, p, and n. Then

$$rm = -2.303n/g \log (1 - (1 - (1 - T)^{1/n})/p) \quad (14)$$

Values for rm were calculated for selected values of p, g, n, and T based on this equation (Table 1). In Table 1, n is the number of units taken in the primary sample, while rm is the number of particles in the subsample, assuming m particles are generated per primary unit:

(1) As n increases, values for rm converge to

$$rm = \log (1 - T)/\log (1 - pg) \quad (15)$$

Table 1. Number of subparticles (rm) required to attain T^a given p, g, and n

		Number of units, n							
p	g	10	50	100	500	1000	5000	10^4	10^5
					$T = 0.95$				
0.8	1	3	3	3	3	3	3	3	3
0.5	1	7	6	6	6	6	5	5	5
0.1	1	—	43	34	30	30	30	29	29
0.01	1	[b]	—	—	454	355	308	304	300
0.001	1				—	—	4,568	3,560	3,041
0.0001	1						—	—	35,605
0.8	0.5	7	7	7	7	7	7	7	7
0.5	0.5	14	12	12	12	12	11	11	11
0.1	0.5	—	87	69	61	60	60	59	59
0.01	0.5		—	—	909	710	617	608	600
0.001	0.5				—	—	9,137	7,120	6,082
0.0001	0.5						—	—	71,211
0.8	0.1	39	37	37	37	37	37	37	37
0.5	0.1	72	61	60	60	60	59	59	59
0.1	0.1	—	435	349	307	303	300	299	299
0.01	0.1		—	—	4,548	3,554	3,088	3,041	3,000

(Continued)

Table 1. (*Continued*)

		Number of units, n							
p	g	10	50	100	500	1000	5000	10^4	10^5
0.001	0.1				—	—	45,685	35,600	30,414
0.0001	0.1							—	356,059
0.8	0.01	390	377	375	374	374	374	374	374
0.5	0.01	729	618	608	600	600	599	599	599
0.1	0.01	—	4,355	3,497	3,079	3,036	3,003	2,999	2,996
0.01	0.01		—	—	45,485	35,542	30,882	30,410	30,001
0.001	0.01				—	—	456,001	356,001	304,147
0.0001	0.01						—	—	3,560,597
					$T = 0.99$				
0.8	1	6	5	5	5	5	5	5	5
0.5	1	13	9	9	9	9	9	9	9
0.1	1	—	105	59	48	47	46	46	46
0.01	1		—	—	1,243	615	482	471	461
0.001	1				—	—	12,666	6,169	4,714
0.0001	1						—	—	61,713
0.8	0.5	12	11	11	11	11	11	11	11
0.5	0.5	26	19	18	18	18	18	18	18
0.1	0.5	—	211	119	96	94	92	92	92
0.01	0.5		—	—	2,486	1,230	965	942	923
0.001	0.5				—	—	25,333	12,338	9,429
0.0001	0.5						—	—	123,426
0.8	0.1	61	58	57	57	57	57	57	57
0.5	0.1	133	96	94	92	92	92	92	92
0.1	0.1	—	1,059	597	480	470	462	461	460
0.01	0.1		—	—	12,432	6,151	4,828	4,713	4,615
0.001	0.1				—	—	126,669	61,694	47,145
0.0001	0.1						—	—	617,133
0.8	0.01	618	582	579	576	575	575	575	575
0.5	0.01	1,339	967	943	925	923	921	921	921
0.1	0.01	—	10,596	5,979	4,807	4,703	4,624	4,614	4,606
0.01	0.01		—	—	124,328	61,518	48,289	47,134	46,157
0.001	0.01				—	—	1,266,694	616,948	471,451
0.0001	0.01						—	—	6,171,330
					$T = 0.999$				
0.8	1	9	8	8	8	8	8	8	8
0.5	1	60	14	14	13	13	13	13	13
0.1	1	—	—	110	73	71	69	69	69
0.01	1		—	—	—	1,166	742	715	693
0.001	1				—	—	—	11,729	7,157
0.0001	1					—	—	—	117,361
0.8	0.5	19	17	17	17	17	17	17	17
0.5	0.5	120	29	28	27	27	27	27	27
0.1	0.5		—	220	147	142	139	138	138
0.01	0.5			—	—	2,332	1,485	1,431	1,386
0.001	0.5					—	—	23,458	14,315
0.0001	0.5							—	234,722
0.8	0.1	97	87	87	86	86	86	86	86
0.5	0.1	604	149	143	139	138	138	138	138
0.1	0.1		—	1,100	737	713	695	692	690
0.01	0.1			—	—	11,660	7,428	7,155	6,931
0.001	0.1					—	—	117,291	71,576
0.0001	0.1							—	1,173,613
0.8	0.01	976	879	871	864	864	863	863	863
0.5	0.01	6,042	1,492	1,432	1,391	1,386	1,382	1,382	1,381
0.1	0.01		—	11,009	7,378	7,132	6,951	6,929	6,909
0.01	0.01			—	—	116,601	74,284	71,553	69,314
0.001	0.01					—	—	1,172,918	715,768
0.0001	0.01							—	11,736,138

[a] p = Probability of a positive unit in n units; T = probability of at least one positive subparticle; g = probability of a positive subparticle from a positive unit.

[b] Blank space indicates no solution.

(*2*) Increasing n does little to change the number of subunits rm required to maintain the same probability of at least one positive if p, g, and T are constant. For example, if $T = 0.999$, $p = 0.1$, and $g = 0.5$, an n of greater than 500 requires rm between 147 and 138. A primary sample n of 500 units would be sufficient to ensure about 140 positive subparticles.

(*3*) The expected number of positive subparticles in the subsample is $rmpg$.

(*4*) Selection of sample size n and rm must consider values for p, g, T, and $rmpg$.

Changes in rm associated with changes in n and T are not large. For a 10-fold decrease in p or g, rm must increase 10-fold. The relationships when $g = 1$ for various n values and T, the probability of at least one positive, have been shown in Figs. 1 and 2. In Table 1, we observe for a given p, g, and T that little change in rm occurs as n increases. If $n = 1000$ units (Table 1) and $T = 0.999$, then the sample size for all combinations of $p < 0.001$ and $g \leq 1$ is insufficient regardless of how small particles are ground. For these points $(1 - p)^n > 1 - T$ and for rm there is no solution. A high probability of positive particles cannot be attained for some combinations of p, n, and g. Table 1 provides insight into the sampling process and specifies minimum values for rm to ensure subsamples with positive subparticles.

Fineness of Grind

To illustrate the determination of how fine to grind units, an example was constructed that shows what m (the number of subparticles generated per r unit) must be to attain the required rm, assuming $p = 0.01$ and $T = 0.999$. If the analysis calls for a subsample equivalent to $r = 100$ units with $g \leq 0.1$, m has to be larger than 71, i.e., at least 71 subparticles have to be generated per unit. The example below shows the extent of grinding required for various g values.

g	Required rm	m (Number of subparticles) $r = 1000$	$r = 100$	$r = 10$
0.5	1,430	2	14	143
0.1	7,100	7	71	710
0.01	71,000	71	710	7,100
0.001	710,000	710	7,100	71,000

If $g = 1$ and $p = 0.01$ with $n = 1000$ and $T = 0.999$, then the required number of subparticles is $rm = 1166$ (Table 1). If $r = 100$, i.e., if the equivalent of 100 units for analysis is taken, then

$$m = 1166/100 \cong 12 \qquad (16)$$

Table 2 provides a rough measure of the number of particles generated for various U.S. mesh sizes. If each unit were 1 cc, a mesh size of 4 would provide 18 subparticles and would satisfy the requirement $m \cong 12$. If each particle were 0.25 cc, the smaller size primary particle would require reduction of the total weight of the sample by a factor of 4 and a mesh size of 6 would be used, since $50/4 \cong 12$. If the same mesh size of 4 were used with 0.25 cc particles, then r would be increased to 400 to maintain the same probability $T = 0.999$ of at least one positive subparticle.

If $p = 0.01$, $g = 0.01$, $n = 1000$, and $T = 0.999$, from Table 1 $rm \cong 120{,}000$. If $r = 100$, then $m = 1200$. For a 1 cc unit, a mesh size of 20 would be required. If the original unit is 0.25 cc and if the same mesh size is retained, r has to be increased to 400. If r is kept at 100, i.e., for the smaller unit a smaller sample weight is taken for the analysis, then a mesh of 25 would be necessary.

Table 2. Number of particles per 1 cc[a] of original particle size

U.S. mesh size	Diameter, mm	Number
4	4.76	18
6	3.36	50
8	2.38	140
10	2.00	240
12	1.68	400
14	1.41	680
16	1.19	1,130
20	0.84	3,220
25	0.71	5,340
30	0.59	9,300
35	0.50	15,300
40	0.42	25,800
45	0.35	44,500
50	0.297	72,900
60	0.250	122,000
70	0.210	173,000
80	0.177	344,000
100	0.149	577,000
140	0.105	1,650,000
200	0.074	4,710,000
270	0.053	12,830,000
400	0.037	37,700,000

[a] Uniform spherical particles having a total volume of 1 cc for a given mesh size.

Detection Limits

The total expected weight of the component in subparticles being assayed is

$$wrmpgY_p \qquad (17)$$

in a sample of weight wrm, where w is the mean weight of a subparticle and Y_p is the true mean concentration in positive subparticles. To detect the compound during analysis, the following equation must hold:

$$wrmpgY_p = Y_A W_A \qquad (18)$$

where W_A = weight of material designated for analysis and Y_A is the true concentration of the component in the sample. The weight of material W_A is fixed by the assay procedure and equals wrm. Considering Y_A as the limit of the assay, a positive analytical result requires

$$pgY_p \geqq Y_A \qquad (19)$$

The expected number of positive subparticles is $K = rmpg$; combining this expression with $pgY_p = Y_A$, the number of positive subparticles required for the assay to detect the compound is

$$m \geqq (Y_p/Y_A)(K/r) \qquad (20)$$

If the limit of detection of Y_A is 10 ppm when positive subparticles have 10,000 ppm (Y_p) of the component, K is the expected number of positive subparticles set at 6.91 in order that $T = 0.999$, and the analytical procedure requires 150 units for analysis, then

$$m = (10^4/10)(6.91) = 46 \qquad (21)$$

If the limit of the assay (Y_A) declined from 10 to 5, m would decrease from 46 to about 20. Half as many positive subparticles would contain a detectable level $Y_A/2$. Thus a coarser grind would be satisfactory. Another consideration involving g and m concerns maintaining the same expected number of positive units in the primary sample and subparticles as in the subsample. Then

$$np = rmpg \qquad (22)$$

or

$$m = n/rg \qquad (23)$$

If g is much less than 1, to maintain the same expected number of positives n/r must be increased by a factor $1/g$. If n is large, then it may be difficult to increase m, since much additional work associated with finer grinding is involved.

Precision of the Assay

To arrive at an approximate expression for the precision with which Y, an assay result based on rm subparticles, is given, we observe that

$$Y = \hat{pg}\hat{Y}_p \qquad (24)$$

where $\hat{pg}$ is the proportion of positive subparticles in a sample of rm subparticles and $\hat{Y}_p$ is the mean concentration per positive subparticle. Positive subparticles after grinding and mixing are assumed to follow a binomial distribution. An exact expression for the variance (square of the standard deviation of Y) is difficult, since the distributions of both pg and Y_p are debatable. Certainly, this point is in need of further study. Assuming Y_p and pg are uncorrelated, an approximate variance for Y, $V(Y)$ is

$$V(Y) = (pg)^2V(Y_p) + Y_p^2V(pg) \qquad (25)$$

where pg and Y_p are the true proportion of positive subparticles and Y_p is the true concentration per positive subparticle. It is convenient to work in terms of the relative standard deviation ($RSD(Y)$) of Y, i.e., the standard deviation expressed as a per cent of the mean.

$RSD(Y_p)$ is the relative standard deviation of the assay for subsamples containing only positive subparticles. $RSD(Y)$ is the relative standard deviation between samples. $RSD(Y_p)$ and $RSD(Y)$ are equal if $g = p = 1$, since then $V(pg) = 0$ and $V(Y) = (pg)^2V(Y_p)$. Since $V(\hat{pg}) = (1 - pg)pg/rm$,

$$V(Y) = (pgY_p)^2(RSD^2(Y_p) + (1 - pg)/rmpg) \qquad (26)$$

and

$$RSD(Y) = \sqrt{RSD^2(Y_p) + (1 - \mathrm{pg})/rmpg} \qquad (27)$$

When $pg = 1$, the relative standard deviation of the reported concentration $RSD(Y)$ is equal to $RSD(Y_p)$.

The term $V(Y_p)$ is composed of laboratory plus analytical variation.

Although the relative deviation may be a minimum for a well developed analytical procedure, extensive variation in the assay result may be associated with the sampling term

$$(1 - pg)/rmpg \qquad (28)$$

If $RSD(Y_p)$ is small,

$$RSD(Y) \cong \sqrt{(1 - pg)/rmpg} \qquad (29)$$

It would be desirable that $(1 - pg)/rmpg < 0.0025$,

$$RSD(Y) = \sqrt{0.0025} = 0.05 \qquad (30)$$

As pg becomes small and $RSD(Y) = \sqrt{0.0025}$, $rmpg$ would have to be about 400. Attaining an expected number of positive subparticles between 1 and 400 should cover most sampling problems from $pg = 1$ to pg approaching zero.

The relative standard deviation of Y for combinations of $rmpg$, the expected number of positive subparticles, and relative standard deviation in Y_p are given in Table 3. As an example, assume that an assay with an $RSD(Y)$ of 0.20 is available. If samples with an $rmpg$ of 1 are assayed, the $RSD(Y)$ would be about 4 times (0.97/0.20) as large when only 10% of the subparticles, i.e., $pg = 0.1$, are positive.

When $RSD(Y_p)$ between assays is high, increasing $rmpg$ has little effect toward improving precision (Table 3). Also as $rmpg$ declines, the assay result becomes less precise. Various combinations of pg and $RSD(Y)$ lead to the same precision depending on the expected number of positives, $rmpg$. The following points are of special importance:

(*1*) As pg declines and with $rmpg$ maintained at the same level, $RSD(Y)$ in the assay becomes constant.

(*2*) The more precise the assay, i.e., the smaller $RSD(Y_p)$, the greater the effect of sample size as reflected by $rmpg$.

(*3*) Increases in $rmpg$ above 50 when $RSD(Y_p)$ is greater than 0.10 have little influence on precision.

Table 3. Predicted relative standard deviations based on assay and sampling variation

	RSD(Y)		
rmpg[a]	0.5	0.1	≤0.01
	$RSD(Y_p) = 0.02$		
0.5	1.00	1.34	1.41
1	0.71	0.95	1.00
5	0.32	0.42	0.45
10	0.22	0.30	0.32
50	0.10	0.14	0.14
100	0.07	0.10	0.10
	$RSD(Y_p) = 0.10$		
0.5	1.00	1.35	1.41
1	0.71	0.95	1.00
5	0.33	0.44	0.46
10	0.24	0.32	0.33
50	0.14	0.17	0.17
100	0.12	0.14	0.14
	$RSD(Y_p) = 0.20$		
0.5	1.02	1.36	1.42
1	0.73	0.97	1.01
5	0.37	0.47	0.49
10	0.30	0.36	0.37
50	0.22	0.24	0.24
100	0.21	0.22	0.22
	$RSD(Y_p) = 0.80$		
0.5	1.28	1.56	1.62
1	1.07	1.24	1.28
5	0.86	0.91	0.92
10	0.83	0.85	0.86
50	0.80	0.81	0.81
100	0.80	0.81	0.81

[a] $rmpg$ = Expected number of positive subparticles; $RSD(Y_p) = RSD(Y)$ when $pg = 1$; $RSD(Y)$ = overall relative standard deviation.

(*4*) Increases in $RSD(Y)$ may reflect decreases in the assumed values for pg and a subsequent decrease in $rmpg$.

DRUGS

Colorimetric Assay of Reserpine in Formulations and Biological Fluids

MOHAMED S. KARAWYA, ABDEL-AZIZ A. SHARAF,* and ALY M. DIAB*
Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt
** Pharmacology Section, Basic Medical Sciences Laboratory, National Research Center, Dokki, Cairo, Egypt*

Two colorimetric methods are presented for determining reserpine. In the first method, an iron hydroxamate complex is formed through the ester group in position 16 in the reserpine molecule. The color is measured at 535 nm (0.5–6 mg/25 ml). This method is useful for routine and control analyses of reserpine formulations. In the second method the tertiary amino group of reserpine reacts with 2% citric acid in acetic anhydride to form a red-violet complex which is measured at 505 nm (5–400 μg/10 ml). This method could be useful in measuring trace amounts of reserpine present in biological fluids.

Many methods have been reported for the assay of reserpine. They include nonaqueous titrimetry (1), ultraviolet spectrophotometry (2), fluorometry (3–5), paper chromatography (6), thin layer chromatography (7–9), ionophoresis (10), and column chromatography (11). Most of the colorimetric methods depend on the formation of an acid-dye adduct (12–14) or reineckate salt (15), or on the reaction with xanthydrol (16), ferric ammonium sulfate in phosphoric or sulfuric acids (17), phenylisocyanate (18), or *cis*-aconitic anhydride (19).

In the AOAC method (20) for determining reserpine, the compound is allowed to react with sodium nitrite and the color produced is measured at 390 nm.

Although the ester function in position 16 in the reserpine molecule is essential for its pharmacological activity (21), no method could be traced in the literature that used this group as a basis for analysis. We sought an assay method in which the pharmacologically active ester group of reserpine is involved in the reaction, and adapted the hydroxamic acid test for esters (22) for this purpose. Moreover, the small amounts of reserpine in formulations required another assay method sensitive to microamounts of reserpine. The citric acid qualitative test for tertiary amines (23) was considered as a promising starting point.

METHOD

Reagents and Apparatus

(a) *Acetate buffer.*—Adjust 0.1*M* sodium acetate solution to pH 1.5 with 70% perchloric acid.

(b) *Citrate buffer.*—pH 3.7. Dissolve 24.6 g citric acid and 21.2 g trisodium citrate dihydrate in 1 L water.

(c) *Citrate buffer.*—pH 4. Dissolve 112 g citric acid and 122 g trisodium citrate dihydrate in 1 L water.

(d) *Citric acid reagent.*—Dissolve 2 g citric acid (analytical reagent grade) in 10 ml absolute anhydrous ethanol and dilute to 100 ml with acetic anhydride. Protect reagent from moisture and light.

(e) *Hydroxylamine-HCl reagent.* — Alkaline, Goddu reagent (22). (*1*) *Methanolic sodium hydroxide.*—12.5% (w/v). Reflux 12.5 g NaOH in absolute methanol for 5 min. (*2*) *Methanolic hydroxylamine-HCl.*—12.5% (w/v). Reflux 12.5 g NH_2OH-HCl in absolute methanol for 5 min. For use, mix equal volumes of solutions 1 and 2 and filter.

(f) *Ferric alum reagent.*—Dissolve 20 g ferric alum ($FeNH_4SO_4.24H_2O$) in 100 ml 70% perchloric acid.

(g) *Reserpine standard solution.*—Dissolve 100 mg accurately weighed reserpine reference material (C. H. Boehringer Sohn, Ingelheim am Rheim, Germany) in 1 ml $CHCl_3$, and dilute to 100 ml with absolute anhydrous ethanol. During experiments, keep this solution and all other reserpine solutions in dark or protect from direct, strong sunlight.

(h) *Colorimeter.* — Spekol, Carl Zeiss, Jena, Germany, or equivalent.

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Estimation of Reserpine in Standards

Carry out entire procedure as quickly as possible without exposing reserpine solutions to direct sunlight.

(a) *Hydroxamic acid method.*—Transfer 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 ml reserpine standard solution to separate 25 ml flasks, dilute each to ca 6 ml with absolute ethanol, and treat similarly. Add 3 ml alkaline hydroxylamine reagent and reflux 5 min at 75°C. Cool to room temperature and gradually add 5 ml ferric alum reagent with continuous stirring. Rapidly wash contents of flask into 25 ml volumetric flask with pH 1.5 acetate buffer and adjust volume to 25 ml with same buffer. Read absorbance at 535 nm against reagent blank.

Calibration curve of 0.5–6 mg reserpine/25 ml vs. color intensity obeys Beer's law with standard deviation of ±0.9%.

(b) *Citric acid method.*—Dilute 5 ml standard reserpine solution to 100 ml with anhydrous ethanol. Transfer 0.1, 0.5, 1.0, 2.0, 3.0, and 4.0 ml dilute reserpine solution to separate 10 ml volumetric flasks and treat similarly. Add 5 ml citric acid reagent, adjust to 10 ml with anhydrous ethanol, and place flask in water bath 15 min at 80–90°C. Remove flask from bath, cool, and measure absorbance of 1 ml solution at 505 nm against reagent blank.

Calibration curve of 5–200 μg reserpine/10 ml vs. color intensity obeys Beer's law with standard deviation of ±1.3%.

Assay of Reserpine in Formulations

(a) *Simple formulations containing no coloring matter.*—For tablets (0.1 or 0.25 mg reserpine/tablet), accurately weigh amount of finely powdered tablets containing 2 mg reserpine and suspend in ca 30 ml 2% aqueous citric acid solution. For ampoules (2.5 mg reserpine/ml), dilute 2 ml injectable solution to ca 30 ml with 2% aqueous citric acid solution. Shake solution 5 min and extract 4 times with 25 ml $CHCl_3$. Add 20 ml 1% aqueous $NaHCO_3$ solution to combined $CHCl_3$ extracts and shake 2 min. Discard aqueous layer and dry $CHCl_3$ extract with anhydrous Na_2SO_4. Evaporate $CHCl_3$ to dryness on ca 50°C water bath and dissolve residue in absolute ethanol. Carry out colorimetric assay as described above.

(b) *Simple formulations containing coloring matter.*—Use this procedure for tablets colored either with indigotin (0.1 or 0.25 mg reserpine/tablet) or with tartrazine (0.1 mg reserpine and 25 mg hydrochlorothiazide/tablet). Accurately weigh amount of finely powdered tablets containing 2 mg reserpine, suspend in 40 ml saturated $NaHCO_3$ aqueous solution, shake well, and extract 3 times with 40 ml $CHCl_3$. Wash combined $CHCl_3$ extracts with 10 ml 2% aqueous citric acid solution. Complete as under simple reserpine formulations, beginning with "Discard aqueous layer . . .".

(c) *Compound reserpine formulations.*—Use this procedure for tablets containing 0.2 mg reserpine, 20 mg rutin, 30 mg ascorbic acid, 4 mg thiamine hydrochloride, and 60 mg phenobarbitone/tablet and tablets colored with yellow-orange, containing 0.15 mg reserpine and 5 mg methylphenidate hydrochloride. Accurately weigh amount of finely powdered tablets containing 2 mg reserpine, suspend in ca 40 ml 2% aqueous citric acid, shake 5 min, and extract twice with 30 ml ether. Discard ethereal extract and re-extract 4 times with 25 ml $CHCl_3$. Add 20 ml saturated $NaHCO_3$ solution and shake 2 min. Discard aqueous layer, wash $CHCl_3$ layer with 5 ml water, discard washings, and complete as mentioned above, beginning with ". . . dry $CHCl_3$ extract . . .".

Application to Biological Fluids

Adjust 10 ml blood or 100 ml urine to pH 4 with pH 4 citrate buffer, add 50 ml $CHCl_3$, and shake well 15 min. Separate and wash $CHCl_3$ with 10 ml pH 3.7 citrate buffer to remove interferences. Dry with anhydrous Na_2SO_4, and filter. Evaporate $CHCl_3$ to dryness on 50°C water bath, dissolve residue in 4 ml anhydrous ethanol, and proceed with citric acid assay.

Results and Discussion

The hydroxamic acid method is the only assay which involves the pharmacologically active (ester) group. The method is also selective and very useful in the routine and control analysis of different reserpine formulations. The hydroxamic acid method, however, is not applicable to blood and urine samples because of relatively small doses of reserpine allowed. The citric acid method, although it depends on the tertiary amino group rather than on the ester group, is applicable to these biological fluids because of its high sensitivity to microamounts of reserpine.

Basic degradation products such as methyl reserpate and acid impurities such as reserpic acid could be removed by shaking the chloroform solution of reserpine with 2% aqueous citric acid and 1% aqueous $NaHCO_3$, respectively (24). Absolute ethanol should be used as a solvent because moisture interferes in the ferric hydroxamate formation by competing with the hydroxamic acid for ferric ion. Other interferences in the hydroxamic acid method are esters, anhydrides, acid chlorides, amides, and lactones, for example, methylphenidate HCl

Table 1. Assay of reserpine in formulations by proposed and AOAC methods

Preparation	Declared, mg/tab.	Found, mg[a] Hydrox. acid	Citric acid	AOAC	Added, mg	Found, mg[a] Hydrox. acid	Citric acid	AOAC	Rec., % Hydrox. acid	Citric acid	AOAC
Simple reserpine tablet	0.25	0.245	0.252	0.255	0.2	0.450	0.454	0.46	102.5	102	102.5
Reserpine ampoule	1/ml	0.97	1.08	1.05	0.1	1.070	1.182	1.15	100	102	100
Colored reserpine tablet	0.25	0.254	0.253	0.255	0.2	0.458	0.450	0.458	102	103	101.5
Colored reserpine tablet	0.125	0.120	0.126	0.126	0.1	0.221	0.226	0.226	101	100	100
Compound reserpine tablet	0.15	0.152	0.153	0.153	0.1	0.252	0.253	0.253	100	100	100
Compound reserpine tablet	0.2	0.200	0.203	0.201	0.2	0.400	0.402	0.402	100	99.5	100.5

[a] Results are averages of 5 experiments.

(Ritalin), thiamine HCl, and phenobarbitone contain an ester group, a tertiary amino group, and a cyclic amide group, respectively. If present, these substances should be removed from the sphere of reaction. Ritalin and thiamine HCl are separated by extraction with 2% citric acid solution, while phenobarbitone is extracted with ether. The coloring matter, viz., indigotin, tartrazine, and yellow-orange which may interfere in the color measurements, is removed by extraction with 2% citric acid and saturated $NaHCO_3$ solutions.

In the citric acid method, the use of normal alkaline glass gave higher results (than the actual amounts present) in comparison with borosilicate neutral glass. Moreover, the sensitivity of the method decreased because of the increased color of the blank when alkaline glass was used.

Other interferences in the citric acid method are salts of potassium, rubidium, cesium, strontium, and barium and salts of alkali and alkaline earth metals with organic acids and quaternary ammonium salts; all these react with citric acid in the same manner. Other possible interferences are Lewis bases such as sodium acetate and sodium phosphate which may be present as impurities in solvents and reagents or as contaminants of glassware. Most of these interferences could be eliminated by using borosilicate glassware, analytically pure reagents and solvents, and distilled water.

Absolute ethanol is also used as a solvent because moisture reduces the sensitivity of the method.

In the citric acid method, the chemistry of the color reaction seems to be complicated and has not been elucidated (23). However, the method shows high sensitivity (Table 1) in comparison with the AOAC method (20). It could be applied to ultramicroamounts such as those present in biological fluids. The efficiency of the method applied to blood and urine was satisfactory. The average concentration of reserpine in the blood of a man who swallowed 2 mg reserpine was 0.034 mg/100 ml after 3 hr, but the average amount of the drug excreted in urine during 6 hr was 0.31 mg (15.5% of the ingested dose).

Table 1 shows that the recoveries from the hydroxamic acid and citric acid methods were quantitative (100.9 and 101.8%, respectively) and the standard deviations are acceptable (± 0.9 and ± 1.3%, respectively). Recoveries from both methods were based on recoveries of standards processed through the entire method, including extraction and evaporation.

The measurable color in both methods remained stable for 25 min after which it faded gradually.

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This convenient laboratory manual covers methods for mycotoxins, marine toxins, and phytotoxins.

Colorimetric Determination of Aconitine in Galenicals and in Biological Samples

MOHAMED S. KARAWYA, ABDEL-AZIZ A. SHARAF*, and ALY M. DIAB*
Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt
** Pharmacology Section, Basic Medical Sciences Laboratory, National Research Center, Dokki, Cairo, Egypt*

A sensitive method is presented for determining aconitine. Aconitine is complexed with Co^{2+}, the aconitine-cobalt complex is extracted with chloroform, and the absorbance is measured at 320 nm. The sensitivity of the method ranged between 0.06 and 3 mg/25 ml, and the color was stable for 6 hr. The method was successfully applied for the quantitative determination of aconitine in animal tissues.

Aconitine in the form of tincture of aconite is used externally for the local relief of neuralgia, sciatica, acute rheumatism, and toothache. The possibility exists of acute poisoning from using such a preparation on an abraded surface because of the danger of absorption (1).

Aconitine gives no specific color reaction, so most of the methods reported for its analysis depend on the determination of its relative toxicity (2–4). Other assay methods are gravimetric (5), aqueous (6, 7) and nonaqueous titrimetric (8), and spectrophotometric (9) techniques. Paper chromatography in combination with ultraviolet spectrophotometry (10) was also reported for the detection of different aconite alkaloids.

We wished to develop a rather sensitive, specific, and simple method for the selective determination of aconitine in the different aconite galenicals. We also wanted to test the applicability and efficiency of the proposed method for the assay of aconitine in biological samples such as blood, urine, and animal tissues.

METHOD

Reagents

(a) *Chloroform saturated with ammonium thiocyanate.* — Distill 1 L $CHCl_3$ over anhydrous Na_2CO_3, shake distillate with excess ammonium thiocyanate to saturate, and filter.

(b) *Cobalt thiocyanate reagent.*—Dissolve 29 mg cobalt nitrate and 45 mg ammonium thiocyanate in 1 L water. Reagent should be freshly prepared.

(c) *Aconitine standard solution.*—Dissolve 2 mg reference aconitine powder (E. Merck, Darmstadt, Germany) in 100 ml 1% HCl.

(d) *Extract, strong tincture, and tincture of aconite.*—Les Laboratoires Givaudan, Lavirotte & Cie, Lyon, France.

(e) *Tincture of aconite suspension.*—Suspend 50 ml strong tincture of aconite (containing 0.2% total alkaloids and 0.14% aconitine) in 150 ml 5% gum acacia in water.

(f) *Citrate-impregnated filter paper.* — Dip Whatman No. 1 paper in 5% sodium dihydrogen citrate solution, blot, and let dry.

Aconitine in Animal Tissues and Urine

Twenty-five male rats (ca 250 g average body weight) were given sublethal doses (4 ml prepared suspension = 1.4 mg aconitine) intragastrically and were divided into 5 subgroups. One hr after administration of the dose, the members of the first subgroup were killed by a sharp blow on the head and immediately dissected; the heart, stomach, and liver were removed for analysis.

The other 4 subgroups were treated similarly after 2, 5, 8, and 10 hr. Members of the fifth subgroup (10 hr) were kept in a metabolism cage prior

Table 1. Assay of aconite galenical preparations by proposed and ultraviolet (UV) spectrophotometric methods (results are averages of 5 replicates)

Preparation	Stated concn, mg/ml	Found, mg/ml		Added, mg/ml	Found, mg/ml		Rec., %	
		Prop.	UV		Prop.	UV	Prop.	UV
Extract of aconite	3.000	3.420	3.500	2.0	5.400	5.480	99.0	99.0
Strong tincture of aconite	1.400	1.440	1.510	1.5	2.925	3.000	99.0	99.3
Tincture of aconite	0.320	0.342	0.343	0.5	0.827	0.841	97.0	99.6

to sacrifice, and their urine was collected and kept for analysis. Twenty-five female rats were treated similarly.

Complexation Reaction

Transfer 10 ml standard aconitine solution (200 μg aconitine) to 50 ml conical flask, heat to 70°C in water bath, add 5 ml cobalt thiocyanate reagent, and let precipitate digest 20 min. Quantitatively transfer contents of flask to separatory funnel, rinsing with 5 ml $CHCl_3$. Extract precipitated aconitine-cobalt complex with successive portions of 10, 5, and 5 ml $CHCl_3$ saturated with ammonium thiocyanate, transferring $CHCl_3$ extracts to 25 ml volumetric flask. Dilute extracts to volume with same solvent and measure absorbance at 320 nm against reagent blank. (Calibration curve of 0.06–3 mg aconitine/25 ml vs. color intensity obeys Beer's law with standard deviation of ±0.50%.)

Determination of Aconitine in Galenicals

Spot 0.1 ml tincture on citrate-impregnated filter paper. Chromatograph 5 hr with *n*-butanol-citric acid-water (870+78+130) developing solvent. Cut out area (R_f 0.62) relative to aconitine standard developed concurrently, add 1 drop ammonia, and extract 4 times with 3 ml $CHCl_3$. Evaporate $CHCl_3$ on water bath, dissolve residue in 2 ml HCl, and proceed with complexation as above, beginning "heat to 70°C. . . ."

Isolation of Aconitine from Biological Samples

Treat each organ (liver, heart, and stomach) immediately after necropsy with 50 ml 1% HCl and blend in homogenizer. Add enough $(NH_4)_2SO_4$ to each homogenized sample and to urine to make saturated solution. Heat mixture to 65°C until proteins coagulate. Filter through Büchner funnel and wash residue with hot water. Cool combined filtrate and washings, make alkaline with NH_4OH, saturate with NaCl, and extract twice with 20 ml ether. Cautiously evaporate ether extracts to dryness and dissolve residue in minimum amount of ethanol. Quantitatively spot solution on citrate-impregnated paper and proceed with determination as above.

For the sake of comparison, the ethanolic solution of recovered aconitine was also measured at 235 nm (10).

Results and Discussion

The proposed method is much more sensitive than other methods reported for aconitine.

The ultraviolet and visible spectra of the chloroform extract of the cobalt complex revealed 2 maxima at 320 and 630 nm; the former

Table 2. Analysis of tissues and urine collected from experimental rats (percentage = (mg found/mg dose) × 100)

	Stomach				Liver				Heart				Urine			
	Male		Female		Male		Female		Male		Female		Male		Female	
Interval,[a] hr	Prop.	UV	Prop.	UV	Prop.	UV	Prop.	UV	Prop.	UV	Prop	UV	Prop.	UV	Prop.	UV
1	7.85	7.50	8.45	6.68	29.30	28.40	28.56	25.20	10.80	10.20	9.98	9.70	—	—	—	—
2	—	—	—	—	22.80	23.10	21.50	20.90	12.90	12.10	9.60	8.60	—	—	—	—
5	—	—	—	—	9.60	8.48	8.44	7.60	6.70	6.20	7.20	7.28	—	—	—	—
8	—	—	—	—	—	3.86	—	2.91	2.20	2.27	—	2.14	22.00	28.80	27.00	25.50
10	—	—	—	—	—	—	—	—	—	—	—	—	28.00	29.50	31.90	30.20

[a] Time between dosing and sacrifice.

Table 3. Testing proposed method for efficiency of recovery from biological samples

Tissue	Found, mg	Added, mg	Found, mg	Rec., %
Stomach[a]				
male	0.10	0.10	0.199	99.0
female	0.12	0.10	0.219	99.0
Liver[a]				
male	0.41	0.40	0.808	99.5
female	0.40	1.00	1.380	98.0
Heart[a]				
male	0.15	0.20	0.348	99.0
female	0.14	0.10	0.237	97.0
Urine[b]				
male	0.31	0.50	0.800	98.0
female	0.52	0.50	1.010	98.0
Av. % rec.				98.2

[a] Sacrificed 1 hr after dosing.
[b] Sacrificed 8 hr after dosing.

was more prominent and highly sensitive (10-fold). Without extraction with chloroform, the complex did not exhibit any absorption maximum in the ultraviolet region in the aqueous phase. The chloroform has to be saturated with ammonium thiocyanate to ensure complete color formation and to increase stability of the complex. Excess ammonium thiocyanate should then be removed to ensure constant absorbance for the complex. Aconine and benzoylaconine interfere in the reaction with cobalt thiocyanate, and thus they should be removed by paper chromatography before aconitine is measured. The method shows good recovery (98.2%) and a reasonable standard deviation (±0.05%) (Table 1).

The method is so sensitive that it was successfully used in the study of the chemical toxicology of aconitine. Tables 1 and 2 show that the results are comparable with those obtained by ultraviolet measurements. Recovery efficiencies from galenicals and biological samples were tested at different concentrations (Tables 1 and 3). Aqueous protein-free filtrates were prepared by adding $(NH_4)_2SO_4$. This technique is particularly suitable for aconitine because the latter, having 2 ester functions in its molecule, is very labile to acid and heat. The toxicity analysis presented in this paper was intended to be used to detect aconitine in the body organs and fluids in cases of acute toxicity or in post-mortem analysis. The study revealed that aconitine was rapidly absorbed from the stomach; after 2 hr the latter was completely devoid of aconitine. At 8–10 hr from the time of administering the drug, aconitine could be traced in the heart and liver. Aconitine could also be detected and estimated in urine samples collected during this period. After 8 hr, aconitine was not detected in any organ.

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Ion-Pair Extraction Method for Quantitation of a Bisquaternary Ammonium Compound, Pancuronium Bromide

ARMAND J. MICHOEL and RENAAT D. KINGET
Laboratory of Galenical and Industrial Pharmacy, Department of Pharmacy, Catholic University of Leuven, Minderbroedersstraat 17-19, B-3000 Leuven, Belgium

Pancuronium bromide (PCBr), a neuromuscular blocking agent, was determined quantitatively in aqueous solution by ion-pair extraction. Separate determination of PCBr and degradation products was possible after thin layer chromatography. The procedure was developed by using a theoretical approach. Favorable conditions were calculated from the ion-pair extraction constant. The drug was determined with bromothymol blue at pH 9.0, using one extraction in the direct procedure and 2 successive extractions in the combined elution-extraction process after thin layer chromatography. In the direct method, 0.100 mg PCBr was determined with a reproducibility of ±1.0%.

Pancuronium bromide (3α,17β-diacetoxy-2β, 16β - dipiperidino - 5α - androstane dimethobromide) (PCBr) is a potent curariform neuromuscular blocking agent of the competitive type. This compound has the structure shown in Fig. 1. Hydrolysis of the 2 acetate groups is responsible for the formation of 3 degradation products: the 2 possible monoacetates and a diol.

A widely used method to determine quaternary ammonium compounds is the acid-dye method, in which an ion-pair is formed with an anionic dye (1). In this study, bromothymol blue (BTB), which was intensively studied by Schill and coworkers (2–5), was used as the counter ion.

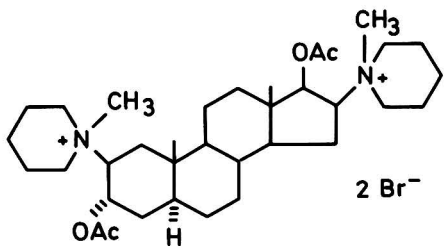


FIG. 1—Structure of pancuronium bromide.

Ion-pair techniques for the determination of PCBr and its degradation products (6–9) do not have much theoretical basis. It is the aim of this paper to predict the influence of several variables, e.g., pH, volumes of organic and aqueous phases, initial dye concentration, and number of extractions, in order to improve the method with regard to simplicity, reproducibility, and speed of execution.

The selective quantitative determination of the unchanged drug and its degradation products, as needed for our kinetic study, presents particular problems. Because of the marked similarity between compounds, especially between the 2 isomeric monoacetates, complete separation is difficult. Another problem is the difficult elution of the polar compounds from silica gel after thin layer chromatography (TLC).

Recently, several papers were published which dealt with the determination of PCBr and metabolites after TLC, using different techniques. Kersten *et al.* (6) used visual comparison after detection of the spots with iodoplatinate reagent. Tanaka *et al.* (7) evaluated the relative amounts of the compounds by densitometry after visualization with Dragendorff reagent. The monoacetyl derivatives were determined together. Buzello (8) used the acid-dye bromophenol blue for the ion-pair extraction. These methods do not always permit a complete resolution of the 2 monoacetates, a fact that is admitted by Buzello. Our paper on improvements of TLC of PCBr appeared recently (10). In combination with the ion-pair extraction method, described here, separate quantitative determination of each of the 4 compounds will be possible.

METHOD

Apparatus

(a) *Spectrophotometer.* — Unicam Model SP 1800 with 10 or 40 mm cells.

(b) *pH meter.*—Model PHM 26 (Radiometer, Copenhagen, Denmark).

Reagents

Use analytical grade reagents without prior purification.

(a) *Pancuronium bromide (PCBr) and derivatives.*—Obtained from N. V. Organon (Oss, The Netherlands). Prepare solutions from products dried at 70°C in vacuum.

(b) *Bromothymol blue (BTB) solution.*—$3.2 \times 10^{-4}M$. Dissolve 0.050 g BTB in 2 ml 0.2 *N* NaOH and dilute to 250 ml with water.

(c) *Borate buffer.*—pH 9.0; 0.2*M* in terms of borate. Dilute 7.628 g $Na_2B_4O_7 \cdot 10H_2O$ and 1.237 g boric acid to 500 ml with water.

(d) *Phosphate buffer.*—pH 10.0; 0.05*M*. Dissolve 4.450 g $Na_2HPO_4.2H_2O$ in 475 ml water. Adjust pH to 10.0 with 0.5*N* NaOH and dilute with water to 500 ml.

Determination of Ion-Pair Extraction Constant

Add 10 ml BTB solution in $CHCl_3$ ($4–12 \times 10^{-5}M$) and 10 ml 0.05*M* phosphate buffer, pH 10.0, containing appropriate amount of PCBr (160–320 μg) to separatory funnel. Shake vigorously 1 min. Let phases separate. Mix 2 phases by swirling before collection. Collect portions of 2 phases and centrifuge 5 min at 4000 rpm. Measure absorbance of organic phase at 420 nm and aqueous phase at 500 nm in 10 mm cell, both against pure solvent.

Determination of Pancuronium Bromide and Degradation Products

(a) *Procedure A: determination of total mixture.*—Add 10 ml $CHCl_3$, 5 ml BTB solution ($3.2 \times 10^{-4}M$), and 5 ml borate buffer, pH 9.0, containing PCBr and degradation products, to separatory funnel. Shake vigorously 1 min. Let phases separate. Mix nonpolar phase by swirling before draining. Collect portion of organic phase and centrifuge 5 min at 4000 rpm. Measure absorbance in 10 mm cell at 420 nm against pure $CHCl_3$. Add 50 μl 0.02*N* NaOH in methanol to cell, and measure absorbance at 620 nm against pure $CHCl_3$.

(b) *Procedure B: separate determination.* — Locate PCBr and degradation products after TLC with iodine vapor (11). Scrape separate silica gel areas, containing bisquaternary ammonium compounds, from plate with razor blade and collect in separatory funnels. Add 10 ml $CHCl_3$, 5 ml BTB solution ($3.2 \times 10^{-4}M$), and 5 ml borate buffer, pH 9.0. Shake vigorously 1 min. Let phases separate. Mix nonpolar phase by swirling before draining. Collect organic phase in 20 ml volumetric flask. Carry out additional extraction with 10 ml $CHCl_3$. Collect organic phase again in flask and dilute to volume with $CHCl_3$. Centrifuge $CHCl_3$ solution 5 min at 4000 rpm. Measure absorbance, *A'*, in 40 mm cell at 420 nm against pure $CHCl_3$. Add 200 μl 0.02*N* NaOH in methanol to cell, and measure absorbance at 620 nm against pure $CHCl_3$. Determine background absorbance, A_0, by similar procedure with equal silica gel area containing no bisquaternary ammonium compounds. Calculate net absorbance, *A*, by subtracting A_0 from *A'*.

Results and Discussion

Determination of Ion-Pair Extraction Constant

The BTB ion-pair with pancuronium ion (P^{+2}) gives a spectrum identical to that of the monovalent anionic form of BTB (HB^-) (3). The absorption maximum lies at 420 nm, which is close to the wavelength of maximum absorbance ($\lambda_{max.}$) of an aqueous solution of the monosodium salt of BTB (NaHB): 430 nm (3). No absorption is shown at 615 nm ($\lambda_{max.}$) of the divalent anion: B^{-2}.

The apparent molar absorptivity of the ion-pair, as calculated from the calibration curve at 420 nm, is 3.66×10^4, which is nearly twice the value for NaHB: 1.82×10^4 (3). This suggests that only an 1:2 ion-pair, $P(HB)_2$, is extracted into chloroform.

The applicable pH range of BTB is limited because of the high partition coefficient of the undissociated species of BTB in water-chloroform systems. At pH $\geqslant$7.5, no significant partitioning of this species occurs. Furthermore, when $C_{BTB} \leqslant 2 \times 10^{-4}M$ (C_{BTB} = total concentration of all BTB species in water) and $C_{Na^+} \leqslant 0.10M$, other side reactions such as dimerization, tetramerization, and micelle formation are not significant (5).

Under these conditions and with the assumption that equal phase volumes are used, the ion-pair extraction constant can be defined by the following equation, according to the principles given by Schill (5) for the concentrations of individual species in the organic and aqueous phases, respectively.

$$E_{P(HB)_2} = [P(HB)_2]_{org}/([P^{+2}] \times [HB^-]^2) \quad (1)$$

In order to use the ion-pair extraction constant without further corrections, e.g., for BTB

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dissociation, it must comprise such side reactions. The extraction constant is then usually called a conditional constant E^x (12) and is expressed by total concentrations. Several illustrations of the method can be found in the literature (5, 12–14).

$$E^x_{P(HB)_2} = C_{BTB,\,org}/(2 \times C_{P^{+2}} \times C^2_{BTB}) \quad (2)$$

where C_{org} and C are total concentrations of all species in the organic and in the aqueous phases, respectively.

The optimal conditions for the determination of E^x are derived from extraction equilibrium diagrams, as outlined by Modin and Schill (12). The extractions are carried out at pH 10.0, using different initial concentrations ($C°$) of BTB and PCBr, as shown in Table 1.

Since $C_{BTB} = [HB^-] + [B^{-2}]$ at pH 10.0, C_{BTB} can be calculated from the absorbance at the isosbestic point of BTB ($\lambda = 500$ nm), A_{500}. The molar absorptivity is obtained from the calibration curve: $\varepsilon_{500} = 5.95 \times 10^3$.

$C_{BTB,\,org}$ can be calculated from the absorbance at 420 nm, using $\varepsilon_{420} = 1.82 \times 10^4$. The concentration of P^{+2}, remaining in the aqueous phase, is obtained using Equation 3:

$$C_{P^{+2}} = C°_{P^{+2}} - (C_{BTB,\,org}/2) \quad (3)$$

According to Equation 2, a straight line is obtained as shown in Fig. 2. The value of E^x can be calculated from the slope of this line: $E^x = (5.55 \pm 1.18) \times 10^8$.

From this E^x, the ion-pair extraction constant can be calculated for the only side reaction which occurs, the dissociation of BTB:

$$[HB^-] = C_{BTB}/(1 + K_{HB^-} \times [H^+]^{-1}) \quad (4)$$

$$\log E_{P(HB)_2} = \log E^x_{P(HB)_2} + 2 \log (1 + K_{HB^-} \times [H^+]^{-1}) \quad (5)$$

Using $pK_{HB^-} = 7.0$ (15), we obtain $\log E = 14.74$, the pH being 10.0.

Pancuronium forms a BTB ion-pair, which is characterized by a high extraction constant in comparison with other bisquaternary ammonium compounds. This can partially be explained by its number of carbon atoms (5, 7).

Procedure Conditions

With the aid of Equation 5, E^x can be calculated at different pH values (Table 2). Knowledge of this E^x permits the estimation of the partition ratio of pancuronium ($D_{(P)}$):

$$\log D_{(P)} = \log E^x_{P(HB)_2} + 2 \log C_{BTB} \quad (6)$$

or

$$\log D_{(P)} = \log E^x_{P(HB)_2} + 2 \log (C°_{BTB} - C_{BTB,\,org}) \quad (7)$$

Equation 7 can be used to calculate $C°_{BTB}$, needed to achieve a desired partition ratio (Table 2). The percentage of pancuronium which remains in the aqueous phase after n extractions (F_n), is easily known from Equation 8, even if the volumes of aqueous and organic phases (V) are not equal. The procedure is easily adapted following the predictions obtained from Equation 8.

$$F_n = 100\ [1/(1 + V_{org} \times D_{(P)} \times V^{-1})]^n \quad (8)$$

In our method a pH value of 9.0 is chosen to avoid any background absorbance and to avoid initial concentrations of BTB which would cause side reactions (Procedure A).

Table 1. Determination of E^x

$C°_{BTB}$ $\times 10^5$ (M)	$C°_{P^{+2}}$ $\times 10^5$ (M)	A_{420}	A_{500}
4	2.13	0.238	0.155
6	2.13	0.353	0.223
7	2.13	0.396	0.268
8	2.13	0.518	0.295
8	2.13	0.468	0.308
10	2.13	0.580	0.395
10	3.20	0.670	0.325
12	4.26	1.058	0.332

Determination of Pancuronium Bromide

Our described ion-pair extraction method followed by spectrophotometric measurement is suitable to determine quantitatively PCBr present in aqueous solution (Procedure A). Beer's law is followed between 1.36 and 27.3 $\times 10^{-6} M$, corresponding to 0.010–0.200 mg PCBr. The molar absorptivity is increased from 3.66×10^4 at 420 nm to 6.76×10^4 at 620 nm by adding alkali to the chloroform solution. The spectrum change suggests the formation of the divalent anionic form. The reproducibility of the method, given in Table 3, is calculated from the results of 6 determinations at 3 concentrations. The absorbance readings of pancuronium and degradation products are inversely related to their mo-

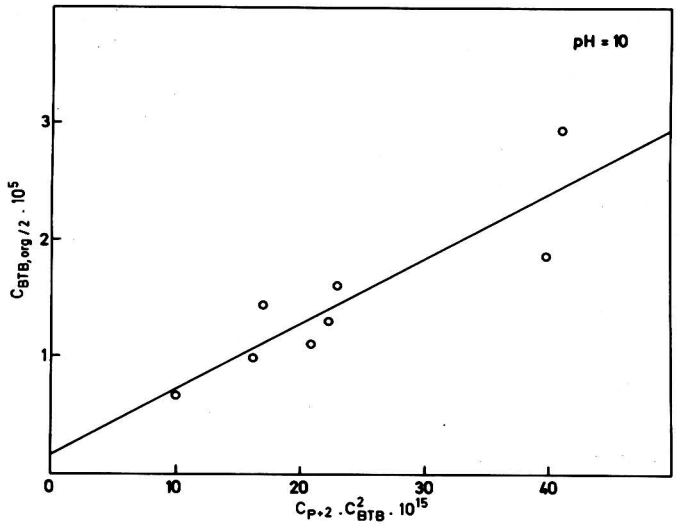


FIG. 2—Determination of ion-pair extraction constant.

Table 2. pH Dependency of extraction parameters[a]

pH	log $E^{\pm}_{P(HB)_2}$	$C^{\circ}_{BTB} \times 10^5$ (M) $F_1 = 0.25\%$	$C^{\circ}_{BTB} \times 10^5$ (M) $F_1 = 0.50\%$	$C^{\circ}_{BTB} \times 10^5$ (M) $F_1 = 1.0\%$	A_{420} (blank) (Proc. A)
8.0	12.74	6.39	6.12	5.93	0.004
8.5	11.74	8.25	7.43	6.85	0.002
9.0	10.74	14.1	11.5	9.75	—
9.5	9.74	32.4	24.5	18.9	—
10.0	8.74	90.6	65.6	47.9	—

[a] $C_{BTB,org} = 5.5 \times 10^{-5} M$; PCBr = 200 μg/10 ml; $V_{org} = V = 10$ ml.

Table 3. PCBr determination (Procedure A)

PCBr $\times 10^6$ (M) (X_1, X_2)[a]	A_{420} (Y_1)	Std dev., % (n = 6)	A_{620} (Y_2)	Std dev., % (n = 6)
1.365	0.045	±6.1	0.062	±4.6
2.730	0.089	±5.0	0.137	±5.9
6.824	0.232	—	0.411	—
13.65	0.488	±0.8	0.914	±1.0
20.47	0.732	—	1.362	—
27.30	0.992	—	1.790	—

[a] Regression equations: $Y_1 = -0.010 + 3.656 \times 10^4 X_1$; $Y_2 = -0.036 + 6.759 \times 10^4 X_2$.

lecular weights. The different products have the same molar absorptivities. Consequently, the same calibration curve can be used for the 4 products if the net absorbances are graphically plotted against the molar concentrations.

The elution of pancuronium from silica gel into water in combination with ion-pair extraction is almost complete after 2 extractions (Procedure B). The absorbances are between 95 and 98% of those obtained from aqueous solutions, considering the different volumes and path lengths of cells used. Taking into account the additional error of applying the solutions to the chromatoplate, this difference is not considered to be significant. Elution is complete because pancuronium must desorb from silica gel in order to restore the equilibrium between adsorbed and dissolved pancuronium, which is disturbed by the high equilibrium constant ($E_{P(HB)_2}$) of the ion-pair extraction process (15).

Conclusions

Our determination method, constructed on a theoretical basis, has the advantage over other published methods that fewer extractions are needed. In addition to a more rapid execution, our procedure uses a minimum amount of organic solvent. After alkali is added to the organic

phase, the same sensitivity is obtained as in the ion-pair extraction methods, using bromophenol blue as the anionic dye (7, 8).

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Collaborative Study of the Spectrophotometric Determination of Procainamide Hydrochloride

JEFFREY C. HAMM
Food and Drug Administration, Winchester Engineering and Analytical Center, 109 Holton St, Winchester, MA 01890

The USP analysis for procainamide·HCl is titrimetric and relatively nonspecific, capsule and tablet dyes may interfere, and the method is not applicable to coated tablets. In the spectrophotofluorometric method the sample deteriorates when exposed to a xenon source. In the ultraviolet spectrophotometric method reported here, the sample is dispersed in acid medium, possible interferences are extracted in chloroform, base is added, procainamide is extracted in chloroform, the residue is dissolved in sodium hydroxide, and the compound is measured by absorption at 272 nm and comparison with a standard. Recoveries of standards added to capsule, tablet, and injection composites ranged from 99.3 to 102%. Twelve collaborators reported duplicate assay results for all 3 dosage forms with per cent standard deviations for 5 samples ranging from 1.01 to 1.27%. The method has been adopted as official first action.

Procainamide·HCl has been analyzed by a variety of methods: nitrite titration, with ferrocyphen internal indicator (1); diazotization and reaction with Bratton-Marshall reagent, *N*-(1-naphthyl)ethylenediamine, with subsequent colorimetric determination (2, 3); reaction with fluorescamine, 4-phenylspiro(furan-2(3H),1′-phthalan)-3,3′-dione (4, 5) or reaction with 9-chloroacridine (6) with subsequent spectrophotofluorometric determination; and gas-liquid chromatographic separation with flame ionization detection (7). In addition, procainamide·HCl possesses natural fluorescence properties at basic pH (8), and an unsuccessful attempt at direct spectrophotofluorometry of procainamide·HCl in blood plasma has been reported (9).

The Associate Referee initially sought to develop a simple spectrophotofluorometric method for procainamide·HCl in pharmaceutical preparations which would eliminate derivatization and use the natural fluorescence of procainamide·HCl directly. Preliminary results based on this approach were reported at the 1974 Annual Meeting of the AOAC, but the technique was abandoned when it became clear that the intensity of fluorescent emissions decreased nonreproducibly on repeated exposure to a xenon excitation source. Exposure to an ultraviolet source, however, had no effect on procainamide·HCl at basic pH.

An ultraviolet spectrophotometric method for determining procainamide·HCl in capsule, tablet, and injection dosage forms was developed. The sample is cleaned up and extracted by solvent partitioning, and determined spectrophotometrically in 0.01*M* NaOH at about 272 nm relative to a standard.

Collaborative Study

Collaborators were supplied with 5 samples. Two samples (1 and 2) consisted of portions of 100-capsule sieved (60 mesh) composites of differing strengths. Two samples (3 and 4) consisted of portions of ground and sieved (60 mesh) 100-tablet coated tablet composites of differing strengths with tablet coatings individually removed before compositing. One sample (No. 5) was an injection solution of procainamide·HCl in a sealed multiple-dose vial. The capsule and tablet dosage forms contained 4–5% magnesium stearate, 1–12% Talc USP, and 0–3% Lactose USP. The injection contained 0.9% (w/v) benzyl alcohol and 0.09% sodium bisulfite. Collaborators also received one vial of standard procainamide·HCl (ICN-K & K Laboratories, Inc., 121 Express St, Engineers Hill, Plainview, NY 11803, No. 17158). All 5 sample materials were produced by the same manufacturer. For calculation purposes, collaborators were to assume 100% potency for the standard material supplied. The instructions specified the approximate amount of each sample to be weighed or pipetted and provided a formula for calculating potency on an mg/g basis in order to eliminate the possibility of estimating potency on an mg/capsule or mg/tablet basis. The collaborators were asked to perform duplicate determinations on each of the 5 samples, using

solutions of standard procainamide·HCl prepared the same day, and to record assay results, wavelength of maximum absorbance, and comments on the reporting forms provided. Collaborators were asked to forward all worksheets and ultraviolet curves to the Associate Referee.

METHOD

38.B08 Principle

Procainamide is extd from acid soln with $CHCl_3$. After evapn of solv., residue is dissolved in alk. soln and max. *A* detd at ca 272 nm.

38.B09 Reagent

Procainamide hydrochloride std solns.—Stock soln.—1 mg/ml. Assay std as in USP XIX. Accurately weigh ca 100 mg procainamide.HCl (ICN-K&K Laboratories, Inc., No. 17158) into 100 ml vol. flask, and dissolve and dil. to vol. with 0.01*N* NaOH. *Working soln.*—1 mg/100 ml. Pipet 1 ml stock soln into 100 ml vol. flask and dil. to vol. with 0.01*N* NaOH. Prep. fresh daily.

38.B10 Preparation of Sample

(a) *Capsules.*—Proceed as in **36.004**. Mix, and transfer accurately weighed portion contg ca 100 ml procainamide.HCl to 125 ml separator. Add 10 ml HCl (1+9) and shake to disperse. Add 15 ml H_2O.

(b) *Tablets.*—Det. av. wt. Reduce tablets to fine powder. (For tablets with coatings that do not reduce to powder, carefully peel off and discard coatings, and reduce tablets to fine powder.) Mix, and transfer accurately weighed portion contg ca 100 mg procainamide.HCl to 125 ml separator. Add 10 ml HCl (1+9) and shake to disperse. Add 15 ml H_2O.

(c) *Injections.*—Dil. soln, if necessary, with HCl (1+9) to give ca 100 mg procainamide.HCl/ml. Pipet 1 ml into 125 ml separator, add 10 ml HCl (1+9), and shake. Add 15 ml H_2O.

38.B11 Determination

(*Caution: See* **51.011** and **51.056**.)

Ext sample soln with three 25 ml portions $CHCl_3$ and discard $CHCl_3$. To aq. soln add 5 ml NH_4OH and ext with five 25 ml portions $CHCl_3$, collecting exts in 250 ml beaker and rinsing tip of separator into beaker after each extn. Evap. $CHCl_3$ to dryness on steam bath with air current. Quant. transfer residue to 100 ml vol. flask with 0.01*N* NaOH, and dil. to vol. with same solv. Pipet 1 ml dild soln into another 100 ml vol. flask and dil. to vol. with 0.01*N* NaOH. Record spectra of std and sample solns against 0.01*N* NaOH and det. *A* at max., ca 272 nm.

mg Procainamide.HCl/capsule or tablet

$$= (A/A') \times C \times D \times (W/W'),$$

where *A* and *A'* refer to sample and std solns, resp.; *C* = concn of std soln in mg/ml; *D* = sample diln factor in ml; *W* = av. mg/capsule or tablet; and *W'* = mg sample.

For injections, mg procainamide.HCl/ml

$$= (A/A') \times C \times D/V,$$

where *V* = ml sample aliquot.

Results

Table 1 summarizes recovery data obtained by the Associate Referee during development of the method. Table 2 summarizes the results obtained by the 12 collaborators.

Table 1. Recovery data obtained by the Associate Referee during method development[a]

Declared, mg	Found, mg	% of Declared	Std added, mg	Std recd, mg	Rec., %
		Capsules			
250	245	98.0	101.2	101	100
375	381	102	100.5	100	99.6
500	508	102	51.7	51.3	99.3
		Tablets			
250	252	101	101.1	103	102
375	362	96.5	101.1	102	101
500	496	99.2	101.8	101	99.6
		Injection			
100	102	102	101.3	101	99.6
500	508	102	100.2	102	102

[a] Recoveries were determined by adding accurately weighed quantities of procainamide·HCl standard to separatory funnels containing sample preparations. Per cent recovered was based on assay value for corresponding unspiked sample preparation. Results represent single determinations for assay and spikes. Lots analyzed were not identical with samples supplied to collaborators.

Table 2. Collaborative results for ultraviolet spectrophotometric determination of procainamide·HCl[a]

	Capsules		Tablets		Injection
Coll.	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
1	780	933	713	717	100
	778	934	712	721	102
2	780	926	716	726	103
	792	942	701	723	103
3	767	910	707	716	99.5
	780	903	710	721	100
4	782	936	715	727	100
	773	927	714	721	100
5	767	929	713	730	101
	762	930	713	726	101
6	789	943	726	728	103
	786	944	721	732	103
7	755	938	717	718	100
	789	937	710	718	100
8	778	933	724	724	101
	782	929	723	721	99.7
	763			733	
	770			718	
9	775	931	689	715	100
	789	934	703	723	99.2
10	791	932	726	738	101
	786	947	728	739	101
11	785	924	703	709	98.8
	782	943	713	712	101
12	782	926	720	722	101
	772	926	716	721	101
Mean	778	932	714	723	101
Std dev.	9.73	10.1	9.07	7.30	1.24
Std error	1.91	2.06	1.85	1.43	0.252
Coeff. of var., %	1.25	1.08	1.27	1.01	1.22

[a] Results are reported as mg procainamine·HCl/g for Samples 1–4 and mg/ml for Sample 5.

Three collaborators expressed reservations about taking the specified 1.00 ml aliquot of injection solution for analysis. Using an NBS calibrated 1.00 ml pipet and reporting results to 3 significant figures seems justifiable based on the 1.22% standard deviation obtained for Sample 5 (injection). One collaborator departed from the instructions and analyzed Samples 1 and 4 in duplicate. He stated that Sample 1 was incompletely ground when received (although grinding was not employed in compositing Sample 1 since it consisted of capsule contents) and he ground it himself prior to the second analysis. He gave no reason for repeating the analysis of Sample 4. One collaborator experienced minor emulsions during the extractions of Samples 1, 3, and 4, while another collaborator obtained cloudy extracts for Sample 1. Centrifugation, although not specified as an option in the method, might have remedied these problems. One collaborator suggested filtering the chloroform extracts through cotton; however, since chloroform occasionally picks up interferences from cotton and since the residue from evaporation of the chloroform is diluted more than 100-fold, this suggestion was not incorporated. One collaborator suggested diluting the samples and reading them in the ultraviolet region directly, without extraction. Although this technique is satisfactory for some dosage forms, the intent of this method is to ensure isolation of the procainamide·HCl from fillers or dyes which may be present, and this suggestion was not incorporated in the method.

No collaborator reported serious difficulty in following the method. Most collaborators stated that the method was straightforward and easy to follow.

Recommendations

On the basis of the collaborative study, the ultraviolet spectrophotometric method for procainamide·HCl is shown to be precise and accurate, and it is recommended that the method

The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee B and were adopted by the Association. *See* (1976) *JAOAC* **59**, 383.

be adopted as official first action. It is also recommended that more advanced techniques for sample extraction be investigated.

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Collaborative Study of a Spectrofluorometric Assay for *Rauwolfia serpentina* Tablets and Powdered Root

WILLIAM M. SMITH and CHARLES C. CLARK[1]
Food and Drug Administration, 900 Madison Ave, Baltimore, MD 21201

Reserpine-rescinnamine group alkaloids are extracted from *Rauwolfia serpentina* preparations into a dimethylsulfoxide (DMSO)-methanol mixture and diluted with 0.5*N* H_2SO_4. The chloroform extract of this solution is passed through a 0.1*N* NaOH-Celite column and then through a silica gel column. The weakly basic alkaloids trapped on the latter column are eluted with a methanol mixture; a portion of the eluate is treated with nitrous acid and the reserpine-rescinnamine content is determined by measuring the intensity of fluorescence of the oxidation product. The following means and standard deviations (11 collaborators) were obtained for the determination of reserpine-rescinnamine group alkaloids in 4 samples of *Rauwolfia serpentina* (NF reference powder, 100 mg and 50 mg commercial tablets, and a 45 mg synthetic tablet formulation): 0.174% ± 0.0112, 0.131% ± 0.0047, 0.160% ± 0.0100, and 0.153% ± 0.0083, respectively.

Rauwolfia serpentina is indicated in the treatment of mild hypertension. It is also useful in adjunctive therapy with other antihypertensive agents in the more severe forms of hypertension. In addition, it is prescribed for the relief of symptoms in agitated psychotic states (e.g., schizophrenia), primarily in those individuals who are unable to tolerate phenothiazine derivatives or who also require antihypertensive medication (1).

Reserpine and rescinnamine are the principal alkaloids in *Rauwolfia serpentina*. However, small amounts of serpentina, yohimbine, reserpinine, ajmalicine, ajmaline, deserpidine, and other oxidation products of reserpine and rescinnamine occur naturally in the plant (2).

In recent years, photometric methods have been introduced for the quantitative determination of both reserpine and *Rauwolfia serpentina* (2–7). These methods are based on reactions with suitable reagents, resulting in the formation of chromophores or fluorogens which can be measured by colorimetric or fluorometric techniques. Colorimetric measurements have been employed for pharmaceutical formulations when the sensitivity is not critical (3). Fluorescence determinations have been used for reserpine in biological or other materials (8) in which the concentration of reserpine is very low. Recently, however, fluorescence determinations have also been extended to *Rauwolfia serpentina* (9, 10).

Identical assay procedures are described for both *Rauwolfia serpentina* tablets and powdered *Rauwolfia serpentina* in the *National Formulary* (11) and *Official Methods of Analysis* (12). This method is based on the Szalkowski-Mader nitrous acid test for reserpine (5). However, the 4-hr Soxhlet extraction, the tedious solvent evaporation steps, and the 30 shakeouts make the method laborious and time consuming.

Both Clark (9) and Kunze *et al.* (13) have published modified versions of this procedure. Kunze *et al.* eliminated the 4-hr Soxhlet extraction and solvent evaporation steps. The 4-hr Soxhlet extraction was replaced by 10 min of homogenization in a definite volume of solvent. The solvent evaporation step was eliminated by adding the developing reagents, sodium nitrite and methanolic hydrochloric acid, directly to aliquots of the chloroform extract. The Kunze *et al.* method shortens the time required to perform an analysis for reserpine-rescinnamine group alkaloids to about 3 hr. However, the method requires a separatory funnel extraction step in which stubborn emulsions are often formed. Clark's modification also eliminates the 4-hr Soxhlet extraction and solvent evaporation steps. In addition, it reduces the 30 shakeouts to just 4 and significantly reduces both the amount of sample required and the analysis time.

The first desired objective was to develop a rapid and quantitative technique for the initial extraction of the reserpine-rescinnamine group alkaloids from the sample matrix. An ideal solvent system would have to readily penetrate the

[1] Present address: Drug Enforcement Administration, 15655 SW 127th Ave, Miami, FL 33157.

W. M. Smith is the present Associate Referee on *Rauwolfia serpentina* (appointed in 1972); C. C. Clark is the past Associate Referee.

plant tissue, completely solubilize the alkaloids, not cause stubborn emulsions in subsequent extractions, and provide an environment in which the alkaloids would be stable. Ethanol, ethanol-acid mixtures, methanol, and dimethylsulfoxide (DMSO)-methanol mixtures were some of the solvents investigated. A 1+1 mixture of DMSO-methanol was found to best meet the requirements for the method. Separatory funnel extraction and column chromatographic separations are used to isolate the weakly basic alkaloids, principally reserpine and rescinnamine.

After a 30 min extraction of the alkaloid by shaking the powdered sample in DMSO-methanol, an aliquot of the supernate is diluted with 0.5*N* H_2SO_4 and extracted with chloroform. Subsequently, the chloroform solution is passed first through a 0.1*N* NaOH column, which traps the acids and possible interfering dyes normally included in tablet preparations, and then through a silica gel column, which temporarily traps the weakly basic alkaloids. Organic-soluble neutral materials elute with the chloroform and are discarded. The alkaloids of interest are eluted from the silica gel column with chloroform-methanol (1+1). An aliquot of this eluate is treated with nitrous acid in a modification of the Szalkowski-Mader nitrous acid test (5) and the resulting fluorescence is measured spectrofluorometrically.

METHOD

Reagents

(a) *DMSO-methanol mixture.*—(1+1). Mix equal volumes of DMSO and methanol; 10 ml per determination is required.

(b) *Sulfuric acid.*—0.5*N*. Add 7 ml concentrated H_2SO_4 to 500 ml water and mix well.

(c) *Sodium hydroxide.*—0.1*N*. Dissolve 2 g NaOH in 500 ml water.

(d) *Silica gel.*—0.05–0.2 mm; *see* 26.015(a) (12) for specifications and activation.

(e) *Sodium nitrite.*—0.3%. Dissolve 300 mg $NaNO_2$ in 50 ml water, dilute to 100 ml with methanol, and mix well. Prepare fresh daily.

(f) *Methanolic hydrochloric acid.*—Mix 25 ml concentrated HCl with 75 ml methanol. Cool to room temperature before use.

(g) *Aqueous ammonium sulfamate solution.*—5%. Dissolve 5 g ammonium sulfamate in water and dilute to 100 ml with water.

(h) *Chloroform-methanol.*—(1+1). Mix equal volumes of chloroform and methanol; 100 ml per determination is required.

(i) *Water-methanol.*—(1+1).

(j) *Reserpine standard solutions.*—(*1*) *Stock solution.*—0.2 mg/ml. Protect solution from direct light. Dissolve 2 mg USP Reserpine, accurately weighed, in ca 50 ml hot methanol. Cool and dilute to 100.0 ml with methanol. (This standard solution when stored in tightly stoppered brown glass bottles is stable for at least 1 month.) (*2*) *Working solution.*—2 μg/ml. Dilute 10.0 ml stock solution to 100.0 ml with methanol. Prepare fresh daily.

Apparatus

(a) *Spectrophotofluorometer.*— Hitachi Perkin-Elmer MPF-2A, or equivalent; excitation wavelength 400 nm (uncorrected); emission wavelength 502 nm (uncorrected). Use excitation and emission slit widths consistent with good quantitation according to manufacturer's recommendations (10 and 12 nm, respectively, for above instrument). Sensitivity setting used depends upon slit widths.

(b) *Chromatographic tubes.*—200 × 22 mm id, and tamping rod; *see* 37.002 (12).

(c) *Wrist-action shaker.*—Burrell, or equivalent.

(d) *Centrifuge tubes.*—50 ml, glass-stoppered.

Preparation of Sample

For tablets, determine average tablet weight from weight of ≥20 tablets. Grind to pass 60 mesh sieve and mix well.

For powdered root samples, use as received.

Preparation of Column

Column 1.—Mix 2 ml 0.1*N* NaOH with 3 g Celite. Transfer to chromatographic tube plugged with glass wool and tamp. Cover Celite with pad of glass wool.

Column 2.—Pour silica gel into chromatographic tube (plugged with glass wool) to height of ca 4 cm. Lightly tap side of tube with tamping rod. Cover silica gel with pad of glass wool. Arrange columns so that effluent from Column 1 flows directly into Column 2. Wet columns with 25 ml water-saturated $CHCl_3$.

Sample Cleanup

(Perform in subdued light.) Accurately weigh amount of prepared sample equivalent to 100 mg *Rauwolfia serpentina* and transfer to 50 ml centrifuge tube. Add 10.0 ml DMSO-methanol (1+1), stopper, and shake vigorously by hand until entire sample is thoroughly wetted. Mechanically shake 30 min at highest setting (wrap tube with aluminum foil if shaker is not located in dark place). Centrifuge tubes 5 min at 1200 rpm. Pipet 5 ml aliquot of supernate (use care to avoid pipetting any of undissolved residue) into 125 ml

separatory funnel containing 50 ml 0.5*N* H_2SO_4. Extract with four 25 ml portions of $CHCl_3$.

In second separatory funnel containing 25 ml 0.5*N* H_2SO_4, individually shake each 25 ml $CHCl_3$ extract and drain each time into Column 1. Let each $CHCl_3$ extract sink entirely into both columns before adding next 25 ml extract. After draining fourth extract into second separatory funnel, rinse tip of first funnel into second with 2–3 ml $CHCl_3$. Subsequently, and in same manner, rinse tip of second funnel into Column 1 and tip of Column 1 into Column 2. Discard all $CHCl_3$ eluted from Column 2.

Place 100 ml volumetric flask under Column 2 and elute with ca 90 ml $CHCl_3$-methanol (1+1). Rinse tip of Column 2 into flask with ca 5 ml $CHCl_3$-methanol (1+1) and dilute eluate to volume with mixture.

Determination

Transfer duplicate 20.0 ml aliquots of $CHCl_3$-methanol eluate to separate 25 ml volumetric flasks. Transfer duplicate 10.0 ml aliquots of working standard solution to separate 25 ml volumetric flasks, each containing 10 ml $CHCl_3$. Add 1.0 ml 0.3% $NaNO_2$ solution to one flask of each set. Mix well. Add 1.0 ml water-methanol (1+1) to the other (blank) flasks. Add 1.0 ml methanolic HCl to *all* flasks, mix well, and let stand in dark 30 min. Add 0.5 ml 5% ammonium sulfamate to all flasks, dilute to volume with methanol, mix well, and determine fluorescence intensity.

Calculations

Calculate per cent reserpine-rescinnamine group alkaloids of labeled amount of *Rauwolfia serpentina* per tablet as follows:

$$\% = (F - F_b/F' - F'_b) \times (W'/W) \times (TW/LW) \times 10$$

where F, F_b, F', and F'_b = fluorescence readings, in arbitrary units, of sample, sample blank, standard, and standard blank, respectively; W' and W = mg standard and g sample, respectively; TW = average tablet weight, g; and LW = label contents, mg/tablet.

Calculate per cent reserpine-rescinnamine group alkaloids in *Rauwolfia serpentina* powdered root as follows:

$$\% = (F - F_b/F' - F'_b) \times (W'/W) [(0.01/(mg/g))]$$

Collaborative Study

Eleven collaborators were supplied with 4 samples of *Rauwolfia serpentina:* an NF reference powder, 50 and 100 mg commercial *Rauwolfia serpentina* tablets, and a 45 mg synthetic tablet preparation. The synthetic tablet preparation was prepared from a commercial formula specificially for this study by the University of North Carolina School of Pharmacy, Chapel Hill, NC. All the tablet preparations were composited by the Associate Referee prior to shipment; thus the collaborators received all samples in powder form. Collaborators were supplied with approximately 6 g of each tablet composite and 0.5 g NF reference powder and were instructed to perform single determinations on each.

Table 1. Collaborative results for the determination of reserpine-rescinnamine group alkaloids (% of labeled amount of *Rauwolfia serpentina* found)

Coll.	NF ref. powder	Tablets 100 mg	Tablets 50 mg	Tablets 45 mg
1	0.163	0.131	0.166	0.155
2	0.194	0.132	0.160	0.163
3	0.160	0.125	0.142	0.144
4	0.172	0.129	0.154	0.144
5	0.166	0.128	0.155	0.146
6	0.177	0.132	0.166	0.153
7	0.181	0.131	0.172	0.160
8	0.186	0.142	0.173	0.165
9	0.170	0.129	0.155	0.146
10[a]	0.194	0.187	0.214	0.201
Mean	0.174	0.131	0.160	0.153
High	0.194	0.142	0.173	0.165
Low	0.160	0.125	0.142	0.144
Std dev.	0.0112	0.0047	0.0100	0.0083
Coeff. of var.	6.42	3.58	6.21	5.46

[a] Three of the 4 results reported by this laboratory were unusually high. Using Dixon's test (14), the 3 high results were confirmed as outliers and were not included in the reported average value or the statistical interpretation.

Results and Recommendations

Ten of the 11 collaborators completed the study. The results of the collaborative study are presented in Table 1. The results of Collaborator 10 were unusually high when compared to the other results. Using Dixon's test (14), 3 of the 4 results reported by this collaborator were confirmed as outliers. From the comments on the procedure from this collaborator, it appears that the instructions for washing the chloroform extract were somewhat ambiguous, thus causing

The recommendation of the Associate Referee was not approved by the General Referee and by Subcommittee B and was not accepted by the Association; both the General Referee and the Subcommittee recommended that the method be studied further to reduce the coefficients of variation obtained in the collaborative study. *See* (1976) *JAOAC* **59**, 382.

this analyst to perform the analysis in a manner slightly different from that intended. In the section under *Sample Cleanup*, the pertinent sentence was changed to read, "In second separatory funnel containing 25 ml 0.5*N* H_2SO_4, individually shake each 25 ml $CHCl_3$ extract and drain each time into Column 1."

One collaborator commented that he developed duplicate aliquots of standard, while performing a preliminary examination of the fluorescence development step, and obtained non-reproducible results. The past and present Associate Referees on *Rauwolfia serpentina* have extensively studied the fluorescence development procedure, including the derivative formation rates for both reserpine and rescinnamine, with linearity checks. No evidence of non-reproducibility of results was obtained from replicate determination of standard solutions. In addition, the derivative formation is the method for reserpine in *The United States Pharmacopeia* and the conditions have been established as optimum for the reserpine assay (15). The Associate Referee could not determine a cause for non-reproducibility of results.

One collaborator suggested that the preparation of all reagents in advance would be expedited by a more complete listing in the reagent section. This section has been revised. At the suggestion of a number of collaborators the method was changed to specify water-saturated chloroform for the pre-wash of Column 2.

All of the collaborators indicated that the method was clearly written (except for the comment on the somewhat ambiguous instruction previously discussed) and easy to follow. Many of the collaborators with experience in analyzing *Rauwolfia serpentina* by the official AOAC method (12) indicated that the proposed procedure greatly reduced or eliminated most of the difficulties they encountered in performing *Rauwolfia serpentina* analyses.

Table 2 shows recovery results obtained by using the proposed method. Recoveries of both individual reserpine and rescinnamine as well as mixtures were studied. These results indicate good recovery of standards by using the proposed method.

Table 3 shows the results of a study conducted by the Associate Referee to compare the proposed and official methods. The study consisted of replicate determinations of 10 different *Rauwolfia serpentina* samples, including both tablet and powdered root preparations. Three things are evident from the data listed: In every instance, higher results were obtained by the proposed method than by the official method;

Table 2. Recovery of reserpine and rescinnamine standards by the proposed method

Std	Added, mg[a]	Rec., mg	Rec., %
Reserpine	0.199	0.196	98.5
Reserpine	0.200	0.197	98.5
Reserpine	0.199	0.195	98.0
Rescinnamine	0.200	0.198	99.0
Rescinnamine	0.199	0.197	99.0
Rescinnamine	0.198	0.194	98.0
Mixed (reserpine-rescinnamine)	0.194	0.190	97.9
Av.			98.4

[a] Standards were dissolved in methanol and an aliquot equivalent to the stated amount of standard was taken for analysis.

Table 3. Assay results for reserpine-rescinnamine group alkaloids in tablets and powdered root

Sample		% Alkaloids, calcd as reserpine, of labeled amt of *Rauwolfia serpentina*: AOAC	% Alkaloids, calcd as reserpine, of labeled amt of *Rauwolfia serpentina*: Proposed	Diff. (proposed − AOAC), %	% Active alkaloids from AOAC extn with proposed detn
Tablets[a]	1	0.147	0.155	+5.4	0.143
	2	0.163	0.186	+14.1	0.159
	3	0.041	0.163	+297.6	0.034
	4	0.076	0.149	+96.1	0.080
	5	0.154	0.167	+9.3	
Powdered root NF		0.113	0.152	+34.5	
	7	0.095	0.150	+57.9	
	8	0.144	0.160	+11.1	
	9	0.131	0.144	+9.9	
	10	0.126	0.152	+20.6	
NF Ref. Std		0.140	0.170	+21.4	

[a] Tablets 1, 3, and 4 were labeled as containing 50 mg/tablet; 2 and 5 were labeled as containing 100 mg/tablet.

instances of unusually low results by the AOAC method suggest the possibility of occasional incomplete extraction; when duplicate analyses are performed in which only the determinative step differs, the results are essentially identical for both methods. In an effort to elucidate some of the reasons for these findings, a thin layer chromatographic (TLC) study and an examination of sample residues from the Soxhlet extractions were conducted.

In a TLC study, an attempt was made to determine if the 30 min DMSO-methanol treatment in the proposed method is comparable to the exhaustive 4-hr Soxhlet extraction used in the AOAC method. In addition, information was needed to determine if any of the known interferences (alkaloids which yield fluorescent products similar to those of reserpine and rescinnamine in the nitrite test) were being removed in the sample cleanup of the AOAC method and not by the proposed method. This possibility was suggested by the data presented in Table 3.

TLC analysis of the final extract from both methods produced essentially identical chromatograms, thus indicating that no additional interfering species were being quantitatively measured by the proposed method. When aliquots of the initial extract obtained by the official method were subjected to the fluorometric determination technique of the proposed method, essentially the same values were obtained as those found by the official method (Table 3). We concluded from these findings that the initial extraction step of the proposed method is more effective than the initial extraction step of the official method in quantitatively extracting the alkaloids.

We also investigated the possible causes for cases in which unusually low results were obtained with the AOAC method. We re-extracted the sample residues from those samples which had produced low results. We found that substantial amounts of the active alkaloids had not been extracted in the initial Soxhlet extraction. Evidence indicates that the relatively large sample (3 g) can cake or clump in the extraction thimble, resulting in non-uniform percolation of the extracting solvent throughout the sample. The ultimate result is incomplete extraction and low values.

At the suggestion of the General Referee on Alkaloid Drugs, we examined the residues remaining in the extraction thimbles from samples analyzed by the AOAC method in an effort to determine if the exhaustive Soxhlet extraction consistently removed all the reserpine-rescinnamine group alkaloids present. The residues were analyzed by using the proposed method with minor modifications, in order to permit the determination of very low concentrations of reserpine-rescinnamine group alkaloids. For each sample examined, we found that reserpine-rescinnamine group alkaloids equivalent to about 5% of the amount extracted by the exhaustive Soxhlet extraction remained in the residue. These findings suggest that the AOAC method in its present form consistently produces results which may be about 5% low because of incomplete extraction.

The proposed method requires about one fourth as much time (2½ hr) and about one sixth the amount of sample material per determination as the AOAC method for *Rauwolfia serpentina*. Because of its simplicity, the proposed procedure improves the precision of *Rauwolfia serpentina* analysis. The method is recommended for adoption as official first action.

Acknowledgments

The Associate Referee thanks the following collaborators from the Food and Drug Administration for their participation in this study: Susan Barkan, Washington, DC; John B. Covell, Minneapolis; Joan Hindley, Philadelphia; Fred R. Krick, Seattle; Helen Naviasky, Baltimore; Richard E. Olson, Kansas City; Carl Ponder, Atlanta; and Edward Skolnick, Boston. Sincere thanks also go to collaborators John A. Kowald, Squibb Institute for Medical Research, New Brunswick, NJ; T. I. Munnelly and the Analytical Research and Development Division, Ciba-Geigy Corp., Suffern, NY; and Joyce E. Wawro, Smith Kline and French Laboratories, Philadelphia, PA.

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DRUG RESIDUES IN ANIMAL TISSUES

Chemical Confirmation of Diethylstilbestrol Residues in Beef Livers

JOHN JOSEPH RYAN and JEAN-CLAUDE PILON
Food Research Division, Health Protection Branch, Health and Welfare Canada, Ottawa, Ontario, Canada K1A 0L2

A simple chemical technique was developed to confirm the presence of diethylstilbestrol (DES) residues in beef tissues at 2 ppb. The DES-trifluoroacetate (TFA) from the previously published method is hydrolyzed in water-methanol at 55°C for 20 min to free DES which is then extracted into benzene. Trimethylamine and a small amount of heptafluorobutyric (HFB) anhydride are added to effect the smooth conversion of DES to its di-HFB at either room temperature or 60°C. DES-HFB is determined by gas-liquid chromatography with electron capture detection on a 6′ × 4 mm id column of 3% OV-210 at 190°C. This procedure yields 2 peaks for DES-HFB (retention times 2.7 and 4.8 min for the *cis* and *trans*, respectively) which are resolved from the 2 DES-TFA peaks (retention times 2.3 and 3.3 min for *cis* and *trans*, respectively). The sensitivity of detection of DES-HFB is increased by a factor of 4–5 over that for DES-TFA. This chemical technique was successfully applied to purified extracts of beef liver fortified with DES at 2–10 ppb with recoveries from 60 to 110%.

One of the main methods for measuring diethylstilbestrol (DES) residues in beef tissues involves the formation and purification of a suitable derivative (1, 2) which can be analyzed by gas-liquid chromatography (GLC) and detected by electron capture. The derivative used in our laboratories and by others (3) is the trifluoroacetate (TFA) because of its ease of formation, high electron-capturing properties, and relatively low temperature of elution. The appearance of the 2 peaks for DES, for both the *cis*- and the *trans*-isomers, is taken as evidence that this estrogen is present in the beef sample. This technique is sensitive to 1–2 ppb, although reproducibility is sometimes low (4).

The appearance of the 2 peaks has been the sole criterion for the presence of DES in tissues; therefore a confirmatory technique was sought with at least the same sensitivity as the original method (1). A definitive procedure has been published (5) for the confirmation of DES residues by GLC-mass spectrometry (MS) of the dichloroacetyl (DCA) derivative.

Heffter *et al.* (6) also used the latter derivative to detect DES residues in tissue by GLC. The compound was then confirmed by thin layer chromatography of DES-DCA on silica gel. The developed spots were made visible by treatment with *p*-toluene sulfonic acid but no detection limits were given. Ponder (7), using the irradiation conversion of DES to its phenanthrenediol, gave details of the thin layer chromatographic separation of the latter on silica gel, using silver nitrate as the detecting reagent. Since the procedure used in our laboratories involves the DES-TFA derivative and because a simpler chemical assay was desired, we investigated the formation and GLC analysis of other electron-capturing derivatives. This paper describes the hydrolysis of the DES-TFA derivative and the formation, GLC analysis, and estimation of the heptafluorobutyrate (HFB) derivative of DES. This new derivative, distinguished from the old by its different retention times on GLC and the increased sensitivity of detection, is used to corroborate the presence of DES in beef livers.

METHOD

Reagents

All solvents are glass distilled, or equivalent. No special precautions were taken with glassware except all reaction manipulations were performed in an all-glass system.

(a) *Trimethylamine (TMA).* — Anhydrous (Eastman Organic Chemicals, Rochester, NY 14650). Cool glass ampoules in ice water and add contents with stirring to cool, tared benzene to give 1.0*M* solution. Dilute with benzene to 0.1*M* in 100 ml volumetric flask.

(b) *Heptafluorobutyric anhydride.* — (PCR, Gainesville, FL or Pierce Chemical Co., Rockford, IL). Reflux 1 hr with phosphorus pentoxide, with

stirring, and distill at 107°C. Store in Teflon-lined screw-capped vials under anhydrous conditions.

(c) *Trifluoroacetic anhydride.*—(Eastman Organic Chemicals). Test for suitability for derivatization. Otherwise purify as in (b).

(d) *Phosphate buffer.*—pH 6.0. Mix 6.15 ml 0.2*M* Na_2HPO_4 and 43.85 ml 0.2*M* NaH_2PO_4 and dilute to 100 ml with water.

(e) *Standard solutions.*—Prepare as in ref. (1).

Apparatus

(a) *Gas-liquid chromatograph.*—Hewlett-Packard Model 5713, or equivalent, equipped with ^{63}Ni electron capture detector, and 6′ × 2 mm id all-glass column with on-column injection and packed with 3% OV-210 on 80–100 mesh Chromosorb W(HP). Conditions: column 190°C; injection port 200°C; detector 250°C; argon-methane (95+5) carrier gas 30 ml/min. Under these conditions, retention times are 2.3 and 3.3 min for *cis*- and *trans*-DES-TFA, respectively, and 2.7 and 4.8 min for *cis*- and *trans*-DES-HFB, respectively.

(b) *Mass spectrograph.*—Varian Model Mat 311A, or equivalent, equipped with Varian gas-liquid chromatograph Model 1440 and Watson-Biemann separator; ionization voltage 70 ev and source 225°C.

Procedure

Evaporate working standard solution or meat extract of DES-TFA to dryness in 15 ml glass-stoppered graduated centrifuge tube under stream of dry nitrogen at room temperature. Dissolve residue in 0.2 ml methanol and let stand 5 min at room temperature. Add 1.0 ml water, stopper, and heat 20 min in 55°C water bath to effect hydrolysis. Cool, extract aqueous phase by shaking 20 sec each with one 2 ml and two 0.5 ml portions of benzene, and transfer each benzene extract with glass pipet to second centrifuge tube. With the last 2 benzene extractions emulsions can be formed which are readily broken by centrifuging at low speed. Transfer benzene washings (ca 3 ml) to third dry 15 ml centrifuge tube and concentrate to 0.5 ml under nitrogen at room temperature.

To benzene solution first add 10 µl HFB anhydride (in humid weather, perform this operation in dry box), and then 0.1 ml 0.1*M* trimethylamine in benzene. Heat 20 min in 55°C water bath. Cool, wash benzene once with 0.5 ml pH 6.0 phosphate buffer, and centrifuge. Inject 2–5 µl benzene solution directly into gas-liquid chromatograph. Compare retention times of DES-HFB to those of standards and quantitate by using total peak height of both *cis* and *trans* peaks.

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

Results and Discussion

The HFB derivative was chosen for confirmation of DES because the electron capture detector was more sensitive to it than to the trifluoroacetate derivative by a factor of 4–5. In turn, the detector was about 2000 times more sensitive to the latter derivative than to the simple acetate. It is possible to form the HFB derivative by reacting DES with 0.1 ml HFB anhydride 1 hr at room temperature, but this necessitated pure HFB anhydride with no free HFB acid present. Adding trimethylamine results in a quick reproducible conversion to the desired product, as reported by Ehrrson *et al.* (8). The trimethylamine base functions primarily as a catalyst but it also prevents interference from HFB acid which can retard the reaction and it eliminates glass adsorptive losses when dealing with small amounts. The phosphate buffer suffices to hydrolyze the HFB anhydride to the acid and remove it in the aqueous phase. This step also eliminates the long evaporation time of the anhydride under nitrogen at room temperature. The presence of small amounts of water dissolved in benzene (<0.1%) did not affect the stability of the derivatives as long as no visible amount of water was present, i.e., drying the benzene before derivatization was not necessary.

Separation, elution, and detection of the 2 DES-HFB peaks were best effected on a 6′ × 2 mm id glass column coated with 3% OV-210 on 80–100 mesh Chromosorb W, as shown in Fig. 1. The *trans* peaks of DES-TFA and DES-HFB were poorly resolved on OV-17, while a lighter loading of OV-210 resulted in poor separation of *cis*-HFB from *trans*-TFA. In addition, the column support mesh size (80–100) was critical; lower values (60–80) resulted in inferior separation, and higher values (100–120) caused gas flow problems. The separation of all 4 peaks from 0.3 ng standards as well as the difference in sensitivity of the electron capture detector are shown in Fig. 1.

The identities of the HFB product and of the already reported (1) TFA derivative were studied by MS. The analysis of samples as noted in Table 1 either directly on the probe or by GLC-MS of both peaks showed a strong peak at m/e 660 for the HFB derivative and

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at m/e 460 for the TFA derivative. These values correspond to the addition of 2 acyl groups in either case to the DES molecule and support the premise that the derivatized products are the simple adducts of DES with acylation on the 2 phenolic hydroxyl groups.

An additional problem arose involving the ratio of the *cis-trans* peaks when small amounts (<500 ng) of DES were derivatized and detected. The ratio of the 2 peaks is known to be variable (9) but generally for DES derived from a solution in polar solvents the ratio of *cis* to *trans* is roughly 1:3. At low levels, from either standards or meat samples, the *trans* peak was often less than *cis*. Since this did not occur when a standard was derivatized at high levels and then diluted before injection, this was probably a concentration-dependent phenomenon as noted previously by Donoho *et al.* (2). A third small (<5% of total) peak for DES-HFB appeared occasionally at longer retention times (e.g., *cis* 2.7, *trans* 4.8, third peak 7.8 min), but its origin was uncertain.

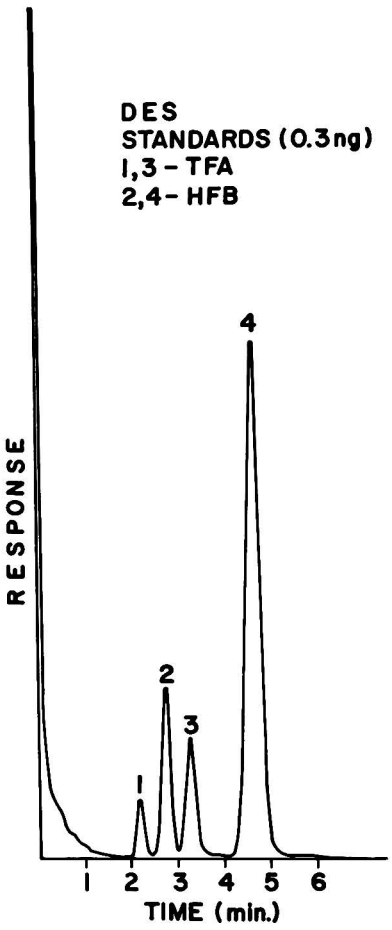


FIG. 1—Gas-liquid chromatogram of DES standards: 1 and 3, *cis*- and *trans*-DES-TFA; 2 and 4, *cis*- and *trans*-DES-HFB; 0.3 ng each.

Hydrolysis of DES-TFA to free DES was relatively facile and was complete in water after 20 min at 55°C or 1 hr at room temperature. Methanol was added as a co-solvent for the nonpolar DES-TFA. At this stage, the presence of free DES in a purified extract requires attention to prevent losses due to evaporation and oxidation. For instance, free DES could not be evaporated and recovered if the solvent were heated or if vacuum evaporation were employed. The polar DES was derivatized in the nonpolar benzene without complete evaporation of benzene to prevent glass-adsorptive losses. The DES derivatives themselves did not show such lability to manipulation. An attempt was made to convert the DES-TFA derivative directly to the HFB derivative by reaction in neat HFB anhydride. This experiment, analogous to transesterification, gave low yields.

Application of these techniques to TFA derivatives of DES from standards and cleaned up liver extracts gave acceptable recoveries as shown in Table 2 and illustrated in Fig. 2. The higher recovery for meat samples when compared with standards treated in the same way at 10 ppb may be caused by the potentiation effect of meat constituents on the GLC column, as was reported by Donoho *et al.* (2).

This procedure has been designed for confirmation and is only semiquantitative in practice. Advantages are that it can be performed

Table 1. Mass spectral data of fluorinated derivatives of DES

Derivative	Conditions[a]	Strong peaks, m/e
DES-HFB	probe	660,[c] 631, 341
	GLC-MS, peak 2[b]	660,[c] 631, 341
	GLC-MS, peak 4	660,[c] 631, 341
DES-TFA	probe	460,[c] 431, 241
	GLC-MS, peak 1	460,[c] 431, 241
	GLC-MS, peak 2	460,[c] 431, 241

[a] GLC-MS conditions as given in the text.
[b] *See* Fig. 1.
[c] Molecular ion peak, M^+.

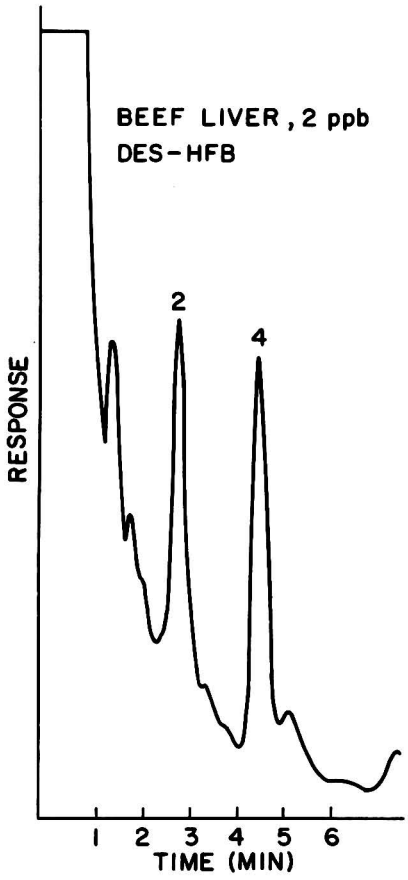


FIG. 2—Gas-liquid chromatogram of purified beef liver extract fortified with 2 ppb DES, purified (1), TFA derivative formed, hydrolyzed, and HFB derivative formed: 2, *cis*; 4, *trans*.

Table 2. Recovery of DES as HFB derivative by hydrolysis and extraction of TFA derivative

Sample	Level, ppb	Amount, ng	Rec., %[a]
Standards	2	100	69
	4	200	74
	10	500	79
Beef liver ext purified as in ref. 1	2	100	60
	4	200	72
	10	500	110

[a] Average of ≥2 determinations.

in a few hours, it uses relatively simple chemical techniques and equipment, and detection of the new derivative so formed is more sensitive than with the original.

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DRUGS IN FEEDS

Gas-Liquid Chromatographic Determination of Diethylstilbestrol at Low Levels in Mixed Feeds

RICHARD G. EINIG
Ralston Purina Co., Checkerboard Square, St. Louis, MO 63188

A gas-liquid chromatographic method for the determination of diethylstilbestrol (DES) at 2 ppb in mixed feeds and ingredients is presented. The DES is extracted into ethanol, the solvent is evaporated, and the residue is redissolved in chloroform. The extract is purified by washing the chloroform solution with carbonate buffer and transferring the DES to sodium hydroxide. The sodium hydroxide is washed with chloroform, the pH is adjusted to 10.3, and the DES is transferred to chloroform. The extract is derivatized with heptafluorobutyric anhydride, chromatographed on an SE-30 glass column, and measured with a ^3H electron capture detector. The method has been used for routine testing of a wide variety of feeds and ingredients.

Diethylstilbestrol (DES) has for many years been incorporated into feed formulations to improve the feed efficiency of steers. Because of its electrostatic nature, cross-contamination of nonmedicated feeds is an ever-present danger and methodology capable of measuring ppb levels is needed. Sensitive methods of analysis for DES in feeds have been reported, using fluorometric, colorimetric, or gas chromatographic measurements (1–5; F. W. Fox & J. L. Koester, 1972, Elanco Products Co., Indianapolis, IN 46206, unpublished results). Only the fluorometric determination (1) has the requisite sensitivity but the extensive purification necessary to remove interfering fluorescent materials limits its usefulness for routine, high volume analysis.

Donoho *et al.* (6) published a method for determining 1 ppb DES in animal tissue which involved measuring the bis-dichloroacetyl ester by electron capture gas-liquid chromatography (GLC). The authors noted that, at low DES concentrations, a "potentiation effect" may occur whereby a standard diluted in solvent may give a lower response than the same standard added to a derivatized control sample. The effect is compensated for by calculating samples against a standard combined with the control sample. When this derivative was applied to the analysis of mixed feeds in our laboratory, the "potentiation effect" was difficult to compensate for because of the variety of matrices encountered, each with its own degree of potentiation.

The method described in this paper combines established extraction and purification techniques with the electron capture GLC determination of the diheptafluorobutyryl ester (DES-HFB). It has been successfully used in our laboratory for quality control of mixed feeds and feed ingredients at a level as low as 2 ppb.

METHOD

Apparatus and Reagents

(a) *Gas chromatograph.*—Varian Model 1400, or equivalent, with ^3H electron capture detector, 6′ × 2 mm id glass column packed with 3% SE-30 on 100–120 mesh Varaport 30. Operating conditions: nitrogen carrier gas, 35 ml/min; temperatures (°C)—injector 225, detector 215, column 170; electrometer range 10^{-9} amp full scale; attenuation as needed.

(b) *Diethylstilbestrol standard solution.*—125 ng USP Diethylstilbestrol (Elanco Products Co.)/ml $CHCl_3$.

(c) *Carbonate buffer (pH 10.5).*—Dissolve 50.6 g $NaHCO_3$ and 21.3 g NaOH in 1 L deionized water and adjust pH to 10.5±0.2.

(d) *Heptafluorobutyric anhydride.*—PCR, Inc., Gainesville, FL 32602.

Extraction

Weigh 20 g ground sample into 300 ml Erlenmeyer flask and add 100 ml ethanol (formula 3A). Cover flask and shake 1 hr on mechanical shaker. If limit of detection is ≥50 ng DES/g sample, pipet 25 ml supernate into 200 ml Erlenmeyer flask. For limit of detection <50 ng DES/g sample, filter contents of flask into 200 ml Erlenmeyer flask and wash precipitate with ca 25 ml ethanol.

Purification

Evaporate solvent just to dryness on steam bath under stream of nitrogen. Quantitatively transfer sample to 125 ml separatory funnel, using 30 ml

$CHCl_3$. Add 30 ml pH 10.5 carbonate buffer and 5 g NaCl to funnel and swirl for 30 sec. Let 2 phases separate completely and drain $CHCl_3$ layer into another 125 ml separatory funnel. Add 25 ml $CHCl_3$ to carbonate buffer, shake 30 sec, let phases separate, and combine $CHCl_3$ fractions. Discard carbonate buffer. Add 10 ml 1*N* NaOH to $CHCl_3$ fraction, and swirl 30 sec. Let 2 phases separate completely and drain $CHCl_3$ layer into another 125 ml separatory funnel. Add 10 ml 1*N* NaOH to $CHCl_3$ fraction, shake 30 sec, let phases separate, discard $CHCl_3$, and combine NaOH fractions. Extract NaOH fraction with 25 ml portions of $CHCl_3$ until it is free of emulsion (3–4 extractions). Let 2 phases separate completely after each extraction and discard $CHCl_3$.

Adjust pH to 10.3±0.1, using ca 50 ml 1*M* $NaHCO_3$. Add 10 ml $CHCl_3$ to separatory funnel and shake 1 min. Let 2 phases separate completely and drain $CHCl_3$ through ca 5 g anhydrous Na_2SO_4 into 50 ml screw-cap centrifuge tube. Repeat 10 ml $CHCl_3$ extractions of sample twice more, wash Na_2SO_4 with 5 ml $CHCl_3$, and combine $CHCl_3$ fractions in centrifuge tube. Evaporate to dryness on steam bath under stream of nitrogen.

Esterification

Add 1 ml hexane, 1 ml tetrahydrofuran, and 0.5 ml heptafluorobutyric anhydride to centrifuge tube and seal with Teflon-lined cap. Heat tube 20 min in 75°±2°C water bath, cool to ambient temperature, add 30 ml 7% $NaHCO_3$, reseal tube, and shake vigorously. Immediately transfer portion of top layer to small screw-cap tube for assay.

Control Sample and Direct Standard

Prepare control sample by fortifying desired aliquot of ethanol extract of blank control sample with 2 ml DES standard solution and continuing with *Purification* and *Esterification*.

Prepare direct standard by transferring 2 ml DES standard solution to 50 ml screw-cap centrifuge tube, evaporating to dryness on steam bath under stream of nitrogen, and continuing with *Esterification*.

Gas-Liquid Chromatography

Inject 5 µl direct standard to determine whether esterification has been successful. Then inject 5 µl control sample and determine recovery of DES against direct standard. If esterification was successful and DES recovery is ≥80%, inject experimental samples. Inject direct standard at least every 4 hr to compensate for slight changes in instrument response.

Calculation

Use peak height of only *trans*-DES peak to determine ppb DES in sample.

ppb DES = (mm peak height sample/mm peak height standard) × (ng standard/1 ml) × (ml final sample volume/g original sample weight) × (ml sample aliquot/ml original sample volume)

Results and Discussion

After the method was developed and evaluated under research conditions, it was introduced into the quality control laboratory for routine use. A mixed feed that was negative at maximum sensitivity was used for a blank sample. Each set of 10–15 samples contained this blank fortified with 2 ml DES standard solution as a control. All of the samples, including the control sample, were calculated against a direct standard. Over a period of a year and under routine conditions, 22 control samples fortified with 50 ppb DES and 5 samples with 12.5 ppb were analyzed. The average recovery of DES in the former was 49.8 ppb and in the latter 12.7 ppb. The standard deviation was 9.04 (per cent relative standard deviation 18.2) and 2.21 (per cent relative standard deviation 17.4), respectively. These data, rather than data collected under research conditions, are presented in order to report a more realistic value for the precision and recovery of the method in daily use. Clearly, the relative variation is not level-dependent and, therefore, the amount of extract carried through purification and esterification is really a matter of convenience. Fifty ppb was selected as the cutoff in order to hasten the purification step and still maintain a reasonable DES concentration.

An emulsion forms when the sample in chloroform is washed the first time with pH 10.5 carbonate buffer. Shaking the separatory funnel at this point will result in a heavy emulsion although sodium chloride partially inhibits it. When 1*N* NaOH is added to the sample, the same problem may occur and swirling the funnel is again recommended. The smaller the aliquot of extract taken, the shorter the time needed to break emulsions.

The average recovery of DES-HFB demonstrates that DES in a mixed feed matrix can be accurately determined when calculated against a standard in solvent only. In a chromatogram of about 1 ng DES-HFB, identification of the 2 isomer peaks can be difficult because of the

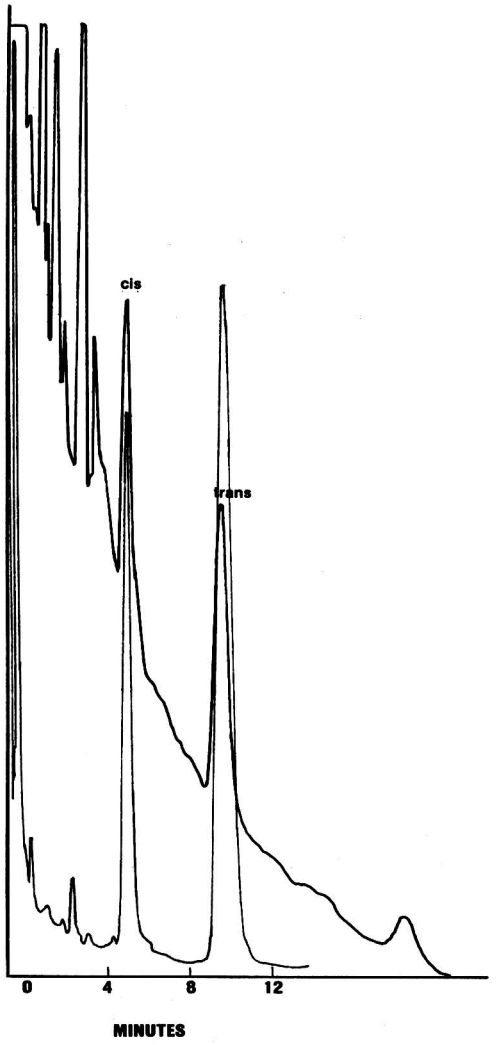


FIG. 1—Gas chromatograms of DES-HFB standards. Dark curve, 125 ng DES derivatized and diluted with 1 ml hexane; light curve, 10 μg DES derivatized and diluted with 25 ml hexane.

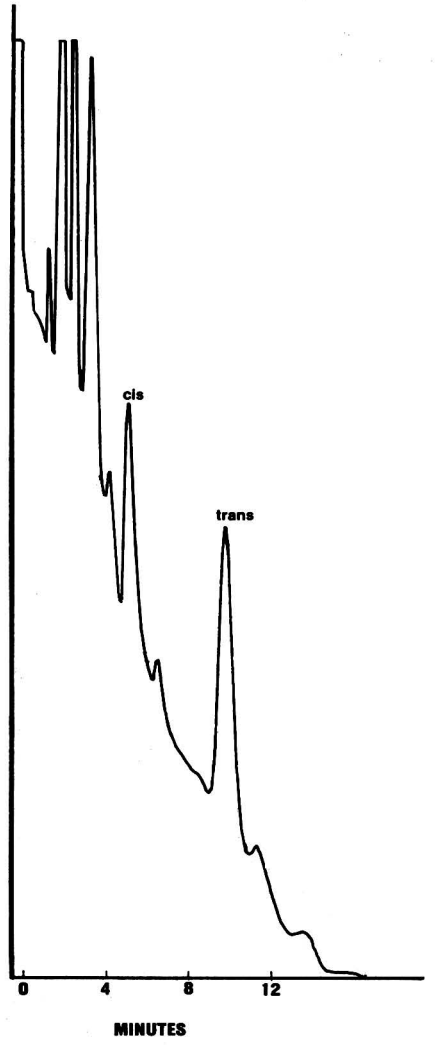


FIG. 2—Gas chromatogram of DES-HFB in fortified mixed feed control sample.

other highly electronegative compounds present due to heptafluorobutyric anhydride. Figure 1 shows a typical standard chromatogram with the *cis* and *trans* isomers labeled. Superimposed on the standard is the chromatogram obtained when 10 μg DES was esterified as described in the method and diluted to 25 ml with hexane before injection. A standard prepared like this facilitates establishing GLC conditions on a freshly prepared column because the chromatogram is free of major peaks other than the 2 isomer peaks. A chromatogram of the control sample (Fig. 2) appears very similar to that of the standard except that most feed matrices contain many compounds that elute for 20–30 min after the *trans*-DES-HFB peak. In our laboratory, the oven temperature is raised after DES has eluted to quickly clear the column.

An analyst trained in this method can perform 6–10 analyses a day, depending on the level of detection desired. It has been successfully used in our quality control laboratory to analyze many different types of mixed feeds and feed ingredients at the very low ppb level.

Acknowledgment

The author thanks Tamara Boling for the analytical data.

Received December 29, 1975.

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ATTENTION

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.

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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EXTRANEOUS MATERIALS IN FOODS AND DRUGS

Collaborative Study of the Extraction of Light Filth From Canned Crabmeat

RUSSELL G. DENT and J. RICHARD GORHAM
Division of Microbiology, Food and Drug Administration, Washington, DC 20204

Canned crabmeat is boiled with mineral oil in a Wildman trap flask. The trappings are transferred to a Corning percolator and cycled at least 3 times. Average recoveries from 7 collaborators were 82.7% for rodent hairs and 98.0% for flies. The proposed method has been adopted as official first action.

There is no official AOAC method for the extraction of light filth from canned crabmeat; therefore, a study was initiated to develop a method for this product. Light filth in canned crabmeat normally consists of contamination originating from the usual insect and rodent pests of packing houses; in addition, crabmeat is also subject to contamination by insects living in the water used in packing procedures. In the method described, minute endoparasitic barnacles were often recovered; they were not counted as contaminants. Rodent hairs, which are striated, were easily distinguished from unstriated crab setae. Rodent hairs and flies were used as the spiking materials in the collaborative study.

Experimental

Seven collaborators were requested to analyze 6 samples each of crabmeat. One additional sample was included as a practice sample. All samples were spiked with light filth which consisted of 2 levels of 10 or 20 whole *Drosophila* sp. fruit flies, and 10 or 20 rodent hairs, each >4 mm.

In some cases, during the development of the method, we noted that the oil layer was deep yellow-orange in different lots of crabmeat. In these instances care must be taken to use the specified detergent concentration and isopropanol as described in the method for cleaning the percolator.

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

METHOD

44.B02 Light Filth—Official First Action

Transfer entire contents of ≤7 oz (200 g) can (or 7 oz portion of larger sample) to 2 L trap flask, **44.002(i)(3)**. Thoroly wash can (and parchment, if present) with tap H_2O and add washings to flask. Add ca 800 ml hot (55–70°) tap H_2O. With mag. stirring, **44.004(b)**, heat to bp. Add 50 ml mineral oil, **44.003(y)**, and stir mag. 3 min while continuing to boil. Remove flask from heat, fill with hot tap H_2O, and let stand 30 min, stirring gently by hand at 10 and 20 min. Trap off into 400 ml beaker. Add 30 ml mineral oil to trap flask. With stirring bar spinning at max. speed, disperse oil throughout aq. (lower) phase, stirring by hand. Stir mag. 5 min at max. speed. Fill flask with hot tap H_2O and let stand 20 min, stirring gently by hand at 10 min. Trap off into same beaker. Wash mouth of trap flask with isopropanol and decant washings into beaker.

Transfer to 2 L percolator, **44.002(i)(2)**, contg ca 250 ml H_2O. Rinse beaker into percolator and bring vol. to ca 1700 ml with H_2O at room temp., ca 20°. Let stand 3 min. Drain oil to 250 ml mark and discard drainings. Repeat fill and drain cycle ≥2 more times. Drain remaining oil and H_2O into original 400 ml beaker. Wash percolator sides with 1% detergent soln, **44.003(q)**, and isopropanol, and collect washings in beaker. Filter onto ruled paper and examine microscopically. If filtering action slows, use new filter paper.

Results and Recommendations

The collaborative recoveries of rodent hairs and flies are given in Table 1. Some collaborators analyzed the samples one at a time, while others analyzed them in groups of 2, 3, or 6. In general, the actual time involved for analysis, excluding reading the papers, was about 2 hr/sample. All collaborators obtained filter papers that were easy to read and which contained very little

The recommendation of the Associate Referee, R. G. Dent, was approved by the General Referee and by Subcommittee F and was adopted by the Association. *See* (1976) *JAOAC* **59**, 397.
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Table 1. Collaborative results for the recovery of added rodent hairs and flies from canned crabmeat

	Flies						Hairs					
	20 added			10 added			20 added			10 added		
Coll.	1	2	3	4	5	6	1	2	3	4	5	6
A	20	20	20	10	10	10	20	20	19	9	10	10
B	20	20	20	10	10	10	20	16	18	10	10	8
C	18	20	20	10	10	10	16	19	14	7	9	9
D	20	20	19	10	9	10	13	17	11	10	7	9
E	19	20	18	10	10	9	20	14	7	8	4	9
F	18	19	20	10	10	10	18	15	18	9	6	9
G	18	20	20	10	10	10	17	16	13	7	10	9
Av. rec., %			98.0						82.7			
Std dev.			3.7						16.0			
Coeff. of var., %												
repeatability			3.77						19.31			
reproducibility			3.88						21.95			

crab tissue. Both the authors and the collaborators read the filter papers for light filth; identical readings were obtained in all cases.

Overall recovery percentages of spike elements by 7 collaborators were 82.7 for rodent hairs and 98.0 for flies. It is recommended that the proposed method be adopted as official first action for the determination of light filth in canned crabmeat.

Acknowledgments

We wish to acknowledge the help of the collaborators: C. E. Highfield, Health and Welfare Canada, Scarborough, Ontario, Canada; and the following Food and Drug Administration personnel: James P. Karpus, Chicago; J. LaRose, Seattle; P. Skyrme, Boston; B. B. Smith, Jr., Atlanta; J. J. Thrasher, Washington, DC; and R. A. Trauba, Minneapolis.



Collaborative Study of a Method for the Extraction of Light Filth from Ground Mace and Ground Caraway Seed

JOEL J. THRASHER
Division of Microbiology, Food and Drug Administration, Washington, DC 20204

A new method has been developed for the extraction of light filth from ground mace and ground caraway seed. The method uses a 2-step chloroform-isopropanol pretreatment followed by separation of the light filth from 40% isopropanol with mineral oil. The collaborative study resulted in clean filter papers with more reproducible recoveries of light filth elements than the official first action method, 44.116(b). The method has been adopted as official first action.

A method for the extraction of light filth from ground mace and ground caraway has been developed. The method consists of a chloroform-isopropanol pretreatment in a filter paper cup, wet sieving, transfer to a Wildman trap flask, boiling, addition of Tween 80-EDTA mixture to the hot flask contents, and then flotation from 40% isopropanol with mineral oil. In developmental work, the method was shown to give reproducible recoveries (Tables 1 and 2).

Collaborative Study

Each of 7 collaborators examined 6 samples of ground mace by the proposed method and 6 samples by the official first action method **44.116(b)**. Samples were spiked with 20 insect fragments (0.20 × 0.20 mm) and cut rodent hairs (ca 1 mm each) at 2 levels (8 and 16). The filth elements on the filter papers were counted by the collaborators and the papers were then sent to the author for additional counting.

METHOD

Reagents

(a) *Tween 80–40% isopropanol.*—To 40 ml Polysorbate 80 (ICI United States, Inc.), add 210 ml 40% isopropanol and mix.

(b) *Tetrasodium EDTA soln.*—Dissolve 5 g Na_4EDTA in 250 ml 40% isopropanol with stirring and filter.

(c) *Mixed reagent.*—Mix equal vols of (a) and (b), (stable for several weeks).

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

Pretreatment

Weigh 25 g sample into 32 cm filter paper cup, 400 ml–1 L, **44.002(k)**. Add 400 ml $CHCl_3$ to cup in sample beaker and boil gently 10 min on hot plate in fume hood. Drain or aspirate to slow drip. Return cup to empty beaker, add 400 ml isopropanol, and boil gently 5 min. Transfer cup to buchner and aspirate to slow drip.

Isolation

Place filter paper cup in No. 230 sieve, **44.002(u)**, and wash sample into sieve with gentle stream of hot tap H_2O. Sieve with forceful spray of hot (55–70°) tap H_2O until rinse is clear. Wet residue on sieve with 40% isopropanol and transfer quant. to 2 L trap flask, **44.002(i)(3)**, using 400 ml 40% isopropanol. Boil gently 10 min with

Table 1. Recovery of background insect fragments and added rodent hairs and elytral squares from ground mace[a]

Sample	Background count	Elytral squares[b]	Rodent hairs[b]
1	30	20	19
2	18	19	19
3	17	20	18
4	24	18	16
5	35	19	18
6	27	18	19
Av.	25.2	19.0	18.2
Rec., %	—	95.0	91.0
Coeff. of var., %	27.69	4.68	6.44

[a] Developmental study by Associate Referee.
[b] Twenty of each added.

Table 2. Recovery of added elytral squares and rodent hairs from clean ground caraway[a]

Sample	Elytral squares[b]	Rodent hairs[b]
1	20	20
2	19	20
3	18	20
4	18	20
5	19	20
6	19	18
Av.	18.8	19.7
Rec., %	94.0	98.5
Coeff. of var., %	3.98	4.17

[a] Developmental study by Associate Referee.
[b] Twenty of each added.

Table 3. Collaborative results for the recovery of insect fragments and rodent hairs from ground mace by the proposed method[a]

	Insect fragments (20 added)						Rodent hairs: 8 added			Rodent hairs: 16 added		
Coll.	1	2	3	4	5	6	1	2	3	4	5	6
A	20	20	20(22)	20	19	20	8	8	7	14	16	16
B	18(19)	20(21)	18(19)	20	20	19	6	8	6	15(16)	15	14(15)
C	14	20	17	18	20	18	8	8	8	16	14	16
D	19(20)	10	17(22)	19	20(21)	17	8	5	7	12	16	15(17)
E	17(13)	20(19)	16(15)	19(15)	19(18)	19(17)	8	7(5)	8	14(15)	14	15(16)
F	19(14)	19	19	19(17)	18(20)	18	8	7	5(6)	13(16)	12	10(11)
G	16(19)	20	17	18	NR[b]	NR	8	7	NR	14(16)	13(16)	NR
Av., %				92.0(91.7)				90.6(90.0)			88.8(93.4)	
Std dev.[a]				1.96(2.49)				1.02(1.05)			1.61(1.64)	
Coeff. of var., %												
repeatability				10.77(12.76)					11.469(13.341)			
reproducibility				10.68(13.68)					13.011(14.038)			

[a] Associate Referee counts; collaborators' counts, if different, are given in parentheses.
[b] NR = not recorded; analyst was aware of deviation from method.

Table 4. Collaborative results for the recovery of insect fragments and rodent hairs from ground mace by the official method[a]

	Insect fragments (20 added)						Rodent hairs: 8 added			Rodent hairs: 16 added		
Coll.	1	2	3	4	5	6	1	2	3	4	5	6
A	0	5	6	19	14	9	6	1	6(7)	3	11	3
B	20	17	14	20	19(20)	17(18)	6	6	7	15(16)	15	14(15)
C	14	18(17)	19	12	17	L[b]	7	6	L[b]	12(13)	13(12)	14(17)
D	11	17	15	16(21)	16	12	7	6	6	9	13(12)	9(10)
E	12(13)	17(16)	15(16)	11	13	16	6	6	5	12(11)	0	4
F	18	19	15	18	13	19(20)	7	8(9)	5	15	13	13
G	17	17	18(19)	20	19(18)	17	8	7	7	12	15	15
Av., %				75.8(76.7)				76.8(78.2)			68.4(69.6)	
Std dev.				4.34(4.46)				1.46(1.55)			4.60(4.79)	
Coeff. of var., %												
repeatability				22.18(22.84)					25.08(26.06)			
reproducibility				29.15(29.55)					33.72(25.17)			

[a] *See* footnote *a*, Table 3.
[b] L = lost.

stirring. Remove from heat and immediately add 100 ml mixed reagent (c) slowly down stirring rod. Hand stir 1 min with gentle rotary motion. Let stand undisturbed 10 min. Dil. to 800 ml with 40% isopropanol, add 50 ml mineral oil, 44.003(y), and stir mag. 5 min, 44.004(b).

Fill flask with 40% isopropanol and let stand 30 min with intermittent stirring. Trap off, and repeat as above, using 35 ml mineral oil for second trapping.

Results and Discussion

No adverse comments were received from any collaborator. The Associate Referee's counts of added spike material, rodent hairs and elytral squares, did not differ appreciably from those reported by the collaborators, as shown in Tables 3 and 4. Because of great variability of background filth, it was not reported.

Recommendation

On the basis of statistical analysis, the proposed method is significantly better in reproducibility and repeatability than the official method for insect fragments and/or rodent hair spikes and, although the average recoveries of insect and rodent hair spikes are greater by the proposed method, the difference was of borderline statistical significance ($P > 0.05$).

The cleanliness of the filter papers, the avoidance of dangerous chemicals, and the mean recoveries of added filth are such that the Associate Referee recommends this method be adopted as official first action to replace method **44.116(b)** for these 2 products.

Acknowledgments

The Associate Referee wishes to express his appreciation to the following collaborators: C. E. Highfield, Health Protection Branch, Searborough, Ontario, Canada; Miranda Weaver, Ohio Department of Agriculture, Reynoldsburg, OH; Mary L. Young, Kraft Foods, Allentown, PA; and the following Food and Drug Administration analysts: R. B. Bradicich and Barbara Dalrymple, Dallas; Maria Chapiot, Boston; R. G. Dent and Ruth Rupp, Washington, DC; Wayne Palmer, Minneapolis.

Foster McClure prepared the statistical evaluation of the data.

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

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In 1969, the AOAC established a registration fee for attendance at the Annual Meeting of the Association. The fee has been instituted to help defray the meeting costs. For the convenience of attendees, special advance prepaid registration forms are printed on the insert at the back of this issue of *JAOAC*.

COSMETICS

Colorimetric Determination of Zirconium in Antiperspirant Aerosols

PAUL BEAVIN, JR.
Food and Drug Administration, 1560 E. Jefferson Ave, Detroit, MI 48207

A rapid direct dilution procedure for the estimation of soluble zirconium and a fusion procedure for the determination of total zirconium (soluble and insoluble forms) in cream base concentrates prepared from antiperspirant aerosols are described. The direct dilution procedure involves extraction of soluble zirconium with HCl (55+45). The filtered extract is reacted with alizarin red S to form a stable colored complex which is measured spectrophotometrically. The fusion procedure involves ashing the aerosol concentrate followed by fusion of the ash with potassium pyrosulfate to form an acid-soluble melt. Zirconium is precipitated from solution as the hydroxide and washed to eliminate interfering ions, particularly sulfate. After redissolving in HCl (55+45) and reacting with alizarin red S, total zirconium is measured. Zirconyl chloride octahydrate, assayed gravimetrically by hydroxide precipitation and conversion to the oxide, is used as the zirconium reference standard. Concentration range of zirconium measured was 200–500 μg/100 ml. Recoveries of standard zirconium added to commercial aerosols labeled to contain aluminum and zirconyl hydroxychlorides ranged from 97 to 101% by the fusion procedure. Analysis of these aerosols by direct dilution gave generally slightly lower results than by fusion. It is recommended that the procedures be collaboratively studied after further testing of their general applicability to a variety of drugs and cosmetics.

Zirconium compounds are formulated in various drug and cosmetic preparations including aerosol sprays, creams, lotions, and ointments. Zirconyl chloride (zirconium oxychloride) is used as an antiperspirant and is usually combined with aluminum compounds for this purpose. Zirconium oxide or carbonate is combined with various other drugs in commercial preparations used for poison ivy and related dermatitis.

The Associate Referee's investigation was initially directed toward the development of analytical procedures for zirconium compounds in antiperspirant creams. However, recent concern with the risk of lung granuloma disease associated with the use of antiperspirant aerosols prompted a diversion to emphasis on the latter preparations at this time.

Newburger *et al.* (1) described an x-ray fluorescence spectroscopic method for zirconium in antiperspirants. The instrumentation needed for this technique is very expensive and not generally available. Grossman and Inglis (2) described an atomic absorption spectrometric procedure for zirconium in biological materials. However, this technique is relatively insensitive to zirconium and is subsequently prone to interferences. Polarography was discarded from consideration because zirconium does not produce waves suitable for direct polarographic analysis.

Mayer and Bradshaw (3) and Wood and McKenna (4) used the sodium salt of alizarin sulfonic acid (alizarin red S) as a colorimetric reagent for determining zirconium in magnesium and titanium alloys, respectively. While various other reagents have been employed to form colored complexes with zirconium, alizarin red S was selected for further investigation because Wood and McKenna showed that it formed a stable color, had more than adequate sensitivity for our immediate needs, and was highly specific for zirconium in the presence of anticipated interferences, particularly aluminum.

Some of the zirconium compounds in use have very limited solubilities in aqueous media. Zirconium oxide, in particular, is insoluble in water and only slightly soluble in mineral acids. The possible presence of insoluble zirconium compounds in antiperspirant preparations and the fact that zirconium oxide and carbonate are used in various other preparations prompted the decision to develop a sample preparation procedure generally applicable to zirconium compounds in drugs and cosmetics.

Newburger *et al.* (1) fused the ashed sample with sodium borate powder which rendered the

ash soluble in citric acid solution. This procedure was somewhat tedious and time consuming because the fused melt was not easily dissolved. Fusion of the ashed sample with potassium pyrosulfate (5) provided a melt readily soluble in dilute sulfuric acid. Since fusion necessarily introduces a high concentration of ions which adversely affect the color development with alizarin red S, a hydroxide precipitation from ammonia (6) was incorporated in the proposed procedure to eliminate those ions. The washed zirconium hydroxide is redissolved in hydrochloric acid solution and subjected to color development with alizarin red S.

Detailed procedures for sample preparation and determination of total zirconium in cream base concentrates prepared from antiperspirant aerosols are presented. In addition, a rapid direct dilution procedure for estimating the soluble zirconium content is presented. Results of the application of these procedures to the analysis of commercial aerosol preparations are also presented.

METHOD

Reagents

(a) *Potassium pyrosulfate.*—Fisher Certified reagent. Grind to fine powder with mortar and pestle.

(b) *Alizarin red S (sodium alizarin sulfonate) solution.*—Dissolve 1.5 g alizarin red S (Allied Chemical indicator) in 300 ml hot water. Cool, and filter through double layer of rapid, medium porosity paper (Whatman No. 12, 24 cm folded filter paper, or equivalent). Dilute filtrate to 1 L with water. Refilter as above. Solution is stable $\geq$1 month.

(c) *Zirconyl chloride octahydrate.*—Fisher Certified reagent containing 31.2% zirconium (determined by standard assay procedure described in this paper) was used to prepare standard zirconium solutions.

Assay of Standard

Accurately weigh 500–600 mg zirconyl chloride octahydrate into 400 ml beaker and dissolve in 50 ml water. Add 4 g NH_4NO_3 and warm on steam bath to ca 50°C. Slowly add, with stirring, 100 ml NH_4OH and continue heating 20 min. Filter while hot through 15 cm Whatman No. 42 (or equivalent) paper. Complete transfer of precipitate with several portions of 2% NH_4NO_3 in NH_4OH (2+98). Carefully fold and place filter paper in platinum crucible (ca 50 ml capacity). Dry in 105°C oven. Partially cover crucible and gently heat with Meker burner until filter paper is well charred. Continue heating at maximum temperature of burner to constant weight.

Zirconium, % = (wt zirconium oxide residue × 74.03)/wt zirconyl chloride octahydrate.

Preparation of Standard Curve

Dissolve zirconyl chloride octahydrate equivalent to 200 mg zirconium in 70 ml water in 200 ml volumetric flask. Add 110 ml HCl, cool, and dilute to volume with water. This stock solution can be used $\geq$1 week. Pipet 2.0, 5.0, 10.0, and 15.0 ml stock solution into separate 100 ml volumetric flasks. Dilute each to volume with HCl (55+45) solution and mix. Pipet 5 ml of each dilute standard and 5 ml HCl (55+45) (blank solution) into separate 100 ml volumetric flasks for color development. Add 10.0 ml alizarin red S solution and 8 ml water to each. Swirl flasks and place in 75±5°C water bath. Monitor temperature of bath with thermometer in 100 ml volumetric flask containing ca 23 ml water, added at room temperature and simultaneously placed in bath. Remove flasks 6.5±1 min after solution temperature reaches 70°C, and cool 20 min at room temperature. Dilute to volume with water and mix. Concentrations of zirconium in color development flasks are 0, 100, 250, 500, and 750 μg/100 ml. Measure color developed at 525 nm against blank solution, using 2 cm cells (on recording spectrophotometer, scan from 700 to 460 nm). Plot absorbance vs. concentration zirconium in 100 ml color development flasks.

Preparation of Sample

Remove cap and any paper wrap from can. Record weight of can to nearest 0.01 g. Replace cap and freeze contents by inverting can in beaker containing mixture of solid CO_2 and acetone for $\geq$1 hr. Transfer can to smaller beaker in exhaust hood and open bottom end with can opener, but keep end attached to can (do *not* sever completely). Let volatile gases escape at room temperature. Remove dip tube after initial thawing when gas evolution has subsided but keep tube with can in beaker. Place beaker on steam bath and heat gently to evolve higher boiling gases. Increase heat slowly and maintain until bubbling subsides. Place beaker containing can and dip tube in 70°C forced draft oven for 45 min. Increase temperature to 115°C and maintain 2.5 hr, stirring can contents occasionally with stainless steel spatula. Remove from oven and cool to room temperature. Thoroughly mix contents of can, including any portion clinging to sides, to form homogeneous concentrate. Weigh capped can plus contents, spatula, and dip tube. Stir contents

ca 1 min and weigh again. Repeat stirring and weighing until weight is constant within 0.01 g. Record weight and immediately transfer contents to glass-stoppered weighing bottle (complete transfer unnecessary). Stopper bottle and protect from further weight loss by opening only when necessary.

Thoroughly remove remaining contents from can, spatula, and dip tube with water and alcohol. Dry cap, can, spatula, and dip tube to constant weight in 110°C oven. Obtain weight of concentrate by subtracting latter weight from weight previously recorded after drying contents. Obtain weight of intact can contents by subtracting weight of clean, dry can, cap, and dip tube from gross weight initially recorded.

Determination

Record gross weight of prepared sample in weighing bottle. Mix sample briefly and remove ca 1 g with spatula. Calculate weight removed as difference in gross weight.

(a) *Direct dilution procedure for soluble zirconium compounds.*—Transfer sample as completely as possible from spatula to lower half of 600 ml beaker. Thoroughly wipe spatula with small piece of filter paper and add paper to beaker. Add 5 ml alcohol and break up sample with glass rod. Slowly add 200 ml HCl while stirring vigorously. Heat to boiling on steam bath. Boil 2–3 min and immediately transfer through funnel to 1 L volumetric flask. Complete transfer with one 200 ml and one 150 ml portion of hot HCl. Warm flask on steam bath and shake vigorously 3–4 min. Rinse beaker with 450 ml water, add rinse to flask, and mix thoroughly. Cool to room temperature, dilute to volume with HCl, and mix thoroughly. Let undissolved material coagulate and settle. Filter portion through double layer of 24 cm Whatman No. 12 (or equivalent) folded paper, discarding first 50 ml. Make appropriate dilutions with HCl (55+45) to obtain zirconium concentration of 40–100 μg/ml. Pipet 5 ml into 100 ml volumetric flask and proceed with color development as in preparation of standard curve. Calculate per cent zirconium in intact can contents:

$$\text{Zirconium, } \% = (C/W_s) \times (W_c/W_i) \times (F/10^4)$$

where C = concentration (μg/100 ml) of zirconium read from standard curve; W_s, W_c, and W_i = weights (g) of sample, prepared concentrate, and intact can contents, respectively; and F = appropriate dilution factor.

(b) *Fusion procedure for total zirconium.*—Transfer sample as completely as possible to lower third of platinum crucible by spreading on bottom and sides. Thoroughly wipe spatula with small piece of filter paper and add paper to crucible. Cover sample with 5 g potassium pyrosulfate. Place platinum cover on crucible, leaving small opening. Place crucible in muffle oven at room temperature. Increase temperature to 120°C and maintain 30 min. Increase slowly in 10° increments (ca 40°/hr) until smoking starts (ca 160°C). If necessary, open oven door during smoking to slow temperature rise and prevent too rapid combustion which may cause sample loss. After smoking ceases, again increase temperature in 10° increments to 315°C, allowing ca 5 min after reaching each temperature or waiting until any resumed smoking ceases before increasing to next higher temperature. Maintain at 315°C for 1 hr. Increase temperature to 450°C in 15–20° increments, allowing 5–10 min after reaching each temperature. Finally increase temperature to 650°C and maintain 1 hr.

Remove crucible and let cool to room temperature. Remove cover and add 8.0 g potassium pyrosulfate. Grasp crucible with platinum tongs, swirl, and heat gently over Meker burner. Apply heat carefully to avoid foaming, spattering, and excessive smoking. Tilt and rotate crucible to incorporate all ash into melt. After obtaining clear melt (no ash particles remaining unincorporated), remove crucible from heat and continue tilting and rotating until melt solidifies on crucible walls. Let cool to room temperature. Place crucible and cover in 800 ml beaker containing 150 ml water and 10 ml H_2SO_4. Place beaker on steam bath and dissolve melt with the aid of a glass stirring rod. Remove crucible and cover, and rinse with water into beaker. Add 4.0 g NH_4Cl and stir to dissolve. Add slowly, with stirring, 100 ml NH_4OH. Let zirconium hydroxide precipitate on steam bath ca 15 min. Filter hot solution through 15 cm Whatman No. 42 (or equivalent) paper. Wash beaker and precipitate on paper with three 30 ml portions of hot 2% NH_4Cl in NH_4OH (2+98) solution. Place paper containing precipitate in original 800 ml beaker. Add 100 ml HCl (55+45) and warm gently on steam bath to dissolve precipitate. Filter warm solution through 15 cm S&S black No. 589 (or equivalent) paper into 200 ml volumetric flask. Wash beaker containing original paper with several small portions of HCl (55+45) and filter washes into flask. (*Note:* Procedure *cannot* be delayed up to this point after precipitation of zirconium hydroxide because acid-insoluble oxide may form on prolonged standing.) Dilute to volume with HCl (55+45) and mix. Make appropriate dilutions with HCl (55+45) to obtain zirconium concentration of 40–100 μg/ml.

Pipet 5 ml into 100 ml volumetric flask and

proceed with color development as in preparation of standard curve. Calculate per cent zirconium in intact can contents, using formula given in *Determination* (a).

Results and Discussion

The accuracy of the fusion procedure applied to commercial products was initially evaluated through recoveries of zirconium added as zirconyl chloride octahydrate to concentrates prepared from 5 different brands of aerosol antiperspirants labeled to contain aluminum hydroxychloride as the only active ingredient (quantity not declared on label). Results are reported in Table 1.

Although aluminum has been reported to have a negligible effect on the alizarin red S color development (3, 4), a study involving the addition of various concentrations of aluminum to zirconium solutions prior to color development was performed to verify these reports. Solutions containing 500 μg zirconium were fortified with 250, 500, 750, 1000, 2000, and 4500 μg aluminum; the zirconium analyses were 499, 499, 502, 502, 501, and 507 μg. No significant effect was found for aluminum levels up to and including 4 times the zirconium concentration. At a 9:1 ratio of aluminum to zirconium, the noticeable increase (about 1%) in the absorbance may be indicative of some interference but is likely due in part to analytical error. Mayer and Bradshaw (3) reported the effect of 100 mg aluminum as equivalent to 0.0075 zirconium (1333:1). The tolerance of the colorimetric step to aluminum far exceeds the anticipated need in antiperspirant preparations.

Two commercial antiperspirant aerosol products (3 different lots of each) labeled to contain both zirconyl hydroxychloride and aluminum hydroxychloride (contents not quantitatively stated) were analyzed by the direct dilution and fusion procedures. The results (Table 2) show reasonable agreement between the 2 procedures; however, results by direct dilution are generally somewhat lower than those obtained by fusion. This is possibly attributable to occasional incomplete extraction of zirconium by the direct dilution procedure because of sample hydrophobicity or the presence of acid-insoluble forms. Recoveries of standard zirconium added to these products and determined by the fusion procedure are reported in Table 3.

Potassium pyrosulfate was chosen as the fusion compound because its fusion product dissolved in 20–30 min compared with several hours for the borax fusion product. Recoveries of zirconyl chloride and zirconium oxide standards from their fusion products with potassium pyrosulfate were excellent. The fusion product is dissolved in dilute sulfuric acid and heated on the steam bath to about 70°C to prevent precipitation resulting from hydrolysis and polymerization (7).

In addition to eliminating interfering ions, especially sulfate, the hydroxide precipitation

Table 1. Recoveries by proposed fusion procedure of zirconium added as zirconyl chloride octahydrate to 2 g samples of concentrates prepared from antiperspirant aerosols containing aluminum hydroxychloride

Product	Zr added, mg	Rec., %
A	32.26	100.9
B	29.18	100.9
C	28.67	101.0
D	28.65	101.1
E	28.39	101.4

Table 2. Analysis of commercial antiperspirant aerosols containing both aluminum hydroxychloride and zirconyl hydroxychloride: comparison of direct dilution procedure results with fusion procedure results

		Zr in intact can contents, %	
Product	Lot	Fusion	Direct diln
F	1	0.528, 0.509, 0.508, 0.507	0.464
	2	0.512, 0.511, 0.491, 0.505	0.490
	3	0.509, 0.504, 0.486, 0.501	0.474
G	1	0.760, 0.758, 0.724, 0.755	0.764
	2	0.727, 0.742	0.725
	3	0.739, 0.739	0.730

Table 3. Recoveries by proposed fusion procedure of zirconium added as zirconyl chloride octahydrate to 1–2 g samples of concentrates prepared from aerosols containing both aluminum and zirconyl hydroxychlorides

Product	Lot	Zr added, mg	Rec., %
F	1	72.78	98.8
		70.05	99.6
Av.			99.2
	2	64.91	101.0
		70.43	99.0
Av.			100.0
	3	72.28	96.9
		66.98	97.7
		62.84	98.2
Av.			97.6
G	1	64.95	99.5
		66.14	97.0
		69.08	98.2
Av.			98.2
	2	72.40	97.4
	3	74.41	99.0

and filtration permit simple adjustment of the proper pH for color development by direct dissolution in HCl (55+45). The strong acidity of this solution also hinders hydrolysis. It is imperative that the hydroxide precipitate does not stand longer than is necessary for complete precipitation because it will become acid-insoluble with aging, probably due to formation of the oxide. The analysis can be delayed following complete dissolution of the precipitate in HCl (55+45).

The zirconium-alizarin red S complex is stable for at least 2 hr. Solutions allowed to stand overnight (about 16 hr) had slightly lower absorbances than when freshly prepared. The color development and spectrophotometric determination are rapid and nearly specific for zirconium. Duplicate reagent blanks carried through the fusion procedure and color development showed baseline absorbance relative to the color development reagent solution. The standard curve for the complex is linear over the range of zirconium concentrations investigated (0–1 mg/100 ml).

The fusion procedure is lengthy but necessary, since the method was developed for general applicability. While the rapid method will give satisfactory results for readily soluble and extractable forms, one cannot always be certain of that situation. The formations of complexes, polymers, or relatively insoluble forms such as the oxide are plausible possibilities in antiperspirants. Furthermore, zirconium is used in other types of preparations which have not been tested in our laboratory but for which the fusion procedure is an absolute necessity at this time.

Conclusions and Recommendation

The proposed fusion procedure is sufficiently specific, sensitive, accurate, and precise for the determination of total zirconium in antiperspirant aerosols. The direct dilution procedure gives results in fair agreement with the fusion procedure when applied to products containing soluble forms and is proposed as a rapid screening method. Although not yet investigated, the fusion procedure should be applicable to all zirconium-containing drug and cosmetic preparations, while the direct dilution procedure should be useful for rapid analysis of most antiperspirant preparations. It is recommended that the procedures be tested on a representative selection of other commercial drug and cosmetic products and that a collaborative study follow.

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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 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**, 399.
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MICROCHEMICAL METHODS

Collaborative Study of the Microanalytical Determination of Phosphorus in Organic Compounds

ROGER A. LALANCETTE, AL STEYERMARK, and NORBERT C. CHERAPAK
Department of Chemistry, Newark College of Arts and Sciences, Rutgers University, Newark, NJ 07102

Seven collaborators studied a specific method for the determination of phosphorus, using wet combustion followed by acidimetric titration of the hydronium ions produced by the precipitation of lanthanum phosphate. Statistical evaluation of the data for 29 determinations on triphenylphosphine gave: $\bar{x} = 11.55\%$, $\bar{x}$ − theory = − 0.26%, $\bar{s} = 0.33$, and $s_m = 0.37$. Omitting the results of one collaborator gave, for 21 determinations: $\bar{x} = 11.66\%$, $\bar{x}$ − theory = − 0.15%, $\bar{s} = 0.18$, and $s_m = 0.30$. Evaluation of the data for 33 determinations on diphenylphosphinic acid gave: $\bar{x} = 14.11\%$, $\bar{x}$ − theory = − 0.09%, $\bar{s} = 0.40$, and $s_m = 0.45$. Omitting the results of the same collaborator mentioned above, for 25 determinations, gave: $\bar{x} = 14.28\%$, $\bar{x}$ − theory = + 0.08%, $\bar{s} = 0.27$, and $s_m = 0.08$. Since the number of collaborators was small, the study will be continued to collect more data.

The present AOAC official method (1, 2) for the microanalytical determination of phosphorus in organic compounds specifies wet combustion (sulfuric and nitric acids) for the conversion of that element to phosphoric acid and gravimetric measurement (ammonium phosphomolybdate). Some analysts have experienced difficulty using this type of combustion for breaking the C—P bond in some types of compounds.

The popularity and speed of oxygen flask combustion for converting various elements in organic compounds to the ionic state (2, 3) made it a desirable alternative for phosphorus determinations. A number of analysts already use this type of conversion and in a previous study (3) an attempt was made to compare it with other procedures (wet combustion and peroxide fusion). However, only 3 of 16 collaborators used oxygen flask combustion, so no comparison could be made. This also indicated that this type of combustion for phosphorus-containing compounds was not as popular as was believed.

Some collaborators (H. J. Francis, Jr., Pennwalt Corp., King of Prussia, PA, and F. J. Scheidl, Hoffmann-La Roche Inc., Nutley, NJ) recommended that perchloric acid along with sulfuric and nitric acids was necessary for efficient cleavage of the C—P bond. The previous study (3) also confirmed that gravimetric measurement was not widely used (4 of 16 collaborators).

Attention had been called to an acidimetric method (4, 5) for the determination of phosphorus based on the titration of the hydronium ions produced by the following reaction:

$$2\ H_2O + H_2PO_4^- + La^{3+} \longrightarrow LaPO_4 + 2\ H_3O^+$$

This titrimetric finish has the advantage over colorimetric methods in that the entire sample is analyzed instead of an aliquot. An exhaustive study of the method was made by one of the collaborators (F. J. Scheidl) who used perchloric, sulfuric, and nitric acids to convert the phosphorus to phosphoric acid. His results were such that it was decided to submit his modified method to collaborative study.

Samples of triphenylphosphine and diphenylphosphinic acid were sent to the collaborators with the request that each sample be analyzed 4 times, using the method given below.

METHOD

Apparatus

(a) *Titrator or pH meter.*—With scale expansion, preferably automatic titrator, which allows pH-stat titrations.

(b) *Piston buret.*—10 ml graduated to 0.01 ml (preferably digital and automatic).

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Reagents

(a) *Digestion acid mixture.*—$HClO_4$-H_2SO_4-HNO_3 (3+1+1). (*Caution:* Observe necessary precautions for using $HClO_4$.)

(b) *Solutions for coarse pH adjustment.*—20% NaOH, 2*N* NaOH (in dropping pipet), 2*N* $HClO_4$ (in dropping pipet).

(c) *Titrant.*—0.02*N* NaOH.

(d) *Lanthanum nitrate solution.*—Dissolve 7 g $La(NO_3)_3.6H_2O$ in 500 ml water.

Procedure

Using weighing (charging) tube, accurately weigh sample containing 0.5–1.5 mg P and transfer to dry 30 ml micro-Kjeldahl digestion flask. Add 2.0 ml digestion acid mixture to sample and digest 40 min on digestion rack at high temperature settings; use slight rocking motion or automatic vibration to avoid bumping. Simultaneously digest blank and standards (some pure phosphorus-containing compound). After digestion is complete and some cooling has taken place, cool flasks in ice water, dilute contents with water, and rinse contents into 100 ml beaker. (Use ≥3 rinses, but do not exceed 60 ml solution.) With pH electrodes in place and solution stirred magnetically, add fixed amount (ca 3.5 ml) of 20% NaOH to neutralize most of digestion acids. Make final adjustment to pH just less than 5 with 2*N* NaOH (or if pH is too high, make adjustment with 2*N* $HClO_4$).

After calibration with standard buffer solution, set titrator, or expanded scale pH meter, to pH 5.00 and titrate phosphate solution with 0.02*N* NaOH to this exact pH (automatically or manually, depending on equipment used). Then add 5.0 ml $La(NO_3)_3$ solution and titrate liberated H_3O^+ with 0.02*N* NaOH to exactly pH 5.00. Volume of 0.02*N* NaOH used for blank must be subtracted from volume used for standard and sample as a correction.

Calculation:

$$\text{Factor} = \frac{(\text{mg standard} \times \%\ \text{P in standard})}{\text{ml } 0.02N\ \text{NaOH (corr.)}}$$

$$\%\ \text{P} = \frac{(\text{ml } 0.02N\ \text{NaOH (corr.)} \times \text{factor})}{\text{mg sample}}$$

Results and Recommendation

The results obtained by the collaborators are shown in Tables 1 and 2. The results were treated the same as in previous studies (3, 6). The tables show *n*, the number of values reported by each collaborator; $\bar{x}$, the mean of the data; *s*, the standard deviation of the data; and $\bar{x}$ − theory, the deviation of the mean from the theory. Also included are the values for $\bar{\bar{x}}$, which is the mean of the $\bar{x}$ values; $\bar{\bar{x}}$ − theory; $\bar{s}$, which is the mean of the *s* values; and s_m, which is the standard deviation of the means ($\bar{x}$ values). A standard deviation of 0.3 and a deviation from the theory of 0.3% are considered to be acceptable.

Table 1 shows the results obtained for triphenylphosphine. Collaborator 7 did not use perchloric acid in the digestion, but his results were both accurate and precise. Statistics were calculated both including and omitting these values. Collaborator 2 used the submitted sample of triphenylphosphine as the standard, so these values are not given. Collaborator 5 obtained erratic results to the extent that one might suspect the samples had become contaminated in transit or otherwise. Therefore, overall statistics were calculated both with and without those values. Collaborator 1

Table 1. Collaborative results for determination of phosphorus in triphenylphosphine,[a] $C_{18}H_{15}P$ (11.81% P)

Coll.	*n*	$\bar{x}$, %	$\bar{x}$ − theory, %	*s*
1	5	11.22	−0.59	0.07
2	–	—	—	—
3	5	11.87	+0.06	0.11
4	4	11.51	−0.30	0.12
5	8	11.02	−0.79	1.04
6	4	11.95	+0.14	0.54
7	3	11.73	−0.08	0.08
Total no. of colls = 6 Total no. of detns = 29 $\bar{\bar{x}}$ = 11.55%; $\bar{\bar{x}}$ − theory = −0.26%; $\bar{s}$ = 0.33; s_m = 0.37				
Omitting results of Coll. 7: Total no. of colls = 5 Total no. of detns = 26 $\bar{\bar{x}}$ = 11.51%; $\bar{\bar{x}}$ − theory = −0.30%; $\bar{s}$ = 0.38; s_m = 0.40				
Omitting results of Coll. 5: Total no. of coll = 5 Total no. of detns = 21 $\bar{\bar{x}}$ = 11.66%; $\bar{\bar{x}}$ − theory = −0.15%; $\bar{s}$ = 0.18; s_m = 0.30				
Omitting results of both Colls 5 and 7: Total no. of colls = 4 Total no. of detns = 18 $\bar{\bar{x}}$ = 11.64%; $\bar{\bar{x}}$ − theory = −0.17%; $\bar{s}$ = 0.21; s_m = 0.34				

[a] Eastman Kodak Co. material recrystallized 6 times from ethanol. Calculated for $C_{18}H_{15}P$: C = 82.43%, H = 5.76%, P = 11.81%. Found: C = 82.44%, H = 5.57%, P = 11.82%, 11.70% (these 2 determinations of phosphorus done by gravimetric method (1, 2)).

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.

Table 2. Collaborative results for determination of phosphorus in diphenylphosphinic acid,[a] $C_{12}H_{11}O_2P$ (14.20% P)

Coll.	*n*	$\bar{x}$, %	$\bar{x}$ − theory, %	*s*
1	4	14.20	0.00	0.22
2	4	14.30	+0.10	0.09
3	5	14.18	−0.02	0.05
4	4	14.30	+0.10	0.13
5	8	13.10	−1.10	1.21
6	4	14.32	+0.12	0.64
7	4	14.38	+0.18	0.47
Total no. of colls = 7 Total no. of detns = 33 $\bar{\bar{x}}$ = 14.11%; $\bar{\bar{x}}$ − theory = −0.09%; $\bar{s}$ = 0.40; s_m = 0.45				
Omitting results of Coll. 7: Total no. of colls = 6 Total no. of detns = 29 $\bar{\bar{x}}$ = 14.07%; $\bar{\bar{x}}$ − theory = −0.13%; $\bar{s}$ = 0.39; s_m = 0.20				
Omitting results of Coll. 5: Total no. of colls = 6 Total no. of detns = 25 $\bar{\bar{x}}$ = 14.28%; $\bar{\bar{x}}$ − theory = +0.08%; $\bar{s}$ = 0.27; s_m = 0.08				
Omitting results of both Colls 5 and 7: Total no. of colls = 5 Total no. of detns = 21 $\bar{\bar{x}}$ = 14.26%; $\bar{\bar{x}}$ − theory = +0.06%; $\bar{s}$ = 0.23; s_m = 0.07				

[a] Ultrex® brand, J. T. Baker Chemical Co., microanalytical reference standard.

obtained poor accuracy ($\bar{x}$ − theory = −0.59%), but excellent precision (s = 0.07). The opposite was the case with Collaborator 6 ($\bar{x}$ − theory = +0.14%, s = 0.54). However, the values of these collaborators were included in all calculations of $\bar{\bar{x}}$, $\bar{\bar{x}}$ − theory, $\bar{s}$, and s_m. Omitting the results of either or both Collaborators 5 and 7 did not drastically change the overall values, although, when these were omitted, the slightly high standard deviations were reduced to acceptable values.

Table 2 shows the results obtained for diphenylphosphinic acid. Essentially, the conditions are the same as for triphenylphosphine. With the exception of Collaborator 5, all obtained excellent accuracy. Collaborators 5, 6, and 7 had poor precision. When the results of all collaborators were included, the standard deviations were not acceptable, but omission of only the results of Collaborator 5 gave acceptable values throughout.

The results indicate that the submitted method is satisfactory, but because so few collaborators participated, the only recommendation that can be made at this time is that the study be continued to include more data.

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F. Moraes and F. J. Scheidl, Chemical Research Department, Hoffmann-La Roche Inc., Nutley, NJ

G. F. Morris, Chemistry and Biology Research Institute, Agriculture Canada, Ottawa, Ontario, Canada

C. S. Yeh, Purdue University, West Lafayette, IN

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COLOR ADDITIVES

High-Pressure Liquid Chromatography of 4,4′-Diazoaminobis-(5-methoxy-2-methylbenzenesulfonic Acid) in FD&C Red No. 40

DANIEL M. MARMION
Buffalo Research Laboratory, Specialty Chemicals Division, Allied Chemical Corp., PO Box 1069, Buffalo, NY 14240

Ten analysts representing 7 laboratories participated in the evaluation of a high-pressure liquid chromatographic procedure for the determination of 4,4′-diazoaminobis(5-methoxy-2-methylbenzenesulfonic acid) (DMMA) in FD&C Red No. 40; each collaborator analyzed 7 samples. Between-laboratory standard deviations ranged from 0.0015 to 0.0033% for samples nominally containing 0–0.05% DMMA. The stability of DMMA vs. pH was also studied. It is recommended that the high-pressure liquid chromatographic method be further studied.

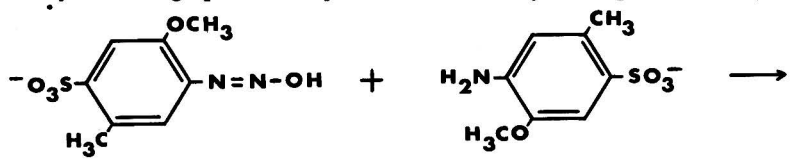
FD&C Red No. 40 (C.I. No. 16035) is a monoazo dye manufactured by coupling diazotized 5-amino-4-methoxy-2-toluenesulfonic acid (CSA diazo) to 6-hydroxy-2-naphthalenesulfonic acid (1). Recently, Bailey and Cox (2) reported that FD&C Red No. 40 can contain small amounts of 4,4′-diazoaminobis(5-methoxy-2-methylbenzenesulfonic acid) (DMMA), a previously unknown impurity formed when CSA diazo combines with undiazotized CSA under certain conditions. This report is the result of a collaborative study of a high-pressure liquid chromatographic (HPLC) procedure developed to determine low levels of DMMA in the colorant.

The HPLC method evaluated is based on that reported by Singh (3). Marmion's gravity-column chromatography procedure (4) was not included in this study, since, by analogy to work reported earlier (5) on a similar compound present in FD&C Yellow No. 6, it was not considered adequate.

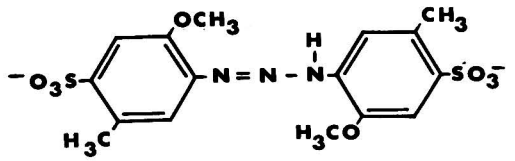
Experimental

Preparation of 4,4′-Diazoaminobis(5-methoxy-2-methylbenzenesulfonic Acid)

Seventy g CSA was weighed into a 3 L beaker containing 550 ml water; then 50% aqueous NaOH was added slowly with stirring until a solution of ca pH 9.5 was formed. Sodium nitrite (22.8 g) was added to the solution which was then cooled with ice to 0–10°C. The solution was acidified by the slow addition of 81 ml 20° Bé HCl, and then stirred 30 min; during this time the temperature of the solution was maintained at <25°C by adding ice. CSA (0.50 g) was



CSA Diazo **CSA**



DMMA

added to the solution and it was stirred 6 min. At this time a spot test for nitrite, using 3,6-thioxanthenediamine - 10,10 - dioxide (EK No. 8776), was negative.

A second solution of CSA was prepared by dissolving 70.7 g amine in 300 ml water containing 17.3 g Na_2CO_3. This solution was added to the solution of diazonium salt and solid Na_2CO_3 was added slowly to pH 8.4. The mixture was agitated at 18–22°C until no diazonium salt could be detected, using a streak test with R-salt. NaCl (406 g) was weighed into the solution and the pH was adjusted to 8.2 by adding ca 10 g Na_2CO_3. The solution was allowed to stand overnight and then filtered. The wet filter cake was washed on the filter with 400 ml water containing 100 g NaCl and 4 g Na_2CO_3, and dried under vacuum at 45°C to constant weight.

Analysis of the sample showed 24.13% NaCl, 5.33% moisture (Karl Fischer), and 0.2% CSA (HPLC). Allowing for the salt, water, and CSA content, the isolated material contained 27.66% carbon, 3.12% hydrogen, 5.85% nitrogen, and 8.93% sulfur. Based on these data, the sample contained 70.34% DMMA as the disodium salt, molecular weight (MW) 489.44.

Preparation of FD&C Red No. 40 Samples

Portions of a sample of FD&C Red No. 40 shown by HPLC to contain no DMMA were fortified as follows: 10 g amounts of the color were dissolved in water and the solutions were adjusted to pH 10 with NH_4OH. Various known quantities of DMMA were dissolved in the solutions which were then dried at ca 50°C under vacuum (20″ Hg). The resulting powders were ground and mixed.

METHOD

Apparatus and Reagents

(a) *High-pressure liquid chromatograph.* — DuPont Model 830 equipped with 1 m × 2.1 mm id DuPont SAX column (strong anion exchange, No. 830950405). DuPont gradient elution accessory, and Model 835 multiwavelength detector fitted with 365 nm filter (No. 835052-907), or any equivalent HPLC system and column. (The collaborators were advised not to use an SAX column that had been heat conditioned as suggested by Singh (3) for fear that this treatment might reduce the amount of sample the column could effectively handle.) Instrument parameters: chart speed 0.5″/min; eluant flow rate 1.50 ml/min; detector sensitivity 0.02 absorbance unit full scale (AUFS); ambient temperature; gradient 0–60% secondary eluant in nonlinear, slow-start mode (Function 5, clockwise) at 2%/min, hold at limit.

(b) *Primary eluant.*—0.01*M* aqueous $Na_2B_4O_7$.

(c) *Secondary eluant.*—0.01*M* aqueous $Na_2B_4O_7$ in 0.50*M* $NaClO_4$.

Preparation of Sample and Resolution

Weigh 0.500 g of each sample into separate 5 ml volumetric flasks. Dissolve each in primary eluant and dilute to volume with same.

Before injecting first sample, run blank (0–60%) gradient; then pump primary eluant through column 14 min. Immediately inject 5.0 µl sample solution into chromatograph, using microliter syringe. Start 0–60% gradient at once and "Hold at Limit" until chromatogram is complete (ca 40 min). Pump primary eluant through column 14 min; then inject next sample.

Method Calibration

Weigh 0.250 g (100% basis) DMMA into 10 ml volumetric flask, and dissolve and dilute to volume with primary eluant. Analyze blank sample (0% DMMA, Sample 7 for collaborators), add 2.0 µl DMMA solution to it with microliter syringe, mix, and re-inject sample. Add 2 separate 4.0 µl portions of DMMA to blank sample, mixing and re-injecting sample into chromatograph after each addition. This procedure provides 3 standards (0.01, 0.03, and 0.05% DMMA), the minimum number of standards which should be analyzed. If time permits, additional standards representing 0.005, 0.020, and 0.060% DMMA should be prepared and analyzed.

Calculation

Prepare plot of peak area (peak height × width at half height, in sq. mm) vs. per cent DMMA for standards and use this to determine per cent DMMA in samples. Report results to the nearest 0.001% as DMMA disodium salt, MW 489.44.

Collaborative Study

The collaborative study involved 10 analysts from 7 different laboratories. The samples of colorant tested included one considered to contain no DMMA; portions of this same sample fortified with DMMA at 5 different levels; and a seventh sample taken from a batch of FD&C Red No. 40 used for various pharmacological studies. The DMMA used was a laboratory preparation.

Each analyst was asked to adhere to the method given as closely as possible and to report in detail any deviations made and the reasons for them.

Results and Comments

All 7 laboratories that were given samples reported results. Three laboratories (A, B, and F) provided data from 2 analysts, and one laboratory (B) submitted repeat results on one sample. Because of the lack of within-laboratory repeats by the same operator, repeatability was not calculated. However, among-laboratory reproducibility was easily measured and is reported below.

In general, there was good agreement among

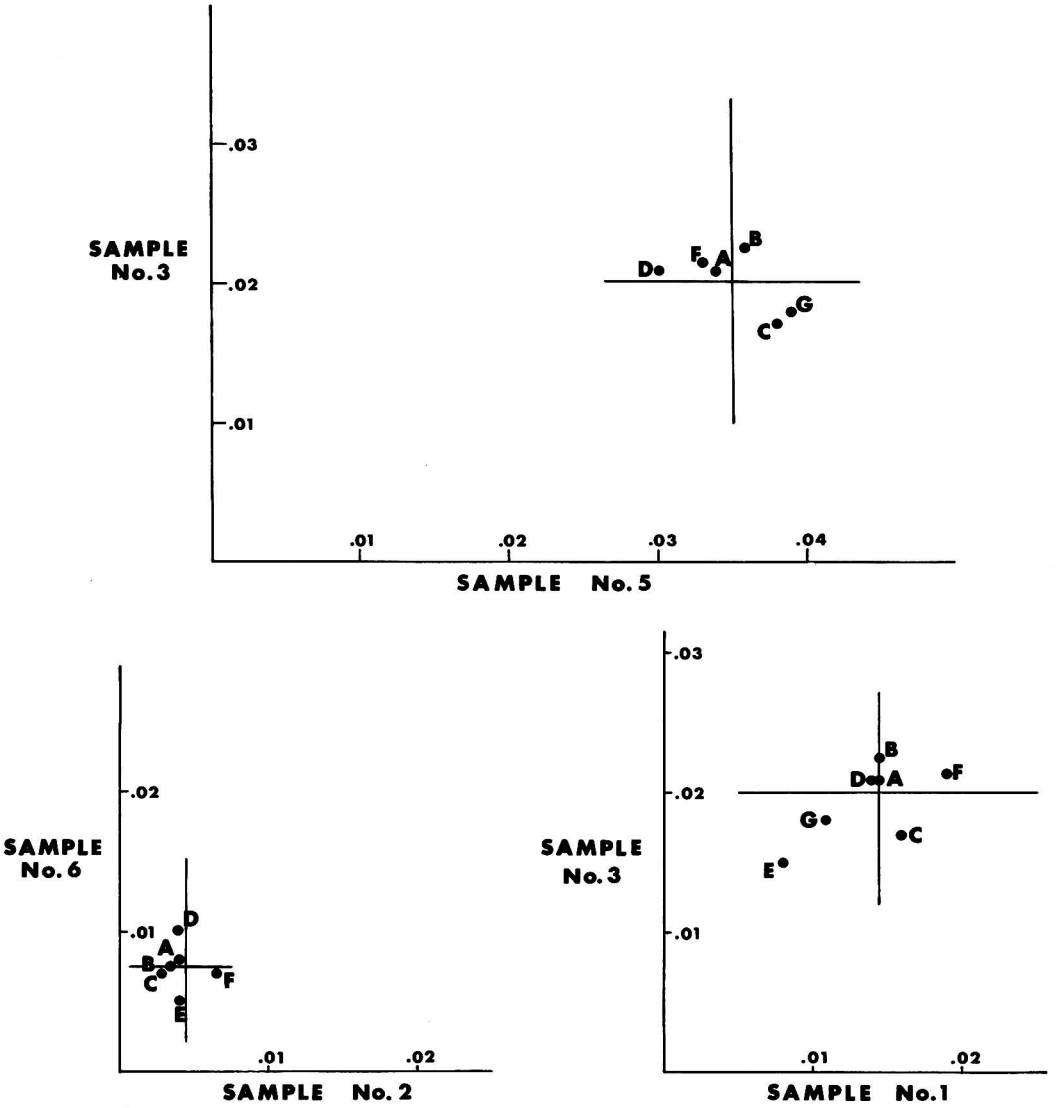


FIG. 1—Two-sample charts demonstrating the role of systematic error (see Youden (6)).

Table 1. Interlaboratory results for determination of per cent DMMA in FD&C Red No. 40

Lab.	Operator	Samples 1	2	3	4	5	6	7
A	1	0.015	0.004	0.020	0.025	0.034	0.007	ND[a]
	2	0.014	0.004	0.022	0.027	0.034	0.009	ND
B	1	0.015	0.003	0.024	0.028	0.033 0.040	0.007	ND
	2	0.014	0.004	0.021	0.028	0.035 0.036	0.008	ND
C	1	0.016	0.003	0.017	0.025	0.038	0.007	ND
D	1	0.014	0.004	0.021	0.021	0.030	0.010	0.002
E	1	0.008	0.004	0.015	0.020	—	0.005	ND
F	1	0.020	0.005	0.025	0.030	0.032	0.008	0.001
	2	0.018	0.008	0.018	0.025	0.034	0.006	0.001
G	1	0.011	0.006	0.018	0.025	0.039	0.030[b]	ND
% DMMA added		0.020	0.005	0.030	PS[c]	0.050	0.010	0

[a] ND = none detected.
[b] Rejected as a spurious result.
[c] Pharmacological sample; DMMA content unknown.

Table 2. 95% confidence limits for per cent DMMA content of samples

Sample	DMMA added, %	Av. of labs.	95% conf. limits[a]
1	0.020	0.0145	0.0115–0.0175
2	0.005	0.0045	0.003–0.006
3	0.030	0.0201	0.017–0.023
4	PS[b]	0.0254	0.0224–0.0284
5	0.05	0.035	0.032–0.038
6	0.01	0.0074	0.006–0.009
7	0	0.0004	0–0.002

[a] The interpretation of these limits is that they enclose the true DMMA value in the sample as measured by HPLC with at least 95% confidence (i.e., there is <5% chance that the limits do not contain the true value).
[b] Pharmacological sample; DMMA content unknown.

the laboratories in analyzing each of the 7 samples. (*See* clustering of results in Youden plots (6), Fig. 1.) For concentrations of DMMA <0.01%, the among-laboratory standard deviation was 0.0015%, while the 95% reproducibility was estimated to be 0.005%. For DMMA values >0.01%, the standard deviation was 0.0033% and the 95% reproducibility was 0.011% (95% reproducibility is defined here as the largest difference between 2 determinations from different laboratories that would be expected at the 95% confidence level). *See* Tables 1 and 2.

For each of the fortified samples the average result reported was estimated to be only 70% of the actual amount of DMMA added to the samples; *see* Fig. 2. It is presumed that this loss in recovery is due to decomposition of DMMA during the preparation of the samples and that this decomposition is a function of time, temperature, and pH.

The pharmacological sample was estimated by this method to contain 0.0254±0.003% DMMA with 95% confidence. In general, the collaborators demonstrated the same skill in reproducing the results for this sample as they did in evaluating the samples containing >0.01% DMMA. This supports the belief that added DMMA was lost during the preparation and helps in discounting the possibility that the samples were continually changing in the different time periods prior to analysis by each laboratory.

Comments provided by the various collaborators follow:

Collaborators B_1 and B_2 both used a DuPont Model 836 detector equipped with a 20 mm long absorption cell and a 325–385 nm filter. This photometer reportedly provided greater sensitivity than the detector recommended and allowed operation at 0.04 AUFS. Collaborator B_1 suggested less concentrated sodium perchlorate in the secondary eluant to permit greater flexibility in the gradient parameters, and recommended that weaker sample solutions be used wherever possible. Collaborator B_2 reported that a single analysis required 75 min instead of the 40 min mentioned in the method distributed.

Collaborator C tried 3 different SAX columns but was unable to obtain adequate resolution on any, using the method conditions given. He made the following parameter changes to effect resolution: primary eluant, 0.01*M* $Na_2B_4O_7$ containing 20 ml secondary eluant/L; secondary

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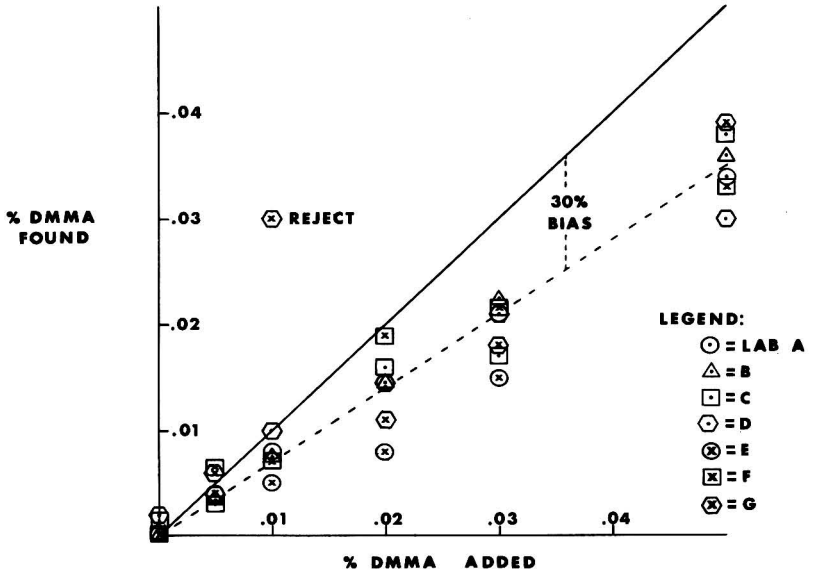


FIG. 2—Collaborative results for analysis of DMMA.

eluant, 0.01*M* $Na_2B_4O_7$ in 0.25*M* $NaClO_4$; eluant flow rate, 0.9 ml/min. The detector was the continuously variable type tuned to 383 nm. Sample size was 5 μl of a 5% solution.

Collaborator D used a linear gradient, programming from 5 to 60% secondary eluant, and a sample injection of 10 μl of a 5% solution (Valco loop injector). The detector was ultraviolet (UV)-sensitive at 254 nm and was operated at 0.01 AUFS. Collaborator D reported that a borate solution (pH 9.1) of DMMA decreased approximately 50% in strength over 10 days.

Collaborator E also used a Valco loop injector, 254 nm detector, and heat-treated SAX column.

Collaborators F_1 and F_2 both used heat-

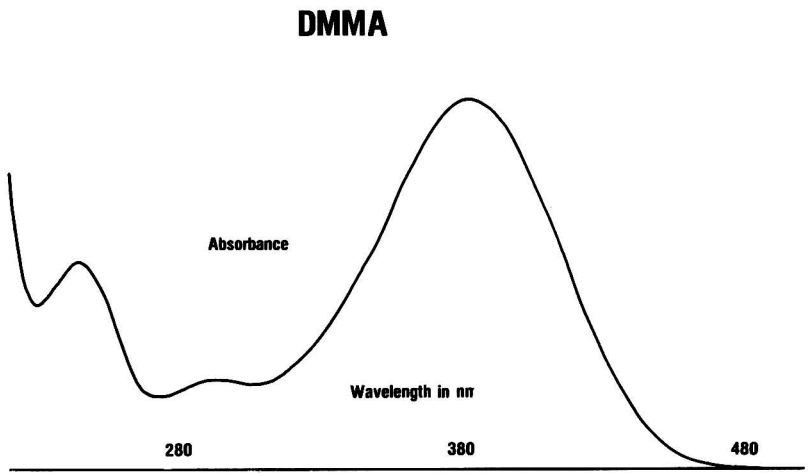


FIG. 3—Ultraviolet spectrum of 14 ppm DMMA in 0.01*M* $Na_2B_4O_7$ (1 cm cell).

treated SAX columns, 5% sample solutions, flow rates of 0.5 ml/min (about 1100 psi), and UV detectors set at 254 nm. The recorder sensitivities used by Collaborators F_1 and F_2 were 0.08 and 0.02 AUFS, respectively.

Collaborator G is the only one who did not use a DuPont Model 830 HPLC. Collaborator G used a Waters Associates instrument equipped with a Model 660 solvent programmer, 2 M-6000 pumps, U6K injector, 254 nm UV detector, and 60 × 0.25 cm (id) column packed with DuPont SAX. The solvent system was that specified, using Waters' program No. 9, 0–60% secondary eluant in 40 min at 1.5 ml/min. The sample injected was 10 μl of a 5% solution.

Discussion

All but one of the laboratories (D) involved in this study participated in the earlier and similar study (5) on the determination of FD&C Yellow No. 6. In general, it appears that the reproducibility and sensitivity exhibited here is superior to that found in the former study, indicating a general improvement in the group's competence in the field of HPLC.

Because of a lack of data, it is uncertain in what way changes made in the procedure by the various collaborators (sample size, method of injection, column conditioning, detection systems, etc.) affected the reproducibility and sensitivity of the method. However, on theoretical grounds alone, it appears that sensitivity and specificity for DMMA should be best with a detector tuned to between 365 and 380 nm (*see* Figs. 3 and 4 and Table 3).

The instability of DMMA in solution reported by Collaborator D was noted by the author prior to distributing the collaborative samples and a study of DMMA stability vs. pH was undertaken at that time.

To determine stability, 0.020% solutions of DMMA were prepared in various buffers and 5 μl amounts of each were injected into a chromatograph at known times. The areas of the peaks obtained were compared vs. elapsed time. The chromatographic conditions chosen were those reported above except that the gradient used was the nonlinear, slow-start Function 2. These conditions were known to resolve CSA (one of the decomposition products of DMMA) from DMMA and, by analogy, were assumed to resolve any other lower sulfonated fragments

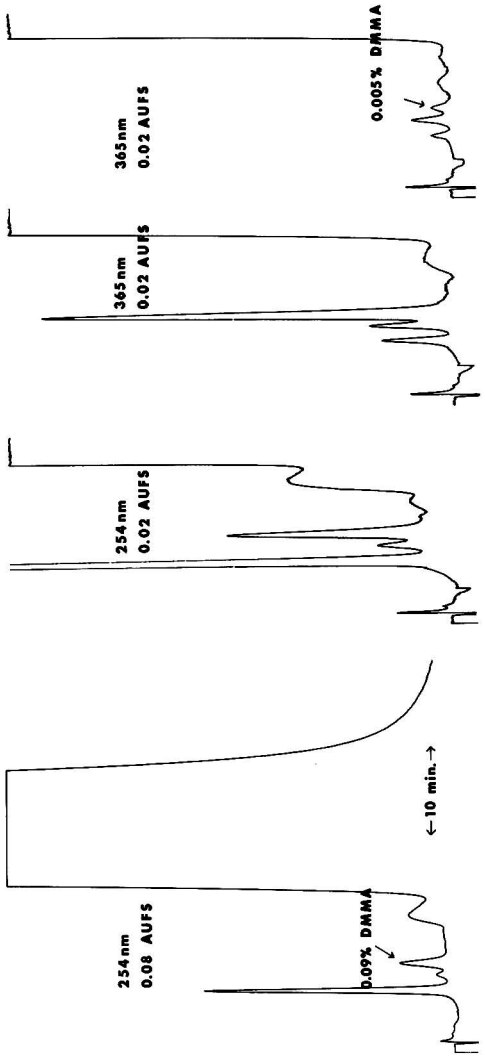


FIG. 4—Chromatograms of DMMA in FD&C Red No. 40 at different wavelengths and sensitivities.

Table 3. Approximate absorptivities in L/g-cm at selected wavelengths

Compound	Wavelength, nm						
	254	280	365	380	400	500	λ max.
Sulfanilic acid, MW 173.2	74.2	10.3	0	0	0	0	86.1 (248 nm)
Cresidinesulfonic acid, MW 217.2	52.1	16.2	0	0	0	0	53.9 (251 nm)
6,6'-Oxybis(2-naphthalenesulfonic acid) disodium salt, MW 474.4	84.0	35.8	0	0	0	0	185 (239 nm)
4,4'-Diazoaminobis(5-methoxy-2-methylbenzene-sulfonic acid) disodium salt, MW 489.4	23.9	3.71	43.2	50.4	43.2	0	50.6 (383 nm)
FD&C Red No. 40	33.4	19.6	7.8	10.9	14.2	53.2	53.2 (499 nm)

produced. The detection system used in the chromatograph was a 365 nm filter-photometer highly sensitive to DMMA and, for the purpose of this experiment, blind to CSA. pH 8, 9, and 10 buffers were prepared from 0.01*M* boric acid and 1*N* NaOH while pH 6 and pH 7 buffers were prepared from 0.01*M* NaH_2PO_4 and 1*N* NaOH.

Because of the inherent day-to-day instability of liquid chromatographs and because of the length of the experiments, peak areas obtained at pH values 8 and 9 were adjusted by a factor equal to the area originally obtained for the pH 10 sample divided by the area obtained for the pH 10 sample at the time of the experiment. (Based on data at pH 10 and on theoretical considerations it was assumed that, for the purposes of this study, DMMA was stable at pH 10.) Data gathered at pH values of 6 and 7 were used as is, since they were obtained in less than one day (*see* Table 4).

At each of the above pH values a plot of log area vs. time was essentially a straight line, indicating a pseudo-first-order reaction with respect to DMMA in the presence of a buffer. The slope of each of these was determined by regression analysis and the logs of these slopes were in turn plotted against pH. The result was also a straight line of the type

$$\log m = -b\text{pH} + c$$

which, by regression analysis, had a slope of 0.9086 and an intercept of 5.1616. Slopes for pH values 6, 7, 8, and 9 were -0.5304, -6.560×10^{-2}, -6.546×10^{-3}, and -1.073×10^{-3}, respectively.

Table 4. Stability of DMMA in solution at different pH values

Time, hr	pH 6		pH 7		pH 8		pH 9	
	Peak area, sq. mm	DMMA decomp., %	Peak area, sq. mm	DMMA decomp., %	Peak area, sq. mm	DMMA decomp., %	Peak area, sq. mm	DMMA decomp., %
0	800	—	970	—	1160	—	1160	—
0.50	418	47.8	783	19.3				
1.00	242	69.8	913	5.9				
1.50	110	86.3						
1.67			810	16.5				
2.00	55	93.1	730	24.7	1120	3.4	1179	0
2.50	44	94.5						
4.25			450	53.6				
4.50	0	100.0						
6.00			370	61.9				
6.75			330	66.0				
12.00			165	83.0				
19.00					860	25.9	1090	6.0
26.00					889	23.4	1090	6.0
28.50			0	100.0				
96.00					276	76.2	921	20.6
120.00					193	83.4	828	28.6
308.00					0	100.0		
309.00							546	52.9

Based on this study it is obvious that DMMA is slightly unstable at the pH at which FD&C Red No. 40 samples are prepared for analysis (pH 9.1) and that samples should be analyzed promptly. It is also reasonable to assume some instability of DMMA at pH 10 and that this instability, enhanced by the elevated temperatures used in preparing the fortified samples, is the reason for the low recovery of DMMA reported here.

Conclusions and Recommendation

HPLC is capable of determining DMMA in FD&C Red No. 40. The method is sensitive, rapid, and repeatable; however, its reproducibility is not as good as one would hope for in a method of this type.

It is recommended that this procedure be studied further to determine the effect of parameter changes and instrument design on reproducibility. Points to consider include sample concentration, pH and age of the sample solution, method of sample injection (syringe vs. valve), column conditioning, eluant concentration and programs, flow rates, and methods of detection.

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M. Gunn, Hilton Davis Chemical Co., Cincinnati, OH

R. Hollister and D. Sommers, Allied Chemical Corp., Buffalo Research Laboratory, Buffalo, NY

F. Penta, H. Kohnstamm & Co., Inc., Brooklyn, NY

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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**, 399.

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

Spectrophotometric Determination of Diphenylamine in Ext. D&C Yellow No. 1

JOHN G. DANTZMAN
Division of Color Technology, Food and Drug Administration, Washington, DC 20204

A separatory funnel procedure is presented that separates diphenylamine (DPA) from Ext. D&C Yellow No. 1. The DPA is extracted from the color with isooctane and the amount of DPA is determined spectrophotometrically. Recoveries of 0.02–1% added DPA ranged from 95.5 to 104%.

Ext. D&C Yellow No. 1 is prepared by diazotizing metanilic acid and coupling it with diphenylamine (DPA) in an acid medium (1). Quite often unreacted DPA is present in the finished product. The *Code of Federal Regulations* (2) states that not more than 0.2% DPA can be present in certifiable batches of the color.

A cellulose column method, a modification of an AOAC method (3), separates DPA and metanilic acid from the color. Low and erratic recoveries are obtained when using this method. A previously published method for this determination presently exists (4), but low recoveries of DPA have been encountered. An alternative method is presented in which recoveries of DPA range from 95.5 to 104%.

METHOD

Apparatus and Reagents

(a) *Spectrophotometer.*—Suitable for use in ultraviolet (UV) region (200–400 nm).

(b) *Isooctane (2,2,4-trimethylpentane).*—Fisher Certified ACS (Fisher Scientific Co., No. 0-299).

(c) *Diphenylamine.* — Fisher Certified ACS (Fisher Scientific Co., No. D-97). Recrystallized from hexane. Melting range 52.5–53.5°C. Prepare standard solution containing 8 mg DPA/L isooctane.

Procedure

Place 5 g NaCl in 1 L volumetric flask, add ca 600 ml water, and let salt dissolve. Add 500 mg dye to volumetric flask and dilute to volume with water. Place 200 ml aliquot of dye solution in 250 ml separatory funnel and extract with three 25 ml portions of isooctane. Combine isooctane fractions. If unable to completely separate aqueous and isooctane emulsion, combine emulsion with isooctane phase. Rinse separatory funnel with small volume of isooctane and combine rinse with isooctane phase. Wash combined organic fraction with 100 ml portions of water until it is colorless. Place organic fraction in 100 ml volumetric flask. Rinse separatory funnel with small volume of isooctane and place rinse in volumetric flask. Dilute to volume with isooctane.

Record UV spectra of the unknown and standard solutions in 1 cm cells from 200 to 400 nm, using isooctane in the reference cell. Draw zero line with isooctane in both cells.

Calculation

$$\text{DPA, } \% = Cstd \times [A281 - 0.5 \times (A241 + A321)] / \{10 \times [A'281 - 0.5 \times (A'241 + A'321)]\}$$

where $Cstd$ = concentration of standard solution, mg/L; $A241, 281, 321$ = absorbances of unknown solution at 241, 281, and 321 nm, respectively; and $A'241, 281, 321$ = absorbances of standard solution at 241, 281, and 321 nm, respectively.

Results and Discussion

Recovery studies were performed in which commercial samples of Ext. D&C Yellow No. 1 were spiked at various levels with DPA. In the first recovery series the dye was spiked with an ethanolic solution of DPA. The amount of dye was determined by the modified cellulose method (3). The modifications are: Use 20 g cellulose, use 100 mg color, and elute column with a 10% $(NH_4)_2SO_4$ plus 1% NH_4OH wash followed by a 10% $(NH_4)_2SO_4$ plus 1% HCl wash. The eluants are analyzed spectrophotometrically in a basic medium. Table 1 shows that recoveries range from 20 to 80%. The interpretation of the spectrophotometric curves of the eluants is difficult when unreacted metanilic acid is present in the dye. Some of the metanilic acid elutes along with the DPA. Metanilic acid absorbs in the same UV region that DPA does.

In the second recovery series the dye was spiked with solid DPA. The amount of DPA added to the dye was determined by the method of Clements and Harrow (4). Recoveries ranged from 79 to 90% as shown in Table 1.

In another recovery study, the dye was spiked with an ethanolic solution of various concentra-

Table 1. Recovery of DPA from Ext. D&C Yellow No. 1, using modified cellulose and Clements and Harrow (4) methods

DPA added, mg	DPA found, mg	Net DPA found, mg	Rec., %
Modified Cellulose[a]			
—	0	0	—
0.05	0.01	0.01	20
0.10	0.08	0.08	80
0.20	0.16	0.16	80
0.40	0.16	0.16	40
—	0.01	0	—
0.05	0.02	0.01	20
0.10	0.06	0.05	50
0.20	0.08	0.07	35
0.40	0.20	0.19	47.5
Clements and Harrow[b]			
—	0	0	—
1.25	1.1	1.1	88
1.25	1.0	1.0	80
2.5	2.1	2.1	84
2.5	2.2	2.2	88
5.0	4.5	4.5	90
5.0	4.2	4.2	84
10.0	8.6	8.6	86
10.0	7.9	7.9	79

[a] 100 mg color used.
[b] 2.5 mg color used.

tions of DPA. The ethanol was evaporated and the DPA content was determined by the proposed method. The recoveries ranged from 95.5 to 104% as shown in Table 2. The recovery of DPA at one level in the dye was examined by using the proposed method. Recoveries ranged from 95 to 102% as shown in Table 3.

An examination was performed to ascertain hat metanilic acid does not interfere in the roposed method. The dye was spiked with thanolic solutions of DPA and metanilic acid. The ethanol was evaporated and the amount of DPA was determined. Recoveries of DPA in the resence of metanilic acid ranged from 97 to 01% as shown in Table 4.

The equation for the calculation of the amount f DPA was derived by using:

$$\text{DPA, mg} = (A \times V)/(S \times l)$$

here A = absorbance of unknown solution; = volume of unknown solution, L; S = standrd absorptivity value of DPA expressed in (aborbance $\times$ l)/(mg $\times$ cm); l = path length, cm.

A must be obtained by a spectrophotometric letermination of the unknown solution. The nethod specifies that V is to be 0.1 L and that

Table 2. Recovery of various amounts of DPA from Ext. D&C Yellow No. 1, using the proposed method[a]

DPA added, mg	DPA found, mg	Net DPA found, mg	Rec., %
—	0.002	—	—
0.020	0.022	0.020	100
0.050	0.054	0.052	104
0.100	0.099	0.097	97
0.200	0.193	0.191	95.5
0.400	0.410	0.408	102
0.800	0.786	0.784	98
1.000	0.974	0.972	97
—	0.230	—	—
0.020	0.250	0.020	100
0.050	0.281	0.051	102
0.100	0.330	0.100	100
0.200	0.430	0.200	100
0.400	0.620	0.390	97.5
0.800	0.990	0.760	95
1.000	1.249	1.019	102

[a] 100 mg color used.

Table 3. Recovery of one level of DPA from Ext. D&C Yellow No. 1, using the proposed method[a]

DPA added, mg	DPA found, mg	Net DPA found, mg	Rec., %
—	0.029	—	—
0.200	0.225	0.196	98
0.200	0.229	0.200	100
0.200	0.225	0.196	98
0.200	0.219	0.190	95
0.200	0.233	0.204	102
—	0.002	—	—
0.200	0.196	0.194	97
0.200	0.198	0.196	98
0.200	0.202	0.200	100
0.200	0.192	0.190	95
0.200	0.200	0.202	101

[a] 100 mg color used.

Table 4. Recovery of DPA from Ext. D&C Yellow No. 1, using the proposed method in the presence of metanilic acid[a]

Metanilic acid added, mg	DPA added, mg	DPA found, mg	Net DPA found, mg	Rec., %
0	0	0.029	—	—
0.1	0.200	0.228	0.199	99.5
0.2	0.200	0.230	0.201	100
0.4	0.200	0.225	0.196	98
0.8	0.200	0.231	0.202	101
1.0	0.200	0.223	0.194	97

[a] 100 mg color used.

l is to be 1 cm. The term to the right of the equal sign in the following equation is substituted for S:

$$S = (A' \times V')/(\text{mg}' \times l')$$

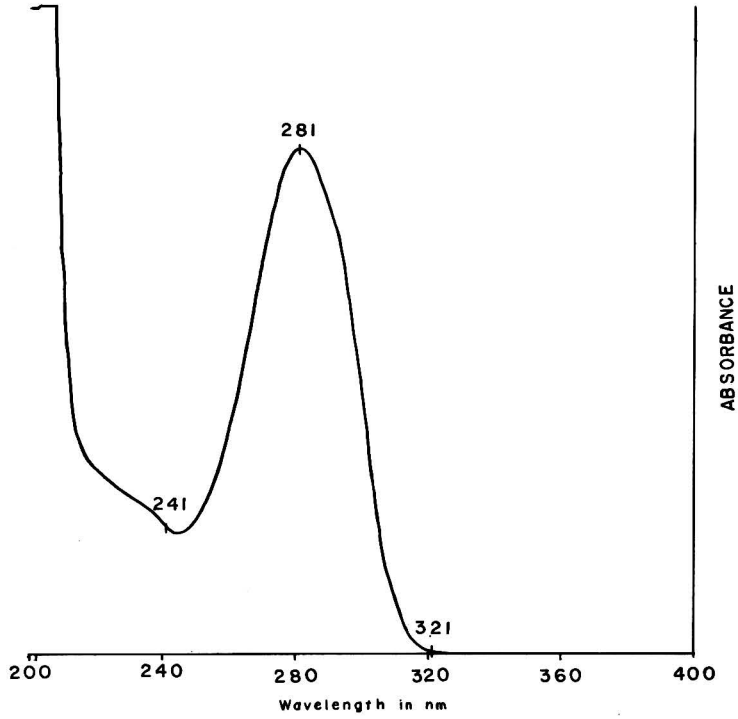


FIG. 1—UV spectrum of DPA in isooctane (8 mg/L).

where A' = absorbance of standard DPA solution; V' = volume of standard DPA solution, L; mg' = mg DPA in final solution; l' = path length, cm.

A baseline correction is necessary, since some weak UV absorber beside DPA bleeds into the final unknown solution. A baseline is drawn from 241 to 321 nm and the absorbances of the standard and sample solutions are read from the peak at 281 nm to this baseline. The parenthetical expressions in the equation for calculation of the per cent DPA are used for that correction.

The UV spectrum of DPA in isooctane is shown in Fig. 1.

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

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ANTIBIOTICS

Screening and Confirmatory Methods for Determining Lincomycin Residues in Animal Tissues

ARTHUR R. BARBIERS and A. WILLIAM NEFF
The Upjohn Co., Kalamazoo, MI 49001

Methods for screening and for confirming residues of lincomycin in animal tissues, both sensitive to 0.1 ppm, are described. In the screening technique, residues are extracted, cleaned up by solvent partition, and detected by microbiological plate assay. In the confirmatory technique, residues are cleaned up on an unfunctionalized macroreticular-type resin column, concentrated, chromatographed on thin layer plates, and detected by bioautography. This system was effective in identifying lincomycin in the presence of 20 other antibiotics and chemicals used in the animal health industry.

Originally, lincomycin was an antibiotic used effectively in humans; in addition, this compound is now used in the animal health field. It has been approved for use as a growth promotion antibiotic in chickens, turkeys, and swine at 2–100 g/ton. As an injectable product, it is approved for treatment of gram-positive pathogens and mycoplasma organisms associated with swine arthritis.

A sensitive assay for 0.5–4.0 g lincomycin/ton animal feed was developed earlier and collaboratively studied at 4.0 g/ton (1). This report presents a tissue residue assay method and a confirmatory technique which will detect as little as 0.1 ppm lincomycin.

Screening Procedure

Assay Organism

Sarcina lutea.—ATCC 9341, maintained as frozen suspension in liquid nitrogen. Organism can also be maintained on antibiotic medium No. 1 slants at refrigerator temperature and transferred to fresh slants in ca 2–3 weeks.

Media

(a) *For maintaining culture*.—Antibiotic medium No. 1 (Difco or BBL penicillin assay seed agar), if slants are used.

(b) *For Roux bottle preparation*.—Antibiotic medium No. 3 (Difco or BBL antibiotic assay broth) and antibiotic medium No. 1, if Roux bottles are used.

(c) *For culture dilution*.—Antibiotic medium No. 3.

(d) *Seed layer*.—Antibiotic medium No. 11 (check that pH is 8.0) (Difco or BBL neomycin assay agar).

(e) *For bioplates*.—Antibiotic medium No. 11 with pH adjusted to 8.0–8.5.

Extracting Solvents

(a) *For tissues other than fat and skin*.—Absolute methanol.

(b) *For fat and skin*.—pH 9.5 buffer-absolute methanol-$CHCl_3$ (1+4+5) (mixed in that order). To prepare pH 9.5 buffer, use 17.42 g K_2HPO_4/L water and adjust pH with KOH.

Preparation of Inoculum

Rapidly thaw frozen vial of test organism at 48°C, and after opening the vial, prepare 1:10 dilution by adding 0.2 ml stock suspension to 1.8 ml antibiotic medium No. 3. Determine inoculum by adding amount of dilution that will give zone of inhibition $\geq$11.00 mm with 0.15 μg lincomycin/ml (usually 0.05–0.1% diluted culture is satisfactory). Alternatively, transfer growth from 2 fresh slants with 1.0 ml antibiotic medium No. 3 each to Roux bottle containing 300 ml antibiotic medium No. 1 and incubate 48 hr at 32–35°C. Harvest growth from surface with sterile, heavy inoculating loop or similar device and a few sterile glass beads. Use total of 18–20 ml antibiotic medium No. 3 in 2 aliquots. Store suspension in refrigerator $\leq$4 weeks. Prepare dilution as with frozen culture and determine inoculum.

Preparation of Plates

Add inoculum to previously cooled antibiotic medium No. 11 (48–50°C), and add 7 ml (all seed, no base layer) to each Petri dish, distribute evenly, and let harden on perfectly level surface. Do *not* use Petri dish with convex bottom. (BBL 1054 stacking dish and most disposable plastic Petri dishes are satisfactory.) Prepare plates just before performing assay. Place 6 steel cylinders and fill in usual manner.

Standard Curve

Weigh enough lincomycin HCl (available from The Upjohn Co.) and dissolve in 0.1*M* pH 8.0 phosphate buffer to obtain final concentration of 100 μg lincomycin base/ml. Alternatively, prepare 1000 μg lincomycin base/ml and prepare intermediate stock solution of 100 μg/ml phosphate buffer. For all tissues, prepare dilutions in 0.1*M* phosphate buffer adjusted to pH 8.5 to obtain final concentrations of 0.15, 0.3, 0.6 (reference concentration), 1.2, and 2.4 μg lincomycin base/ml. Since samples will be concentrated 3:2, plot points as 0.1, 0.2, 0.4, 0.8, and 1.2 ppm. For serum and urine, use pH 8.0 phosphate buffer and concentrations of 0.1, 0.2, 0.4 (reference concentration), 0.8, and 1.6 μg lincomycin base/ml (in this case, μg/ml = ppm). Use 2 plates for each concentration. Incubate 18 hr at 32°C. Read zone size of inhibition and calculate as usual for 5-point curve.

Preparation of Samples

(a) *Tissues other than fat and skin.*—Place 30 g tissue, 40 ml absolute methanol, and proper amount of 1*N* NaOH (pH ca 8.0–9.0) (*see* Table 1) in homogenizer and homogenize 3 min. Filter through Whatman No. 4 paper in Büchner funnel into 500 ml round-bottom flask. Return tissue to blender along with filter paper and 40 ml solvent (do not add more NaOH) and re-extract. Extract and homogenize 3 times. Adjust combined extracts to pH ca 3.5–4.5 with proper amount of 2*N* HCl (*see* Table 1). Reduce volume on rotoevaporator to ca 100 ml and transfer to separatory funnel. Rinse round-bottom flask with two 45 ml aliquots of hexane and add each rinse to separatory funnel. Shake 1 min and let phases separate. Place small pledget of glass wool just above stopcock to retain material formed at interface. Collect lower phase into original round-bottom flask, place on rotoevaporator, and concentrate to ca 1–2 ml. Add 5.0 ml pH 8.5 buffer and transfer material to 25 ml graduated cylinder. Wash flask with 2 more 5.0 ml aliquots of buffer and add buffer to cylinder. Adjust to pH 8.5 with 3.5*N* NaOH and adjust final volume to 20 ml with pH 8.5 buffer. Assay against standard curve, using 2 plates for each sample. After incubation, measure zone of inhibition and, by using standard curve, determine concentration in usual manner. For each set of samples assayed, fortify control tissues known to be negative for lincomycin and assay as above. From these, determine per cent recovery and whether the procedure is working properly. *See* Fig. 1 for flow sheet.

(b) *Fat and skin.*—Use 30 g tissue, 60 ml extraction solution, and no NaOH. Extract as above. Add proper amount of 2*N* HCl (Table 1), place on rotoevaporator, and reduce volume to ca 80 ml. Transfer to 500 ml separatory funnel with aid of two 45 ml portions of hexane. Shake, and let phases separate (stirring with glass rod some-

Table 1. Adjustment of pH for each tissue to be processed

Animal	Initial pH adj., ml 1*N* NaOH	Second pH adj., ml 2*N* HCl
	Muscle	
Poultry	2.3	2.0
Calf	1.3	1.2
Lamb	1.5	1.5
Swine	1.7	1.9
	Liver	
Poultry	1.1	1.0
Calf	0.7	0.9
Lamb	1.4	1.5
Swine	1.3	1.4
	Kidney	
Poultry	1.0	1.3
Calf	0.9	0.9
Lamb	0.9	1.0
Swine	1.3	1.4
	Skin/Fat	
All animals	none	0.6

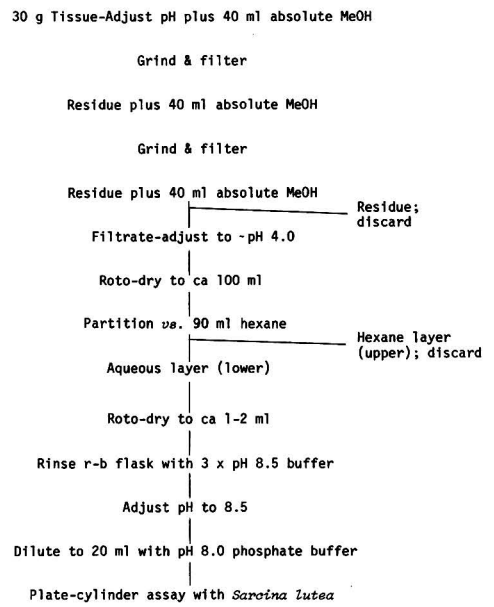


FIG. 1—Schematic of lincomycin tissue residue techniques.

times helps). Use glass wool pledget as specified above. Proceed with assay as in (a).

Confirmatory Procedure

Apparatus and Reagents

(a) *Chromatographic columns.—(1) To contain XAD-2 resin.*—Any large column with stopcock, e.g., 10 × 120 cm column. (*2*) *For sample cleanup.*—Glass column, 10–20 mm id × 500 mm with coarse porosity frit and Teflon stopcock. Fit top of column with 24/40 ⅋ female ground glass joint to which 500 ml reservoir can be attached.

(b) *Thin layer chromatographic (TLC) sheets.*—Brinkmann Polygram® Sil N-HR. *Note:* In this study, Eastman chromatogram sheets No. 13179, silica gel absorbent without fluorescent indicator, 20 × 20 cm, were used. These sheets consist of 100 μm coating of silica gel on inert, flexible poly(ethylene teraphthalate), with polyvinyl alcohol as binder. However, because of a change in the binder, these are no longer satisfactory.

(c) *Thin layer chromatographic tank.*—To accommodate 20 × 20 cm thin layer plates.

(d) *Bioplates.*—Plastic or glass tray to accommodate 1 or 2 TLC sheets, e.g., 50 × 20 × 0.8 cm.

(e) *Synthetic ion exchange resin.*—Amberlite XAD-2 (Rohm and Haas Co., Independence Mall W, Philadelphia, PA 19105) unfunctionalized divinylbenzene-cross linked polystyrene known as macroreticular-type. Pretreat before use as follows: Pack ca 10 cm glass wool (to prevent passage of fines) into bottom of large glass column (about 10 × 120 cm) with stopcock. Add ca 5 kg XAD-2 resin to produce bed height of 80 cm and place plug of glass wool on top of resin bed. Wet column with deionized water and wash resin with ca 3 gal. acetone at 60–65 ml/min. Wash column with acetone until effluent changes from cloudy to clear when added to water. Wash resin with 2 gal. methanol at the same rate. Wash overnight with deionized water at same rate to insure removal of all solvents. Store as water slurry.

(f) *Solvents.*—Analytical reagent quality acetone, methanol, and chloroform (Mallinckrodt Chemical Works); methyl ethyl ketone and ethyl acetate (Burdick & Jackson Laboratories, Inc.); diethylamine (Eastman Organic Chemicals).

(g) *Assay organism.—Sarcina lutea,* ATCC 9341. Maintain as frozen suspension in liquid nitrogen or on antibiotic medium No. 1 slants at refrigerator temperature and transfer to fresh slants ca every 2–3 weeks.

(h) *Media.—(1) For maintaining culture.*—Antibiotic medium No. 1 (Difco or BBL penicillin assay seed agar), if slants are used. (*2*) *For culture dilution.*—Antibiotic medium No. 3 (Penassay broth). (*3*) *For bioplate.*—Antibiotic medium No. 11 (Difco or BBL neomycin assay agar), adjust to pH 8.0–8.5 with NaOH.

Preparation of Sample Extract for Thin Layer Chromatography

About 15–17 ml extract remains after plate-cylinder assay. Store this extract in freezer (or refrigerator overnight), pending outcome of screening assay. If results are positive, treat extract as follows:

Column Chromatography

Add water slurry of washed XAD-2 resin (ca 100 ml) to 20 × 500 mm glass column (stopcock open) to obtain ca 300 mm bed height. Rinse sides of column with water to settle resin. Put small pledget of glass wool on top of resin and pass 100 ml water through column. Set flow rate at 8–10 ml/min. Add extract to column, rinse container with two 25 ml portions water, and add rinsings to column. Attach reservoir and wash column with 200 ml water. Discard all effluent water. Just before column goes dry, add 200 ml methanol to elute lincomycin from column. (Last portion of water on column can be discarded by noting temperature increase as methanol-water interface moves down resin.)

Dry methanol solution by rotary evaporation. Dissolve residue in 3 ml methanol-water (3+1) in preparation for TLC. Do *not* attempt to recharge XAD-2 for reuse.

Thin Layer Chromatography

Six samples can be spotted on each plate. Apply 50 μl sample on plate 2.5 cm above lower edge. Add sample in 5–10 μl amounts and let spot dry in order to obtain as small a spot at origin as is practical. Pass stream of warm air over spot between applications to speed solvent removal. Apply fortified sample containing lincomycin on each plate to establish R_f value of lincomycin. Prepare plates in duplicate. Develop one plate in methyl ethyl ketone-acetone-water (186+52+22) and the second plate in chloroform-methanol (4+1). Let solvent front move to 2–3 cm of top edge of plate. Systems 1 and 2 require 1–2 and 2–3 hr, respectively. Remove plates from tanks and let solvent evaporate.

Preparation and Use of Bioplate

Grow inoculum of *S. lutea* ATCC 9341 in antibiotic medium No. 3 overnight with shaking. Dilute 2 ml broth to 5 ml with antibiotic medium

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No. 3 and add 0.05 ml diluted culture to each liter of melted antibiotic medium No. 1 (48°C) which has been adjusted to pH 8.0–8.5. Pour ca 125 ml seed agar (depending on plate size) per plate (gives agar thickness of 1–1.5 mm). Use bioplate same day it is prepared and store in refrigerator until ready for use.

Late in afternoon, expose TLC film to bioplate. If necessary, trim off bottom edge of film to fit bioplate. Carefully place film face down on one-half of bioplate. (Two films may be placed on bioplate side by side.) Remove air bubbles by rubbing back of film with finger. Mark center of each visible TLC spot (at origin) and any additional identification on back of the bioplate. Let TLC film contact agar 30 min, remove film, and incubate plates overnight at 32°C. The following morning, compare R_f values of zones of inhibition of unknown samples with that of lincomycin. Photograph bioplates to serve as permanent record.

Results and Discussion

Average per cent recoveries from chicken and swine tissues fortified at 0.1 ppm were as follows: liver, 82.7 and 77.8; kidney, 90.5 and 78.7; muscle, 92.0 and 94.8; and fat/skin, 93.8 and 91.3, respectively. Many residue studies in chickens, turkeys, and swine indicated that lincomycin is eliminated from the body very rapidly and residues do not accumulate (Table 2). Following 7 daily injections at the same site, lincomycin decreased to <0.1 ppm at the injection site within one day post-injection. The described procedure is very workable and does the job adequately.

After the lincomycin tissue assay was developed, all potential interfering antibiotics and related compounds used in swine (2) were assayed in buffer against *S. lutea*. The estimated amounts (μg/ml) of antibiotic necessary to produce an inhibition zone equivalent to lincomycin at 0.15 μg/ml (14.0 mm zone) concentration were: ampicillin 0.015, penicillin 0.017, erythromycin 0.025, oleandomycin 0.072, tylosin 0.13, carbomycin 0.14, virginiamycin 0.20, novobiocin 0.75, gentimicin 0.88, tetracycline 1.2, neomycin 1.9, streptomycin 2.5, bacitracin 5.0 (the latter 2 are extrapolated estimations). Ampicillin had the highest specific activity. Antibiotics considered to be inactive against *S. lutea* and produced no zones at 10 μg/ml are: furaltadone, furazolidone, hygromycin, kanamycin, monensin, nitrofurazone, and spectinomycin. The antibiotics of po-

Table 2. Summary of lincomycin residue studies (ppm detected)

Rate, g/ton	Duration, days	Tissue	Withdrawal time, days 0	1	2	3	4
		Oral Administration					
100	76	liver	0.165	—	—	<0.1	—
		kidney	0.51	—	—	<0.1	—
		muscle	<0.1	—	—	<0.1	—
		fat	<0.1	—	—	<0.1	—
1000	37	all tissue	4.18	—	—	—	<0.1
2600	28	all tissue	16.50	—	—	<0.1	—
		Intramuscular Administration					
5 mg/lb body wt	7	liver	8.43	0.13	<0.1	—	—
		kidney	21.00	0.24	<0.1	—	—
		site of injection	93.33	<0.1	—	—	—

Table 3. Antibiotics interfering in the basic lincomycin tissue residue procedure

Antibiotic	Antibiotic, ppm	Apparent lincomycin, ppm	Observed residue as lincomycin, ppm	Rec. through procedure, %
Erythromycin	0.33	1.60	0.45	28.0
Oleandomycin	0.67	1.06	0.15	14.0
Carbomycin	0.67	0.60	0.10	17.0
Lincomycin	0.67	0.67	0.51	76.0
Tylosin	3.3	0.93	0.55	59.0
Virginiamycin	6.3	1.25	0.16	13.0
Novobiocin	6.7	0.63	0.45	71.0

tential concern were assayed according to the procedure so their effect on lincomycin recovery could be determined. The results (Table 3) indicated that apparent lincomycin recoveries ranged from 13 to 71% for virginiamycin and novobiocin, respectively. Therefore, a confirmatory procedure was required to determine whether the activity observed in the microbiological assay was indeed due to lincomycin.

The use of paper chromatography and TLC to separate chemicals and antibiotics is well known and is useful to separate many closely related compounds (3, 4). Since lincomycin and many antibiotics cannot be made visible on TLC, a bioplate is used. All of the active antibiotics are spotted on a TLC plate; the plates are developed and placed on bioplates seeded with *S. lutea*. Methyl ethyl ketone-acetone-water (186+52+22) was quite effective in separating all the antibiotics (Table 4). However, with tissues present, tylosin and lincomycin and high levels of penicillin and lincomycin sometimes caused the zones of inhibition to become one large zone. Penicillin presented no problem as penicillinase would remove the activity. Tylosin interference was avoided by using chloroform-methanol (6+1).

Table 4. TLC of representative antibiotics in comparison with lincomycin

Position No.	Antibiotic	Amt on plate,[a] μg/ml	R_f[b] System 1	R_f[b] System 2
1	neomycin	10.0	0.00	0.00
2	bacitracin	100.0	0.00	0.00
3	novobiocin	25.0	0.54	0.40
4	penicillin	0.05[c]	0.26	0.17
5	tetracycline	20.0	0.00	0.00
6	lincomycin	2.0	0.40	0.51
7	virginiamycin	19.0	0.62	0.72
8	carbomycin	5.0	0.66	0.75
9	erythromycin	1.0	0.13	0.38[d]
10	oleandomycin	5.0	0.16	0.41[d]
11	tylosin	2.0	0.57	0.71
12	lincomycin	2.0	0.39	0.48

[a] Applied as 50 μl aliquot of indicated concentration.
[b] System 1 = methyl ethyl ketone-acetone-water (186+52+22); System 2 = chloroform-methanol (4+1).
[c] Unit/ml.
[d] Merged with lincomycin.

Table 5. Confirmation of lincomycin in the presence of potential interfering antibiotics in swine liver tissue

Antibiotic	Fortification level, ppm	Apparent lincomycin, ppm	Separation[a] TLC 1	Separation[a] TLC 2
Erythromycin	0.4	0.1	yes	no
Oleandomycin	4.0	0.075	yes	no
Tylosin	1.0	0.48	no	yes
Carbomycin	2.0	0.62	yes	yes
Virginiamycin	11.0	0.35	yes	yes
Lincomycin	0.1	0.08	—	—

[a] System 1 = methyl ethyl ketone-acetone-water (186+52+22); System 2 = chloroform-methanol (4+1).

In order to test the confirmatory procedure under conditions where a second antibiotic may be present at a concentration much higher than the lower limit of detection for lincomycin, a series of swine liver samples were fortified with 0.1 ppm lincomycin plus the second antibiotic at an elevated level. The results of this test (Table 5) indicated that lincomycin can be confirmed at 0.1 ppm in the presence of erythromycin, oleandomycin, carbomycin, and virginiamycin in one TLC system, whereas the second TLC system is required if tylosin is present. Actual tracings of TLC-bioautograms of various antibiotics plus lincomycin are shown in Figs. 2–5.

If tylosin B (desmycosin, a metabolite of tylosin A) is present, it must be separated from lincomycin by a third solvent system, ethyl acetate-diethylamine (9+1). In this system, lincomycin and tylosin A and B have R_f values of <0.1, 0.54, and 0.61, respectively.

In developing a confirmatory procedure for antibiotics in animal tissues, it should be pointed out that the analyst's general knowledge of each

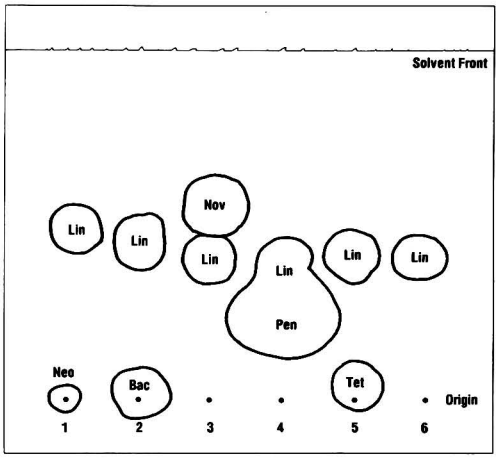


FIG. 2—Actual tracing of TLC bioautograph: methyl ethyl ketone-acetone-water (186+52+22).

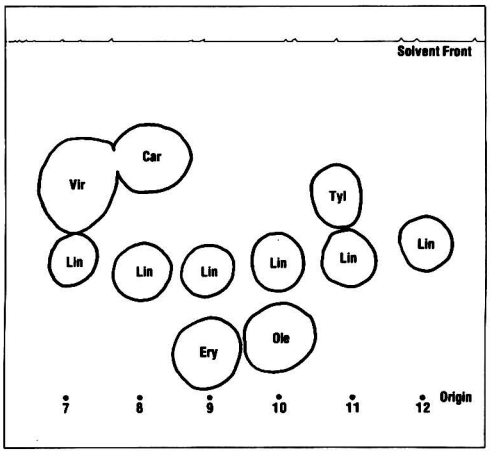


FIG. 3—Actual tracing of TLC bioautograph: methyl ethyl ketone-acetone-water (186+52+22).

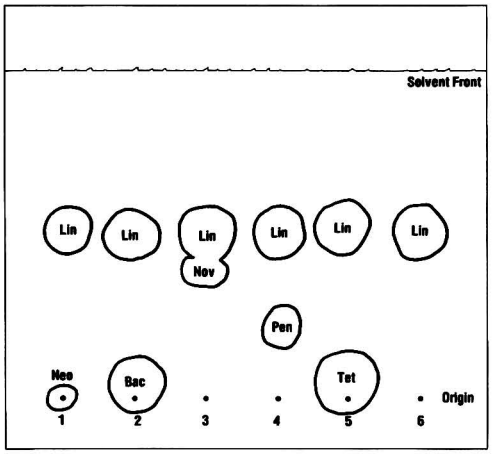


FIG. 4—Actual tracing of TLC bioautograph: chloroform-methanol (4+1).

antibiotic and its likelihood of being present in a tissue will be of inestimable value in identifying the unknown antibiotics in a tissue sample. Also, the confirmatory procedures for other suspect antibiotics will be available for positive

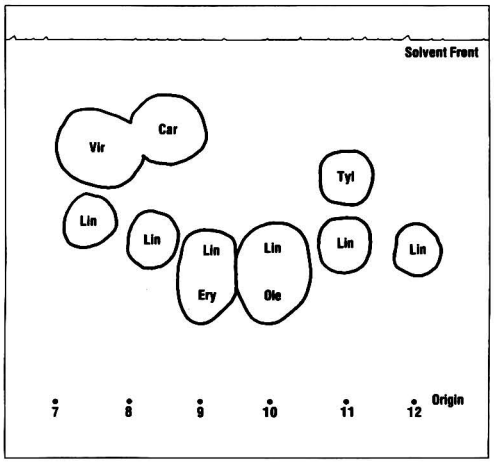


FIG. 5—Actual tracing of TLC bioautograph: chloroform-methanol (4+1).

identification of the offending antibiotic. It is also quite possible that the TLC systems used here may be useful in confirming the presence of some of the other antibiotics which are of concern to the analyst.

It is recommended that the study of lincomycin be continued.

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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**, 398.

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

OILS AND FATS

Gas-Liquid Chromatographic Determination of C_{22} Fatty Acids for the Certification of Low Erucic Acid Varieties of Rapeseeds

JEAN P. BARRETTE
Laboratory Services Section, Plant Products Division, Agriculture Canada, Ottawa, Ontario, Canada K1A 0C5

A rapid gas-liquid chromatographic (GLC) procedure was developed for the determination of C_{22} fatty acids (mostly erucic acid) in rapeseeds containing less than 5% erucic acid. The method involves an oil extraction from a representative sample of rapeseeds, a vigorous boron trifluoride-methanol transesterification of the oil to methyl esters, and analysis by GLC with the aid of an electronic integrator. Six laboratories participated in a monthly check sample program. By adjusting the GLC detector response with 2 synthetic reference standard mixtures containing 1.0 and 3.0% methyl behenate, interlaboratory agreement within 0.3% C_{22} was consistently obtained.

When the low erucic acid varieties of rapeseeds appeared on the Canadian markets in the early 1970s, standards were set for foundation and certified grades of rapeseeds which limited the maximum permissible levels of C_{22} fatty acids to between 0.8 and 3.2%, depending on the variety. In order to enforce these standards and help the seed trade to comply, a simple and rapid procedure was developed to harmonize laboratory results from the government and private sectors. Furthermore, since regulatory laboratories are faced with a rapidly increasing load of work, commercial laboratories were eligible to apply for authorized establishment status, provided they successfully participated in a monthly check sample program. Six laboratories have shown an average standard deviation of 0.15% on 5 monthly check samples over a 10-month period, for C_{22} fatty acid analysis.

Gas-liquid chromatography (GLC) has long been recognized as the most accurate procedure for determining fatty acid content of oils. Classically, however, chromatography on polar columns, which separates saturated and nonsaturated homologs, is rather lengthy, requiring up to 1½ hr. Regulatory activities require the analysis of thousands of samples each year, so the use of polar columns which separate erucic acid from other C_{22} fatty acids is considered prohibitive. The content of C_{22} behenic acid in the lower erucic acid rapeseeds is low, generally around 0.2–0.3%. It can either be taken into account and systematically subtracted or neglected, and nonpolar GLC columns which reduce the analysis time to <10 min may be used. This view is now shared by other workers in the field (1).

Preparing methyl esters from rape oil is an important step which requires time and skillful manipulations. Prevot *et al.* (1) recently reported a procedure which reduces this step to 2.5 min; however, samples must be prepared individually, and stopwatch precision is necessary to ensure uniformity. Base-catalyzed transesterification methods can be more rapid than acid-catalyzed reactions, but timing is important because of the 2 competing reactions which take place at different rates, namely, the rapid formation of methyl esters and the simultaneous, but slow, saponification, of these esters.

Although acid-catalyzed transesterifications seem to be less popular with oil chemists because of the time required, the boron trifluoride-methanol procedure (2) described in this paper is nevertheless simple and reproducible, and, most important, 25–30 samples/hr can be analyzed simultaneously without rigorous supervision. Once the reaction is completed, no competing reactions can upset the equilibrium. The reaction time was reduced to about 1 hr by vigorously agitating the samples at 65°C in an ultrasonic bath. The possible loss of the volatile fatty acid methyl esters was completely eliminated by carrying out the reactions in vials sealed with air-tight screw caps fitted with Teflon liners.

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METHOD

Reagents and Apparatus

All solvents are reagent grade.

(a) *Boron trifluoride.*—14% in anhydrous methanol.

(b) *Reference standard solutions.*—*Standard A (3% C_{22}).*—Weigh to nearest mg: 0.05 g methyl palmitate (C_{16}), 0.85 g methyl stearate (C_{18}), 0.07 g methyl arachidate (C_{20}), and 0.03 g methyl behenate (C_{22}) and dissolve in 100 ml *n*-hexane. (Pure reference fatty acid methyl esters were obtained from Applied Science Laboratories, State College, PA.) *Standard B (1% C_{22}).*—Prepare similarly to Standard A, but add only 0.01 g methyl behenate.

(c) *Extraction and transesterification equipment.*—Micromill (Krups Model 25); rotary evaporator (Buchi Rotovapor); ultrasonic bath (Branson Model D-150).

(d) *Gas chromatograph.* — Hewlett-Packard Model 5700 (Palo Alto, CA) or equivalent, fitted with dual flame ionization detectors and 6′ × ⅛″ diameter stainless steel columns containing 10% U.C. W98 or equivalent on 80–100 mesh Chromosorb W (Applied Science Laboratories, Inc., State College, PA). Operating conditions: temperatures (°C)—injector 280, detector 300, column 250; flow rates (ml/min): helium carrier gas 30, hydrogen 50, air 500. Peak areas are measured by electronic integrator (Hewlett-Packard Model 3370B, or equivalent).

Preparation of Sample

Grind ca 10 g rapeseeds 10 sec in micromill. Transfer rapemeal to 250 ml beaker. Add 50 ml petroleum ether (bp 60–80°C, or *n*-hexane), swirl 5 sec, and let stand 15 min. Filter supernate through Whatman No. 1 paper into 125 ml round-bottom flask. Evaporate solvent on rotary vacuum evaporator until oil residue is practically free of solvent.

Transesterification

Transfer ca 0.1 g oil (ca 5 drops) to 50 ml, 85 mm × 35 mm diameter, screw-cap vial. Add 10 ml anhydrous methanol and 2 ml 14% BF_3 in methanol. Place sealed vial in 65°C ultrasonic bath with generator activated ca 1 hr. Under this vigorous agitation, reaction mixture becomes emulsified, but when reaction is complete, mixture is clear. Let vials cool, add 5 ml water and, by pipet, 5 ml petroleum ether. Seal and vigorously shake each vial ca 5 sec. Let stand 2–3 min; petroleum ether phase separates cleanly from aqueous methanol phase. Inject 1 μl aliquot of upper phase (petroleum ether or hexane layer) directly onto GLC column.

Calibration of the Chromatographic-Integrator System

Alternately inject 1 μl of each reference standard solution A and B. From the integrated total areas for C_{16}, C_{18}, C_{20}, and C_{22}, calculate per cent composition corresponding to each peak. Adjust, if necessary, hydrogen-air flow ratio so that agreement is as close as possible between calculated and actual percentages for C_{16}, C_{18}, and C_{20} but well within ±0.1% for C_{22} for both reference standards. When this adjustment is achieved for the standards, the GLC system is ready for the analysis of samples.

Analysis of Esterified Samples

Inject 1 μl upper phase from each sample vial. While exact quantity of methyl esters can vary slightly from sample to sample, sum total of all integrated peak areas for reference standards and samples must agree within ±10%. Keep injection volume constant. If necessary, adjust sample concentration by either diluting or concentrating the petroleum ether solvent incrementally. In most samples a measurable peak for C_{24} will appear and must be included in the sum total peak areas for calculating per cent C_{22} present. Calculate per cent C_{22} by multiplying ratio of integrated count for C_{22}/the sum total count for all peaks times 100, and correcting to the nearest 0.1%.

Interlaboratory Evaluation of Method

Ten 25 lb samples of Span rapeseed, with erucic acid content ranging between 3.0 and 4.0%, were carefully subdivided into about 10 g subsamples by using a commercial fertilizer divider. For the first distribution, 6 laboratories were provided with a complete set of 10 samples, 10 ml reference standard solutions A and B, and an outline of the proposed method. Each laboratory was requested to conduct duplicate analyses of the oil from each sample on 2 different days. Subsequently, 5 of the participating laboratories agreed to analyze 5 samples in duplicate each month by using the above method only. For 10 months (Jan.–Oct. 1973), 5 samples selected at random from the 10 original samples, and identified differently, were distributed each month. Participants were supplied with standard solutions A and B on request.

Results and Discussion

Of the 6 laboratories contacted, Laboratories 5 and 6 were unable to test the proposed method for the first distribution of samples because of

delays in obtaining equipment. Table 1 summarizes results obtained by 4 laboratories. Table 2 gives results reported by Laboratories 4, 5, and 6 for the same instrumental method but with alternative transesterification procedures, all of which were variations of base-catalyzed reactions. All participants adjusted their GLC equipment with the standard solutions supplied and obtained results within 0.05% of the actual per cent C_{22} for both standard solutions.

Comparison of results in Tables 1 and 2 shows that the proposed method generally gave more consistent results. A mean range of 0.49 and mean standard deviation of 0.146 as against 0.96 and 0.442 for the alternative procedure was sufficient to encourage 5 of the laboratories to agree to participate in a monthly check sample program to further evaluate the proposed method.

Table 3 shows results reported by 5 laboratories on the same 10 rapeseed samples, redistributed monthly, 5 at a time, over 10 months. Some samples were distributed 6 times while Sample 8 was sent only 3 times. Each result in Table 3 represents a sample distribution and an average of duplicate analyses.

Comparison of results in Tables 1 and 3 shows excellent agreement of the sample mean for all 10 samples. This is remarkable, since results in Table 1 represent analyses by 4 laboratories with a new and unfamiliar method. Although the mean range in Table 3 is slightly higher (0.64), it is still acceptable. By applying a rejection limit of sample mean ±2 standard deviations,

Table 1. Interlaboratory results for proposed transesterification and GLC analysis of C_{22} fatty acids (%) in rapeseed oil

	Samples									
Lab.	1	2	3	4	5	6	7	8	9	10
1	3.3	4.0	3.5	3.7	3.2	4.2	3.5	3.4	3.1	3.7
	3.1	4.1	3.6	3.7	3.2	4.0	3.4	3.3	3.0	3.8
	3.4	3.9	3.6	3.8	3.3	4.2	3.4	3.4	3.1	3.7
	3.2	4.2	3.5	3.7	3.2	4.0	3.4	3.3	3.1	3.8
2	3.3	4.4	3.7	3.8	3.3	3.8	3.5	3.3	3.2	4.0
	3.3	4.3	3.5	3.9	3.3	3.9	3.5	3.3	3.2	3.9
	3.2	4.3	3.6	3.7	3.2	3.8	3.5	3.3	3.2	4.0
	3.3	4.2	3.6	3.8	3.2	3.9	3.6	3.3	3.2	3.9
3	3.3	4.1	3.3	4.2	3.3	3.8	3.2	3.1	3.0	3.9
	3.5	4.0	3.2	3.7	3.3	4.1	3.5	3.3	3.3	4.0
4	3.2	4.2	2.9	3.5	3.3	3.6	3.4	3.2	2.8	3.5
	3.3	4.0	2.8	3.5	3.1	3.7	3.4	3.2	2.8	3.6
Mean	3.28	4.14	3.41	3.75	3.24	3.91	3.44	3.28	3.08	3.82
Range	0.4	0.5	0.9	0.7	0.2	0.6	0.4	0.3	0.5	0.5
Std dev.	0.10	0.15	0.27	0.18	0.07	0.19	0.10	0.08	0.16	0.16
Mean range	0.49									
Mean std dev.	0.146									

Table 2. Interlaboratory results for alternative transesterification methods for analysis of C_{22} fatty acids (%) in rapeseed oil

	Sample									
Lab.	1	2	3	4	5	6	7	8	9	10
4	3.2	3.9	3.2	3.6	2.9	3.7	3.1	3.0	3.2	3.6
	3.2	4.1	3.1	3.5	3.1	3.8	3.5	2.8	3.2	3.5
5	3.6	3.1	3.8	4.0	3.7	2.0	3.1	2.5	2.2	4.9
6	2.9	3.4	3.6	3.0	2.8	3.4	3.1	2.8	2.5	3.1
Mean	3.23	3.63	3.43	3.53	3.13	3.23	3.20	2.78	2.78	3.78
Range	0.7	0.8	0.7	1.0	0.9	1.8	0.4	0.5	1.0	1.8
Std dev.	0.29	0.46	0.33	0.41	0.40	0.83	0.20	0.21	0.51	0.78
Mean range	0.96									
Mean std dev.	0.442									

Table 3. Interlaboratory results for proposed analysis of C_{22} fatty acids (%) in rapeseed oil—monthly check sample program

Lab.	Sample 1	2	3	4	5	6	7	8	9	10
1	3.3	4.2	3.5	3.8	3.2	4.1	3.4	3.3	3.2	3.8
	3.4	4.1	3.4	3.6	3.4	3.9	3.5	3.1	3.1	3.9
	3.2	4.2	3.5	3.7	3.4	3.9	3.4	3.4	3.1	3.8
	3.2	4.0	3.7	3.7	3.3	4.0	3.4		3.0	3.7
			3.6	3.7	3.0		3.3		3.2	3.8
			3.4				3.6		2.9	
2	3.2	4.0	3.2	3.6	3.2	3.9	3.4	3.5	3.0	3.6
	3.4	4.1	3.4	3.6	3.2	4.0	3.4	3.2	3.1	3.6
	3.4	4.1	3.3	3.8	3.3	3.8	3.4	3.3	3.3	3.9
	3.0	4.1	3.7	3.5	3.4	3.9	3.4		3.0	3.7
			3.5	3.7	3.3		3.4		3.1	3.8
			3.4				3.6		3.1	
4	3.4	4.1	3.3	4.0	3.1	3.6	3.1	3.6	3.0	4.1
	3.3	4.1	3.4	3.8	3.3	3.9	3.1	3.2	3.6	3.8
	2.9	3.6	3.3	3.7	3.3	3.7	3.4	3.2	3.2	3.8
	3.1	4.0	3.1	3.9	2.9	4.0	3.0		3.2	3.9
			3.3	3.7	3.2		3.4		3.1	3.7
			3.5				3.3		3.2	
5	3.5	4.1	3.3	3.7	3.2	3.9	3.4	3.5	3.1	3.9
	3.3	4.1	3.3	3.8	3.3	3.9	3.4	3.2	3.4	3.8
	3.3	3.9	3.5	3.7	3.2	4.1	3.6	3.7	3.1	3.9
	3.4	3.9	3.4	3.5	3.3		3.6		3.1	4.0
			3.8		3.6		3.9		3.2	
							3.8			
6	3.3	4.0	3.3	3.8	3.1	3.7	3.6	3.0	3.2	3.9
	3.4	4.0	3.4	3.6	3.7	4.2	3.8	3.4	3.3	3.7
	3.5	4.1	3.4	3.7	3.2	3.8	3.5		3.0	3.8
	3.2	4.0	3.4	3.6	3.3	3.8	3.8		3.1	3.7
			3.5		3.2		3.4		3.2	
							3.4			
Mean	3.29	4.04	3.42	3.70	3.26	3.90	3.46	3.33	3.15	3.81
Range	0.6	0.6	0.7	0.5	0.7	0.6	0.9	0.7	0.7	0.5
Std dev.	0.16	0.13	0.15	0.12	0.17	0.15	0.20	0.19	0.14	0.12
Mean range	0.64									
Mean std dev.	0.153									

only 9 of 230 results (3.91%) in Table 3 would be rejected, indicating an acceptance probability of 96%.

Fulk and Shorb (3) reported that the boron trifluoride-methanol transesterification procedure can produce artifacts which cause a 30% conversion loss of oleic acid. However, in isolated experiments, we have found that the transesterification procedure proposed in this paper, at time intervals of 45 min to 2 hr, did not alter the peak areas of C_{16} or C_{22}. Under the conditions employed in this procedure, no artifacts have been observed; this confirms the findings of Morrison and Smith (4).

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PESTICIDE RESIDUES

Gas-Liquid Chromatographic Determination of Alachlor (2-Chloro-2′,6′-diethyl-*N*-(methoxymethyl)-acetanilide) Herbicide Residues in Corn and Soybeans

BARBARA DOHMAN AMMANN, DANIEL J. CALL, and HANS A. DRAAYER
Department of Chemistry, South Dakota State University, Brookings, SD 57006

A method has been developed for the extraction and determination of alachlor (2-chloro-2′,6′-diethyl-*N*-(methoxymethyl)-acetanilide) residues in green corn and soybeans. Residues are extracted with acetonitrile and cleaned up on a Florisil column. The methylene chloride extract is sufficiently clean for electron capture gas-liquid chromatographic analysis and for verification by thin layer chromatography. Average recoveries of spiked samples (0.2 ppm) were 69 and 82% for corn and soybeans, respectively. This procedure could be useful for the detection of the parent compound in these crops soon after field application, but it does not detect metabolites.

Alachlor (2-chloro-2′,6′-diethyl-*N*-(methoxymethyl)-acetanilide, Lasso) has become a commonly used herbicide for weed control in midwestern crops such as corn and soybeans. Present methods of residue analysis involve either acid-base or acid-pressure hydrolysis of the parent compound to yield 2,6-diethylaniline, and subsequent determination of this derivative by flame ionization gas-liquid chromatography (GLC) (*Pesticide Analytical Manual* (1969) Vol. II, Monsanto Chemical Co., St. Louis, MO, sec. 120.249).

This laboratory developed a rapid method for determining the herbicide which eliminated the hydrolysis procedure. The parent compound in green corn and soybeans is detected by electron capture GLC, and verified by thin layer chromatography (TLC).

METHOD

Reagents

(a) *Alachlor.* — Analytical standard, 99.9+% (Monsanto Chemical Co., St. Louis, MO).

(b) *Solvents.*—Nanograde quality methylene chloride, acetonitrile, and hexane; methanol; and distilled analytical grade ethanol (Mallinckrodt Chemical Works, St. Louis, MO).

(c) *Florisil.*—60–100 mesh, commercially activated at 649°C (Fisher Scientific Co., Pittsburgh, PA). Heated 16 hr at 130°C and deactivated by adding 6% (w/w) water before use.

(d) *Thin layer adsorbent.*—Silica gel G (EM Reagents Division, Brinkmann Instruments, Inc., Westbury, NY).

(e) *Chromogenic agent.*—0.2 mg Rhodamine B (Aldrich Chemical Co., Milwaukee, WI)/ml methanol.

Apparatus

(a) *Flash evaporator.*—Buchler portable Model PTFE (Buchler Instruments, Fort Lee, NJ).

(b) *Chromatographic columns.* — 20 mm id × 400 mm Pyrex glass tubes with sealed-in fritted disks (Curtin Matheson Co., Minneapolis, MN).

(c) *Homogenizer.*—Sorvall Omni-Mixer (Norwalk, CT).

(d) *Thin layer equipment.* — Standard applicator, 20 × 20 cm glass plates, and developing chamber (Desaga-Brinkmann Instruments, Inc., Westbury, NY).

(e) *Gas chromatograph.* — Varian Aerograph Series 2100, equipped with ^{63}Ni electron capture detector, and 1 mv recorder (Beckman Ten Inch). Borosilicate glass column 1/16″ id × 6′, packed with 15% QF-1 silicone (fluoro)/10% DC-200 silicone on 80–100 mesh Chromosorb W (HP) (acid-washed and dimethylchlorosilane-treated). Nitrogen carrier gas 40 ml/min; operating temperatures for injector, column, and detector 210, 200, and 290°C, respectively.

Extraction

Place 20 g chopped sample in Petri dish. Air-dry 24 hr at room temperature; then place in pint jar. Add 150 ml acetonitrile and blend in homogenizer 5 min. Filter through S&S No. 589 black ribbon paper in Büchner funnel into 500 ml suction filtration flask. Rinse jar with two 25 ml portions of acetonitrile, and filter rinses. Transfer

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extract to 500 ml round-bottom flask and evaporate to dryness on flash evaporator. Transfer extract to 10 ml test tube, using 3 rinses of ca 3 ml hexane. Dilute to 10 ml.

Cleanup

Place 5 ml sample on 10 g anhydrous Na_2SO_4 mixed with 5 g Florisil in 100 ml beaker, mix thoroughly, and dry gently with nitrogen until material is free flowing. Prepare chromatographic column by placing 30 g deactivated Florisil into chromatographic column, rinse column with 150 ml hexane, and discard rinse. Place sample on column when hexane is ca 3″ from top of column packing. Rinse beaker with 10 ml hexane and place rinse on column. Elute sample with 500 ml methylene chloride, discarding first 100 ml. Collect remaining 400 ml in 1 L round-bottom flask. Function of column should be checked with each new batch of Florisil to confirm that no alachlor is eluted in first 100 ml or after last 400 ml. Evaporate sample to dryness on flash evaporator, quantitatively transfer to test tube, and dilute to 10 ml with hexane. Inject 1–3 μl sample, equivalent to 1–3 mg original crop. Determine peak area as ½ base × height in recorder paper units. Using this value, quantitate alachlor from standard curve in μg/ml (Fig. 1). Each laboratory should prepare its own standard curve, because of GLC and column discrepancies.

For verification by TLC, prepare 0.5 mm thick plates by adding 120 ml water to 60 g silica gel G and apply sorbent slurry with adjustable applicator to 20 × 20 cm glass TLC plates. Let plates dry overnight at room temperature, and store in desiccator 2–3 hr before use. Evaporate sample to ca 0.1 ml with nitrogen, and spot sample on plate beside 40 μl 1.0 mg alachlor/ml standard. Develop 15 cm with absolute ethanol. Remove plate and dry, cover sample section of plate, and spray standard section with Rhodamine B solution. Visualize under shortwave ultraviolet light. Scrape off sample area at same R_f value as alachlor standard (ca 0.73). Collect sample in glass Pasteur pipet plugged with glass wool. Elute sample from silica gel with ca 10 ml methylene chloride. Evaporate just to dryness with nitrogen and dilute to 10 ml with hexane. Inject 1–3 μl aliquot onto chromatographic column.

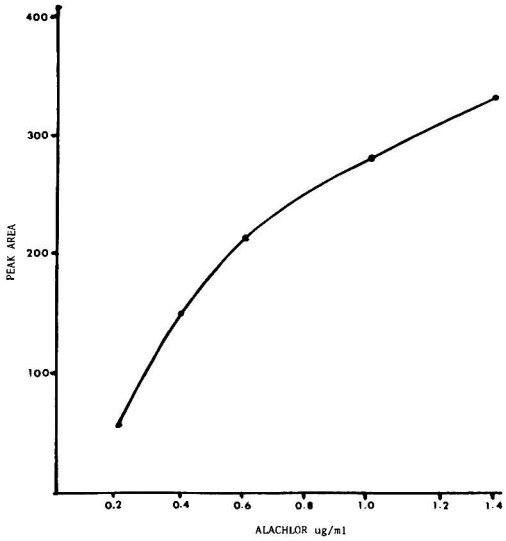


FIG. 1—^{63}Ni electron capture response to a range of alachlor concentrations.

Results and Discussion

Recoveries were determined by adding 0.2 ppm alachlor to 10 samples each of corn and soybeans (Table 1). Per cent recoveries and standard deviations were 69±6.5 and 82±6.5% for corn and soybeans, respectively. The lower limit of detection for this procedure was 0.05 ppm. The Monsanto procedure gives recoveries of 70–90%, and a lower limit of detection of 0.05 ppm for crops (personal communication, Richard Trammer, Monsanto Chemical Co., St. Louis, MO, Aug. 1975).

The gas-liquid chromatograms of the blank corn and soybeans showed no peaks with the same relative retention time as alachlor (about 1.09). Typical chromatograms of blank and spiked (0.2 ppm) corn and soybean samples, and 0.6 ng alachlor standard, are shown in Fig. 2. Alachlor was verified by TLC.

The response of the ^{63}Ni electron capture detector to alachlor was determined over a range 0.2–1.4 μg/ml as a check for linearity. This is shown in Fig. 1.

The authors feel that this method is rapid and

Table 1. Per cent recoveries of 0.2 ppm alachlor added to corn and soybeans

Analysis	Corn	Soybeans
1	65	72
2	70	72
3	70	83
4	62	82
5	77	90
6	63	90
7	63	83
8	67	80
9	81	84
10	75	89

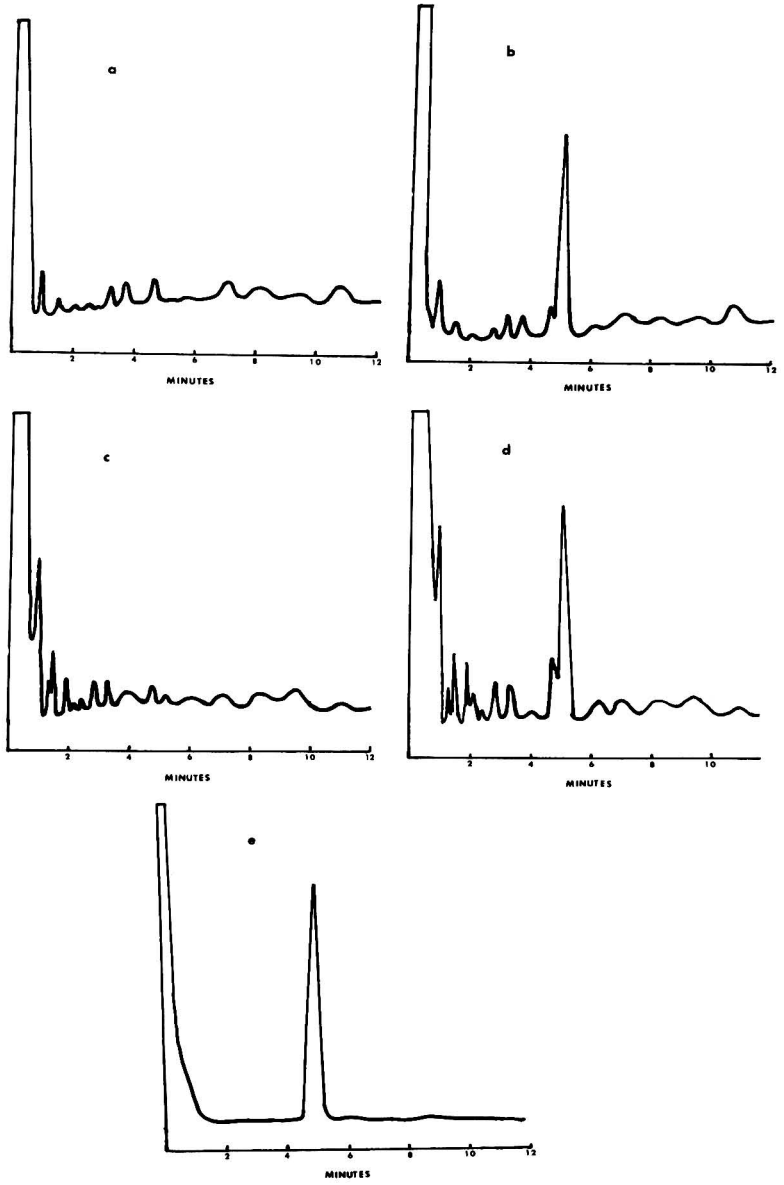


FIG. 2—Chromatograms for a, corn blank; b, corn with 0.2 ppm alachlor added; c, soybean blank; d, soybean with 0.2 ppm alachlor added; and e, 0.6 ng alachlor standard.

efficient for determining alachlor in corn and soybeans. Compared with previous methods, this procedure is considerably shorter and omits steps which involve a safety hazard. This method does not determine the metabolites of alachlor, whereas one of the Monsanto procedures does (*Pesticide Analytical Manual*, Vol. II, sec. 120.249). Samples were sufficiently cleaned up on the Florisil column for GLC determination and TLC verification.

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Gas-Liquid Chromatographic Determination of 3-Trifluoromethyl-4-nitrophenol in Natural Waters

JOHN A. COBURN and ALFRED S. Y. CHAU
Water Quality Branch, Canada Centre for Inland Waters, 867 Lakeshore Rd, Burlington, Ontario, Canada L7R 4A6

A procedure for the analysis of 3-trifluoromethyl-4-nitrophenol (TFM) in natural waters is described. The lampricide is extracted from acidified water samples on the macroreticular resin XAD-7 and eluted from the column with ethyl ether. The ether extract is dried, concentrated, and partitioned with potassium carbonate. TFM is acetylated in the aqueous alkaline solution and the acetate derivative is extracted into benzene for analysis by electron capture gas-liquid chromatography. Recoveries of TFM from natural waters exceeded 90% and as little as 0.01 μg TFM can be quantitated in a 1 L sample.

The continuing use of 3-trifluoromethyl-4-nitrophenol (TFM) for the control of sea lamprey in the Great Lakes necessitates the development of sensitive analytical procedures for the routine analysis of TFM in fish, sediments, and water. Allen and Sills (1) reported a sensitive method for the determination of TFM in fish by using the methyl ether derivative for gas-liquid chromatographic (GLC) quantitation. The methods available for the analysis of water samples are colorimetric analyses (2, 3) which are less sensitive and less specific than GLC analysis. The acetylation procedure we used is a modification of Chattaway's method (4) of acetylation in aqueous alkaline solution that has been reported previously for the analysis of pentachlorophenol from natural waste and waters (5, 6). Bothwell *et al.* (7) reported using XAD-2 macroreticular resin in their study on the degradation of TFM.

METHOD

Reagents

Distilled in glass, pesticide residue grade solvents were used throughout.

(a) *3-Trifluoromethyl-4-nitrophenol.* — Purified grade (Alfred Bader Library of Rare Chemicals, Division of Aldrich Chemical Co., Milwaukee, WI 53233).

(b) *Acetic anhydride.*—99+% (Matheson, Coleman and Bell, East Rutherford, NJ 07073). Purified as detailed in ref. 5.

(c) *Potassium carbonate.*—0.1*M*. Dissolve 13.8 g K_2CO_3 (Analar analytical reagent) in 1 L XAD-2-purified distilled water. Details on the purification of distilled water with XAD-2 resin are reported in ref. 8.

(d) *Macroreticular resin.*—XAD-7 (obtained from Rohm and Haas through British Drug Houses Chemical Co., Toronto, Ontario). Purify according to ref. 8 and store in closed glass container under methanol.

(e) *Anhydrous sodium sulfate.*—Reagent grade; heat 12 hr at 350°C and store in closed container in desiccator.

(f) *Silanized glass wool.*—For use in resin columns (Altech Associates Inc., Arlington Heights, IL 60004).

Apparatus

(a) *Gas chromatograph.* — Hewlett-Packard Model 5713 equipped with linearized ^{63}Ni electron capture detector. Operating conditions: column 200°C, detector 300°C, argon-10% methane carrier gas flow 65 ml/min, electrometer settings ×8–×32.

(b) *Columns.*—1.8 m × 3.5 mm glass columns packed with (*1*) 4% SE-30/6% OV-210 on 80–100 mesh Gas-Chrom Q; (*2*) 1.5% OV-17/1.95% OV-210 on 80–100 mesh Gas-Chrom Q. (Column materials obtained from Applied Science Laboratories, Inc., State College, PA 16801.)

(c) *Resin columns.*—30 cm glass column, 12 mm od with Teflon stopcock and 19/38 ⅀ female ground-glass joint and separate 250 ml capacity glass reservoirs with 19/38 ⅀ male ground-glass joint (Emerald Glass Co., Toronto, Ontario).

Extraction and Derivatization

Prepare resin column for each sample as follows: Place small wad of silanized glass wool in glass column, add purified XAD-7 in methanol to packed height of 6 cm, and place silanized glass wool on top of resin. Maintain level of methanol above resin bed at all times during preparation. Wash column with 20–30 ml methanol followed by

100–150 ml XAD-2-purified water to remove all traces of methanol; let water level reach just above top of resin.

Adjust each 1 L sample to pH 1–2 with H_2SO_4 and pass water sample through resin column at $\leq$50 ml/min. Let resin column drain after entire sample has passed through column. Place 125 ml separatory funnel under column and add 25 ml ethyl ether to sample container. Rinse sample container, transfer solvent to column, taking care to wash down reservoir with solvent, and let ether drain through resin until 2 phases are evident in separatory funnel. Cap column, close stopcock, and let ether remain in contact with resin 20–30 min. Place reservoir back onto column and elute column with 50 ml ether. Let solvent drain completely into separatory funnel. Discard aqueous phase and dry solvent by suction through 10–20 g column of Na_2SO_4, wash Na_2SO_4 with 50–60 ml benzene, and collect ether and benzene in 300 ml round-bottom flask. Evaporate to ca 10 ml on rotary evaporator (40°C water bath), transfer to 125 ml separatory funnel, rinse flask with 30–40 ml benzene, and add rinse to separatory funnel.

Partition solvent with two 50 ml aliquots of 0.1*M* K_2CO_3, transferring aqueous phase to 125 ml Erlenmeyer flask after each partitioning. Pipet 10.0 ml benzene into flask and add 1 ml acetic anhydride. Stir reaction mixture 1 hr, remove benzene, dry with 1–2 g Na_2SO_4, and inject aliquot onto GLC column. For samples containing <0.1 μg TFM/L, concentrate final benzene extract to 1–5 ml for GLC analysis.

Quantitation

Standards should be prepared and analyzed with each group of samples, along with method blank (including the resin column) if samples containing <0.1 μg TFM/L are to be analyzed. Prepare series of 3 standards to cover expected concentration range by adding 0.01–10.0 μg TFM to 100 ml 0.1*M* K_2CO_3 solution and derivatizing exactly as detailed for samples. Determine sample concentrations either by comparing with a standard curve or by directly comparing areas of sample and closest standard, using the following formula:

$$\text{TFM, } \mu g/L \text{ (ppb)} = (A \times W \times EV \times 10^{-3})/(A' \times IV \times SV)$$

where A and A' = peak area of sample and standard, respectively; W = weight (pg) TFM that produces the peak area A'; IV = injection volume (μl); SV = sample volume (L); EV = extract volume (ml).

Samples and standards should be analyzed on at least 2 different GLC columns to ensure correct identification and to minimize effects of any co-extractives on quantitation.

Results and Discussion

The acetylation reaction for determining TFM was chosen on the basis of selectivity, speed, sensitivity, and lack of reagent background of this procedure. All of these factors have been discussed previously (5, 6). The derivatization procedure was tested at 2 concentrations of TFM in order to determine the repeatability of the technique. Nine replicates at each concentration were analyzed by adding 0.250 or 0.050 ng TFM to 100 ml 0.1*M* K_2CO_3, acetylating, drying, and, for the lower level, concentrating the extract 5-fold so each solution contained 25 pg TFM phenol/μl. The mean peak areas and relative standard deviations were 13,200±2.0% and 12,900±5.1% for the high and low levels, respectively. These results show an increase in the relative standard deviation and a slight decrease in the recovery (−2%) when compared with results for the higher level; this indicates that loss of TFM and decreased precision are minor when the extra concentrating step is required for low residue levels.

The extraction of TFM from various water samples was studied by using XAD-2 and XAD-7 resins and ether, benzene, and potassium carbonate as eluting agents. The first experiments revealed that both resins removed TFM from the acidified water samples.

The samples were partitioned with benzene after column extraction. No trace of TFM was found in the XAD-7-extracted waters, but 5–10% of the residue remained in the XAD-2-extracted samples. The XAD-7 column was selected for all further tests on the basis of these results. Elution of TFM from the XAD-7 resin column with 0.1*M* K_2CO_3 solution was tested initially with the idea that intermediate steps using organic solvent elution and partitioning with the alkaline solution might be eliminated. However, as much as 100 ml 0.5, 0.1, and 0.05*M* K_2CO_3 failed to recover >30% of the TFM from the resin column. Other basic solutions were not tested because the derivatization step required the use of potassium carbonate, so organic solvents were studied next.

Benzene elution of the XAD-7 resin recovered only 50% of the TFM.

Table 1. Precision and accuracy of TFM analysis

True value, μg/L	Mean rec.,[a] μg/L	Mean error, μg/L	Std dev., μg/L	Rel. error, %	Rel. std dev., %	Total error, %
0.250	0.240	0.010	0.023	4.0	9.5	22.4
0.050	0.047	0.003	0.006	6.0	12.8	30.0

[a] Mean of 10 determinations at each level.

Ethyl ether elution of the resin recovered 90–100% of the TFM in all types of water tested, including distilled, tap, Lake Ontario, and Hamilton Harbour waters. These recoveries were consistent for 30–100 ml ether. We chose 75 ml eluant to ensure good recoveries from a wide variety of samples and to minimize losses on subsequent extract manipulations.

The precision and accuracy of the entire method were determined by performing replicate analyses on Hamilton Harbour samples, which were spiked at 0.250 and 0.05 μg/L and analyzed the following day. Total error calculations were performed on the data, as proposed by McFarren *et al.* (9), and the results are shown in Table 1. According to the categories proposed by McFarren *et al.*, the method is excellent for the higher level tested (total error $\leqslant 25\%$) and acceptable for the lower level (total error $\leqslant 50\%$).

The chromatographic characteristics of TFM acetate derivative were studied on the 2 GLC columns described under *Method*. The SE-30/OV-210 column resulted in longer retention times and good separation of TFM acetate derivative from all other phenolic acetates tested. Their retention times relative to pentachlorophenyl-acetate are given in Table 2.

Several samples were taken from Duffin Creek approximately 2 weeks after the application of TFM by personnel of the Sea Lamprey Control Centre. The samples were collected from low-flow areas along the creek and analyses were performed 3 days later. The samples from 2 locations contained μg/L levels of TFM. Chromatograms of pure TFM, crude TFM (33%) used in stream treatment, and one of the water sample extracts, all derivatized according to the procedure, are shown in Fig. 1. The TFM acetate derivative and the derivatized sample extract were submitted to Dupont Instruments (B. N. Colby, Monrovia, CA, Aug. 1975) and Finnigan Corp. (J. B. Knight, Sunnydale, CA, Aug. 1975) for analysis by GLC-mass spectrometry. Their results confirmed the presence of TFM along with a trichlorophenol, a tetrachlorophenol, and pentachlorophenol. Results obtained by our analysis with the GLC-Autolab® integrator and Finnigan's analysis by GLC-mass spectrometry in the chemical ionization mode and selected ion recording agree very well, 39.6 and 41.2 μg TFM/L, respectively.

Table 2. Retention times of phenolic acetates, relative to pentachlorophenol[a] on SE-30/OV-210 column

Acetate deriv. of compd	Rel. retention time
Phenol	no peak
p-Chlorophenol	0.19
4-Chloro-3-methylphenol	0.24
2,3-Dichlorophenol	0.29
2,4-Dichlorophenol	0.24
2,5-Dichlorophenol	0.24
2,6-Dichlorophenol	0.24
3,4-Dichlorophenol	0.29
2,3,6-Trichlorophenol	0.38
2,4,5-Trichlorophenol	0.38
2,4,6-Trichlorophenol	0.38
p-Cyanophenol	0.44
TFM	0.50
4-Chloro-2-nitrophenol	0.57
p-Nitrophenol	0.57
p-Nitro-*m*-cresol	0.59
2,4,5,6-Tetrachlorophenol	0.71
Pentachlorophenol	1.00

[a] Retention time = 365 sec.

These analyses show significant levels of TFM in environmental samples 2 weeks post-application, and would suggest that TFM is not degraded as completely as previously reported (3). Bothwell *et al.* (7) have also found no significant degradation of TFM under aerobic conditions for up to 2 months.

Lake Ontario water was spiked with 1.0 μg TFM/L and stored at 4°C, or acidified to pH 1–2 and stored at 4°C. The recoveries of TFM from these samples were 90 and 92%, respectively, after 15 days and 86 and 84%, respectively, after 30 days, indicating no significant measure of preservation was achieved by acidification of these samples.

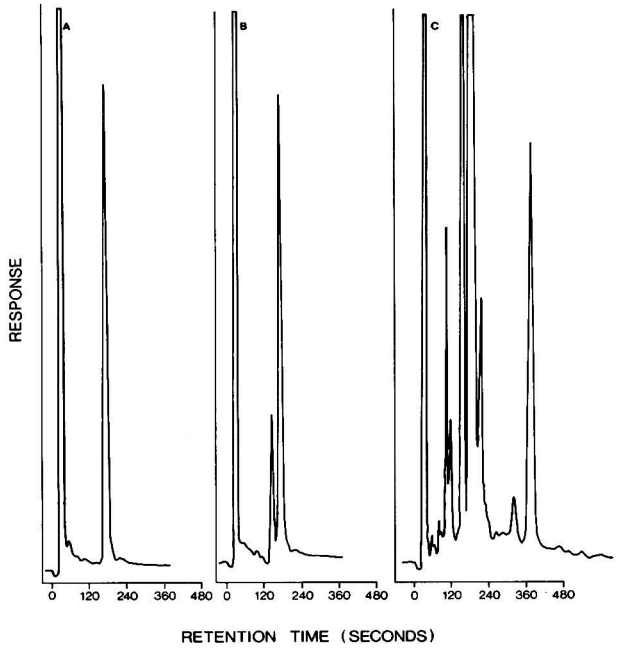


FIG. 1—Gas chromatograms of acetate derivative of TFM: A, pure TFM; B, crude TFM; C, Duffin Creek water extract containing 39.6 μg TFM/L.

Acknowledgments

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IUPAC Commission on Terminal Pesticide Residues

PHILIP C. KEARNEY, *Secretary to the Commission*
Agricultural Environmental Quality Institute, U.S. Department of Agriculture, Beltsville, MD 20705

The eighth meeting[1] of the above Commission of the Pesticide Section of the Applied Chemistry Division of IUPAC was held at ICI Plant Protection Ltd., Jealott's Hill Research Station, Bracknell, England, Oct. 2, 1974, under the Chairmanship of K. R. Hill. The following account of the proceedings is based on the minutes of the meeting and on the appendices drawn up by members and associate members of the Commission as indicated.

The Commission considered the report of the Joint FAO/WHO Meeting on Pesticide Residues held in Geneva in December 1973, in which some problems concerning terminal residues were discussed.

1. Terminal Organochlorine Compound Residues

The Commission received a report on terminal residues of cyclodiene insecticides covering metabolic pathways, metabolites, and photoproducts of chlordane, aldrin, dieldrin, and heptachlor. There is considerable debate about the environmental aspects of several of the cyclodiene insecticides, and several countries are currently reviewing these compounds. It was recommended that the occurrence and true significance of products mentioned in the report be assessed at next year's meeting. An evaluation of these products in the environment to assess their toxicology would be useful. It was further recommended that an authoritative article be published on the latest advances in cyclodiene pesticide chemistry. It was pointed out that different results for rates of degradation are obtained with photodecomposition on leaves and silica gel. The Commission concluded that there is a need for strictly comparable metabolic studies to discover the real significance of metabolites.

A. Evaluation—Friedhelm Korte (Institut für Ökologische Chemie der Gesellschaft für Strahlenforschung mbH München, D. 5201 Schloss Birlinghoven, W. Germany) and P. E. Porter (Shell Development Co., PO Box 4248, Modesto, CA 95352)

Chlordane

Interest in the terminal residues of the *cis* and *trans* isomers of chlordane has been stimulated by the recent development of a high purity chlordane by Velsicol Chemical Corp. This product, which has been designated HCS-3260, contains approximately 98% of a 3:1 mixture of the *cis* and *trans* isomers. Residues of the chlordane isomers themselves have been thoroughly discussed in the past and do not appear to present problems in relation to proposed uses of chlordane. Concern has arisen, however, about the important metabolite which is commonly called oxychlordane. This metabolite seems to have been first encountered as a contaminant in milk products from cows consuming hay containing chlordane residues (1, 2). It was subsequently found and identified as an animal metabolite of *cis*- and *trans*-chlordane (1, 3–7).

Mammalian metabolism studies with chlordane have demonstrated that both *cis* and *trans* isomers are rapidly converted to hydrophilic metabolites which have been characterized as mono-, di-, and trihydroxylated derivates and their conjugates (3–6, 8, 9). These metabolites are quickly eliminated. A minor part of each chlordane isomer is converted to oxychlordane; however, because this metabolite is eliminated slowly, measurable tissue residues accumulate. When animals are fed *cis*- or *trans*-chlordane over long periods, the principal residue in tissues is oxychlordane, particularly in fat. In general, more storage of oxychlordane resulted from ingesting the *trans* isomer than the *cis* isomer. Male rats stored less than female rats. When feed free of chlordane is given to rats whose adipose tissue contains oxychlordane and chlordane isomers, the compounds appear to be completely eliminated in the form of hydrophilic metabolites. In rats, the oxychlordane elimination is similar to that for dieldrin. The rate of elimination in females is slower than in males, and in both the rates appear somewhat faster than that for dieldrin.

The animal feeding data suggest that conver-

[1] For a report of the seventh meeting, *see* (1975) *JAOAC* **58**, 1257–1285. A report of the ninth meeting, Madrid, September 1975, will be published in *Compt. Rend.*

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sion rates of chlordane to hydrophilic metabolites are proportionally greater at higher feeding levels than at low. This is particularly evident in the results reported by Dorough and Hemken (5) for cows fed HCS-3260. At average feeding levels of 0.06–0.1 ppm for 30 days, the fat storage ratio (oxychlordane in fat/chlordane . fed) was about 0.25; at the 1 ppm level the ratio was 0.16; at the 10 ppm level the ratio was 0.10; and at the 100 ppm level the ratio decreased to 0.0196. Similarly, milk fat had storage ratios (oxychlordane in milk fat/chlordane fed) of 0.34, 0.21, and 0.038 at 60 days of feeding 1, 10, and 100 ppm HCS-3260, respectively. In all cases, proportionally less oxychlordane was stored as the feeding level increased.

The importance of oxychlordane as a metabolite of chlordane was further increased by the confirmation and consistent finding of this compound in adipose tissue samples from the general population, obtained through the United States National Human Monitoring Program. Biros and Enos (10) examined 27 specimens and found oxychlordane contents ranging from 0 to 0.40 ppm with a mean of 0.14 ppm. Twenty-one of 27 samples contained $\geq$0.03 ppm. Computer printouts from the Environmental Protection Agency monitoring studies which were made available to Shell Chemical Corp. show that, in 1970, the mean oxychlordane content of human adipose tissues was 0.08 ppm with 97% of 132 samples positive ($\geq$0.01 ppm); in 1971 the average was 0.10 ppm with 97% of 3328 samples positive; and in 1972 the average was 0.11 ppm with 97% of 2707 samples positive

Aldrin and Dieldrin

Experiments on the fate of ^{14}C-aldrin used in the soil under simulated normal agricultural conditions, which have been conducted by the Institut für Ökologische Chemie in West Germany, are nearing completion. Results have been reported in past summaries and are summarized in 3 papers (11–13). A brief review is presented below:

In 1969, an open air experiment was started with soil application (2.9 kg/hectare) of ^{14}C-aldrin in several culture boxes; in 1970, one box was retreated with ^{14}C-aldrin. No further retreatment was performed after that time. Several crops have been grown each year in these boxes from 1969 to 1972. The results from the years 1969 to 1970, pertaining to crop analyses and to analyses of different soil samples, showed that aldrin incorporated into the soil is converted to a number of metabolites. Besides dieldrin, most of the radioactivity recovered from soils and plants was due to a group of hydrophilic metabolites. One major metabolite of this group is dihydrochlordene dicarboxylic acid. In addition, photodieldrin and photoaldrin were also detected in small amounts in the treated soils, as well as another, yet unidentified nonpolar component (X). Photodieldrin and component X were also found in small amounts in the plants, in addition to dieldrin and the hydrophilic group of metabolites.

The analyses of the samples from 1971 and 1972 gave the following results: The radioactivity, as compared with earlier years, decreased in all soil and crop samples, especially in the upper soil layer (0–10 cm from the surface) which had been treated; in most cases, the total residues were $<$1 ppm. Although, in the first year after the application the main portion of the radioactivity was due to hydrophilic compounds in most of the samples, in 1971 and 1972 dieldrin was the major radioactive product. Small amounts of unchanged aldrin, photodieldrin, and unidentified products were present, as well as 20–40% hydrophilic compounds, mainly dihydrochlordene dicarboxylic acid.

The leaching water draining from soil below 60 cm in the experimental boxes was collected and analyzed. Over the 3-year period, approximately 10% of the aldrin initially applied was found as dihydrochlordene dicarboxylic acid. The radioactivity of the leaching water was 0.05 ppm in 1970 and has been decreasing since then. At the end of 1972, the water contained only 0.014 ppm radioactivity. The radioactivity of the water results from, beside dihydrochlordene dicarboxylic acid, very small amounts (approximately 2%) of *trans*-dihydroxy-dihydroaldrin and traces of still unknown compounds.

The influence of the leaching of aldrin and its metabolites on nontreated soils and crops near treated areas was investigated. ^{14}C-Aldrin was applied in the open air to a 30 $\times$ 60 cm area at a rate of 2.4 kg/hectare and incorporated immediately to a depth of 10 cm. A nontreated 30 $\times$ 60 cm area was separated from the treated area by a glass plate fixed in the soil to a depth of 40 cm. One and 3 years after application, carrots and potatoes, respectively, were planted in the untreated soil; after harvest, crop and soil samples were analyzed. The same compounds contained the radioactivity as in the outdoor experiments described above.

The results of this experiment (Table 1) show that, after aldrin is applied, aldrin and its metabolites are translocated only in very small portions to untreated soils and to crops cultivated therein.

The 1970 and 1971 outdoor experiments with dieldrin were continued in 1972, with carrots as the crop. The initial soil residue in 1972 was 2.628 ppm radioactivity resulting from dieldrin treatments in 1970 and 1971. The radioactive residues

Table 1. Residues of ^{14}C-aldrin and its conversion products in potatoes and soil, following soil application; results expressed as equivalent ppm aldrin

Sample	Aldrin + dieldrin	Photo-dieldrin	Hydrophilic metabolites[a] (extd)	Unextractable radioactivity	Total residue
Treated soil	0.591	0.014	0.057	0.153	0.815
Untreated soil	<0.004	<0.001	<0.001	0.004	0.008
Peeled potatoes	<0.002	<0.001	<0.001	0.001	0.001
Potato peels	<0.010	<0.005	<0.005	0.002	0.007
Potato roots	<0.186	<0.093	<0.093	0.001	0.094
Potato foliage	<0.006	<0.003	<0.003	0.011	0.014

[a] Principal product is dihydrochlordene dicarboxylic acid.

in soil, carrots, and weeds consisted mostly of unchanged dieldrin, beside small amounts of photodieldrin, hydrophilic metabolites, and traces of unextractable materials. In the carrot leaves, the radioactive residues were low; the radioactive substances could not be determined separately because of the presence of high amounts of biological coextractives. The residues in the 1972 samples are shown in Table 2.

The leaching water drained from the experimental box was collected the first year after application and analyzed. Its radioactivity was below the detection limit (0.001 ppm). Nevertheless, it was extracted with ether after acidification. The ether was concentrated, and its radioactivity was 0.5 ppb, corresponding to 0.1% of the applied radioactivity. The leached metabolite was isolated; after methylation with diazomethane, it was identified as dihydrochlordene dicarboxylic acid dimethyl ester by mass spectrometry.

Further studies in other laboratories have demonstrated the metabolism of aldrin by plants and by homogenates of plant roots. Earl and Kennedy (14) showed that pea plants and extracts are subject to induction of aldrin-metabolizing activity. The induced enzyme system appeared to lead to production of more hydrophilic metabolites, rather than an increased rate of expoxidation to dieldrin. McKinney *et al.* (15–17) and Mehendale (18), in an important study with pea and bean root preparations, demonstrated that aldrin was converted to dieldrin, *cis*-aldrin diol, *trans*-aldrin diol, the ketone isomer of dieldrin, and *exo*-4-hydroxy-4,5-dihydroaldrin. They found that none of the products was an intermediate in the formation of the others; in particular, dieldrin was not metabolized and the *cis* diol was not epimerized to the *trans* diol. They postulated that one enzyme was responsible for the hydration of aldrin to the monohydroxy compound, and that a second enzyme produced the other 4 compounds through a common intermediate (15).

McKinney *et al.* (16) discussed the stereochemical aspects of the metabolism and toxicity of some chlorinated pesticide systems. Their experiments with mammalian tissues *in vitro* (17) showed that the *cis* as well as the *trans* diol is a metabolite of dieldrin, which suggested that the *cis* form may be an intermediate in the formation of the *trans* diol. They demonstrated that the *cis* diol is converted by mammalian tissue preparations to the *trans* isomer. The epimerization is stereoselective, resulting in optical activity in the *trans* isomer. They postulated, on the basis of their evidence, that the epimerization may involve the formation of α-hydroxyaldrin ketone which is, again, reduced to both *cis* and *trans* diols. They observed no transformation of the *trans* diol in their system so that the cycle of oxidations and reductions would result in conversion of the *cis* to the *trans* isomer but not the reverse. The possibility was not ruled out that hydroxyaldrin ketone is formed directly from dieldrin by microsomal hydroxylation at one of the epoxide carbons.

An attractive scheme for the metabolism of dieldrin in animals would result from hydrogen

Table 2. Residues of ^{14}C-dieldrin and its conversion products in carrots and soil, following soil application; results expressed as equivalent ppm dieldrin

Sample	Dieldrin	Photo-dieldrin	Hydrophilic metabolites[a] (extd)	Unextractable radioactivity	Total residue
Soil, 0–10 cm depth	0.634	0.035	0.003	0.018	0.690
Carrot roots	0.125	0.005	0.002	0.003	0.134
Carrot leaves	<0.033	<0.033	<0.033	0.002	0.035
Weeds	0.343	0.045	0.006	0.007	0.401

[a] Principal metabolite is dihydrochlordene dicarboxylic acid dimethyl ester.

or hydride extraction, followed by introduction of an hydroxyl group. This scheme, proposed by Hutson and Bedford (Tunstall Laboratories, Sittingbourne, England), and by McKinney *et al.* (16), would account for the known metabolites, including the glucuronide intermediate found in *in vitro* preparations by Matthews and Matsumura (19). This scheme is also compatible with that proposed by McKinney and Mehendale (15) for aldrin metabolism in plants.

Metabolites

The Institut für Ökologische Chemie is working on a number of aspects of cyclodiene metabolism and environmental degradation. The work on photochemical breakdown is discussed under *Photodecomposition*. Some of their recent unpublished results in other areas are outlined below:

1. Aldrin Metabolites. — *trans*-Dihydroxy-dihydroaldrin is known to be a major metabolite of aldrin and dieldrin in mammals. Some years ago, it was shown that it is not the end product of aldrin metabolism. After the radiolabeled diol was administered to mammals, it was oxidized to dihydrochlordene dicarboxylic acid; in microorganisms, too, a highly hydrophilic metabolite was formed.

To elucidate the fate of *trans*-dihydroxy-dihydroaldrin in higher plants and soil, the ^{14}C-labeled compound was applied to soil and to lettuce leaves in a plant growth chamber. In soil, after 7 weeks, >70% of the recovered radioactivity was due to hydrophilic compounds, mainly to dihydrochlordene dicarboxylic acid. In lettuce leaves, 4 weeks after foliar application, about 40% of the radioactivity was due to hydrophilic metabolites; the main product was again dihydrochlordene dicarboxylic acid.

Since the metabolism experiments with aldrin and its primary metabolites resulted in dihydrochlordene dicarboxylic acid, experiments were initiated to determine whether this compound is indeed the final product of aldrin metabolism. The acid was synthesized, labeled with ^{14}C, administered to rats, and applied to mosquito larvae, soil microorganisms, and corn. In rats, after intravenous injection, 87% of the radioactivity was excreted within 5 days. The excreted radioactivity consisted of the unchanged acid as well as several metabolites, some of which, however, are only conjugates of the unchanged acid. The main metabolites were 2 isomeric dechlorination products, with dechlorination at the dichloromethylene bridge. Both isomers were also detected in *in vitro* experiments with rat liver homogenate fractions. Several breakdown products of the dihydrochlordene dicarboxylic acid with low molecular weights were detected in smaller amounts; their structures, however, could not be elucidated.

Less than 10% of the dihydrochlordene dicarboxylic acid applied to water was metabolized by mosquito larvae within 72 hr; <10% was metabolized by soil microorganisms within 3 weeks. Dihydrochlordene dicarboxylic acid was applied to the leaves of young corn plants in a plant growth chamber; the plant tissues were analyzed at the cob stage. The unchanged acid and conjugates of the unchanged acid were detected, as well as conjugates of a degradation product; the last substances, however, amounted to only 2% of the total radioactivity recovered.

All these experiments show that dihydrochlordene dicarboxylic acid is not the final product of aldrin metabolism, but that it is a persistent intermediate which is degraded very slowly in the environment.

2. Aldrin, Heptachlor, and Lindane Metabolism in Algae.—After application of ^{14}C-aldrin (2.5 ppm) to the nutrition solution of the green alga *Chlorella pyrenoidosa*, 48% of the radioactivity applied was metabolized by the algae within 14 days. About half of this radioactivity consisted of a metabolite which was identified as 1,2,3,4,9,9-hexachloro-1,4,4a,5,6,7,8,8a-octahydro-1,4-methanonaphthalene.

^{14}C-Heptachlor and ^{14}C-lindane were also applied to the nutrition medium of the same green algae. With heptachlor (1–5 ppm in the medium), about 66% of the applied radioactivity was metabolized by the algae within 14 days. The algae extract contained unchanged heptachlor, heptachlor epoxide, 3 minor hydrophilic metabolites which were not identified, and a conversion product which constituted 7% of the radioactivity detected in the algae extract. This compound, identified by mass spectrometry, was 4,5,6,7,8,8-hexachloro-2,3-epoxy-4,7-methano-3a,4,7,7a-tetrahydroindan-1-on. The same compound was detected in the algae extract after ^{14}C-heptachlor epoxide was applied to the nutritive medium; thus, this compound might be formed from heptachlor via heptachlor epoxide.

After application of lindane (1 ppm in the medium), a conversion product was detected in small amounts in the medium as well as in the algae. It was isolated and identified as 2,3,4,5,6-pentachlorocyclohex-1-ene.

3. Photodieldrin Metabolism in Soil.—^{14}C-Photodieldrin was incorporated into the top 10 cm soil in a tube 50 cm long and 11 cm in diameter; a layer of activated charcoal was placed at the bottom of the tube to prevent loss of water-soluble compounds. The tube was sunk into the soil and exposed to outdoor conditions for about 15

months. Overall recovery of radioactive carbon was 84.5%. In the 10–35 cm soil layers, 30.2% of the radioactivity was recovered, and only 1.9% was leached to greater depth. In the 0–10 cm layer, 16.3% of the activity was in the form of metabolites and unextractable materials, while in the 10–35 cm layer, 25.6% was metabolites and unextractable materials. Three metabolites were found in the soil as shown in Fig. 1. The diol and the diacid, 1 and 3, respectively, were confirmed by comparison with reference standards. The third structure, 2, was proposed from mass spectra.

4. Heptachlor Metabolism in Soil.—The following greenhouse experiments were carried out to investigate heptachlor metabolism:

^{14}C-Heptachlor was applied to the leaves of young cabbage plants (1 mg/plant, in acetone). After 4 weeks, only 7.4% of the applied radioactivity was recovered in and on the plants and 0.3% in the soil; the rest of the radioactivity probably evaporated into the atmosphere. The recovered radioactivity consisted of 7% unchanged heptachlor, 17% heptachlor epoxide, 6% of an unknown metabolite, and 70% hydrophilic substances. Two components of this hydrophilic group were 1-hydroxychlordene and prillic acid.

Forty wheat seeds were treated individually with 11.4 μg ^{14}C-heptachlor in acetone and were planted in a box with 21 kg soil (5 cm intervals, 2 cm depth). Upon harvesting (3½ months later), 2% of the applied radioactivity was recovered in the plants and 80% in the soil. Whereas the concentration of radioactive compounds in the plants was too low to isolate metabolites, the following substances were isolated from the soil and identified: 18% unchanged heptachlor, 35% heptachlor epoxide, 12% 1-methoxychlordene, and 35% hydrophilic substances consisting of 1-hydroxychlordene and a monohydroxy derivative of dihydroheptachlor.

Photodecomposition

Graham *et al.* (20) used a KBr disk technique to study the photochemical decomposition of heptachlor epoxide. They identified 3 compounds as primary products after sunlight or ultraviolet light (254 nm) irradiation. Independently, Knox *et al.* (21) found these compounds as products of the decomposition of heptachlor epoxide. Knox *et al.* (21) irradiated acetone solutions of *trans*-chlordane and *trans*-nonachlor with ultraviolet light and found new half-cage isomers which have different bridging than that previously observed for the *cis* isomer.

Parlar and Korte (22) irradiated *cis*-chlordane in acetone solution with light above 300 nm; they found an additional product as well as the half-cage structure already known. Under the same conditions, 1-hydroxy-dihydrochlordene also underwent transformation.

Ivie (23) reported that oxychlordane is converted into 2 major photoproducts when exposed to sunlight as deposits on silica gel chromatoplates. One of the products is isomeric with oxychlordane. The other products appear to be one or more of 4 additional isomers.

The work of Crosby and Moilanen on vapor-phase photodecomposition of aldrin and dieldrin has been published (24). As they have pointed out, the photochemical reactions of pesticides in vapor phase may be quite different from those occurring on surfaces or in condensed phases. It is important that both situations be studied, since both conditions are represented in the environment. In this regard Korte and his associates have obtained evidence that aldrin, dieldrin, and photodieldrin, when adsorbed on silica gel, can be degraded completely to CO_2 and HCl by light of wavelengths above 300 nm in the presence of a stream of oxygen.

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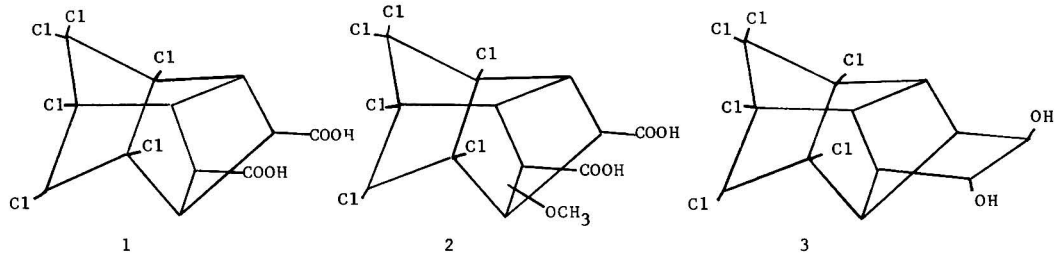


FIG. 1—Metabolites of photodieldrin: 1 and 2, bridged isomers of dihydrochlordene dicarboxylic acid; 3, bridged isomer of aldrin-*trans*-diol.

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B. Metabolism of Endrin in Mammals in Relation to Its Acute Toxicity—Contributed by D. H. Hutson and C. T. Bedford (Tunstall Laboratory, Shell Research Ltd., Sittingbourne, England)

It has been known for more than a decade that endrin is readily excreted by mammals. Research by several groups in the 1960s confirmed that endrin was transformed in rats and rabbits and excreted as a mixture of metabolites, but the structures of these metabolites were not elucidated. In addition, experiments in the mid-1950s indicated that the insecticide fed to cows did not present a residue problem in milk (1). The problem was investigated in our laboratory in the late 1960s in order to compare the metabolism of endrin with that of dieldrin, which had been under intensive study. Information on the chemical identity of endrin metabolites in a mammal was published in 1970 (2).

We have recently been re-investigating the metabolism of endrin in rats, rabbits, cows, and humans in relation to industrial hygiene and to the consequences of agricultural use with particular reference to metabolites rather than the parent pesticide. The studies have been aided by our recent synthesis (3) of authentic reference compounds which have not hitherto been available. Progress in our recent work on endrin metabolism is summarized below, together with findings which shed some light on the nature of the acute toxicant in endrin poisoning.

Comparative Metabolism in Endrin

Rat.—When endrin is ingested orally by the rat (2 mg/kg), the major pathway of metabolism is hydroxylation at the 12-position to give *anti*-12-hydroxyendrin. This compound, probably formed in the liver, is excreted in the bile as its glucuronide conjugate, deconjugated by bacterial enzymes in the gastrointestinal tract, and excreted in the feces. Endrin is also found in the feces, together with a trace of a hydrolysis product, isodrin-*trans*-diol. There is a sex difference in these processes in the rat. Male rats excrete 66% of an oral dose and female rats, 37%, over a 3-day period. Less than 5% of the dose is excreted in the urine. Urinary products are composed of 12-ketoendrin, endrin, and 12- and 3-hydroxyendrins, together with polar metabolites (probably the glucuronide of *anti*-12-hydroxyendrin, the glucuronide of isodrin-*trans*-diol, and the 12-*O*-sulfate conjugate), and traces of unknown compounds. Endrin and 12-ketoendrin are found in fat, liver, and kidney of male animals.

Rabbit.—The rabbit excretes a single oral dose of endrin (2 mg/kg) in a rather different way compared with the rat. Only unchanged endrin is excreted in the feces, mostly within 24 hr of

dosing. Approximately 40% of the dose is excreted in the urine over a 13-day period. The major metabolic products in the urine were *anti*-12-hydroxyendrin-*O*-sulfate and the 12-*O*-glucuronide. There is evidence for a small amount of *syn*-12-hydroxyendrin, both free and conjugated.

Cow.—This mammal presents another excretion pattern which differs from that of the rat (although in this case the dose used was 0.004 mg/kg/day for 21 days). About 70% of a daily dose (fed in diet) appears in the urine and 25% in the feces. Most of the urinary metabolite is *anti*-12-hydroxyendrin; traces of its isomer (*syn*-12-hydroxyendrin) and of 12-ketoendrin are also found together with some unidentified polar metabolites (about 8%) which could be the 12-*O*-sulfate. It is likely that the hydroxylated metabolites are excreted as glucuronides which are deconjugated by bacterial β-glucuronidase in the urine. This is a feature of excretion in cows. The fecal excretion products are composed of a 1:1 mixture of endrin and 12-hydroxyendrin.

Human.—*Anti*-12-hydroxyendrin has been detected at low concentrations in the feces of some men in a group working in the endrin plant at Pernis, England. The urine of some of these operatives contained *anti*-12-hydroxyendrin glucuronide. Studies have not been performed on humans under experimental conditions.

These interspecies comparisons have, of necessity, been made under nonidentical conditions. However, in spite of differences in routes of excretion, the major metabolic step (12-hydroxylation) operates in each of the species studied. The differences that do arise are probably differences between the species in the biliary excretion of metabolites. There are differences between rat and rabbit in the molecular weight requirements for biliary excretion of foreign compounds and, in general, lower amounts of drug and pesticide metabolites appear in rabbit feces. The extent of sulfate conjugation in the rabbit, however, is a unique observation.

Studies in vitro

Several of the metabolic steps discussed above are observable *in vitro*, using tissue enzyme preparations. Endrin can be hydroxylated by rat liver microsomes under conditions in which mono-oxygenation occurs; glucuronide synthesis has been observed by using rabbit liver microsomes in the presence of *anti*-12-hydroxyendrin and the glucuronic acid-donating cofactor, uridine diphosphoglucuronic acid. The 12-*O*-glucuronide has been hydrolyzed by β-glucuronidase of mollusk (limpet), bovine liver, and the bacterium *Escherichia coli* (present in the gastrointestinal tract of rats). A particularly interesting mechanism has been elucidated for the formation of 12-ketoendrin. This metabolite is apparently *not* formed by a dehydrogenase acting on a hydroxyendrin, but via microsomal mono-oxygenation—the same process that leads to the formation of *anti*-12-hydroxyendrin from endrin. Moreover, this enzyme will convert only the *syn* isomer of 12-hydroxyendrin to 12-ketoendrin. This is an interesting observation of stereochemical specificity. *In vitro* experiments have revealed a 14-fold sex difference between male and female rat liver microsomes in their ability to catalyze the reaction, the male possessing the greater activity.

Observations on the acute toxicity of endrin, *anti*-12-hydroxyendrin, *syn*-12-hydroxyendrin, and 12-ketoendrin in male and female rats, together with analysis of certain rat tissues for these compounds suggest that: (a) 12-ketoendrin is probably produced *in vivo* from *syn*-12-hydroxyendrin, and (b) this reaction may account for the acute toxic effects of endrin, i.e., 12-ketoendrin is a toxic metabolite of endrin.

The acute toxicity to a male rat of 12-ketoendrin (1 mg/kg) and endrin (6 mg/kg) would seem to confirm the hypothesis. Thus, the same biochemical reaction which ultimately leads to the detoxication of endrin (hydroxylation, which is then followed by conjugation) is also responsible for the acute toxicity of endrin to rats. The balance between the 2 reactions and alterations in this balance by extraneous factors could be critical in the onset of endrin poisoning.

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C. Terminal Residues of Toxaphene: Evaluation—Kenneth R. Hill (Agricultural Environmental Quality Institute, U.S. Department of Agriculture, Beltsville, MD 20705)

During the past year there has been a considerable increase in research, reported or in progress, on all aspects of toxaphene chemistry. This resulted in a somewhat premature half-day Symposium on Toxaphene Composition and Environmental Fate on Sept. 10, 1974, at the 168th Meeting of the American Chemical Society at Atlantic City, NJ. Although progress was noted, most of the work is incomplete. At this stage there are insufficient data to provide a complete

evaluation of the mechanisms of toxaphene degradation.

Technical toxaphene consists of at least 175 C_{10} polychloro compounds (1–3). By means of sequential liquid-liquid partition chromatography and liquid-solid adsorption chromatography, followed by preparative gas chromatography and fractional crystallization, 2 components of toxaphene were isolated. One component, $C_{10}H_{10}Cl_8$, comprising 6% of technical toxaphene, was 14 times more toxic to mice than toxaphene. The other component, $C_{10}H_{11}Cl_7$, 3% of technical toxaphene, was identified as 2,2,5-*endo*-6-*exo*-8,9,10-heptachlorobornane and was 6 times more toxic to mice than technical toxaphene.

Oral administration of ^{14}C-toxaphene and ^{36}Cl-toxaphene to rats resulted in the excretion of 50–60% of the ^{36}Cl in the urine and 20–30% in the feces as chloride ion within 14 days (1, 2, 4). The tissue content of ^{14}C was very low, indicating that toxaphene components do not persist in rats.

No evidence of metabolism was detected when ^{36}Cl-toxaphene was applied to cotton plants maintained in a closed all-glass system for 7 days (5). The residue on the plants was identified as toxaphene; the mechanism of loss was volatilization.

Research in progress on the fate of toxaphene in soil, air, and water was reported by Plimmer (6). Anaerobic conditions, such as in flooded soils or sediment, appear to accelerate reductive metabolism. Otherwise, the key factors in its disappearance appear to be photolysis and volatilization.

The following conclusions were reached at a Toxaphene Workshop, held in New Orleans, LA, in Jan. 1974: "The most extensive metabolism of toxaphene seems to occur in mammalian systems where there is little apparent accumulation of toxaphene *per se*. In fish there appears to be considerable accumulation and little evidence of metabolism. Avian systems are intermediate in this respect. Our knowledge of toxaphene metabolism in mammals, birds, and fish is undoubtedly limited."

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D. Photodecomposition of Chlorinated Pesticides: Evaluation—Donald G. Crosby (Department of Environmental Toxicology, University of California, Davis, CA 95616)

Previous reviews have included the photochemistry of chlorinated insecticides (1), chlorinated herbicides (2), the fate of such compounds in the atmosphere (3), and their transformation by photosensitizing processes (4). The photochemistry of cyclodiene insecticides, reviewed in part by Benson (5), is covered in Section A of this report.

Chlorinated Aliphatic Hydrocarbons.—Many simple chloroalkanes receive wide use as fumigants—chloroform, carbon tetrachloride, 1,1,1-trichloroethylene, and *sym*-dichloroethylene, for example. However, they do not absorb light at wavelengths above 290 nm, the sunlight region of the spectrum at the earth's surface (6). While radical reactions could be expected to occur in the upper stratosphere (above about 40 km) where the necessary radiant energy is available (7), their association with terminal residue concerns presently is speculative.

Both as vapor (8) and liquid (9), 1,1,2,2-tetrachloroethylene (perchloroethylene) is photooxidized to its epoxide, followed by rearrangement to trichloroacetyl chloride or, in the presence of water, trichloroacetic acid. The use of these chlorinated hydrocarbons as space fumigants probably precludes photoproducts as terminal residues in food, but their annual billion-pound use as cleaning solvents almost certainly results in atmospheric transformation to gaseous products. The closely related soil fumigants telone and D-D mixture (isomeric 1,3-dichloropropenes) appear to be unreactive at sunlight wavelengths (D. G. Crosby and K. W. Moilanen (1974) unpublished results).

Chlorinated Aralkyl Compounds.—Pentachlorobenzyl alcohol, a rice fungicide, is rapidly degraded under ultraviolet (UV) irradiation in aqueous methanol (10), probably to the corresponding aldehyde and acid analogous to the

mono-, di-, and trichlorobenzyl alcohols (11). The simultaneous formation of a large proportion of ionic chloride also is consistent with the replacement of Cl by H and OH observed with the less chlorinated analogs as well as many other aromatic halides (12). Composition of the terminal residue would depend primarily upon the absorbance of the resulting isomeric chlorobenzoic acids in the sunlight region and the subsequent reduction and hydroxylation (13).

DDT long has been known to decompose in sunlight or laboratory UV light (14). The initial products from homolysis of the aliphatic C—Cl bond, DDD (TDE), and DDE, are prominent products of most DDT photolysis. With filtered mercury arc light, DDT in methanol formed a variety of oxidation products (detected only by mass spectroscopy) (15), and these and other substances also were formed by UV irradiation of DDT or DDE in water (16). Photodecomposition was slow in sunlight, but sensitization allowed extensive breakdown to oxygenated products, including DDT acid, dichlorobenzophenone (DCB), and *p*-chlorobenzoic acid within 24 hr. These products could be explained by formulation of a (still hypothetical) intermediate, DDE epoxide (16); DDMU (1-chloro-2,2-bis(*p*-chlorophenyl) ethylene) also is commonly observed.

A number of natural and synthetic substances act as photosensitizers for DDT (17), although DDE and DCB were the only products detected from sensitized photolysis on leaf surfaces (18). However, irradiation of unsensitized DDT or DDE as vapor showed surprisingly facile dehydrochlorination, oxidation, and fragmentation to provide lower members of the polychlorinated biphenyl series (PCBs) (19). 2,7-Dichlorofluorenone, readily formed from crystalline DDE in sunlight (20), has not been isolated under other circumstances, but a "DDE isomer" probably containing 3 chlorines on the aromatic rings was detected, along with the usual products, from the irradiation of DDE as a solid, in solution, and in the vapor phase (21).

Methoxychlor solutions were easily photolyzed in sunlight to yield the DDE-DCB analogs as well as *p*-methoxybenzoic acid and *p*-methoxyphenol (22, 23). Therefore, terminal photolysis products from other insecticides of the DDT series may be expected to include the olefin, ketone, and acids; the environmental presence of these compounds has been verified in field experiments with DDT (24, 25). The methoxychlor analog MPA (1,1,1-trichloro-2,2-bis(5′-chloro-2′-methoxyphenyl)ethane) decomposed at sunlight wavelengths to give the expected products (26), and its olefin absorbed strongly in the sunlight region to give, primarily, 2,5-chloro-3-(5′-chloro-2′-methoxyphenyl) benzofuran (27).

Chlorinated Aromatic Hydrocarbons.—Although irradiation of a methanol solution of hexachlorobenzene, the major impurity in Dacthal (dimethyl tetrachloroterephthalate) herbicide, resulted only in isolation of an artifact, pentachlorobenzyl alcohol (*see* above) (28), the facile photoreduction of pentachlorobenzene and isomeric tetrachlorobenzenes to less chlorinated homologs in organic solvents would be expected to apply (29).

The polychlorinated biphenyls also undergo photoreduction when irradiated with sunlight or UV light under a variety of conditions (30–34). In solution or suspension, oxidation, replacement of Cl by OH, and polymerization to complex products were also observed (31–35), although pure PCB isomers were stable as vapor (19). The hydroxylated derivatives were as toxic to insects as common insecticides such as aldrin (35). Considering the alleged stability of PCBs, these results are surprising; an acute toxic hazard from PCB photoproducts could possibly exist, interpretation of a PCB terminal residue to represent any particular commercial product (such as Aroclor 1254) may well be erroneous, and the lower PCBs probably are terminal products of DDT photolysis only as long as they remain in the atmosphere.

Other Chlorinated Pesticides.—A sizable proportion of the common pesticides contain chlorine, so a complete review obviously lies outside the scope of this discussion. However, several examples which illustrate reactions of the C—Cl bond are particularly pertinent to the question of photochemical terminal residues.

The phenoxy herbicides such as 4-CPA (4-chlorophenoxyacetic acid) (36) are photodecomposed in water at sunlight wavelengths by common processes of side-chain oxidation and the usual replacement of the halogen by hydroxyl and by hydrogen. The photooxidation is readily sensitized, causing appreciable conversion of 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) to the corresponding phenol within a few hours (37). Pentachlorophenol (PCP) solutions undergo analogous photoreduction and hydroxylation (28, 38), followed, in the latter case, by oxidation to chloranil (tetrachloro-*p*-benzoquinone), chloranilic acid, and further products of both condensation and rearrangement.

The photonucleophilic generation of phenols with hydroxide ion (13) suggests that *o*-chlorophenols might condense to form chlorinated dibenzo-*p*-dioxins. While this transformation has been demonstrated with solutions of sodium pentachlorophenate (39), the dioxins themselves are

so readily dechlorinated and cleaved in light (40) that lower chlorinated members of the series—such as tetra-chlorodibenzo-*p*-dioxin—could not be detected. Replacement of the *m*-chlorine of PCP by hydroxyl provides relatively stable tetrachlororesorcinol, while the corresponding tetrachlorohydroquinone and tetrachlorocatechol form a stable chlorocyclopentanedione (41). Displacement of the aromatic nitro substituent in PCNB (pentachloronitrobenzene) to give tri- and tetrachlorobenzenes accompanies the displacement of chlorine (38).

Environmental Photochemistry and Terminal Residues.—At present, the exact relation of photochemical processes to pesticide dissipation and terminal residues remains unknown. While pesticide photodecomposition by sunlight often is demonstrable under idealized conditions, classical photochemistry generally has not provided predictability for real environments.

This is due largely to the dependence of photolysis pathways on the immediate chemical environment of the pesticide. The previous examples reveal the action of both radical reagents (oxygen and organically bound hydrogen) and ionic ones (hydroxide ion and other nucleophiles), often working in concert as with 4-CPA. While laboratory experiments allow the formation of general mechanisms, accurate knowledge and recognition of the natural microenvironments usually are lacking. For example, the toxic dioxin impurities of 2,4,5-T are rapidly photoreduced *in vitro* to harmless products in the presence of organic solvents but otherwise appear quite stable (e.g., as a crystalline solid) (38). But in what form does the dioxin actually exist on the sprayed surface of a leaf—as a stable solid, dispersed in cuticular leaf wax, or still dissolved in the mixture of hydrogen-donating solvent and pesticide in which it was inadvertently applied?

The chemical nature of the formulation, then, is of immediate importance to pesticide photodecomposition and terminal residues. The hydrocarbon solvents used in emulsifiable concentrates provide hydrogen atoms for photoreductions (42). Dust particles, usually represented by silicic acid in the laboratory, provide sites which can cause significant changes in both the reactivity and light absorption of adsorbed pesticides (1). Photosensitization, often by common natural products such as chlorophyll or riboflavin, occurs in both aqueous and organic solutions as well as on leaf surfaces (4). Photochemical reactions in organic surface films and micelles stabilized by surfactants often occur at greatly accelerated rates (43).

The ability of the C—Cl bond to undergo homolytic cleavage in light as well as photonucleophilic displacement of chloride ion by nucleophiles causes chlorinated pesticides to be exceptionally reactive. However, terminal residues actually may be dictated by light absorption, for example, PCB isomers are photoreduced only to the stable monochlorobiphenyl which no longer absorbs in the sunlight region (31). Terminal residues also may be dictated by subsequent nonphotochemical processes; photooxidation and photonucleophilic hydroxylation of 2,4-D (2,4-dichlorophenoxyacetic acid) eventually form 1,2,4-benzenetriol which undergoes rapid air oxidation to polymeric humic acids even in the dark (44).

The identity of photolysis products with products of metabolism—in DDT, for example—compounds the confusion over the significance of photodecomposition to terminal residues (45). However, the current surge of interest in environmental photochemistry promises eventual clarification of many of these important issues.

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2. Terminal Organophosphorus Residues

The Commission received 3 reports on terminal residues of organophosphorus insecticides covering diazinon, cyanofenphos, and leptophos and an overview on these insecticides. Leptophos was reported to be rapidly eliminated, fairly stable in the environment, but metabolized in animals. The Commission pointed out that the diazinon pathway may have to be redrawn to include additional products. It was also pointed out that desbromoleptophos is an important metabolite. The FAO representative pointed out that there was no significance assigned to any of these metabolites and that no further work on diazinon is required of the Commission, since it has been reviewed and an acceptable daily intake has been established.

Evaluation—Roy Greenhalgh (Chemistry and Biology Research Institute, Agriculture Canada, Ottawa, Ontario, Canada K1A 0C6)

Developments which have occurred during the past year are summarized in the following sections.

Animals

Lichtenstein *et al.* (1) incubated ^{14}C-(2,6-ring)-parathion and paraoxon with highly purified subfractions of rat liver mitochondria and microsomes and showed that different enzymes are responsible for degradation and detoxification. Both parathion and paraoxon were primarily degraded by the soluble enzyme fraction (105,000–500,000*g*) to water-soluble metabolites together with small amounts of aminoparathion and aminoparaoxon, respectively. The microsomes showed some activity

in degrading parathion. Paraoxon was degraded to 4-nitrophenol by particulate associated enzymes.

Fuhremann *et al.* (2) showed that ^{14}C-(ring)-parathion and paraoxon in blood were rapidly degraded by isolated perfused rat livers. Parathion degraded to give 68% of the radioactivity as water-soluble metabolites and 2.5% converted to organic-soluble paraoxon and traces of 4-nitrophenol. Paraoxon gave 98% water-soluble products, none of which were toxic to mosquito larvae.

Potter *et al.* (3) administered ^{14}C-dichlorvos (2,2-dichlorovinyl dimethyl phosphate) formulated as slow release (polyvinyl chloride) PVC pellets to pigs and recovered 62% of the radioactivity as pellets in the feces, 5.6% in the feces, 3.6% in the urine, 14% in expired air, and 9.6% in the carcass. The dichlorvos level in the tissues ranged from 2 to 33 ppm; no dimethyldichlorvos, dichloroacetaldehyde, or dichloroacetic acid was found.

Janes *et al.* (4) dosed sheep with diazinon (1000 mg/kg) by stomach tube and characterized 4 metabolites found in blood, urine, and tissues. Hydroxydiazinon was found in blood to the extent of 25–75% diazinon, but less was found in other tissues. The amounts of 2 other metabolites were small and were not measured. These 3 compounds were distributed in tissues according to their relative polarity. The fourth compound was obtained only by ultraviolet irradiation.

Plants

Hosokawa and Miyamoto (5) found that ^{14}C-fenitrothion (*O,O*-dimethyl *O*-(4-nitro-*m*-tolyl) phosphorothioate) rapidly disappeared from the surface of apples, decreasing from 3.95 to 0.21 ppm in 21 days. The major degradation products on the surface were fenitrooxon, which reached a maximum of 0.017 ppm after 3 days, 4-nitrocresol, and some *S*-methyl fenitrothion. In the apple pulp, the water-soluble metabolites, which included desmethyl fenitrothion and 4-nitrocresyl β-glucoside, gradually increased with time.

Greenhalgh *et al.* (6) studied the uptake of fenitrothion by germinating pine seeds and showed it was translocated into the embryo through the seed coat and endosperm. Metabolic products were detected in the embryo after 8 days. Desmethyl fenitrothion (25 ppm) was the main metabolic product and was also postulated as a precursor for the *S*-methyl fenitrothion. Other metabolites included 2.1 ppm fenitrooxon and 2.4 ppm *S*-methyl fenitrothion present after 14 days.

Chiba *et al.* (7) topically applied ^{14}C-Cyanox (cyanophos) to bean leaves and found that it was translocated to all parts of the plant. Cyanophos rapidly disappeared from the leaves, decreasing from 106 to 3.5 ppm in 7 days, giving desmethyl cyanophos as the major metabolite, together with cyanophosoxon, desmethyl cyanophosoxon, and 4-cyanophenol. Stem injection of cyanophos gave the same metabolites, while cyanophosoxon gave only desmethyl cyanophosoxon and 4-cyanophenol.

Chiba *et al.* (7) also showed that ^{14}C-Surecide (cyanofenphos) was more stable than cyanophos on bean leaves; 74% remained after 1 week, and it was not translocated. Two metabolites (desethyl cyanofenphos and 4-cyanophenol) were isolated, but no cyanofenphosoxon. Injection of the oxon into the stem indicated rapid decomposition to desethyl cyanofenphosoxon and 4-cyanophenol.

Aharonson and Ben-Aziz (8) determined the persistence of leptophos on tomatoes and grapes: 20% was lost in 3 weeks from tomato leaves, and from 12 to 7.5 ppm in 44 days on grapes. The oxon analog did not accumulate, but the phenol was present to the extent of 1–10% of that of the parent compound. There was no translocation from leaves to fruit in grapes.

Soil

Sethunathan (9) examined the persistence of parathion in 5 acid soils and showed that rapid decomposition took place in flooded soils with a high organic content because of biological action. The rate of hydrolysis to *p*-nitrophenol was enhanced by repeated addition of parathion. A *Bacillus* sp. capable of decomposing 4-nitrophenol was isolated from parathion-treated soil.

Sethunathan and Yoshida (10) also found that the rate of parathion disappearance to give aminoparathion was enhanced under submerged conditions in 4 Philippine soils. Soils innoculated with *Flavobacterium* sp. exhibited a high capacity for the conversion of parathion to aminoparathion.

Walker and Stojanovic (11) tested 18 soil bacteria in mineral salt medium for their ability to utilize malathion. The *Arthrobacter* species were the most effective in degrading malathion, giving malathion half ester, malathion dicarboxylic acid, potassium dimethyl phosphorothioate, potassium dimethyl phosphorodithioate, and one unidentified metabolite. Potassium desmethyl malathion was also produced but a nonbiological mechanism was involved.

Takimoto *et al.* (12) found that ^{14}C-(3-methyl)-fenitrothion was converted to aminofenitrothion as a result of microorganisms in 4 different submerged soils. Aminofenitrothion was found in 50–70% yield in 10 days. Some ^{14}C-carbon dioxide was detected from oxidation of the ring methyl group.

Chiba *et al.* (7) attributed the difference in the

loss of radioactivity between ^{14}C-cyanophos-treated soil (6% remained after 6 weeks) and ^{14}C-cyanofenphos-treated soil (53% remained after 3 weeks) to differences in the volatility of the 2 insecticides.

Photodecomposition

Ohkawa *et al.* (13) irradiated Sumithion (fenitrothion) with ultraviolet (UV) light in various solvents in the presence of air. The solvent determined the rate of decomposition and the products formed; the main product was carboxyfenitrothion together with fenitrooxon, carboxyfenitrooxon, 3-methyl-4-nitrophenol, and 3-carboxy-4-nitrophenol. Exposure of an aqueous solution of fenitrothion (14 ppm) to sunlight gave the same products plus some *S*-methyl fenitrothion. UV irradiation of fenitrothion on silica gel plates gave these same 4 metabolites; in sunlight some 3-carboxy-4-nitrophenol was also obtained. These results corresponded closely with the effect of sunlight on ^{14}C-fenitrothion-treated bean leaves, except for the absence of carboxyfenitrooxon. After 6 days, only 0.5% of the recovered radioactivity was fenitrothion.

Independently, Greenhalgh (unpublished data) isolated 10 photodecomposition products of fenitrothion. In methanol, the main product detected by gas chromatography was carbomethoxyfenitrothion; in addition to the other compounds mentioned above, formyl fenitrothion and *O,O*-dimethyl-*O*-3-methylphenyl phosphorothioate were identified.

Joiner and Baetcke (14) applied ^{14}C-parathion to cotton leaves in the laboratory and under field conditions. After 28 days, only 11–15% of the total radioactivity applied was recovered and 58–68% of this was unchanged parathion. Photoalteration products present included paraoxon, *S*-ethyl parathion, *S*-phenyl parathion, and 4-nitrophenol.

Joiner and Baetcke (15) showed that the UV irradiation of parathion (neat) for 35 days induced the formation of 15 products, 12 of which they characterized. Apart from the 4 products previously mentioned, they identified 4-amino parathion, diethyl phenyl phosphate, *O,O*-diethyl-*O*-phenyl phosphorothioate, ethyl bis(4-nitrophenyl) phosphate, *O*-ethyl *O,O*-bis(4-nitrophenyl) phosphorothioate, diethyl phosphate, and monoethyl phosphate.

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Terminal Residues: Evaluation—Junshi Miyamoto (Research Department, Pesticides Division, Sumitomo Chemical Co., Ltd., Osaka, Japan)

Diazinon

Mammalian metabolism of diazinon, *O,O*-diethyl *O*-(2-isopropyl-4-methyl-6-pyrimidinyl) phosphorothioate, has been extensively studied since its advent as an insecticide. Several earlier studies reported rapid and complete elimination of the compound in urine, feces, and milk in cows (1, 2) and goats (3). Urine was the main route of excretion. Fresh milk obtained from cows ½, 1, and 2 days after spraying with diazinon emulsion contained diazinon residues, with a maximum of 0.35 ppm. By day 7 the residue was the same as in milk from the control cattle (3).

The distribution and excretion of ^{32}P-labeled diazinon was also followed in the dog (4), guinea pig (5), and rat (6). Considerable breakdown of diazinon in these species was confirmed. In the guinea pig, elimination of radioactivity ceased

within 7 days, when 87% of the orally administered dose was recovered in the urine (5). In the rat (6), the biological half-lives of ring-^{14}C-labeled and ethoxy-^{14}C-labeled diazinon were 12 and 7 hr, respectively. The pyrimidine ring did not cleave to form radioactive carbon dioxide, whereas a small portion of ^{14}C-ethoxy (approximately 6%) was expired as carbon dioxide. The ring-radiocarbon was eliminated substantially completely (95–98% of the dose) in 168 hr, with the major quantity present in the urine (80% in males and 69% in females).

Most of the urinary ^{32}P-metabolites were polar products and, in the cow, 50 and 45% of the urinary ^{32}P-compounds were identified as dimethylphosphorothioic acid and dimethylphosphoric acid, respectively (1). In the rat, 71% of the ^{32}P was phosphorothioate and 24%, phosphorate (7).

Urinary and fecal metabolites of ring-^{14}C-diazinon in the rat were resolved into 4 fractions by thin layer chromatography; 3 of these, representing 70% of the total radioactivity applied, were identified by spectroscopic analysis (6): 2-isopropyl-4-methyl-6-pyrimidinol and its oxidation products at the primary and tertiary carbon atom of the isopropyl side chain. Intravenous administration of these compounds revealed that the oxidation products were derived from 2-isopropyl-4-methyl-6-pyrimidinol. One product was excreted unchanged, but the second gave rise to the metabolites in fraction 4. 2-Isopropyl-4-methyl-6-pyrimidinol and the oxidation product of the tertiary carbon atom were not cholinesterase inhibitors and were 10-fold less toxic to rats than diazinon (6).

Several other ^{14}C- or ^{32}P-metabolites were separated and identified with the ester bond intact, although the quantity is presumably small in each case. Metabolites of diazinon in chloroform or acetone extracts of sheep urine were chromatographed on silica gel columns and were analyzed spectroscopically, using authentic reference compounds for comparison. The structures of 3 were confirmed (8–10): hydroxydiazinon or *O,O*-diethyl *O*-(2-(2′-hydroxy-2′-propyl)-4-methyl-6-pyrimidyl) phosphorothioate; *O,O*-diethyl *O*-(2-isopropenyl-4-methyl-6-pyrimidinyl) phosphorothioate; and 4-hydroxy analog of diazinon. Hydroxydiazinon was detected in various tissues and blood of sheep given diazinon, but the amount was <10% of the diazinon content. Hydroxydiazinon, *O,O*-diethyl *O*-(2-isopropenyl-4-methyl-6-pyrimidinyl) phosphorothioate and 4-hydroxy analog of diazinon, as well as diazoxon, were detected in mice urine *in vivo* shortly after oral administration of diazinon. The presence of the 4-formyl analog of diazinon was presumed. *O,O*-Diethyl *O*-(2-isopropenyl-4-methyl-6-pyrimidinyl) phosphorothioate was also formed when hydroxydiazinon was administered (11). These phosphorothioate esters inhibited human plasma cholinesterase similarly to diazinon and the oral LD_{50} value of hydroxydiazinon in mice was 110 mg/kg (11), so they are likely to be toxicologically significant metabolites.

In vitro guinea pig liver slices converted diazinon to hydroxydiazinon (8). Although rat liver microsomal fractions were active in oxidatively cleaving the ester bond of diazinon to yield dimethylphosphorothioic acid and the corresponding pyrimidinol (12, 13), similar enzyme systems oxidized diazinon to hydroxydiazinon in the presence of nicotinamide-adenine dinucleotide phosphate and oxygen (13). The enzyme systems were also capable of converting diazinon to diazoxon and hydroxydiazinon to hydroxydiazoxon. On the other hand, diazoxon and probably hydroxydiazoxon were hydrolyzed by rat liver microsomal esterase *in vitro* to yield the pyrimidinol and the oxidation product at the tertiary carbon atom (14). The latter compound is assumed to be derived from hydroxydiazinon by oxidative cleavage of the ester linkage.

In the presence of reduced glutathione the rat liver supernatant fraction transferred the pyrimidinol moiety of diazinon to glutathione to form still another product (15).

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Cyanofenphos

The metabolism of cyanofenphos (Surecide®, *O*-ethyl *O*-4-cyanophenyl phenylphosphonothioate) in mice (1) and in bean plants (S. Kato & I. Yamamoto, private communication) was investigated with the 4-cyano-^{14}C preparation. Proposed pathways of biodegradation are presumed to be essentially the same in both mice and bean plants.

^{14}C-Labeled cyanofenphos, orally administered to male and female dd-strain mice, was rapidly absorbed from the intestinal tract. Radioactivity was present 30 min post-treatment in 10 major organs, fat, muscle, and blood. The tissue radioactivity reached a maximum 30 min after treatment and then decreased rapidly. Several radioactive metabolites were detected including a trace amount of cyanofenphos-oxon as well as unchanged cyanofenphos.

The ^{14}C-cyanofenphos given to mice was eliminated quite rapidly, mostly within 24 hr, and during 3 days approximately 95 and 5% of the applied radioactivity was excreted, respectively, in the urine and feces. No detectable radioactive carbon dioxide was expired. The urinary radioactive metabolites of cyanofenphos were separated by silica gel thin layer chromatography and compared with authentic reference compounds for identification. The following radioactive metabolites were presumed to be present: unchanged cyanofenphos (5% of the urinary radioactivity), desethyl cyanofenphos (10%), and free 4-cyanophenol (15%). No cyanofenphos-oxon was detected and desethyl cyanofenphos-oxon was not positively confirmed. The polar radioactive products amounted to approximately 70% of the urinary radioactivity: they were acid-labile and yielded primarily 4-cyanophenol from aryl-sulfatase digestion.

These biodegradation patterns appear to be essentially similar to those of the corresponding *O,O*-dimethyl phosphorothioate ester of 4-cyanophenol (Cyanox®, cyanophos) in rats (2). Cyanofenphos is likely to be quite rapidly metabolized in mammals similar to another phosphonothioate insecticide, leptophos (3).

Preliminary studies on the plant metabolism of radioactive cyanofenphos were conducted in bifoliated bean plants (*Phaseolus vulgaris* L.) under greenhouse conditions. The radioactivity was neither taken up from the root to aerial parts nor translocated to untreated plant parts from the leaf surface.

Cyanofenphos appears to be resistant to decomposition or volatilization under experimental conditions. In the plant, samples obtained 2 weeks after leaf application contained more than two-thirds of the total radioactivity and 50% of the cyanofenphos initially applied. In contrast, <5% of the ^{14}C-cyanophos remained under similar conditions (S. Kato & I. Yamamoto, private communication). The persistence of cyanofenphos was compared with that of cyanophos in several field samples, including rice grains, vegetables, and fruits. (Sumitomo Chemical Co., Ltd., unpublished report).

Radioactive 4-cyanophenol and desethyl cyanofenphos-oxon, which were extracted by acetonitrile, were detected by thin layer chromatography. However, neither cyanofenphos-oxon nor desethyl cyanofenphos has been positively confirmed.

Thus, cyanofenphos can be regarded as a persistent, biodegradable compound and is possibly useful as a residual insecticide without problems of biomagnification in the food chain.

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Leptophos

The metabolism of leptophos, *O*-methyl *O*-4-bromo-2,5-dichlorophenyl phenylphosphonothioate, in mice and cotton plants was investigated with 2 different radiolabeled preparations (^{14}C-phenoxy and ^{14}C-phenyl) (1). The metabolic patterns were essentially the same in both mice and cotton plants.

^{14}C-Leptophos orally administered to female Swiss mice was rapidly degraded and eliminated. The biological half-life of ^{14}C-phenoxy was 7 hr and elimination of radiocarbon was virtually complete 48 hr after administration. In comparison, ^{14}C-phenyl was excreted at a slower rate and radioactivity was detected in urine as long as 144 hr post-treatment. The recovery of radioactivity was essentially quantitative. The urine

contained major portions of the radioactivity (90–96% of the recovered radiocarbon). A small amount of intact leptophos and a trace of leptophos-oxon (<1% of the recovered radioactivity) were present in feces.

With phenoxy-labeled leptophos, >90% of the urinary metabolites was in the form of conjugated 4-bromo-2,5-dichlorophenol, although approximately 7% of the free radioactive phenol was also present. The exact nature of the conjugates was not established but both enzymatic (β-glucuronidase/arylsulfatase) digestion and acid hydrolysis yielded only the phenol.

The phenyl-labeled leptophos gave *O*-methyl phenylphosphonothioic acid, the principal degradation product (about 57% of the recovered radioactivity) and, as minor products, *O*-methyl phenylphosphonic acid and phenylphosphonic acid in nearly equal amounts. These metabolites were present in the urine.

The unidentified product(s) was detected by cochromatography with an authentic sample of *O*-(4-bromo-2,5-dichlorophenyl) phenylphosphonothioic acid.

Thus, the metabolic pathways of leptophos in mice are similar to those proposed for other organophosphorus esters in mammals, such as bromophos (2) and ronnel (3).

Radioactive leptophos applied to leaf surfaces of young cotton plants under greenhouse conditions did not penetrate into the leaf tissues, but remained on the surface and was gradually lost mainly by volatilization. For example, 73–75% of the applied radioactivity was recovered 1 week after application compared with 11% after 9 weeks. Relatively small amounts of radioactivity were found inside the leaf or bound to the pulp after benzene or methanol extraction.

The major radioactive compound recovered 1 week after application was unchanged leptophos (91–96%) and the amount gradually decreased with time to approximately 29% in 9 weeks. Most of the leptophos remained on the surface, although a significant but small amount was evidently absorbed into the leaf. Little, if any, leptophos-oxon (<0.1% of the applied radiocarbon) was detected. Alteration products such as *O*-methyl phenylphosphonothioic acid, *O*-methyl phenylphosphonic acid, phenylphosphonic acid, and the phenol were detected. Among the acids, the phenol was predominant at the end of the experiment. No conjugated phenol but a salt of the phenol was present. A small amount of the radioactivity from both types of labeled leptophos was bound to the pulp.

Because of its stability on and in cotton plants, together with its rapid metabolism in mice, leptophos can be regarded as a persistent, slowly biodegradable organophosphorus insecticide.

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3. Terminal Carbamate Residues

The Commission received a report on terminal residues of methylcarbamate insecticides, including carbaryl, carbofuran, methomyl, aminocarb, mexacarb, and meobal. The significance of nitrosocarbaryl was reviewed in detail. It was concluded that any comments relating to the environmental significance of nitrosocarbaryl are to be viewed with some caution, since the conditions under which nitrosocarbaryl is formed are atypical of natural conditions.

Carbamate Pesticides: Evaluation—Ronald L. Baron (Pesticide and Toxic Substances Effects Laboratory, Environmental Protection Agency, Research Triangle Park, NC 27711)

Several reviews on carbamate metabolism have recently appeared in the literature. Two reports initially presented in 1971 at the IUPAC International Pesticide Congress have been published in their entirety (1, 2) and one new report on carbamate metabolism has been published (3).

Reports of new terminal residues for carbaryl include a suggestion of a highly mutagenic derivative, nitrosocarbaryl, which may be formed by the interaction of nitrate and carbaryl (4–6). It has been suggested that the compound would also be carcinogenic, based on a similarity of structure to *N*-nitrosomethylurethane. Previous studies reporting an *N,O*-conjugate of carbaryl appear to have been resolved with the consideration that there is small possibility for such a reaction. The significance and actual presence of nitrosocarbamates need to be resolved.

The metabolism of carbofuran in mugho pine (7) was qualitatively the same as in a model ecosystem (8), reported for other organisms. Carbofuran was metabolized more slowly in the pine than in other plants and animals, and again apparently yields 3-hydroxycarbofuran, 3-ketocarbofuran, and cleavage products. Studies designed to evaluate the interaction of carbofuran with herbicides showed that atrazine enhanced the toxicity

of this carbamate to insects (9) and that its metabolism in barley was inhibited by chlorobromuron (10).

The metabolism of methomyl in rats, plants, and soil has been reported in depth (11–14). The primary metabolic pathway of methomyl follows a pattern of metabolism different from that of other oxime carbamates. No residues of the corresponding *S*-oxide or *S,S*-dioxide were observed. In plants, methomyl was rapidly converted to acetonitrile and carbon dioxide without accumulation of detectable intermediates. These fragments were reincorporated into natural components such as lipids and tricarboxylic acids. In rats, methomyl was rapidly eliminated in the ratio of 1 part carbon dioxide to 2 parts acetonitrile to 1 part urinary metabolite. In soils, methomyl was hydrolyzed to the corresponding oximino compound, followed by total decomposition to carbon dioxide without formation of acetonitrile. Aldicarb, as previously reported and confirmed, follows a different pathway of metabolism, yielding a sulfoxide and sulfone prior to total degradation (14).

m-Tolyl *N*-methylcarbamate is widely used in Japan for insect control on rice. The metabolism of the compound in rats is different from that of most carbamates because <5% of an oral dose is hydrolyzed and 70% of the urinary metabolites are in the free form (15, 16). The predominate metabolites in the urine were free 3-carboxyphenyl *N*-methylcarbamate and conjugated 4-hydroxy-3-methylphenyl *N*-methylcarbamate. The dose was nearly totally excreted within 24 hr by male rats but only 75% was excreted by females. However, there was no difference in the nature of the metabolites in the urine of males and females. When *m*-tolyl *N*-methylcarbamate was applied to bean leaf surfaces, there was rapid loss of the insecticide (86% after one day) and little metabolism. No hydrolysis products and only small amounts of carbamate-type materials were detected. Oxidative metabolites formed by rats, houseflies, and plants and by photolytic reactions (17) were less potent anticholinesterase agents than the parent compound.

An *in vitro* artificial oxidation system degraded aminocarb through demethylation and hydroxylation of the aromatic ring, oxidation of the methyl to the corresponding methylol, and cleavage of the carbamate to its phenol. All these reactions were similar to those previously described for the biodegradation of aminocarb and other carbamate insecticides (18, 19). Further studies on methiocarb reconfirm the metabolic pattern reported previously (oxidation, hydrolysis, etc.) (20).

Mexacarbate was decomposed photolytically (296.7 nm wavelength) in solution to yield 4-dimethylamine-3,5-dimethylphenol, 4-hydroxy-2,6-dimethyl *N*-methylbenzamide, and 4-monomethylamine-3,5-xylyl methylcarbamate. This study suggests that new and previously unknown materials may occur in the environment as a result of photolytic degradation (21). In studies with meobal, photolytic products include: 3,4-dimethylphenol, 2-hydroxy-4,5-dimethyl-*N*-methylbenzamide, and 2-hydroxy-5,6-dimethyl-*N*-methylbenzamide (22). Aminocarb decomposed to 4-dimethylamino-3-methylphenol. Landrin decomposed to the trimethylphenol (23). In these studies, the *in vitro* photoproduct did not correspond to photoproducts found on beans sprayed and exposed to sunlight.

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4. Terminal Fumigant Residues

The Commission received 3 reports covering methyl bromide, ethylene oxide, and phosphine. A report was discussed which suggested that residues from ^{32}P-phosphine in several foodstuffs were persistent and that 25% of the parent material was recovered in residue analysis. Impurities in the labeled materials used to prepare ^{32}P-phosphene are responsible for these reported residues in foodstuffs.

Evaluation—Gero E. Mayr (DEGESCH, PO Box 2644, D-6000 Frankfurt/Main, W. Germany)

Methyl Bromide

Methyl bromide is one of the most important fumigants. In Germany methyl bromide is used in about 50% of all mill fumigations, as well as fumigations of stacked boxes, sacks, and grain ships (1). Brown (2) described the technique of fumigating under gas-tight sheets. Fumigation of flour, particularly when done repeatedly, leads to odor problems. However, when treated flour is mixed with several times its volume of untreated flour, the influence of a single fumigation is slight (3). Methyl bromide, unlike other gases, has no narcotic effect and there is usually a considerable difference between the doses tolerated by plants and doses lethal to pests. Absorption is slight, except in fatty substances (4). Because the gas is highly dangerous, Hunold and Pietrulla (5) discuss methyl bromide from the viewpoint of industrial safety. They give the main safety precautions which should be observed in handling the gas, and methods for determining the presence of the substance in air, e.g., detecting it with a halide test lamp. A comprehensive paper on methyl bromide by Thompson (6) gives details of its properties, various applicational fields, fumigation techniques, appropriate safety precautions, and analytical methods for determining concentrations and residues.

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Ethylene Oxide

Ethylene oxide is an excellent fumigant for the destruction of pests of all types—insects, bacteria, viruses, and phagocytes—in both their vegetative and spore forms. However, it is highly toxic to mammals, and humans in particular, so suitable precautions must be taken. Only trained staff should work with the compound. The formation of explosive gas-air mixtures can be prevented by adding inert gases (carbon dioxide, freons, etc.), but this method is still inadequate because these mixtures are also inflammable. The vacuum process is safer because the formation of explosive ethylene oxide-air mixtures is prevented from the very outset.

An important feature of ethylene oxide is the high rate of penetration through all conventional packing materials so that it is possible to pack a bacteria-tight product, sterilize it, and subsequently store it in a sterile condition with undamaged packaging. Ethylene oxide forms compounds with practically all organic substances, so undesirable reaction products can occur. Thorough chemical, physical, medical, or organoleptic tests are therefore necessary (1). The equipment for sterilizing with ethylene oxide was described at a symposium of the British Pharmaceutical Society (2).

Numerous publications emphasize the importance of the minimum moisture content of treated goods, since sterilization is not possible without moisture. Equally important is "preclimatizing." The moisture content of the ambient air, on the other hand, has practically no effect on the success of the treatment (1–8). Among the products sterilized were dry gelatin and dried egg powder (9); dried therapeutical plants and herbal preparations (10); glass, plastics, rubber, wood, cellulose, paper, linen, metal, nutrient media, reagents, sausage skins, meat, and sausage mix (11); membrane filters and nutrient cardboards (12); and medical instruments (13). Salmonella and enterobacteriaceae, found in large numbers in dried egg products,

coconut scrapings (14), and bone meal (15) before fumigation, were no longer detectable. Infected shoes, stockings, and inner soles, which are practically inaccessible for fungicides, are sterilized without damaging the leather (16).

There is extensive literature on cold sterilization with ethylene oxide of spices, three-quarters of which are bacteriologically impure (17, 18). After fumigation, the residues generally disappear after 2–3 weeks or are insignificant, as spices are always used in a greatly diluted form (in exceptional cases, 50–100 ppm were still found). The flavoring capacity, aroma, and color of the spices remain practically unchanged, with the exception of mustard (19, 20). In the case of black pepper, its origin (probably as a result of differing composition), grain size, water content, and load factor of the fumigation chamber determine the distribution of the ethylene oxide. Residues increase in proportion to grain size, as does the metabolized component. At higher water contents, one finds fewer residues, while the metabolized component changes very little. In unventilated pepper, one still finds 1773–1865 ppm, of which 24.6% reacts with the substances of the residue (cellulose), 11.8% with the water extract (sugar, proteins, amino acids), 2.6% with the starch, 1.3% with the methylene chloride extract, and only 0.05% with essential oils. From 54.6 to 57.3% forms volatile metabolites, mainly ethylene chlorohydrin (657–761 ppm). Under practical conditions, the values are considerably lower: total quantity 866.9 ppm, reactions with 11.6, 4.8, 1.9, 0.8, and 0.4%, respectively. About 52.6% or 456 ppm is adsorptively bound and 27.9% is volatile metabolites (ethylene chlorohydrin 302 ppm) (21). Changes in color, pH value, etc., if undesirable, can be prevented by the use of Etoxiat (a 1:1 mixture of ethylene oxide and methyl formate); the residue problem is also reduced by this method (22).

Seven ppm ethylene oxide was found in wheat after fumigation of a silo. Control experiments confirmed that damp wheat absorbs more ethylene oxide than dry wheat. Ethylene oxide is distributed between the phases, i.e., "gas space" and the water contained in the wheat. The coefficient of distribution is determined as a special material constant of the wheat, which depends on the water content. A relationship can be formulated which forms the basis for calculating the metabolizable or already metabolized components of ethylene oxide in the wheat. These components depend on the water content of the grain, the distribution constants, the intensity of ventilation, the duration of ventilation, and the quantity of ethylene oxide used.

The problem of residual ethylene oxide is small from toxicological and food law aspects, because it disappears through metabolism after 4–6 weeks; e.g., residue analyses can no longer determine, after this time, whether a given sample had been fumigated or not. Fumigation with radiolabeled ethylene oxide and determination of the concentration through radioactivity measurements showed a linear regression between the ash content of the flour fractions and ethylene oxide content (bran 54 ppm, white flour 6.8 ppm, wheat germ 62 ppm, mean residue 12–15 ppm) (4, 5, 23). A comparison of applied and recovered ethylene oxide via radioactivity measurements revealed that between 22.8 and 28.0% of the ethylene oxide was metabolized, which confirmed the results of earlier experiments (27.9%) (4, 5, 23).

About 75% ethylene oxide in the grain reacts with the water-soluble components or with the moisture content of the individual grain; approximately 28% reacts with water to form ethylene glycol, about 15% reacts with the water-soluble substances of albumen, about 16% reacts with the saccharides, about 12% reacts with organic acids, and about 4% reacts with minerals and vitamins. After conventional fumigation, about 11–14 ppm of the relatively nontoxic ethylene glycol was found; on the other hand, about 3–4 ppm of the more toxic ethylene chlorohydrin was detected. The activity of the dehydrogenase is reduced only by 4-fold overdosing (24). With cocoa powder, the metabolism rate of ethylene oxide apparently depends on the moisture content of the foodstuff; the residual values are inversely proportional to the water content. The water content, however, exerts no influence on the quantity of the metabolized ethylene oxide which, under practical conditions, is on the average of 11.5% of the quantity used, but increases with greater concentrations. From this, one can conclude that cocoa powder has an adsorption mechanism and not a distribution mechanism, as does wheat. If the reaction products are extracted, the toxic substances ethylene glycol and ethylene chlorohydrin which may have been formed are found in the ether extract only, namely, 9.7 ppm or $<1.5\%$. In the chloroform extract 155 ppm metabolites are found, in the water extract 421 ppm, and in the residue 105 ppm. The metabolized component of the original weight of cocoa is on the average of 690 ppm (25).

If tobacco is fumigated with ethylene oxide to remove the nicotine, an *N*-hydroethyl compound is formed (26); the nicotine content, however, is only slightly reduced (0.02–0.12%). Nicotine in the smoke, dry condensate, and benzopyrene content did not differ significantly from that of untreated samples (27). Only after treatment under extreme

conditions (3000 g/cu m, 45°C, 4 hr) was it possible to detect *N*-methyl aminoethanol; it was, however, not possible to detect ethylene glycol (28). Ethylene oxide (0.02 μg/ml) was found in the smoke of untreated cigarettes; fumigation increased this amount (29). No ethylene oxide residues were found in the tobacco 5 weeks after fumigation. Smoke, on the other hand, contained a higher ethylene oxide concentration, even after several weeks (reaction with nicotine and reversion due to pyrolysis). Chlorohydrin is found in small quantities (12 μg/g) in the tobacco after fumigation; this rapidly disappears under normal storage conditions and practically none is found in the smoke (0.5 μg/g). The smoke of untreated tobaccos already contains a small quantity of chlorohydrin (1 μg/g) (30).

Experiments concerning deactivation and the initiation of mutation of phagocyte Kappa of *Serratia marcescens* indicated that at least part of the damage can be attributed to DNA lesion. The humidity before and during fumigation has a decisive influence. The effects are evident after several reaction stages with temperature-dependent rates (6). While the classical sterilization processes (heat and steam) are based on momentary, irreversible physical reactions with proteins and nucleic acids, gas sterilization is a chemical reaction with the same cell components, which can, however, only take place under suitable conditions and after a longer period of treatment. A cell must contain sufficient moisture if the gas molecule is to penetrate it. Vegetative cells usually fulfill this requirement (without artificial drying), while spores dry out to a greater degree and are therefore more resistant. Not all alkylating products are lethal for the cell; this means that ethylene oxide must be present in the cell in sufficient concentration to irreversibly alkylize certain essential groups. Increasing the concentration and raising the temperature or moistening the material will not lead to successful sterilization; the only method is to ensure adequate conditioning. The use of bio-indicators to determine the success of sterilization is of value only if the method of preparing the indicator is adapted to the climatic conditions of the product and the indicator can be tested before use with the product. On the other hand, the preparations can also be manufactured in such a way that sterilization is absolutely impossible and their use as indicators thus becomes pointless.

The ethylene oxide-carbon dioxide mixture damages some plastics (e.g., polystyrene); the mixture with methyl formate (Etoxiat) on the other hand, does not (7, 8). Apart from drastically reducing the bacterial count, or even freeing the material of bacteria, Etoxiat can also considerably reduce the aflatoxin content.

There is a direct relationship between the desorption of the physically bonded ethylene oxide and the formation of ethylene chlorohydrin (31). Not only are the values for ethylene chlorohydrin residues found by Wesley *et al.* (32) too high, but those stated by Heuser and Scudamore (33–36) are also excessive; large amounts of ethylene chlorohydrin (up to 800%) which were not previously present are formed from ethylene oxide during the analysis when the acetone extract is dried with sodium and calcium chlorides. On the other hand, the existing ethylene glycol is lost (37).

While hostaphan, rigid and plasticized polyvinyl chloride (PVC), and cellophane are in some cases considerably more permeable to Etoxiat than to pure ethylene oxide, the differences for polyethylene, polypropylene, polyamide, and nitrocellulose are insignificant (38). Sorption in polyethylene, polyamides, silicones, aluminum membranes, and glass beads is noticeably less than in polypropylene, polystyrene, PVC, and paper. Desorption is more rapid in all cases if Etoxiat is used in place of pure ethylene oxide (39–41).

No toxic effects from ethylene oxide-fumigated nutritional products were observed in a 2-year feeding test with rats; their appearance, behavior, body weight, mortality, and organic structure remained unchanged, even with relatively high residues (500–1400 ppm initially, 53–400 ppm after 6 days) (42).

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Phosphine

Analytical methods for phosphine are generally based on the method proposed by White and Bushey (1) and modified to meet specific requirements, e.g., the method may be automated (2). Residues in grain stock, although they rapidly disappear, can be determined with a sensitivity of 0.005 ppm (3). One can even determine 0.0005 ppm by gas chromatographic methods (4, 5). A colorimetric method is sensitive enough to analyze phosphine at residue tolerance levels and with smaller samples (6).

Analyses of fumigated grain, other foodstuffs, and animal feedstuffs revealed residues of $\leq$0.01 ppm phosphine. Vitamin analyses gave no indications of any changes as a result of application of phosphine to the raw materials (7). Metabolic analyses of fumigated grain also revealed no noticeable changes in the glutamic acid decarboxylase content (8), intensity of respiration, and enzyme activity (9).

The effect of phosphine varies with the moisture content of the grain above about 18%. Up to about 20% moisture, the effect was slight, but increased rapidly until the effect was lethal at 25% (10). The characteristic odor of phosphine may be absent while considerable gas quantities are still present, because very small odor-generating fractions are probably chemically bonded (11).

Very little gas is adsorbed on wheat or corn. Sorption increases slightly with space utilization and higher moisture content (12). Quantities added to grain products and inert materials can be recovered in air-tight systems; losses occur only at elevated temperatures in leaky systems. Chemisorption therefore does not occur (13). Even a 0.05 mm thick polyethylene film (14) suffices as a seal; polycarbonate, polypropylene, or cellophane are also practically impermeable, while Kraft paper, even the 2-ply or the resin-coated type, is moderately permeable (15).

Most of the toxicological properties of phosphine have been investigated. Representative publications are refs. 16–18.

Three-month feeding experiments with rats revealed no deleterious influences from fumigated grain. Inhalation tests (8 hr) with houseflies and grain weevils proved that the tablet residues were not toxic (19). Chronic feeding studies over 2 years with rats fed a diet which had been fumigated at high concentration also revealed no toxic effect (20). In a suicide attempt, death was pre-

vented by extracorporeal hemodialysis and medication with agents which support the heart and circulatory system (21).

The effect of phosphine on insects is the theme of numerous publications. The resistance of insects differs, depending on their stage of development, e.g., the larvae of *Trogoderma sternale* are most difficult to destroy. With these products, a short-term application of the gas is less effective than longer exposures (22). In larder beetles, the egg stage is the most resistant. With the exception of *Trogoderma sternale,* the adult insects were most susceptible, but even these showed greater tolerances than the adult lesser rice weevil, grain weevil, and confused flour beetle. There is no agreement among the tolerances of the individual stages of the different species (23).

For *Anthonomus grandis,* 9–10-day-old adults were less susceptible than 2–4-day-old insects (24). For various dried fruit pests, the eggs were the most difficult to destroy while the larvae and adults were easy to eliminate (25).

The different stages of development of the grain beetle, *Sitophilus granarius,* show increasing resistance: adult>larva>egg>pupa. Within one stage there are also differences, e.g., one-month-old insects can be eliminated with half the exposure time which is needed for freshly emerged beetles (26).

Fumigation with low concentrations of phosphine (0.01–0.024 mg/L) will not prevent the development of insect stages and even viable eggs can be laid. From this, one can conclude that the stages which are resistant at this concentration develop to less-resistant stages during the fumigation period and are then destroyed if the fumigation period is at least 10 days (27). Sublethal fumigations, when repeated, lead to cumulative effects which vary with each species. *Tribolium confusum* recover after 10 days, while *Sitophilus granarius* do not recover after 40 days; *Tenebroides mauritanicus* behave differently (28).

The absorption of phosphine occurs only in the presence of oxygen. Without this, phosphine is not toxic, even when respiration fails, e.g., because of the effects of hydrocyanic acid or when the insects were previously treated with nitrogen or carbon dioxide. The lethal action of phosphine does not increase with concentration (from 5 to 20,000 ppm). High concentrations have a paralyzing action similar to a narcotic effect and decrease the respiration rate and thus the mortality. Lower concentrations have a more toxic effect on the insect; the increased oxygen consumption may have accelerated the phosphine permeability in membranes of the cell and mitochondria. Then, phosphine would inhibit the respiration chain of the mitochondria, possibly cytochrome a_3 and b. On the other side, revival of insects after application of higher concentrations of phosphine may be due to paralysis and reduced oxygen consumption, and it is likely that phosphine does not reach the toxic site of the cell. Twenty-four hr after treatment with 10,000 ppm, 50% of the insects were active, and the 90% mortality within 10 days indicates that they had absorbed a lethal dose of phosphine in a very short time before they became paralyzed. The last 10% of the insects (exposed to 10,000 ppm for 12 hr) survived for one month and more, behaving normally as far as activity and reproduction were concerned. It is also possible that phosphine may be converted in the insect to nontoxic compounds, e.g., orthophosphate, because oxy-acids of phosphorus have been found in the tissues of insects (29). Klimmer indicated the possibility of detoxification and excretion of phosphine in inhalation tests with warm-blooded animals, which was reported by Nakakita *et al.* (30).

Cockroaches go into irreversible spasms after fumigation, the degree apparently depending on the concentration. The absorption differs with the insect species; in no case was desorption observed (31). In laboratory experiments with *Sitophilus granarius,* a triple tolerance was chosen after 28 selection processes; this decreased somewhat after 9 untreated generations but it was stabilized once more after 3 further treated generations (32). *Spodoptera littoralis* have particularly resistant eggs; if these were, however, preconditioned for 3 days, their tolerance became equal to that of the larvae (33).

A summary by the American National Pest Control Association, containing notes on the physical properties, application methods, insecticide properties, toxic effects, warning posters, and dosage recommendations, emphasizes the international importance of the agent Phostoxin (34).

With various types of grains, pulses, and seeds, fumigation does not harm the germination capacity in any way, in fact, it may improve it (35–38).

The effectiveness of phosphine as a fumigant for the control of pests in several commodities has been studied. The fumigation of sacked rice under gas-tight sheets (39) did not affect organoleptic quality (40). Natural raisins have been treated under Kraft paper (41). Groundnuts (42–46) and soybeans (47) were successfully treated; germination of the seeds was not affected. Farinaceous products and processed commodities have been freed of insects (48–50). A specially designed cocoa warehouse, not gas-tight, was treated with phosphine with good results and no

injuries (51–55). Good results have also been reported for the fumigation of sacked milho (56).

Tobacco was fumigated with phosphine in several different packages, in warehouses, and in containers during transit, and the fate of the residues was determined (57–67). Empty railway freight cars and cars carrying bagged groundnuts, barley and other grains, and processed cereal products were successfully fumigated (68–70). An entire mill was also fumigated with phosphine (71).

The common stored product pests, the pink bollworm (*Pectinophora gossipiella*) (72), and termites living within teaplants (73) have been controlled.

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5. Terminal Fungicide Residues

The Commission reviewed a report on terminal residues of dithiocarbamate fungicides, which dealt primarily with the ethylenebisdithiocarbamate compounds and the decomposition product, ethylenethiourea (ETU). Analytical problems with ETU are still unresolved. The Commission recommended that, because ETU is a by-product in many commonly used fungicides and because of its toxicity, this problem should be drawn to the attention of the Joint Meeting of FAO/WHO. A dependable residue method is needed particularly for poultry and eggs.

Evaluation—N. Drescher (Badische Anilin- und Soda-Fabrik AG, Landw. Versuchsstation, Postfach 220, D-6703 Limburgerhof, W. Germany) and R. Engst (Zentralinstitut für Ernährung, Arthur Scheunert Allee, 1501 Bergholz-Rehbrucke, E. Germany)

Dithiocarbamates, especially the ethylenebisdithiocarbamates (EBDCs), are still the most extensively used organic fungicides. Since the IUPAC Commission meeting in 1973, some studies concerning the metabolic fate of EBDCs, particularly the formation and behavior of ethylene thiourea (ETU) have been published. Very little data have been reported on terminal residues of these compounds.

Hylin (1) has investigated the oxidative decomposition of nabam and maneb in soil as well as in solution and suspension, respectively. He proposed the following mechanism of EBDCs breakdown:

EBDCs → ethylene thiuram disulfide →
[transient intermediate] →
ethylene thiuram monosulfide + H_2S
and/or ETU + CS_2 + S^0

Lyman and Lacoste (2) reported that ETU was highly susceptible to photodecomposition and microbial degradation (formation of carbon dioxide in soil) and, consequently, does not accumulate in soil.

Soil tube leaching studies, carried out in our laboratory (N. Drescher, unpublished data) with metiram have shown that the active ingredient is strongly adsorbed in soils. No leaching could be observed with 3 different soils and amounts of rain as high as 200 mm within a 48 hr period.

Sijpesteijn and Vonk (3) investigated the metabolism of ^{14}C-labeled nabam. After root and leaf treatment of cucumber, ETU, ethylene urea (EU), and 2-imidazoline were found in the plant, in addition to a fair quantity of identified material. ETU disappeared within 19 days and was almost quantitatively converted in the plant into 2-imidazoline and EU. 5,6-Dihydro-3H-imidazo[2,1-c]-1,2,4-dithiazole-3-thione, a spontaneous transformation product of nabam in aqueous solution, is not taken up by the plant.

The effect of boiling on the formation of ETU in zineb-treated carrots, spinach, and tomatoes was studied by Newsome and Laver (4). Cooking promoted decomposition of the parent compound to ETU. Much of the ETU formed is removed in the cooking water but, compared with the uncooked samples, increased amounts remain in the crop. Engst and Schnaak (5) reported similar tests with maneb-treated fruits. According to the authors, ETU residues can increase 10-fold compared with the starting material during the thermal treatment.

Vogeler and coworkers (6) reported on preliminary experiments with ^{14}C-labeled propineb applied to apples and grapes under practical conditions. Relatively large amounts of the radioactivity were found in the peel and pulp of apples and in the juice and solid material of grapes. They concluded that propineb metabolizes by splitting into oligomeric fractions which are similar to the parent compound.

According to Lyman and Lacoste (2) there is negligible uptake of ETU from soil and no significant translocation of ETU from leaf surfaces.

Kuhr *et al.* (7) analyzed soil samples from an apple orchard treated 4 times with metiram (total amount of active ingredients, 20 lb/acre). Metiram residues accumulated in the top 2″ soil layer up to a maximum of 6 ppm during the spray period. For the following 4 months, residues, mostly <1 ppm, were occasionally detected in the upper soil layer. No metiram was found in soil lower than 2″ from the surface.

Newsome (8) found ETU residues ranging from 0.02 to 0.04 ppm in commercial apple samples.

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6. Terminal Herbicide Residues

The Commission received 2 reports, one on phenylamide-type pesticides in soil, with special reference to the formation of aniline-containing condensation products, and the other on chlormequat metabolism in wheat. The need for additional and rigidly controlled investigations in phenylamide-type pesticides was stressed.

A. Phenylamide-Type Pesticides

Evaluation—Hans Geissbühler (Agrochemicals Division, Ciba-Geigy Ltd., Basel, Switzerland)

Numerous phenylamide-type pesticides (phenylcarbamates, anilides, phenylureas, formamidines, etc.) are hydrolyzed to the corresponding anilines under certain laboratory and field conditions (for background material, *see* ref. 1). In many of these studies the methods used to establish the identity of the anilines and their rate of formation were not rigorously controlled. However, in spite of lack of reliable and sufficient data, these preliminary observations provoked a host of experiments dealing with the further fate of substituted anilines in various biological media.

In 1967, Bartha and Pramer (2) reported that, upon application of high concentrations (500 ppm) of propanil (*N*-(3,4-dichlorophenyl) propionamide, 3′,4′-dichloropropionanilide) to soil samples, the herbicide decomposed to carbon dioxide and 3,4-dichloroaniline. The latter compound was condensed to form 3,3′,4,4′-tetrachloroazobenzene. Soil microorganisms were involved in both transformation reactions.

Symmetrical azobenzenes were subsequently formed with or from high concentrations of phenylcarbamates, anilides, formamidines, and free, substituted anilines in a variety of different model systems, including soil suspensions, microbial cultures, and photolytic media (for review, *see* ref. 3). In addition, further condensation products, such as asymmetrical (hybrid or mixed) azobenzenes, azoxybenzenes, anilinoazobenzenes, and triazenes, were formed, depending on the concentrations of the anilines, the positions of their substituents, and the conditions chosen for the experiments (for extensive lists of pertinent literature, *see* refs. 4–6). To elucidate the potential pathways of formation of these condensation products, a number of studies were conducted, both with biological (including enzymatic) and chemical (sensitized/photosensitized) systems. These experiments indicated that the intermediates were dependent on the relative concentrations of the

various potential reactants. However, most of the proposed transformation mechanisms involved one or more oxidative steps and the formation of radicals (for summary of reactions, *see* ref. 5).

Although the various laboratory and model experiments were normally carried out with concentrations of anilines and aniline-containing parent compounds far beyond those applied in practice, conclusions were drawn in many papers and even in press releases with regard to the environmental significance of condensation products derived from phenylamide pesticides. In regulatory terms, these implications were amplified by allusions made to toxicological effects of certain azobenzene derivatives (7, 8).

A number of experiments were also conducted in which the concentration of phenylamide pesticides and free aromatic amines approached those to be expected in practice. These studies were facilitated by the high sensitivity of gas chromatographic methods, by which condensation products of anilines, especially halogenated azobenzenes, can be determined (9; Ciba-Geigy Ltd., internal report, 1974). Free 3,4-dichloroaniline was applied by Sprott and Corke (6) to 4 different Canadian soils. Even at the relatively high concentration of 100 μg/g soil (100 ppm), the conversion to 3,3′,4,4′-tetrachloroazobenzene was minimal (0.0–0.6% in terms of applied 3,4-dichloroaniline). These data, which indicated that the formation of condensation products was strongly dependent on the concentrations of anilines and that the formation was significantly reduced or non-existent at levels actually used, were confirmed by several studies (10–12; D. D. Kaufman and C. L. Fletcher, 1974, private communication).

Extensive investigations with phenylureas, both in the laboratory and under field conditions, demonstrated that no detectable amounts of condensation products, such as azobenzenes, were generated from these herbicides in soils or plants (9, 13, 14; Ciba-Geigy Ltd., 1974). Apparently the rate of formation of free anilines from phenylureas was too small to allow sufficient buildup for condensation reactions. The same situation applied to formamidines (15).

A recent report from the Pesticide Monitoring Laboratory, Bay St. Louis, MO shows that, from among 99 different soil samples derived from rice fields treated with propanil for one to several years, only 6 soils were found to contain traces of residues of 3,3′,4,4′-tetrachloroazobenzene (0.01–0.05 ppm).

From present evidence it is concluded that the condensation products discussed above do not represent significant terminal residues of anilides, phenylcarbamates, phenylureas, and formamidines in agricultural soils. From recent reports in the literature (11, 12) it appears that certain binding or conjugation reactions with soil constituents are more important for aniline-containing pesticides. These reactions deserve further attention.

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Evaluation—Harold M. Dekhuijzen (Institute for Organic Chemistry, T.N.O., Organization for Applied Scientific Research, Utrecht, The Netherlands)

B. Chlormequat

Fate of Chlormequat in Wheat Plants.—Recent work on chlormequat, (2-chloroethyl) trimethylammonium chloride, showed that this plant growth regulator, when taken up by the roots of wheat seedlings (for 6 hr), is rapidly translocated to the above-ground parts and converted to choline. Choline was further metabolized to betaine which was demethylated to glycine, serine, and ^{14}C-CO_2 as end products (1, 2). Half of the total amount of chlormequat originally present in whole wheat

seedlings was metabolized after 7.5 days. These findings are consistent with those reported by Mooney and Pasarela (3) and by Jung and El-Fouly (4) after spraying chlormequat on wheat plants. These results, however, are not in agreement with those of Birecka (5) and Bohring (6, 7), which showed no decrease of chlormequat after root or leaf application over a 4-week period.

These discrepancies are difficult to explain and may be due to the use of different wheat varieties or different environmental conditions during the experimental period. Another explanation may be that chlormequat is taken up more slowly by the leaves than by the roots and, consequently, a deposit remains for a long time on top of the leaves (*see also* ref. 8).

The breakdown of chloromequat in potato and poinsettia plants is slow. A recent publication (9) suggested that a stepwise demethylation of the quarternary ammonium group was involved. This then would imply the formation of 2-chloroethyl dimethyl amine, a monofunctional alkylating agent (10). Consequently, the latter compound could have some public health problems. After reviewing the article the group expressed its doubts whether the results obtained by Intrieri and Ryugo supported their suggestion of a possible stepwise demethylation of the quarternary ammonium group of chlormequat.

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Effect of Site of Application on Metabolism.— The problem of the rate of disappearance of chlormequat raises several general questions. For chemical identification of a terminal residue it is often necessary to increase the amount of compound applied as high as possible. For that purpose it is possible to use root application, stem injection, or uptake into an excised leaf via the cut end of the petiole. This, however, does not tell anything about the rate of disappearance under practical conditions.

To obtain quantitative information on the rate of disappearance of the parent compound and various transformation products, a method of treatment of the plant (leaf application, in the case of most herbicides and growth regulators), which parallels the actual field application as closely as possible is recommended.

Metabolism of a pesticide is dependent on the (physiological) age of plants, at least in quantitative terms. Thus the terminal residues in mature plants (for example, grain and straw) are normally quite different from those found in young, immature seedlings.

These problems are well known to laboratories which normally deal with regulatory authorities (especially in the United States), although often they are not considered or recognized in publications on pesticide metabolism.

C. Aniline Formation and Aniline-Containing Pesticides

According to Geissbühler and Voss (1) and Frear and Swanson (2), degradation of urea herbicides to their anilines does not represent a major pathway of transformation in animals and plants. Aniline derivative formation seems to be more important in soils, although a detailed balance of radioactivity derived from ring-labeled ureas is lacking. This becomes more urgently needed because aniline-containing fungicides are used for seed treatment (oxathiins).

One example is carboxin (5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide) which is rapidly oxidized. However, this compound is not hydrolyzed in the water draining from the soil to which it was applied (3). This contrasts with results obtained by Bochoffer *et al.* (4) in which soil bacteria of the species *Nocardia* (enrichment culture) were able to grow on a medium supplemented with carboxin or aniline as a sole carbon and nitrogen source.

Recently McClure (5) demonstrated that microorganisms grew and respired rapidly on nonchlorinated aniline herbicides, but ring chlorination depressed respiration and inhibited growth.

Hydrolysis of the side chain, yielding the corresponding aniline derivative, followed by metabolic degradation of the phenyl ring, could be shown in a number of cases.

A more detailed study on the fate of aniline-containing pesticides in soil and an elucidation of the pathway for the breakdown by microorganisms are needed.

Snel and Edgington (6) and Chin *et al.* (7) found complex formation between carboxin and plant lignins. It is unknown whether the whole molecule is involved in complex formation or whether aniline is first produced and then bound to polymers. According to Geissbühler (personal communication, 1973), free anilines may be bound to certain lignin-type or humic acid-type constituents, as has been demonstrated previously. However, this has not been shown to apply to phenylurea-derived anilines.

For ureas, there is definite evidence from the work of Frear and Swanson (2) and Lee and Fang (8) that monuron forms a glucoside in plants. The free aniline is not conjugated, but rather the parent compound or its demethylated and/or hydroxylated derivative. Ring hydroxylation followed by glucosidation has been reported by Lee and Fang (8). On the other hand the results show a monuron polypeptide complex formation.

Further studies on complex formation of aniline-containing pesticides may be recommended.

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IUPAC Commission on the Development, Improvement, and Standardization of Methods of Pesticide Residue Analysis

PETER SLADE,[1] *Secretary of the Commission*
ICI Plant Protection Division, Jealott's Hill Research Station, Bracknell, Berkshire, England

The eighth meeting[2] of the above Commission of the Pesticide Section of the Applied Chemistry Division of IUPAC was held at Jealott's Hill Research Station, Bracknell, in September 1974, under the Chairmanship of H. Frehse. The following summary of the proceedings is based on the minutes of the meetings and on the reports presented by members and associate members of the Commission as indicated.

The Commission considered the reports of the FAO/WHO Joint Meeting on Pesticide Residues (JMPR) held in 1973 and the meeting of the Codex Commission on Pesticide Residues (CCPR) held in 1974 insofar as they affected the Residue Analysis Commission, and arrangements were made for members to cover some of the work considered as desirable by the meetings.

Some progress had been made during the previous year in preparing the Commission's recommendations on analytical methods for publication as IUPAC Technical Reports, and work on this would continue.

1. Organochlorine Compounds

The Commission discussed recent work on the multiresidue method recommended by the Commission in 1969. Studies have included a comparison with other multiresidue methods, extraction from soil and blood, and a rapid method for milk. There has been important progress during the year toward solving the problem of the separation and estimation of polychlorinated biphenyls (PCBs). Perchlorination with antimony pentachloride gives the decachlorobiphenyl, which is separated from the organochlorine insecticides. Other studies have included work on cleanup adsorbents, electrolytic conductivity detectors, and analysis of polychlorinated dioxins. Difficulties in the use of antimony pentachloride for PCBs were discussed.

Evaluation—Kenneth E. Elgar (Shell Research Ltd., Woodstock Agricultural Research Centre, Sittingbourne, Kent, England)

Further studies to expand and strengthen the multiresidue method for organochlorine residues (1) have been made. Much of this progress was under the auspices of the AOAC, and the reports of the General Referees, J. A. Burke on chlorinated pesticides (2) and P. A. Corneliussen on multiresidue methods (3), summarized their recent work.

General

Additional general studies included that by Smart *et al.* (4) who compared the AOAC, De Faubert Maunder *et al.* (5), Wood (6), and an unpublished European Economic Community method. They concluded that there were no gross differences between results obtained with these methods, although for certain foodstuffs and in certain situations some methods appeared preferable to others. Holden (7) has reported an international cooperative study with organochlorine residues by 26 laboratories in 12 countries. The analytical method was based on extraction, cleanup, and gas-liquid chromatographic (GLC) determination, but no 2 laboratories used exactly the same procedure. Nonetheless, there was reasonable agreement in the analyses of test samples with coefficients of variation for different organochlorines ranging from 10 to 17%.

Extraction

Nash *et al.* and Woolson (8, 9) have reported 2 studies on the extraction of organochlorine compounds from soil. In the first, shake, Soxhlet, and column methods were compared, and in the second, soil premoistened with 0.2*M* ammonium chloride, and then shaken with hexane-acetone (1+1), was studied collaboratively in 7 laboratories. Recovery of 6 organochlorines from spiked samples was 91% with a coefficient of variation of 13.5%.

Cleanup

Claeys and Inman (10) have compared Florisil, alumina, and silicic acid for their ability to retain lipids and found that basic alumina retained more than Florisil, which in turn held more than silicic acid. Also, they found that deactivation and elu-

[1] Present address: Plant Protection Limited, Fernhurst, Haslemere Surrey, England GU27 3JE.
[2] For a report of the seventh meeting, *see* (1975) *JAOAC* **58**, 1244–1255.
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tion with less polar solvents gave a superior separation of organochlorines from lipids than activated adsorbents and more polar elution solvents.

Analysis and Confirmation of Residues

The multiresidue method has been extended to the analysis of whole blood (11). Pesticides are freed from the substrate with sulfuric acid. Analysis of the data from 11 collaborators revealed a large systematic error and the method will be studied further. A rapid version of the multiresidue method for milk has been studied by Gabica *et al.* (12) and was useful for screening large numbers of samples.

Dolan and Hall (13) investigated the electrolytic conductivity detector as a possible alternative to the electron capture detector. They found that after modification the detector has a usable response to subnanogram amounts of a number of organochlorine insecticides. Optimization of several factors enabled the organochlorines to be determined in the presence of PCBs with a selectivity of the order 1000 to 1.

Chemical reactions that could aid confirmation of residues continued to be studied, for instance, by Cochrane and Maybury on BHC isomers (14) and by Chau *et al.* on heptachlor (15).

Polychlorinated Biphenyls

The determination of organochlorine insecticides in the presence of PCBs has now become an established procedure. The separation of the higher chlorinated members from DDE in particular has been carried out by column chromatography on silica gel (16) and has been incorporated into the general analytical schemes (17, 18). However, separations were not easy to achieve and investigations into column chromatography (19), oxidation of DDE to dichlorobenzophenone (20), and gas chromatography-mass spectrometry (21) have been published in the last year. Another procedure that assisted the analysis of residues of PCBs was the perchlorination procedure published by Berg *et al.* (22) and further investigated by Armour (23) and Huckins *et al.* (24). The chlorination was performed with antimony pentachloride to give a single derivative from all the members of the group, the fully chlorinated decachlorobiphenyl. The procedure increased the sensitivity of the determination and the ease of identification of the group.

Polychlorinated Dibenzo-p-dioxins and Dibenzofurans

A review of the USDA studies on the sources, analysis, and environmental monitoring of these toxic materials has been published by Helling *et al.* (25). Further analytical studies with GLC with detection and identification by mass fragmentography have been reported by Buser and Bosshardt (26). The method, with a limit of determination in the μg/kg range, was used to monitor samples of 2,4,5-trichlorophenoxyacetic acid from European manufacturers.

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2. Organophosphorus Compounds

Recent advances in residue analytical methodology for organophosphorus pesticides were discussed by considering 2 papers presented to the Commission. In the first of these, H. Frehse selected 5 analytical procedures and indicated to which compounds each method was applicable. All of the compounds have been of interest to either the FAO/WHO, JMPR, or CCPR. Three main points emerging from the discussion were that multiresidue methods were not usually suitable for metabolites, so it was necessary to look separately for the parent compound and metabolites, that the method should be validated in the worker's own laboratory, and that differences in results were more likely to arise from sampling problems than from the analytical methods themselves.

In the second paper, V. Batora presented recent work reported in the COMECON countries. Thin layer chromatography (TLC) with enzymatic and/or chemical detection was much preferred for routine screening and estimation of organophosphorus residues.

Evaluation — Helmunt Frehse (Pflanzenschutz ATEA, Biologische Forschung, D-5090 Leverkusen Bayerwerk, W. Germany)

Table 1 gives a complete list of organophosphorus insecticides, acaricides, and nematocides of interest to the JMPR and/or the FAO/WHO Codex Alimentarius Commission on Pesticide Residues, up to and including the 1974 JMPR. Capital letters attached to the individual compounds are explained in the text (see *Multiresidue Methods*).

This review is confined to recent advances in residue analytical methodology concerning the compounds listed in Table 1, aimed at producing a critical selection of available methods. Publications not resembling complete methods of analysis or methods based on TLC or other techniques not in wide usage for routine analysis have been neglected. This principle was followed in order to underline the advisory character of the Commission's activity. In the future, authors of pertinent analytical procedures would be asked whether their method would meet with the validation requirements as outlined in this paper. At future meetings, the list would be expanded.

Table 1. Analysis of organophosphorus insecticides, acaricides, and nematocides (see text for identification of methods)

Compound	Method					
Azinphos ethyl				D[a]	E	
Azinphos methyl				D[a]	E	
Bromophos ethyl				D		
Bromophos methyl				D	E	
Carbophenothion	A[b]	B		D	E	
Chlorfenvinphos				D	E	
Chlorpyrifos						S
Coumaphos						S
Crufomate						S
Demeton				(D)[c]		S
Demeton methyl and oxydemeton methyl				(D)[c]		S
Diazinon	A	B	C	D[a]	E	
Dichlorvos				D		
Dimethoate				D	E	
Dioxathion				D	E	
Disulfoton				D[d]	(E)[c]	
Ethion	A	B		D[d]	E	
Fenamiphos						S
Fenchlorphos	A			D	E	
Fenitrothion				D	E	
Fensulfothion						S
Fenthion				D[d]	(E)[c]	
Formothion				D	E	
Malathion	A	B	C	D[a]	E[a]	
Methidathion						S
Mevinphos				D	E	
Monocrotophos						S
Omethoate				D		
Oxydemeton methyl	*see* demeton methyl					
Parathion	A	B	C	D[a]	E	
Parathion methyl	A	B	C	D[a]	E	
Phosalone				D		
Phosphamidon				D		
Pirimiphos methyl						S
Thiometon				D[d]		
Trichlorfon				D		
Trichloronat						S
Vamidothion						S
Additional compounds covered by some of these methods:						
Dichlofenthion				D		
Dimefox				D		
Ethoate methyl				D		
Mecarbam				D[d]		
Naled (dibrom)				D		
Phenkapton				D	E	
Phorate				D[d]		
Pyrimithate				D		
Sulfotepp				D		
Zinophos (thionazin)				D[a]	E	

[a] Oxon included.
[b] Low recoveries might be encountered.
[c] Significant oxidative metabolites *not* included.
[d] Significant oxidative metabolites included.

Multiresidue Methods

There is an increasing number of publications dealing with the simultaneous determination of several residues. A great number of these procedures have probably not yet been validated in laboratories outside the authors' premises, and so cannot be recommended for general use. Another

prerequisite for recommending a multiresidue procedure is that adequate recoveries can be demonstrated and that significant metabolites, i.e., those known to contribute to the biological efficacy of a product (e.g., sulfoxides and sulfones deriving from thioether compounds, some oxons deriving from dithio- or thiono-parent compounds) or to be of toxicological significance, are included in the analytical procedure.

The following procedures have been considered. The capital letters in Table 1 indicate the compounds to which the method was demonstrated to be applicable.

A. General method for chlorinated and phosphated pesticides, AOAC (11th Ed.) **29.001–29.027 (1).**

B. Multiple residue method for phosphated pesticides, AOAC (11th Ed.) **29.028–29.033 (1).**

C. Single sweep oscillographic polarographic confirmatory method (for **A**), AOAC (11th Ed.) **29.034–29.038 (1).**

D. A method designed for total diet studies and therefore applicable to a great variety of food commodities (2). This method was still valid in basically unaltered form. For more recent published versions of the method as applied to cereals, *see* Crisp and Tarrant (3) and the Panel Report (4). Concerning the extraction and cleanup steps, the following modifications are in use (2):

Group 1 crops (cereals): Take 10 g sample instead of 50 g, mix with 5 g anhydrous Na_2SO_4 (except for cereal grain), and macerate with 3 aliquots of methanol (50 ml each). Collect 3 fractions in centrifuge bottle and centrifuge 4 min, pour through glass wool plug, and evaporate to 2 ml, using air or micro-Snyder column. Dilute to 10 ml with acetone.

Group 2 commodities (meats and fish): Take 10 g sample instead of 50 g.

Group 3 commodities (fats and butter): Take 5 g sample instead of 50 g and extract 4 times with petroleum ether (60–80°C) (50 ml each). Collect extracts in 500 ml separatory funnel and shake with 3 aliquots of acetonitrile (50 ml each).

Groups 4, 5, and 6: Take 20 g sample instead of 50 g.

Group 7 (milk): Take 30 g milk instead of 50 ml.

E. Becker, G., Method S8 in Deutsche Forschungsgemeinschaft (Lieferung 1974) *Ruckstands Analytik von Pflanzenschutzmitteln,* No. 3, Verlag Chemie, Weinheim/Bergstr., W. Germany.

S. Special methods (*see* below).

Special Methods

The significance of special methods and their relation to multiresidue procedures have been discussed or outlined repeatedly in recent years (5–8). Multiresidue procedures are usually preferred for all practical purposes of food inspection or tolerance enforcement. For academic studies on the behavior of a given pesticide in the environment, or, in particular instances, in cases of dispute, it might be preferable to use a special method. However, these methods hardly lend themselves to generalized recommendations because they are often subject to frequent revisions or modifications to keep them adjusted to current or extended demands. It might, therefore, be advisable for institutions or interested individuals to contact laboratories known to have the best information about the latest developments in methodology (*e.g.,* the residue laboratories of a main manufacturer) rather than rely on such methods in the form in which they were published.

For these reasons, the validation aspect as expressed earlier could not always be maintained when selecting or proposing special methods. The other requirements which were outlined as governing the selection of multiresidue procedures did, of course, also apply here.

The Commission might, therefore, wish to adopt a policy of recommending or proposing special methods for general use, solely for 2 reasons: (*1*) a suitable and validated multiresidue method was not available for the compound in question; (*2*) the occurrence of significant metabolites which, for various possible reasons, were not normally included in a multiresidue method, made it necessary to use a special method for clarification.

Chlorpyrifos.—This compound was extensively evaluated by the 1972 JMPR. Numerous methods for various substrates were listed in the monograph, including a flow diagram of 3 extraction and cleanup procedures generally suitable for water, wet tissues and soil, and dry and fatty tissues, followed by GLC (alkali flame ionization detection). The JMPR concluded that these procedures "would be suitable for most regulatory purposes." Unfortunately, they were described only qualitatively in the monograph.

Recent methods not covered by the JMPR monograph were:

(a) Sweep codistillation followed by GLC with alkali flame ionization detector (9, 10). This procedure was suitable for plant material. It was essentially identical with the AOAC method (**B**, Table 1). The oxon was not detectable by this procedure, but it very rarely occurred in plants and animals.

(b) Determination of parent compound and its oxon in cattle and turkey tissues and organs by GLC with flame photometric phosphorus detector

(11, 12). A Russian GLC method (electron capture detector or alkali flame ionization detector) for determination of the parent compound, together with diazinon, in milk and organs and tissues of animals was also published (13).

(c) Determination of parent compound and its oxon in wheat plants, kernels, and straw by GLC with flame photometric phosphorus detector (14). With certain liquid phases, poor resolution of the 2 compounds (parent and oxon) or poor response of the oxon was encountered. Struble (15) studied these effects, comparing 10 polyalkyl glycol liquid phases. The most suitable material was Ucon 75-H-90,000.

Braun (16) avoided this problem with a different procedure. After partitioning, the parent compound was eluted from a silica gel column prior to eluting the oxon in the form of its hydrolysis product (3,5,6-trichloro-2-pyridinol). Both compounds were then determined by GLC, the parent with a flame photometric detector (phosphorus or sulfur mode), the pyridinol, after silylation, with an electron capture detector. The method allowed also for the determination of free pyridinol, if present, in the same manner. Good recoveries were reported for the 3 compounds from vegetables. This method was also suitable for the simultaneous determination of leptophos and its corresponding metabolites.

All of these recently published methods appeared to be recommendable.

Coumaphos.—This compound was evaluated by the JMPR in 1968. At that time, the GLC method published by Bowman *et al.* (17) appeared to be the method of choice. However, the meeting considered collaborative studies desirable "to evaluate its suitability as a referee method." Apparently this was not done (*cf.* reevaluation by the 1972 JMPR). However, another GLC method with flame photometric detection was developed for determination of coumaphos and its oxon in eggs (18). It could also be applied to animal tissues and milk (19). The liquid phases used in these 3 methods were 10% DC-200, 5% DC-200, and 5% QF-1, respectively, on Gas-Chrom Q.

Crufomate.—This compound was evaluated by the JMPR in 1968 and 1972. Reference was made to an unpublished GLC method for residues in animal tissues (20) and the following statement was made (1972): "The method has many features in common with existing multiresidue methods so it would appear reasonable that crufomate would be detected by these methods; this assumption, however, must be verified by collaborative testing."

A method for the determination of residues in milk (GLC with flame photometric detection) (21) would be suitable for regulatory use. Another GLC procedure, employing an alkali flame ionization detector for the determination of residues in bovine blood was published by Greenhalgh *et al.* (22).

Demeton and Demeton-S-methyl and their Oxidative Metabolites (Including Oxydemeton Methyl).—Methods which could be recommended for wider regulatory usage were not yet available. The referee might be able to make suitable suggestions for the 1975 meeting. For a discussion of the methods currently in use, *see* demeton-S-methyl and related compounds and disulfoton monographs of the 1973 JMPR (23).

Fenamiphos. — Thornton (24) developed a method by which the compound and its sulfoxide were oxidized to the sulfone, and the sulfone was determined by GLC with an alkali flame ionization detector. Bowman (25) published a procedure by which the 3 compounds could be determined separately in grass (GLC with flame photometric detector). For practical purposes, the determination of the combined residues as a single peak seemed preferable.

Fensulfothion.—Available GLC methods were discussed and evaluated by the 1972 JMPR, which concluded that they "are suitable for regulatory purposes as required." This statement did not refer to methods which were developed for the determination of individual metabolites. The other methods were based on the principle of oxidizing fensulfothion, its sulfone, and its oxygen analog to the oxon-sulfone, so that the combined residues were specifically measured as a single peak with an alkali flame ionization detector.

(a) Corn (kernels, cobs, greens), peanuts, soil: Katague and Olson (26), Olson (27), and Olson (28), respectively.

(b) Bovine tissues and organs: Thornton and Olson (29).

(c) Tobacco: Olson (30).

Methidathion.—The method of Eberle and Hörmann (31), according to the JMPR 1972, appeared satisfactory for regulatory purposes.

Monocrotophos.—According to the 1972 evaluation of the JMPR, the GLC method of Bowman and Beroza (32) (with flame photometric phosphorus detector) was still the method of choice for regulatory purposes. Modifications of this method for application to other crops were demonstrated in various unpublished reports of Shell Research Ltd.; similarly, Shell had available reports on the use of the alkali flame ionization detector for residue analysis in crops. Beynon *et al.* (33) published a method for determining neutral conjugates of an *N*-hydroxymethyl derivative of monocrotophos.

Pirimiphos Methyl.—A GLC method for this compound and its metabolites has been published by Bullock (34).

Trichloronat.—Because the oxon would occur, if at all, only in low concentrations, determination of the parent compound alone was sufficient for regulatory purposes. The methods developed by Möllhoff for determining residues of pesticides belonging to the parathion group, using electron capture (35) or thermionic (36) detection, are recommended.

Vamidothion.—A GLC procedure had been developed by the manufacturer's laboratories. After suitable cleanup, the parent compound and its sulfoxide were oxidized to the sulfone which was determined with an electron capture detector (37). Identity could be confirmed and the parent compound and the sulfoxide could be determined separately and semiquantitatively by TLC (38). The GLC method was considered suitable for regulatory purposes by the 1973 JMPR (23).

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Evaluation—Vojteck Batora (Vyskumny Ustav Agrochemichej Technologie, Bratislava-Prejmeste, Czeckoslavakia)

Residue Analysis of Individual Compounds

A number of papers on routine use of TLC for organophosphorus residues have been published. Methods were reported for residues of phosalone and phenthoate in apples (1, 2) and for phosalone, trichlorfon, parathion, methyl malathion, and dimethoate in soil (3–5). A TLC method for phosalone and phosmet (6), and paper chromatography combined with colorimetry for fenchlorophos were used in forensic analysis (7). Residues of parathion, malathion, fenitrothion, dichlorvos, trichlorfon, fenthion, demeton methyl, dimethoate, and their toxic metabolites were determined in fruit and vegetables with a sensitivity of 0.01–1.0 mg/kg (8). A modified NBP reagent was reported (9) for the detection of omethoate, demephion, and metabolites. TLC methods were described for residues of diazinon (10), butiphos (DEF) (11, 12), and trakephon (*O,O*-dibutyl-1-butalaminocyclohexylphosphonate) (13).

A voltammetric oscillopolarographic method with a sensitivity of 0.2 mg/kg was used for phosphorodithioate and carbamate residues in apples, following alkaline hydrolysis (14). A method of enzymatic titration was described (15) and used for the determination of butiphos and folex, respectively, in plants. The limit of detection was 2 μg for butiphos and 1 μg for folex. The sum of trichlorfon and dichlorvos residues in waste waters and natural water (16), and residues of dichlorvos in milk (17) were determined colorimetrically with sensitivities of 0.05 and 0.1 mg/L, respectively. Rapid colorimetric methods were proposed for the determination of dimethoate and phosalone in air (18).

Multiresidue Methods

A procedure for the column extraction of organophosphorus residues based on the elution of a homogeneous mixture of the blended plant material with deactivated silica was used for 7 pesticides on 15 crops (19). The recoveries were evaluated by means of GLC and a radioactive isotope technique, respectively, and ranged from 90 to 100%. Organophosphorus residues in citrus juice (20) and animal tissues (21) could also be extracted by this procedure.

In connection with the GLC determination of organophosphorus residues, the GLC apparatus for the sweep codistillation cleanup of extracts was adapted (22). The recovery was evaluated for a mixture of diazinon, malathion, fenitrothion, and trichloronat on 10 fruits and vegetables and in milk. The mean values (in %) for plants were as follows: diazinon (94.2), malathion (87.9), fenitrothion (84.1), and trichloronat (86.4); for milk the recovery was within the range 96–107%.

A GLC (thermionic ionization detection) analysis following acylation for phosphonic acid esters was reported (23). An alkylation procedure at the microgram level was developed as a chemical confirmatory test for phosphate and carbamate insecticides, and *s*-triazine and urea herbicides with reactive NH moieties (24). Thermal ionization, electron capture, and flame ionization detectors were evaluated for phosphated and chlorinated pesticides (25).

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3. Carbamate Compounds

A. Insecticidal Carbamates

The Commission discussed recently published work. The formation of electron-capturing derivatives for GLC continued to be the favored technique for both single and multiple carbamate residue determinations. For multiresidues the successful application of fluorogenic labeling combined with liquid chromatography has been reported. Pirimicarb and 2 of its metabolites could be analyzed by GLC, using a nitrogen detector (*Analytical Methods for Pesticides and Plant Growth Regulators* (1973) G. Zweig (Ed.), Academic Press, Inc., New York, NY, pp. 7, 399).

There is a need for a reliable analytical method for carbaryl (the 1973 FAO/WHO JMPR had requested a method for carbaryl in milk, but the problem was a general one), and also a need to determine which residues should be measured. In addition, requests are being made for analysis of hydroxyl-containing metabolites of carbamates.

Evaluation—Derek C. Abbott (Laboratory of the Government Chemist, Stanford St, London S.E.1, England)

The determination of residues of carbofuran and its metabolites has received attention from various workers. Williams and Brown (1) published a GLC method for residues on soft fruits, which uses a modified form of the extraction method of Cook *et al.* (2). Following methylene dichloride extraction and silica gel and alumina column chromatography, samples were analyzed by GLC with a Coulson conductivity detector in the nitrogen mode. Recoveries of both carbofuran and 3-hydroxycarbofuran from fortified samples were greater than 86% over the 0.2–5.0 mg/kg range. For carbofuran residues in soil, Caro *et al.* (3) used acid ammonium acetate extraction and GLC with an electron capture detector. Determination at the 0.1 mg/kg level without cleanup was possible by formation of the dinitrophenyl-ether derivative. Recoveries from various soils were in the 61–92% range. This method might prove useful because Landrin, propoxur, and Bux (bufencarb) insecticides might also be determined. Moye (4) studied the reaction between the phenolic portions of hydrolyzed carbamate pesticides and various aromatic sulfonyl chlorides. The resulting esters were highly sensitive to the electron capture detector. A method for carbofuran in lettuce at the 0.01 mg/kg level resulted from this work. TLC and autoradiography techniques (5, 6) have been used to detect the breakdown products of carbofuran, such as carbofuranphenol, 3-ketocarbofuran, and 3-hydroxycarbofuran.

The direct GLC of insecticidal carbamates is normally difficult because of thermal decomposition. A direct GLC method, without cleanup, for carbaryl and its hydrolysis product 1-naphthol, has been published (7). This method is useful for quantities in the 0.2–15 ng range. A column (0.3 m × 4 mm) of 3% SE-30 on 80–100 mesh Gas-Chrom Q was employed and no decomposition below 160°C was detected. The short column with a low loading was used to avoid errors resulting from decomposition. When examining water extracts for residues, no cleanup was necessary.

Carbaryl was generally determined by decomposing to 1-naphthol, forming a suitable derivative, and estimating by gas chromatography with an electron capture detector. The pentafluorobenzyl esters and ethers have been used for the analysis of trace organic acids and phenols in water supplies, but the need to remove excess pentafluorobenzyl bromide reagent is a drawback. Johnson (8) used the same derivatives, but removed excess reagents with a silica gel column. He determined 1-naphthol and *sec*-amyl phenols, which were hydrolysis products of the insecticides carbaryl and Bux, respectively. The colorimetric method of the AOAC (9), depending on the formation of the *p*-nitrobenzenediazonium fluoroborate derivative, was used by Kurtz and Studholme (10) to detect residues of carbaryl in birds; the minimum amount that could be detected was 0.1 mg/kg.

The results of a useful study on the stability of carbaryl in aqueous media were reported by

Wauchope and Haque (11). Carbaryl was stable in weakly acidic solutions, but hydrolyzed to 1-naphthol under basic conditions. A similar study was conducted by MacNeil *et al.* (12) for the compounds carbaryl and Mobam. The analyses for the decomposition products were carried out *in situ* on silica gel thin layers by reflectance spectroscopy after the developed chromatograms had been sprayed with CNTNF (9-dicyanomethylene 2,4,7-trinitrofluorene) to form visible pi complexes. Clegg (13) reported a GLC method for residues of promacyl in the tissues of cattle. The phenol produced by hydrolysis of the carbamate was coupled with fluoro-2,4-dinitrobenzene and determined by electron capture GLC; the procedure was similar to that of Cohen *et al.* (14). However, important differences in the cleanup and reaction conditions during derivatization were developed in order to separate promacyl from its metabolites promecarb and isothymol. Recoveries for spiked samples were in the 80–90% range with a detection limit of 0.1 mg/kg. This method should be applicable to other aryl esters of carbamic acid.

A specific GLC method for determining residues of mesurol (mercaptodimethur) and its sulfoxide and sulfone metabolites in plant and animal tissues was reported by Thornton and Draeger (15). After initial extraction and precipitation, the extract was oxidized with potassium permanganate to convert mesurol and its sulfoxide to the sulfone. The sulfone was detected by a sulfur-specific flame photometric detector, thereby avoiding interference from tissue coextractives. The detection limit was 0.03 mg/kg.

To aid the analysis of pesticidal carbamates, an understanding of the metabolism and degradation is both desirable and useful. Meikle (16) reviewed the metabolism of mexacarbate (Zectran) in biological systems and Benezet and Matsumura (17) determined the major metabolites of this compound, using ring-labeled mexacarbate and silica gel TLC. Unger and coworkers (18–20) studied the photochemical breakdown of mexacarbate, metacrate, aminocarb, and Landrin. A detailed analysis of the metabolites and residues resulting from the soil application of aldicarb under field conditions has been reported by Andrawes *et al.* (21). The procedure was based on determination of the aldicarb sulfoxide and sulfone metabolites as the sulfones after oxidation by perchloric acid. Two-dimensional TLC was used to resolve the various metabolic components present in organic extracts of plant samples. The methods described were useful for characterizing aldicarb residues in cotton foliage and for determining the total toxic residue at 0.01 mg/kg in cottonseed. The flame photometric detector was also used by Woodham *et al.* (22) for examining the effect of aldicarb sprays on grasses and wildlife. Whitten and Bull (23) investigated the fate of a recently introduced carbamate insecticide, Diamond Shamrock DS 15647 (3,3-dimethyl-1-(methylthio)-2-butanone *O*-(methylcarbamoyl) oxime), in cotton plants and soil, using the labeled insecticide. The breakdown metabolites were separated and identified by TLC and radiography.

Reeves and Woodham (24) noted a detection limit of 0.05 mg/kg for analysis of residues of methomyl in soil, sediment, and tobacco by GLC with a flame photometric detector after Florisil cleanup. Mendoza and Shields (25) reported a method for methomyl in rapeseed oils by GLC with electron capture detection of the 1-fluoro-2,4-dinitrobenzene-methylamine reaction product after cleanup on a silica gel minicolumn. To ensure complete hydrolysis of the carbamate, sodium hydroxide was added immediately before reaction with DNFB. Recoveries of 0.01–1 mg/kg were 80–105% and no GLC interference was obtained from the oil. The optimum conditions affecting the DNFB and methylamine reaction were also studied. Details of the minicolumn cleanup technique for the rapid cleanup of the reaction product extracts were subsequently published (26). Mendoza *et al.* (27) developed a TLC enzyme inhibition technique for the semiquantitative detection of methomyl residues in rapeseeds, oils, and meals. The method was suitable for the detection of 20 ng methomyl, but not the oxime, in the presence of extractives. TLC with iodo- or chloroplatinate reagents was also used for confirmation; although 50 times less sensitive than the enzyme reaction for methomyl, these reagents detected the oxime.

Data on the metabolism and residues of methomyl in the rat, tobacco, corn, cabbage, and soil provided at the time of registration of this compound in the U.S., have been published in 3 papers by Harvey *et al.* (28–30). The authors used temperature-programmed GLC, with TLC with mass spectrometry-infrared spectroscopy for identification.

Cheng and Casida (31) used TLC with a variety of solvent systems and visualizing agents to identify the metabolites and photoproducts of radio-labeled BPBSMC (3-(2-butyl)-phenyl *N*-benzenesulfenyl-*N*-methyl carbamate) and BPMC (3-(2-butyl)-phenyl *N*-methyl carbamate). Product confirmation involved a variety of procedures including infrared, mass, and nuclear magnetic resonance spectrometry. Radioactivity was also measured by liquid scintillation spectrometry. The value of this paper was enhanced by the inclusion

of procedures for the radiosynthesis of the parent compounds together with their degradation products.

Multiresidue Methods

Since the 1973 report there has been considerable activity in the development and application of analytical techniques and systems for the multiresidue determination of carbamates.

Frei *et al.* (32) developed a scheme for carbamate insecticides by combining fluorogenic labeling and high-performance liquid chromatography. The formation of the dansyl chloride derivatives allowed a wide range of *N*-methyl carbamate insecticides to be separated at detection limits between 1 and 10 ng per 4-μl injection (fluorescence detector); soil and water samples containing mesurol and carbofuran could be analyzed without preliminary cleanup. Development of a multiresidue method based on the technique of Frei *et al.* would be desirable because this would be useful for total diet samples. A procedure for pesticide residues including phenyl and methyl carbamates in plant material has been published by Thier (33). Following separation from other pesticides by Florisil column chromatography, the carbamates were determined by GLC with electron capture detection after bromination. Suzuki *et al.* (34) developed a systematic procedure for the separation and identification of a mixture of 13 carbamate pesticides. After separation on silica gel TLC plates, trifluoroacetates were prepared and identified by GLC. Ishikawa *et al.* (35) established an analytical method for the separation and determination of insecticidal carbamate residues in rice grain. Following hexane-acetonitrile partitioning and alumina column cleanup, the carbamates were hydrolyzed to their corresponding phenols and coupled with a 4-nitrobenzenediazonium ion to produce colored compounds which could be separated and identified by TLC. Recoveries of 60–80% were obtained for 0.1 mg *N*-methyl carbamates/ml except 1-naphthyl *N*-methyl-carbamate. Aromatic sulfonyl chloride esters (4) and fluoro-2,4-dinitrobenzene derivatives (2) previously mentioned were also suitable derivatives for multiresidue separation and identification of certain insecticidal carbamates by GLC.

Recommendations

The major problem with carbamate determination has been the thermal instability of the compounds, which makes direct GLC difficult. The formation of halogenated derivatives overcomes this problem somewhat, but derivatization is often time consuming and involved. The method of Holden (36), as mentioned in the previous report, involving the GLC of the 1-fluoro-2,4-dinitrobenzene derivatives of methyl carbamates, appears promising and should be tested collaboratively. There is a great need for a method for multiple residues which is both rapid and specific. Finally, whenever chlorinated hydrocarbons are used in the extraction, any water-soluble and conjugated metabolites which might be more potent cholinesterase inhibitors should also be extracted.

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B. Herbicidal Carbamates

The Commission discussed the fact that direct GLC of these compounds, using a Coulson conductivity detector, had been developed for residues of these compounds; this was an important advance. Other analytical work was also discussed.

Evaluation—Chiam Resnick (Plant Protection Department, Ministry of Agriculture, POB 15030, Jaffa, Israel)

Development of satisfactory multiresidue methods for the carbamate herbicides by direct chromatography is difficult (as it is for other nitrogen-containing pesticides) mainly because of the tendency of these compounds to degrade on GLC columns (1–3). Derivatization of either the carbamate compounds or their hydrolysis products, followed by GLC determinations, has been suggested in the past and used successfully (4–9).

Recently, direct chromatography of a number of the carbamate herbicides was reported by Laski and Watts (10). By employing carefully controlled chromatographic conditions, the Coulson electrolytic conductivity detector, and 5 and 10% DC-200 columns, these authors successfully minimized the degradation and adsorption of carbamate herbicides and other organonitrogen pesticides on the GLC columns. Table 2 summarizes the GLC data for the standard compounds chromatographed in these studies.

Procedures for cleanup of carbamate herbicide residues in crops for measurements by the Coulson detector are currently used by the U.S. Food and Drug Administration. For residues of carbamate herbicides that were water-insoluble and acetonitrile-extractable, it seems possible that the FDA procedures (11) for multiresidues of chlorinated pesticides might usefully be employed. The development of a highly deactivated column packing material—an acid-washed Chromosorb W support surface-modified with Carbowax 20M (12)—has enabled the direct GLC analysis of some *N*-methyl carbamate pesticides, using an alkali flame detector (13). In principle, this column might also prove to be useful for the direct GLC analysis of *N*-methyl carbamate herbicides (e.g., Sirmate (dichlormate) terbutol, metham). A rapid screening method for chlorpropham (CIPC, 3-chlorophenyl-isopropyl carbamate) at low residue levels in plant materials was described by Friestad

Table 2. GLC data for herbicidal carbamates chromatographed on 5 and 10% DC-200 columns, with Coulson electrolytic conductivity detector

Compound	Chemical name	Rel. retention time[a]	50% Full-scale defl., ng
EPTC	*S*-ethyl dipropylthiocarbamate	0.13	10
Propham	isopropyl *N*-phenylcarbamate	0.16	10
Butylate	*S*-ethyl diisobutylthiocarbamate	0.17	10
Vernolate	*S*-propyl dipropylthiocarbamate	0.17	10
Pebulate	*S*-propyl butylethylthiocarbamate	0.18	20
Cycloate	*S*-ethyl cyclohexylethylthiocarbamate	0.31	20
Chlorpropham	isopropyl *N*-(3-chlorophenyl) carbamate	0.31	25
Diallate	*S*-2,3-dichloroallyl diisopropylthiocarbamate	0.37; 0.39	50
Tandex	*m*-(3,3-dimethylureido)phenyl-*tert*-butylcarbamate	0.12; 0.42	75[b]
Sirmate (2,3-isomer)	2,3-dichlorobenzyl *N*-methyl carbamate	0.54	40
Sirmate (3,4-isomer)	3,4-dichlorobenzyl *N*-methyl carbamate	0.55	40
Triallate	*S*-2,3,3-trichloroallyl diisopropylthiocarbamate	0.62	50

[a] Parathion = 1.00.
[b] Peak tailing occurs.

(14). This method utilized a preliminary cleanup with a slightly modified Mills-Onley-Gaither procedure (15), hydrolysis of CIPC to the aromatic amine, automatic diazotization and coupling, and colorimetry. Long and Thompson (16), in a study of some herbicide residues in tobacco, could not detect any residues of the carbamate herbicide Pebulate (*S*-propylbutylethylthiocarbamate), which was applied at planting and was persistent in the soil. The limit of detection was 0.02 mg/g dry weight, with a 60% recovery. Complete metabolism by the tobacco plant of the thiocarbamate herbicide was suggested as an explanation for the absence of residues.

Chang *et al.* (17) reported a radioassay technique to study the uptake and metabolism by corn seedlings of the carbamate herbicide EPTC (ethyl-*N*,*N*-di-*n*-propylthiocarbamate) to elucidate the mechanism of protection to corn from EPTC injury by the presence of *N*,*N*-diallyl-2,2-dichloroacetamide. Plants treated with ^{14}C-EPTC were extracted, extracts were centrifuged, and aliquots of the supernates were used directly for radioassay. Radioactivity in the pelleted residues was estimated by combustion and subsequent counting of the liberated ^{14}C-CO_2. Radiolabeled metabolites in the aqueous phase were analyzed by TLC on silica gel. Radioassay techniques were also used in a study on the isolation and identification of propham (isopropyl *N*-phenyl carbamate) metabolites from animal tissues and milk (18).

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4. Fumigants

The Commission discussed developments in methods of analysis of residues from phosphine, methyl bromide, ethylene oxide, and other fumigants. Collaborative testing of a multiresidue method for chlorinated hydrocarbon and other fumigant residues among 8 English and Dutch laboratories was successfully completed (Report by the Panel on Fumigant Residues in Grain (1974) *Analyst* **99**, 570–576). The group will conduct collaborative tests on a GLC method for bromide ion in cereals.

Evaluation—Stanley G. Heuser (Pest Infestation Control Laboratory, Ministry of Agriculture, Fisheries and Food, London Rd, Slough, Bucks, England)

Residues from Use of Phosphine

The aeration method of Bruce *et al.* (1) has been used to determine free phosphine in commodities by other workers (2) and appeared to be the method of choice. Dumas and Bond (3) have shown, however, that when aluminum phosphide-generated phosphine was passed through a chromatographic column consisting of Apiezon L on firebrick, odorless phosphine was eluted, suggesting that there was also present an unidentified foul-odor volatile compound capable of producing a residue for which no analytical method yet existed.

Residues from Use of Methyl Bromide

(a) *Bromide Ion.*—Brown *et al.* (4) determined bromide in wheat, potato tubers, and haulm, using x-ray fluorescence spectrometry of pressed powdered samples, with a claimed sensitivity of 1 mg/kg (10 mg/kg haulm). The GLC method of Heuser and Scudamore (5) for specific determination of bromide ion in cereals was being tested collaboratively by 8 English and Dutch official laboratories. This method has also been

adapted by W. B. F. Grevenstuk (personal communication, 1974) for the determination of bromide ion in milk.

(b) *Alkylation Reaction Products.*—Asante-Poku *et al.* (6) have determined methylation sites in free amino acids and those from protein hydrolysates of cocoa bean shell and nib after fumigation with ^{14}C-labeled methyl bromide, using a Beckman amino acid analyzer for separation and liquid scintillation counting of the separated fractions. Methylated basic amino acids were separated and provisionally identified by TLC.

Ethylene Oxide Residues

Two methods for determining residual ethylene oxide in sterilized pharmaceutical materials, such as polymers and cotton, were compared by Romano *et al.* (7). Results obtained by extraction or solution with dimethyl formamide, with analysis of aliquots by GLC with flame ionization detection, were similar to those obtained by subjecting the sample to heating at 100°C in a sealed vial, followed by GLC analysis of the headspace gas on Porapak R stationary phase. The latter method was sensitive to 0.1 mg ethylene oxide/kg. White and Bradley (8) determined residual ethylene oxide in similar materials by high-vacuum distillation followed by gas chromatography with flame ionization detection.

Residues of Halogenated Hydrocarbon Fumigants

Eight laboratories collaboratively studied the multiresidue detection method of Heuser and Scudamore (9), modified by use of a gas chromatographic column of 15% (w/w) polypropylene glycol on Chromosorb W. Two series of tests, the first using wheat and corn fumigated with carbon tetrachloride and the second in which a mixture of chloroform, trichloroethylene, and ethylene dibromide residues was similarly recovered, have been described for publication in *The Analyst* in 1974.

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5. Systemic Fungicides

There was discussion on the recent work in this field although only a few new methods have been published. The residue analytical method for ethirimol, using GLC with a nitrogen detector following derivative formation, was to be published shortly (*Analytical Methods for Pesticides and Plant Growth Regulators*, G. Zweig (Ed.), Academic Press, Inc., New York, NY, in press). There has been a recent review of analytical methods for systemic fungicides (P. B. Baker and R. A. Hoodless (1974) *Pestic. Sci.* **5**, 465). It was suggested that there might be an increasing need for analytical methods for metabolites of systemic fungicides.

Evaluation — Siegbert Gorbach (Farbwerke Hoechst AG, Postfach 800320, D-6230 Frankfurt (Main 80), W. Germany)

The first GLC method for the determination of benomyl has been published by Rouchaud and Decallonne (1). Residual benomyl and 2-benzimidazole carbamic acid methyl ester (MBC) were extracted with benzene and partitioned into 0.1*N* HCl. The acidic layer was washed several times with chloroform, and then neutralized. All benomyl was converted to MBC by the extraction and cleanup and partitioned into ethyl acetate. MBC was then trifluoracetylated and measured by GLC with an electron capture detector. The lower limit of detection was reported to be 0.02 mg/kg, with 80–100% recovery.

Ahronson and Ben-Aziz (2) analyzed benomyl in the presence of thiabendazole by using a spectrofluorometric method after column chromatographic cleanup with a magnesium oxide-Celite-alumina column. Detection limits of 0.1 mg benomyl and 0.02 mg thiabendazole/kg were reported. Tjan and Burgers (3) reported a TLC determination after cleanup by partitioning with ethyl acetate. Thiabendazole could be determined along with benomyl. For the simultaneous detection of benzimidazole fungicides in the presence of carboxin, oxycarboxin, tridemorph, dimethirimol, and ethirimol, Baker *et al.* (4, 5) also recommended TLC on silica or alumina plates. Thiabendazole could be determined by the UV spectrophoto-

metric procedure described for benomyl and MBC (6).

Ethyl acetate is the best extractant for benomyl and MBC. The residue is reextracted with hydrochloric acid (pH 4.5) and partitioned into ethyl acetate, reextracted with hydrochloric acid, and determined by UV spectroscopy. This procedure leads to satisfactory results for most crops. TLC assays give necessary confirmation and indicate the presence of interfering substances. The new GLC determination is a promising alternative with a more specific determination.

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6. Dithiocarbamate Fungicides

The Commission discussed recent work in the analysis of dithiocarbamates, including a microbiological method. Such methods require a cleanup procedure, and the difficulty of making them both specific and quantitative was pointed out. The importance of ethylenethiourea (ETU) as a terminal residue was discussed. ETU is in fact present in technical dithiocarbamates. It was recommended that the FAO/WHO JMPR should consider the possibility of assigning a specific residue limit to ETU, in view of its constant occurrence following crop treatments with the dithiocarbamate fungicides and its toxicological significance.

Evaluation—Chiam Resnick (Plant Protection Department, Ministry of Agriculture, POB 15030, Jaffa, Israel) and Rudolf Engst (Zentralinstitut für Ernährung der Akademic der Wissenschaften der DDR, Arthur-Scheunert Allee, 1501 Bergholz-Rehbrücke, E. Germany)

Knapek and Chmiel (1) have modified the colorimetric step of the CS_2-evolution method for dithiocarbamates. CS_2 is absorbed in potassium hydroxide to form xanthate. When this is heated, H_2S is evolved and reacts with *p*-aminodimethylaniline to form methylene blue, which is measured spectrophotometrically. The sensitivity of the method is 0.2–1.0 mg/kg for 10 g samples.

The specific analysis of thiram (tetramethylthiuram disulfide) has been reported in a few studies. Kettmann and his coworkers (2) applied infrared multiple attenuated total reflectance spectrometry to the identification and measurement of thiram residues on lettuce. By comparing spectra of extracts obtained from lettuce leaves free from thiram or enriched with thiram (80 μg/100 g leaves), and the spectrum of 20 μg thiram deposited directly on a KRS-5 crystal, the fungicide could be identified satisfactorily at a concentration of 1 mg/kg in the sample.

Rappe *et al.* (3) described a microbiological method for the determination of thiram residues. Using *Saccharomyces carlsbergensis* ATCC 9080 as the test organism, as little as 25 ng thiram/well or 200 ng/disk could be detected. This method was highly sensitive and could be applied when the nature of the fungicide being investigated was known. Other dithiocarbamate fungicides with antibacterial properties, such as nabam and ziram, could also be determined by this method but at higher concentrations than with thiram, and thus would not interfere with the thiram assay. In the chemical methods for the determination of thiram, such as the colorimetric method reported by Rangaswamy *et al.* (4), ziram and ferbam in the sample analyzed would interfere.

Laski and Watts (5), in their investigation on the determination of 95 organonitrogen pesticides in a GLC system with the Coulson electrolytic conductivity detector, could not obtain a response to 200 ng ziram, used as a model in this study for the behavior of other metallic dithiocarbamate fungicides.

Engst and Schnaak (6) developed a polarographic technique for measuring ETU (a decomposition product common to several major dithiocarbamate fungicides) residues in crops and animal substrates with a reported sensitivity of about 0.05 mg/kg. ETU is cleaned up by column and paper chromatography, nitrosated, and measured by a cathode ray polarograph. A TLC detection technique for ETU was described whereby ETU, after nitrosation, was reacted with the Bratton-Marshall reagent (*N*-(1-naphthyl)-ethylenediamine dihydrochloride) for visible TLC spotting (7).

Newsome (8) has developed a method for determining ETU in apples. Reaction with benzyl chloride gives 2-benzylmercapto-imidazoline, which is subsequently treated with trifluoroacetic anhydride to yield 2-benzylmercapto-*N*-trifluoroacetylimidazoline which is measured by GLC with an electron capture detector. The procedure of Haines and Adler (9) for measuring ETU residues in various crops involves purifying a methanol extract on an alumina column and derivatizing

the ETU with 1-bromobutane in the presence of dimethylformamide and sodium borohydride. The resulting derivative, 2-*n*-butylmercapto-imidazoline, is measured by GLC with a flame photometric detector; the method is sensitive to 0.01 mg/kg.

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7. Rethrins and Synergists

Although it has been stated that the requirements of the FAO/WHO JMPR for methods of residue analysis of rethrins and synergists have been met (D. C. Abbott (1970) *JAOAC* **53**, 1004–1009), the renewed interest in pyrethroids as environmentally acceptable replacements for more persistent and more toxic pesticides might result in the need for new methods of analysis. The Commission discussed recent advances in this field.

Evaluation—Kenneth R. Hill (Analytical Chemistry Laboratory, Agricultural Environmental Quality Institute, U.S. Department of Agriculture, Beltsville, MD)

The photodecomposition of resmethrin and related pyrethroids has been studied by Ueda *et al.* (1), who found that the mammalian toxicity of photodecomposed resmethrin depended on the route of administration. The toxicity to mice of mixed photoproducts via intraperitoneal administration was greater than resmethrin, but less when administered orally. From these results it was concluded that residue analysis for the parent compound only continued to be valid. Olive (2) developed a TLC procedure for the identification and quantitation of pyrethrins and piperonyl butoxide. The TLC plate was treated with a mixture of phosphoric, tannic, and acetic acids in acetone, resulting in pink spots for pyrethrins and a blue spot for piperonyl butoxide. Although the method was developed primarily for formulation analysis, 1 μg of either compound could be detected; therefore, it should be suitable for residues with adequate cleanup.

Bhavnagary and Ahmed (3) developed a spectrophotometric method for the microdetermination of piperonyl butoxide in the presence of pyrethrins. Treatment of the mixed residue extract with 18% nitric acid converted the piperonyl butoxide to a soluble yellow compound with an absorption maximum at 370 nm. The method was sensitive to 4 μg piperonyl butoxide/ml measurement solution. A novel procedure for determining resmethrin in peanuts was given in an application note for plasma chromatography (4). A cleaned up extract of peanuts containing 0.1 mg resmethrin/kg was analyzed by gas chromatography interfaced with a plasma chromatography (PC) as a selective detector. When 5 μl of extract containing 1.25×10^{-10} g resmethrin was injected with the plasma chromatograph tuned to the characteristic drift time of resmethrin, a peak was observed at a gas chromatographic retention time of 6 min. A signal-to-noise ratio of 25:1 was obtained, whereas the flame ionization detector for the gas chromatograph gave no response at the low levels. No information was given as to whether the response (area) was proportional to concentration.

George and McDonough (5) developed a microanalytical procedure for determining residues of Bioethanomethrin ((5-benzyl-3-furyl)methyl *trans*-(+)-3-(cyclopentylidenemethyl)-2,2-dimethyl-cyclopropanecarboxylate) a new synthetic analog of pyrethrin. The molecule was saponified in alcoholic sodium hydroxide and a trichloroacetyl derivative was made from both the resultant acid and alcohol. The carboxyl group of the acid was protected during esterification with trichloroethanol by pretreatment with dicyclohexylcarbodiimide in pyridine. The derivatized esters were then determined by GLC on an SE-30 column with an electron capture detector. As little as 0.6 ng of either ester could be detected. Analysis of crop extracts is being investigated.

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8. Selective Gas Chromatographic Detectors

Review—Walter A. Aue (Dalhousie University, Halifax, Nova Scotia, Canada)

Sensitivity, selectivity, linearity, speed, and ruggedness are the criteria generally used to judge detector performance. These categories have been significantly improved for a variety of detectors in recent years. Yet, these did not always filter through from the instrument manufacturers to the users as fast as would be desirable. It is not uncommon to find some detectors, notably electron capture and alkali flame ionization, operating at less than optimum conditions in analytical laboratories.

Electron Capture Detector

This detector could be operated in dc, pulsed, or a new mode called linearized, frequency-modulated, or constant current electron capture (1). Calibration curves for the latter are linear over 4 decades with argon-methane as a carrier gas. In order to use nitrogen, or to work at the highest possible sensitivity, some of this linearity has to be sacrificed. Several firms now sell the electronics necessary for this new mode, and the detector is used increasingly in residue laboratories. Although it is too early yet for a final judgment on its performance in routine analysis, prospects look good. The detrimental effect of oxygen—contained in the carrier gas or diffusing in from the atmosphere—should again be stressed (2). A suitable oxygen scavenger and a clean chromatographic system are most important for good performance. The condition of any electron capture detector can easily be judged by I/V plots in the dc mode or I/frequency plots in the pulsed mode. It is deplorable in this context that some detectors on the market do not provide for easy variation and readout of the potential used in dc mode. This subject has been reviewed recently by Aue and Kapila (3).

Alkali Flame Ionization Detector

This is most useful for phosphorus- and nitrogen-containing compounds, in competition with the flame photometric and the electrolytic conductivity detectors, respectively. The alkali flame ionization detector is generally the cheapest, most sensitive, least selective, and least rugged of the 3. Thus, further industrial development centers on the latter 2 parameters, and includes the long known use of a mobile collector electrode (4, 5), the noise-reducing function of an auxiliary polarizing electrode (4), and the development of more stable alkali sources, for instance, a rubidium glass bead (6). This is the one detector most in need of frequent optimization and constant surveillance. Two older reviews of its constructions and functions can be found in refs. 7 and 8.

Flame Photometric Detector

Since the pioneering work of Brody and Chaney and of Bowman and Beroza, very little detector development *per se* has taken place. Noteworthy, however, is the recently described approach to switch (and carefully adjust) the gas flows so that hydrogen is mixed with the carrier gas before combustion. The signal-to-noise ratios are not significantly changed, but the bothersome extinguishing of the flame—which calls for bypassing the solvent peak or relighting the flame—is now eliminated (9). Although used with *liquid* effluents, the successful wedding of the flame photometric detector to a wire transport system for determining phosphorus pesticides should be mentioned (10).

Electrolytic Conductivity Detector

In this area improvements are the most pronounced. Peak broadening has been curtailed and sensitivity has been markedly improved (11, 12). The company which marketed the Coulson detector is now promoting the Hall detector. It is apparently quite convenient to use and at least 10 times more sensitive than the older models (R. Greenhalgh, personal communication). From what is known now, the improved conductivity detector might well find increased use in the analysis of nitrogen- and sulfur-containing compounds at the expense of alkali flame ionization and flame photometric detectors, respectively. Its sensitivity is similar to that of the other detectors, and selectivity against carbon is much higher. For polychlorinated hydrocarbons, the Hall detector competes well with the electron capture detector in cases where the superior sensitivity of the latter is not required, but where polar coextractants call for improved selectivity.

Other Detectors

A wide variety of selective gas chromatographic detection devices applicable to residue analysis have been described, but these are rarely found in typical pesticide laboratories. This might be due to the cost, complexity, or novelty of equipment or approach. A current listing might include the variety of setups utilizing emission from

heteroelements in discharges or flames (Hg, B, Sn, Pb, N, P, S, Cl, Br, I, and others), single ion monitoring by mass spectrometry, plasma chromatography, fluorescence measurements, and several more.

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METALS AND OTHER ELEMENTS

Selenium in Foods: Evaluation of Atomic Absorption Spectrometric Techniques Involving Hydrogen Selenide Generation and Carbon Furnace Atomization

MILAN IHNAT
Chemistry and Biology Research Institute, Agriculture Canada, Ottawa, Ontario, Canada K1A 0C6

The performance of hydrogen selenide generation- and carbon furnace atomization-atomic absorption spectrometry, with a semiautomated trace metal accessory and a carbon rod atomizer, respectively, was evaluated for the determination of selenium in foods. Samples were digested with nitric, perchloric, and sulfuric acids for both procedures. Hydrogen selenide was generated with stannous chloride/potassium iodide/zinc from 20 ml aliquots in a hydrochloric-sulfuric acid medium and directed into an argon-hydrogen-entrained air flame. Sample matrix interferences in the carbon furnace technique were minimized by isolating selenium by precipitation with ascorbic acid and redissolution in nitric-perchloric acid prior to taking 5 μl aliquots for estimation; precision was significantly improved by incorporating 5000 μg nickel/ml into analytical solutions. For the hydrogen selenide and carbon furnace techniques, respectively, the following data were obtained for untreated standard solutions: sensitivity, 0.13 and 10.0 ng/ml; absolute sensitivity, 2.6 and 0.05 ng; detection limit, 0.4 and 90 ng/ml; absolute detection limit, 7 and 0.45 ng. The detection limit and absolute detection limit for samples were 2.5 ng/ml and 50 ng, respectively, for the hydrogen selenide method, and 25 ng/ml and 0.13 ng, respectively, for the carbon furnace method. Taking into consideration these figures and the practical aspects of both methods, the overall performance of the hydrogen selenide method was superior.

Fluorometry has gained wide acceptance as a sensitive technique for determining selenium at the microgram and submicrogram/gram levels in a variety of materials; fluorometry forms the basis for 2 current official AOAC methods for selenium in plants and foods (1). These methods, although applicable to large classes of samples, are still limited: Samples containing nanogram quantities of the element cannot be conveniently analyzed, and the techniques are fairly tedious.

The advent of atomic absorption spectrometry (AAS) 2 decades ago brought into the analytical laboratory a powerful and relatively simple technique which has become firmly established as the mainstay of many laboratories. A number of publications (2–26) dealing with or mentioning the estimation of selenium by solution aspiration into various flames have appeared, and detection limits ranging from 0.082 to 42 μg/ml for aqueous solutions have been reported. This technique clearly lacks the sensitivity required for most biological applications where nanogram or microgram levels of selenium are encountered. Sensitivity can be somewhat improved by using a sampling boat or cup for introducing the solution into the flame (10, 16). However, the hydrogen selenide technique (27–44), whereby a gaseous form of the element is swept into the flame, substantially improves detection limits to the range 0.1–1.8 ng/ml. Flameless modes of atomization, with electrically heated carbon furnaces, and their application to selenium estimation (45–58) have similarly improved detection limits to the range 0.25–400 ng/ml. (The term carbon furnace is used as a generic term for all such atomization devices using carbon/graphite.) Table 1 presents a comparison of sensitivities and detection limits obtained by several different atomic absorption spectrometric techniques.

It appeared that both the hydrogen selenide generation and carbon furnace atomization techniques might be suitable determinative steps for the estimation of nanogram quantities of selenium in food samples. Consequently, the performance of both techniques was investigated, and this report presents the results obtained with 2 commercially available instruments.

Contribution No. 883 from the Chemistry and Biology Research Institute.

Table 1. Sensitivities and detection limits for selenium determination by atomic absorption spectrometry[a]

Technique	Soln vol. required	Sensitivity,[b] ng/ml	Absolute sensitivity,[b] ng	Detection limit,[c] ng/ml	Absolute detection limit,[c] ng	Refs
Solution Aspiration into Flame						
Air-acetylene	—[d]	500–3000 (1000) $n = 19$[e,f]	—	82–3200 (360) $n = 18$	—	3–13, 16, 17, 20, 21, 23–25
Nitrogen or Ar-H_2-entrained air	—	150–1150 (500) $n = 7$	—	50–1150 (100) $n = 8$		6–8, 13, 18, 19, 22, 25
Air-H_2	—	400–1000 (500) $n = 4$	—	1000, $n = 1$	—	6, 9, 14
Air-propane	—	500, 1000 $n = 2$	—	—	—	5, 6
N_2O-acetylene	—	1500–14,000 (5800) $n = 12$	—	260–42,000 (5000) $n = 21$	—	17, 20, 21
Discrete Sampling						
Tantalum sampling boat and flame[g]	1 ml	—	—	10, $n = 1$	10, $n = 1$	10
Delves sampling cup and air-acetylene flame	100 μl	—	—	1000, $n = 1$	100, $n = 1$	16
H_2Se generation	1–50 ml	0.07–20 (0.8) $n = 11$	0.7–500 (9) $n = 11$	0.1–1.8 (0.25) $n = 8$	1.7–50 (5) $n = 7$	27, 30–32 34–36, 39, 41–44
Furnace	0.5–200 μl	0.9–144 (65) $n = 6$	0.009–0.4 (0.050) $n = 5$	0.25–400 (25) $n = 11$	0.025–2 (0.1) $n = 10$	45–49, 51, 53–56, 58

[a] Data were obtained using a variety of instruments, equipped with hollow cathode or electrodeless discharge lamps, at a wavelength of 196.0 nm. The hydrogen selenide generation technique includes commercial Perkin-Elmer and Instrumentation Laboratory hydride generators, but usually laboratory-constructed apparatus; a variety of reductants were used to liberate H_2Se which was swept into various flames, electrically heated tubes, and, in one case, a graphite furnace. Furnace data were generated with commercial Varian-Techtron and Perkin-Elmer instruments and with furnaces constructed and used by Massmann and L'vov. All data were obtained with aqueous solutions and usually with standard solutions. Some sensitivities and detection limits were calculated from data in the references, and virtually all data refer to the performance of the determination step only.

[b] Sensitivity and absolute sensitivity are defined as concentration and quantity of selenium giving an absorbance of 0.0044 or 1% absorption.

[c] Detection limits do not have consistent definitions and many times are undefined by the authors; common definitions of detection limit and absolute detection limit include the concentration and quantity of selenium, respectively, giving an instrumental response equal to once or twice the peak-to-peak noise of the baseline, or twice the standard deviation of the blank.

[d] Continuous aspiration.

[e] Range of n values; median is in parentheses.

[f] A sensitivity of 5000 ng/ml obtained by Allan (2) at the less sensitive line of 203.99 nm has not been included.

[g] Presumably air-acetylene.

Experimental

Apparatus

(a) *Hydrogen selenide generator.*—Jarrell-Ash trace metal accessory (Jarrell-Ash Division, Fisher Scientific Co., Waltham, MA, No. JA 82-732).

(b) *Carbon furnace atomizer.*—Carbon rod atomizer Model CRA 63 (Varian-Techtron Pty. Ltd., N. Springvale, Victoria, Australia).

(c) *Atomic absorption spectrometer.*—(*1*) *Hydrogen selenide-AAS.*—Varian-Techtron Model AA3 equipped with R106 photomultiplier tube. Introduce 25 psig argon and hydrogen (tank pressure 30 psig, flow meter reading 4) into sprayer-burner assembly to produce an Ar-H_2-entrained air flame supported on AB 52 burner head having 100 × 1.0 mm slot. Direct radiation from neon-filled selenium hollow cathode lamp (Varian-Techtron) operated at 7 ma through flame at height of 7 mm above burner surface. Set monochromator slit widths at 50 μm, corresponding to spectral band pass of 0.17 nm. Monitor selenium absorbance without scale expansion and with minimum damping, at 196.0 nm wavelength, and display on Varicord Model 43 strip-chart recorder (Photovolt Corp., New York, NY). Introduce H_2Se into spray chamber via 3 mm id, 4 mm od, 13 cm long steel tube inserted into chamber through hole in plastic bung. (*2*) *Carbon furnace-AAS.*—Varian-Techtron Model AA5 equipped with R446 photomultiplier tube. Position carbon furnace

work head on burner holder in place of burner head and align on optical axis to permit source radiation from selenium hollow cathode lamp (Varian-Techtron or Beckman Instruments Inc., Fullerton, CA) operated at 10 ma, or hydrogen hollow cathode lamp (Varian-Techtron) operated at current to match emission intensity of selenium lamp, to pass through tube or cup furnace (bulk of work was carried out with the tube) and impinge on monochromator entrance slit. Position masking plate with 3 mm diameter circular aperture immediately after sample cell to block out light not passing through furnace. Use nitrogen as inert gas sheath at 4 L/min. Set monochromator wavelength to pass 196.0 nm selenium line, and set slit width at 300 μm (0.99 nm spectral band pass) and slit height at 2 mm with attachment fitted to slit mechanism drum. Operate amplifier in high gain mode; use scale expansion as required, with A damping (least signal damping). Record absorbance signals with strip-chart recorder (Hitachi Model 056, Perkin-Elmer, Norwalk, CT) having 0.5 sec full-scale pen response time.

Reagents

(Analytical grade reagents and singly distilled water are used throughout.)

(a) *Stannous chloride-potassium iodide.*—Dissolve 300 g $SnCl_2 \cdot 2H_2O$ in 580 ml HCl. Dissolve 40 g KI in 200 ml water. Mix the 2 solutions and dilute to 1 L with water.

(b) *Zinc slurry.*—Disperse 227 g zinc dust (Fisher Scientific Co., No. Z-5) in 250 ml water.

(c) *Saturated ammonium oxalate solution.*—Mix enough $(NH_4)_2C_2O_4 \cdot H_2O$ with water so that excess solid remains.

(d) *Sulfuric acid-hydrochloric acid solution for hydrogen selenide-AAS.*—To 500 ml water, add 50 ml H_2SO_4 and 450 ml HCl and dilute to 1 L with water.

(e) *Nickel nitrate solution.*—50,000 μg Ni/ml. Dissolve 24.8 g $Ni(NO_3)_2 \cdot 6H_2O$ in water and dilute to 100 ml.

(f) *Selenium standard solution.*—(*1*) *Stock solution.*—100 μg/ml. Dissolve 0.100 g black Se^0 (purity ≥99.9%) in ca 5 ml HNO_3 and warm to dissolve. Dilute with water to 1 L. Alternatively, use commercially prepared 1000 μg selenium/ml stock solution. (*2*) *Working solutions for hydrogen selenide-AAS.*—Serially dilute stock solution with H_2SO_4-HCl solution to give 3–30 ng selenium/ml. (*3*) *Working solutions for carbon furnace-AAS.*—Serially dilute stock solution with water, HNO_3, $HClO_4$, and $Ni(NO_3)_2$ solution to give 0.02–10 μg selenium/ml in a solution 1*N* with respect to $HClO_4$, 0.5*N* with respect to HNO_3, and containing 5000 μg nickel/ml. Omit nickel when standard solutions are processed as samples, but add to final analytical solution.

Sample Digestion

(a) *Carbon furnace-AAS.*—Digest samples with HNO_3-$HClO_4$-H_2SO_4 essentially according to procedure developed for fluorometry (59) with several minor changes. Heat 1 g sample with 10 ml HNO_3 in 30 ml Kjeldahl flask until volume is reduced to ca 3 ml; after cooling, add 6.0 ml 70% $HClO_4$ and 3.0 ml H_2SO_4 and heat contents to SO_3 fumes. Complete treatment by adding three 1.0 ml portions of 30% H_2O_2 and dilute cool solution in flask with 3.0 ml water.

(b) *Isolation of selenium from sample matrix for carbon furnace-AAS.*—Add 2.0 g L-ascorbic acid to digest in Kjeldahl flask and shake 5 min on wrist-action shaker. Let stand 25 min, add 20 ml water, and shake again to dissolve remaining ascorbic acid. Filter solution through 25 mm diameter, 0.2 μm, Type EG Millipore membrane filter, and rinse filter and digestion flask with several portions of water. Return filter, with its contents of elemental selenium, to Kjeldahl flask, and digest with 1.0 ml HNO_3 and 0.26 ml $HClO_4$ to fumes of $HClO_4$. Transfer solution to 3 ml volumetric flask containing 0.10 ml HNO_3 and 0.30 ml $Ni(NO_3)_2$ solution and dilute to volume with water. Dilute samples containing ≤1 μg selenium/g to final volume of 1 ml. In these cases, 90 μl $HClO_4$ with 1 ml HNO_3 is used for digestion of filter, and 30 μl HNO_3 and 0.10 ml $Ni(NO_3)_2$ solution are added. The final matrix is 1.0*N* $HClO_4$, 0.5*N* HNO_3, and 5000 μg nickel/ml. Treat selenium standard solutions similarly.

(c) *Hydrogen selenide-AAS.*—Follow published procedure (59) with either 30% H_2O_2 or 5 ml saturated $(NH_4)_2C_2O_4$ finish. Transfer digest to 100 ml volumetric flask, add 45 ml HCl, and dilute contents to volume with water. Process appropriate selenium standard solutions and reagent blanks through both digestion procedures.

Determination

(a) *Hydrogen selenide-AAS.*—Before proceeding with determination, aspirate 10 μg selenium/ml standard solution into flame through nebulizer (which, with aspiration tubing, is left in spray chamber) to set spectrometer parameters (although a 50 μm slit width was used, later experiments showed that a somewhat better signal/noise ratio could be obtained with a larger slit width), and operate trace metal accessory unit according to manufacturer's manual. Pipet 20 ml aliquot of acidified digest into 100 ml, ⅁ 24/40, round-bottom flask and attach to reaction head of trace

metal accessory unit. Depress start button to activate 60 sec automatic cycle to dispense $SnCl_2$/KI and zinc into flask and sweep generated H_2Se into flame. The Ar-H_2 flame is invisible; use caution. In typical analysis, analyze 6 standard solutions including reagent blank before and after set of sample digests, and calculate selenium content from calibration curve drawn through mean standard absorbances.

(b) *Carbon furnace-AAS.*—Introduce 5 µl aliquot of solution into tube or cup furnace with Autopette pipet fitted with an Eppendorf polypropylene tip. Furnace is sequentially and automatically taken through the drying, ashing, and atomization steps. Adjust power supply voltage settings and heating times for each furnace as described in operating manual; typical voltage settings and durations of drying, ashing, and atomization stage are: 5 (0.9 v), 8 sec; 8 (3.0 v), 5 sec; and 10 (9 v), 1 sec, respectively. Take analytical absorbance signal from height of transient peak recorded during atomization step. Measure non-atomic absorbance with hydrogen continuum lamp, and correct all absorbances for non-atomic and reagent blank readings. Compare net sample absorbance with net absorbance of appropriate standard bracketing sample determination to give selenium content of sample. Perform decontamination firings occasionally to check on volatilization of selenium. Incorporate untreated selenium standard solution into set to monitor performance of furnace. Figure 1 schematically depicts typical determination sequence.

Results and Discussion

The biological samples used in this study were selected from those used in previous fluorometric investigations (59, 60). Levels of selenium determined by fluorometry (59, 60) and the certified values for NBS orchard leaves (SRM 1571) and bovine liver (SRM 1577) were taken as references for evaluating the performance of the analytical procedures.

Hydrogen Selenide-Atomic Absorption Spectrometry

Precautionary information in the trace metal accessory operating manual together with experiences of other workers (personal communication) using similar units led us to construct a Plexiglass cabinet to fit over the entire instrument. This cabinet had a removable top to permit access to the top of the trace metal accessory, and a vent which could be connected to a suction hood to remove acid fumes. This safety feature counteracted the consequences of the rubber tubes, carrying strongly acidic solutions under 5 psig, either pulling loose from the connections or bursting due to deterioration. Although no such problems were encountered, this safety precaution should be taken.

The reaction head as supplied by the manufacturer had all 3 glass delivery tubes extending 3 mm below the bottom of the ground-glass joint.

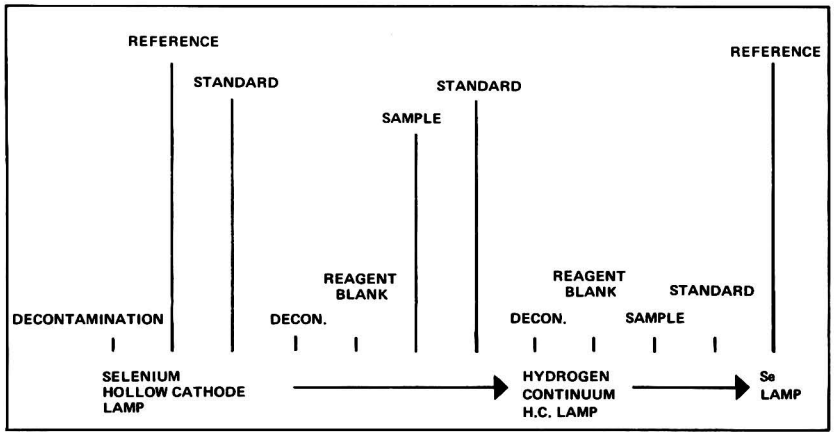


FIG. 1—Schematic representation of a determination sequence with the carbon furnace.

Vertical lines represent absorbances; standard refers to a selenium standard solution treated the same as the sample; reference is an untreated standard solution.

The stannous chloride/potassium iodide reagent, dispensed through the 2 mm id tube, tended to spatter onto the adjacent 3 mm id tube carrying the zinc slurry, resulting in frothing of the slurry and blockage of the zinc tube. Lengthening the first tube by 6 mm minimized this problem. Occasional blockage of the zinc tube due to drying of the slurry was minimized by interchanging the zinc and argon delivery so that the zinc was dispensed through the larger 4 mm id tube. Other problems with the zinc reductant involved premature dropping of small quantities of zinc into the reaction flask, and settling and caking of the powder in the storage bottle. The hydrogen selenide outlet tube leading from the head was straightened to prevent moisture build-up which was thought to trap the hydrogen selenide. The position of the steel inlet tube inserted into the spray chamber had no effect on absorbance and a distance of 1 cm was chosen for the location of the tip on the flame side of the hydrogen inlet.

In early trials, the instrument was operated both without desiccant and with charges of several different drying agents. Without desiccant, moisture collected on the glass check-valve and absorbance tended to drop with successive determinations. Drierite or $CaCl_2$, 4 mesh and freed of small particles and powder, appeared to be satisfactory for about 25 determinations, after which the desiccant was replaced with a fresh charge and the check-valve was dried.

During the automatic cycle, the following sequence of events occurs: The initial argon flush is followed at 15 sec by the introduction of about 2 ml stannous chloride/potassium iodide reagent; at 28 sec, a 3.5 ml aliquot of zinc slurry is dispensed and the hydrogen selenide (together with the generated hydrogen) is swept into the flame to produce a transient absorbance peak as shown in Fig. 2. The advantage of the technique is that the chemical reactions occur rapidly and the analyte is released from the sample matrix, presumably leading to fewer chemical interference problems in the cool argon-hydrogen-entrained air flame. The instrument in its semiautomatic (it does not sample) operation takes 30 sec to generate an absorbance signal, and 60 sec to accept the next sample.

Within- and between-run precision data for the determinative step obtained with standard solutions are listed in Table 2. The reagent blank gave stable long-term readings as indicated by the low between-run standard deviation. The mean long-term reagent blank absorbance of 0.034 (degrees of freedom = 45) seems somewhat high, and may result from a trace of selenium in the 3 g charge of zinc and/or a change in the flame composition resulting from the surge of gas. The former problem might be alleviated by substituting sodium borohydride (31, 34, 35, 39, 40, 43, 44) for zinc as the reductant; the latter effect can be eliminated by making simultaneous non-atomic absorbance measurements. A solution of sodium borohydride could be more conveniently handled and would eliminate the mechanical problems encountered with zinc. A standard curve through average absorbances obtained

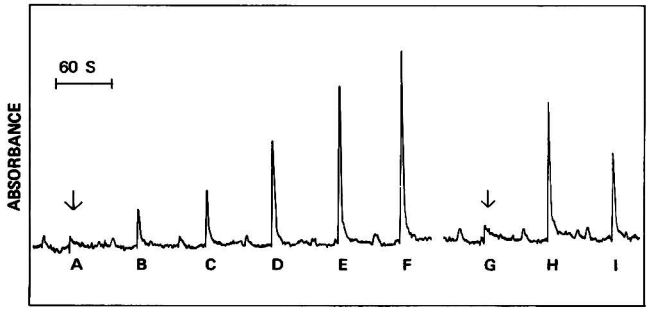


FIG. 2—Absorbance traces for H_2Se-AAS technique.

A and G, processed reagent blanks; B–F, processed selenium standard solutions containing 3.0, 5.0, 10.0, 15.0, and 20.0 ng selenium/ml, respectively; H and I, respectively, digested halibut (0.5 g, 2.83 μg selenium/g) and flour (1.0 g, 0.72 μg selenium/g) samples diluted to 100 ml. Small signals before each analytical absorbance result from purging reaction vessel with argon. A–F and G–I, consecutive runs.

Table 2. Precision of selenium determination using hydrogen selenide-atomic absorption spectrometry[a]

	Within-run[b]			Between-run[c]		
Se, ng/ml	s_{WR} (absorbance units)	df	CV	s_{BR} (absorbance units)	df	CV
0.0	0.0059	22	17.0	0.0022	21	6.4
3.0	0.0194	24	14.5	0.0191	23	14.2
5.0	0.0241	14	11.1	0.0259	13	12.0
10.0	0.0236	20	6.2	0.0324	19	8.5
15.0	0.0261	11	4.9	0.0746	10	14.2
20.0	0.0324	13	5.3	0.0456	12	7.5

[a] Data refer to performance of determinative step over 4 months. s = standard deviation; df = degrees of freedom; CV = coefficient of variation.

[b] A run is described as a set of measurements comprising the sequence: standards-samples-standards. Within-run variability, s_{WR}, was obtained from absorbance measurements for standard pairs containing one measurement prior to and one measurement after sample readings, from the relation:

$$S_{WR} = \left[\left(\sum_{i=1}^{m}\sum_{j=1}^{2} x_{ij}^2 - \frac{\sum_{i=1}^{m} x_{i}^2}{2}\right)\Big/ m\right]^{1/2}$$

where x_{ij} is the jth replicate of absorbance in the ith run, m is the number of runs, and $x_{.i} \sum_{j=1}^{2} x_{ij}$.

[c] Between-run variability, s_{BR}, was calculated from the same absorbance measurements as s_{WR}, from the relation:

$$s_{BR} = \left[\left\{\left[\left(\frac{\sum_{i=1}^{m} x_{.i}^2}{2} - \frac{\left[\sum_{i=1}^{m}\sum_{j=1}^{2} x_{ij}\right]^2}{2m}\right)\Big/ (m-1)\right] - S_{WR}^2\right\}\Big/ 2\right]^{1/2}$$

over 4 months, depicted in Fig. 3, exhibits linearity over the 0–15 ng/ml concentration range.

Carbon Furnace-Atomic Absorption Spectrometry

Details of our experiments with the carbon furnace have been published elsewhere (58); only some relevant results dealing with the analytical performance of the system will be mentioned here.

An obvious characteristic of the carbon furnace was variability of absorbance signals. An example of this is given in Fig. 4 where a sequence of absorbance tracings for a standard solution is presented as a function of firing number; both short- and long-term variabilities are evident. This behavior was typical over the lifetime of the furnace (average 300 firings per tube) after the cup or tube was conditioned by several dozen firings. It was thought that part of the variability could be attributed to analyte volatility which could be decreased by adding nickel to the analytical solution. This improved precision by a factor of 2.4 and sensitivity by about 3-fold, and all solutions related to the analysis of samples contained 5000 μg nickel/ml. Signal variability was still, however, relatively high and a problem for quantitative analysis.

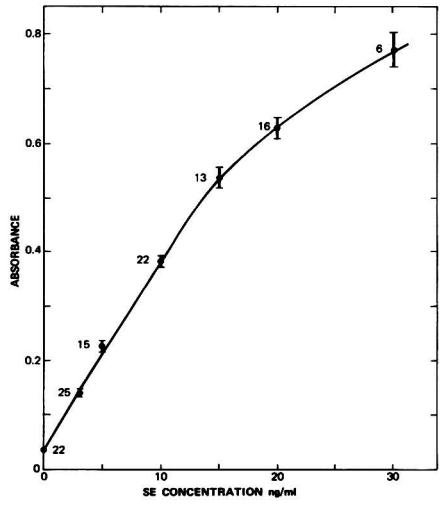


FIG. 3—Calibration curve for the H_2Se-AAS technique. Absorbance of untreated selenium standards is plotted vs. concentration in analytical solution. Each point is mean of number of determinations indicated by adjacent numerals, obtained for 4 months. Vertical bars denote standard error of mean.

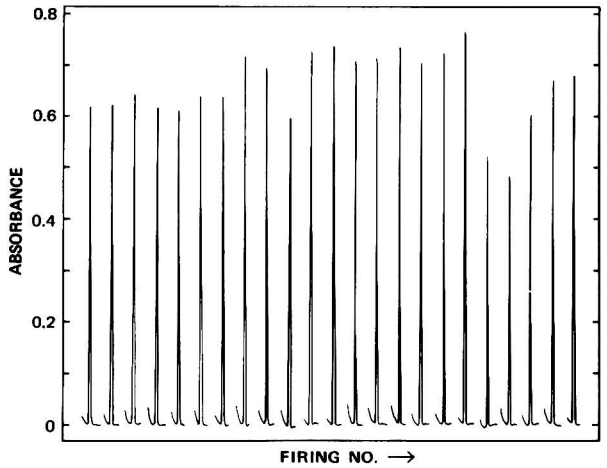


FIG. 4—Typical sequence, as function of firing number, of atomization step absorbances, depicting behavior of carbon tube furnace.

These 23 peaks for 1.0 μg selenium/ml standard solution were obtained during 120th–204th firing of one carbon furnace, and show both short- and long-term reproducibility. Matrix: 1.0*N* $HClO_4$ + 0.5*N* HNO_3 + 5000 μg nickel/ml.

This aspect of the performance precluded the use of standard curves and made mandatory the bracketing of sample runs with standards to offset the incursion of error from long-term signal variations. A standard curve of average absorbances could be constructed, however, and used to calculate sensitivity (Fig. 5). With nickel in the system, the data fitted a straight line over the concentration range 0–1.3 μg/ml; the mean non-atomic absorbance was 0.068 (standard deviation = 0.013, degrees of freedom = 135, 9 tubes).

Experiments with selenium standards in several different acid solutions demonstrated the dependence of absorbance on the bulk matrix (this aspect of the study was conducted by using 20 μl aliquots in carbon cups; no nickel was present). The good absorbance of selenium dissolved in 1*N* H_2SO_4 was impaired when the standard solution was digested with nitric-perchloric-sulfuric acids, ending in 1*N* H_2SO_4. The reason was deduced to be the possible presence of small traces of residual perchloric acid. When perchloric acid was deliberately introduced (Fig. 6), the signal was severely depressed. Behavior was improved in either nitric or perchloric, or mixtures of the 2 acids. At a constant concentration of $HClO_4$ (0.5*N*), the signal maximum occurred at 0.3–0.6*N* HNO_3 as shown in Fig. 7. This behavior, together with an absorbance plateau at

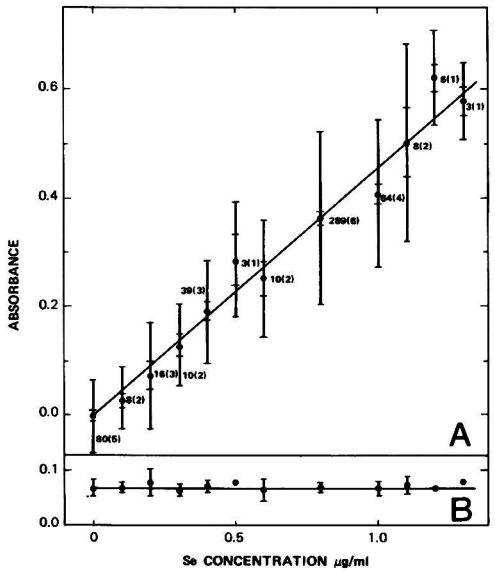


FIG. 5—Net absorbance of selenium standard solutions in carbon tube furnace as function of analyte concentration in the presence of 5000 μg nickel/ml.

Solutions contain 1.0*N* $HClO_4$ and 0.5*N* HNO_3. A, Absorbance measured with selenium hollow cathode lamp. Total number of measurements is indicated beside each data point; number of different tubes used is in parentheses. Vertical bars to first and second horizontal lines represent standard error of mean and standard deviation, respectively. B, Absorbance measured with hydrogen hollow cathode lamp. Vertical bars represent standard error of the mean.

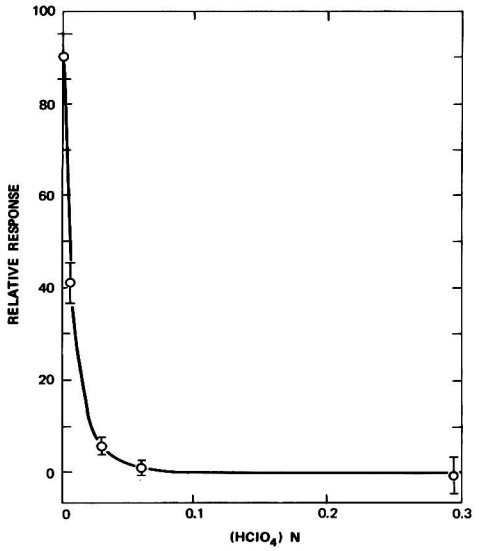


FIG. 6—Effect of $HClO_4$ on absorbance of 1.0 μg selenium/ml standard solution in 1.0*N* H_2SO_4 medium.
Carbon cup form of furnace; no nickel present.

0.2–>1.4*N* $HClO_4$ obtained at a constant nitric acid concentration, led to the choice of 1*N* $HClO_4$ plus 0.5*N* HNO_3 for the acid matrix in the analysis.

Small amounts of inorganic matrix remaining after digestion sometimes severely depressed the selenium signal. These effects were evident in studies when the analyte was not separated, and depended on the quantity and nature of the sample (56, 58). Such interferences appear to be common features of carbon furnace performance and depend on construction. We felt that separating the selenium from the sample matrix before measurement would improve the performance of the determinative step. This was conveniently accomplished by selectively precipitating the selenium with ascorbic acid and redissolving the precipitate in acid. This treatment simplified and normalized the drying and ashing absorbance pattern, enhanced the atomization absorbance (56), and made it possible to use identical voltage and time settings for standard solutions and samples. Absorbance patterns resulting during the 3 heating stages, together with a partial determination sequence, are depicted in Fig. 8.

Comparison of the Two Methods

Sensitivities and detection limits for the 2 methods, determined with standard solutions, are listed in Table 3. Additional detection limit data based on actual analyses of samples containing native and added selenium are also included. These detection limits, defined as concentration and weight, respectively, that have an accompanying coefficient of variation of 50%, are from more realistic analytical schemes. The de-

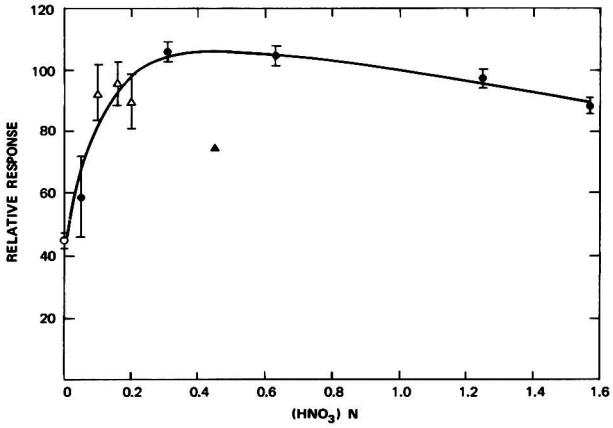


FIG. 7—Dependence of absorbance signal of selenium standard solution on HNO_3 concentration in carbon cup furnace without nickel.
$HClO_4$ concentration is constant at ca 0.5*N* in all solutions. Data were obtained with 4 different cups.

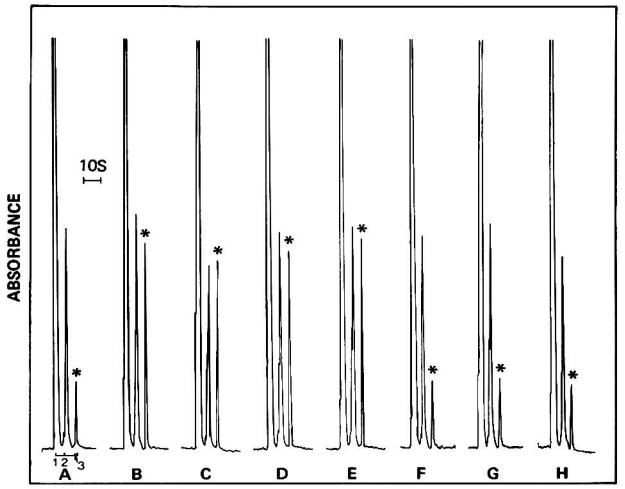


FIG. 8—Example of determination sequence showing absorbances at 3 heating stages of carbon tube.

A and F, reagent blank; B, E, and G, selenium standard solution containing 0.400 μg selenium/ml; C, D, and H, oat cereal sample solution containing 1.0 g sample/ml. Absorbances for A–E were measured with selenium hollow cathode lamp; F–G were measured with hydrogen continuum lamp. Identical procedures of digestion-precipitation-redigestion were followed for all solutions. Matrix is 1.0*N* $HClO_4$ + 0.5*N* HNO_3 + 5000 μg nickel/ml. Three absorbance peaks in each firing are as depicted in A: (1) drying (5 sec), (2) ashing (7 sec), and (3) atomization (1 sec); atomization peaks are also indicated by asterisks.

Table 3. Sensitivities and detection limits for selenium determined by hydrogen selenide generation- and carbon tube furnace-atomic absorption spectrometry[a]

Parameter	H_2Se-AAS	Carbon furnace-AAS
Sensitivity, ng/ml[b]	0.13	10
Absolute sensitivity, ng[c]	2.6	0.050
Detection limit, ng/ml[d]	0.35 (2.5)[e]	90 (25)[e]
Absolute detection limit, ng[c]	7.0 (50)[e]	0.45 (0.13)[e]

[a] Based on untreated standard solutions in appropriate bulk acid matrices.

[b] Sensitivity defined as concentration of selenium giving absorbance of 0.0044, and based on the limiting slopes (at 0 concentration) of curves through averages of pooled results.

[c] Absolute sensitivities and absolute detection limits based on 20 ml and 5 μl sample volumes for H_2Se-AAS and carbon furnace-AAS, respectively.

[d] Detection limit defined as concentration of selenium giving an absorbance equal to twice the standard deviation of blank readings (in this work, df = 22 for within-run readings for H_2Se-AAS; df = 79 for within-/between-run readings for carbon furnace-AAS).

[e] From analyses of samples containing native and added selenium; equal to concentration and weight of selenium which can be determined with 50% coefficient of variation.

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tection limit and absolute detection limit for the carbon furnace technique are improved because long-term variability problems are removed by bracketing sample readings with those of standard solutions. The deterioration, on the other hand, of the hydrogen selenide figures is more in line with behavior normally expected; this technique has less severe long-term variability. Whereas both sensitivity and detection limits for the hydrogen selenide-AAS method are substantially lower than the corresponding figures for the carbon furnace method, due to the much lower volume requirements (5 μl vs. 20 ml, a factor of 4000) of the carbon furnace technique, absolute detection limits by the latter method are superior. On a practical basis, however, assuming a 1 g sample is processed to give a 1 ml volume of solution and considering the real situation detection limits, the carbon furnace method has a sample detection limit of 25 ng/g, based on the analytical solution detection limit of 25 ng/ml. Similarly, getting the sample into the 20 ml required for hydrogen selenide-AAS leads to a detection limit of 50 ng/g on a sample basis. Thus, both techniques have comparable detection limits when applied to real situations which are somewhat poorer than the 10 ng/g

figure for fluorometry. It should be noted that the 20 ml for hydrogen selenide-AAS is sufficient for only one determination, whereas about 200 determinations can be performed with the 1 ml for carbon furnace-AAS.

A point of interest is interjected. The concept of detection limit has led to several definitions for this term. The definition for the determinative step detection limit for standard solutions adopted in this work is: the concentration of the element in solution that gives a reading equal to twice the standard deviation of a series of at least 10 determinations *at or near* the blank level. The detection limits for both techniques, given in Table 3, were computed from standard deviations of blanks. As can be seen in Table 2, however, the within-run and between-run standard deviations of the blank for hydrogen selenide-AAS measurements are several-fold lower than the standard deviations of any of the standards. If we had selected the lowest standard for detection limit calculation, a value of 1.15 ng/ml (23 ng) from the within-run standard deviation would have been obtained vs. the 0.35 ng/ml (7.0 ng) calculated from blank variability. The hydrogen selenide-AAS detection limits in Table 3 should, therefore, be considered as lower limits. Such a problem was not found with the carbon furnace (cf. Fig. 5 which shows comparable standard deviations for blank and standards).

Selenium levels in several foods and biological samples determined by the 2 methods are given in Table 4 together with fluorometrically determined levels and NBS values for comparison. In general, the data generated by both techniques agree well with fluorometric levels; the exception is the low carbon furnace result for swordfish B. The very good agreement of 1.11 μg/g by hydrogen selenide-AAS and 1.00 μg/g by carbon furnace-AAS for the NBS bovine liver sample, with the 1.04 μg/g calculated from the certified NBS value, is encouraging. Agreement of the hydrogen selenide-AAS level for the NBS orchard leaves is also good.

Conclusions

The hydrogen selenide-AAS technique looks promising as a rapid method, convenient for routine application. The encouraging results obtained from this preliminary assessment, the placing on the market of several commercial apparatus designed specifically for hydride generation, and the work and custom development of related instruments by a number of investigators attest to the potential of this technique for the estimation of selenium. Once digested, a large number of samples can be processed rapidly; this is the main advantage over fluorometry.

Pronounced sample matrix effects and signal variability in the carbon furnace-AAS method place constraints on its usefulness in the direct estimation of selenium in sample digests. Isolation of selenium from the matrix considerably enhances the potential of this method, although

Table 4. Levels of selenium (μg/g) in biological samples determined by hydrogen selenide generation- and carbon tube furnace-atomic absorption spectrometry[a]

Sample	H_2Se-AAS			Carbon furnace-AAS			Fluorometry		
	Mean	n[b]	$s_{\bar{x}}$	Mean	n[b]	$s_{\bar{x}}$	Mean	n	$s_{\bar{x}}$
Halibut	2.74	3	0.08	2.90	3	0.16	2.83[c]	17	0.07
Swordfish A	2.58	3	0.04				2.79[c]	16	0.05
Swordfish B	—	—	—	2.64	5	0.32	3.44	3	0.16
NBS liver (SRM 1577)	1.11	3	0.06	1.00	3	0.02	1.04[d]	—	—
Flour	0.68	3	0.08	(0.76, 0.75)[e]			0.72[c]	16	0.01
Oat cereal	0.45	3	0.04	(0.35, 0.38)[e]			0.40[c]	17	0.01
Round steak	0.20	3	0.02	(0.22, 0.18)[e]			0.24[c]	17	0.01
Skim milk powder	0.11	3	0.04	—	—	—	0.11[c]	18	0.01
NBS orchard leaves (SRM 1571)	0.082	3	0.024	—	—	—	0.076[d]	—	—

[a] One g samples digested and diluted to 100 ml in HCl-H_2SO_4 for H_2Se-AAS method; for carbon furnace-AAS method, selenium in digests of 1 g samples was precipitated, dissolved, and diluted to 3 ml (first 3 samples) or 1 ml (last 3 samples) in an HNO_3-$HClO_4$-nickel matrix.

[b] n denotes the number of separate analyses; $s_{\bar{x}}$ is the standard error of the mean. In the H_2Se method, one determination was performed per analysis, whereas about 6 determinations per analysis were performed for the carbon furnace method.

[c] Data from interlaboratory study (60); n denotes number of laboratories.

[d] Certified NBS value corrected for moisture content.

[e] Individual values.

this does not improve the reproducibility or reduce the effort required to generate acceptable data.

Based on the performance data on hand, from this investigation and previous work (59, 60), of the 3 methods, the hydrogen selenide-AAS method seems to be the most rapid and most convenient for routine application to samples containing 50 ng selenium/g and higher levels; carbon furnace-AAS is troublesome; and fluorometry provides the lowest (best) detection limit.

Recommendations

It is recommended—

(*1*) That the hydrogen selenide-AAS technique be studied further to take full advantage of the potential of this method; this should include evaluation of sodium borohydride as a reductant, optimization of hydrogen selenide generation and AAS parameters, and a study of interferences in the reaction steps and flame determination step.

(*2*) That the carbon furnace-AAS technique be evaluated further, including comparison of the performance of the commercial models of the carbon furnace, and assessment of analyte separation and concentration procedures.

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FERTILIZERS

Collaborative Study of the Modifications of the Official AOAC Method for the Determination of Biuret in Fertilizers

LUIS F. COROMINAS
Guanos y Fertilizantes de México, S.A., Morena Núm. 804, México 12, D.F.

Six collaborators participated in the second part of the study of the official AOAC method for the determination of biuret in fertilizers. Four samples of fertilizers containing 1.5–3.0% biuret were analyzed by using 2 standard curves prepared from biuret obtained from 2 different sources and having different melting points. Biuret contents higher than expected were obtained from the curve prepared from the low melting biuret. This confirms the necessity of checking the purity of the standard to be used and justifies a recrystallization step.

In the first part of this study (1), 3 methods for determining biuret in fertilizers were compared: the 1970 official first action method, 2.073–2.075 (2), the same method with some proposed modifications (3), and the nickel sulfate method (4). Five samples containing from 0.4 to 0.9% biuret were collaboratively studied. On the basis of that study, it was concluded that the weight of the sample should be increased to 25 g (instead of 2–5 g for urea and 10–20 g for mixed fertilizers) and the standard biuret should be recrystallized.

In the present study, 4 samples with the following composition were sent to 8 collaborators:

Sample 10: urea (46-0-0)

Sample 11: 50% urea + 50% K_2SO_4 (23-0-24)

Sample 12: 70% urea + 30% DAP (38-14-0)

Sample 13: 60% urea + 20% DAP + 20% K_2SO_4 (31-9-10) (DAP = diammonium phosphate)

Two samples of biuret standards from different sources were included. One had a low melting point (Standard B) and the second had a melting point near the theoretical (Standard A).

The collaborators were instructed as follows:

(*1*) Analyze the fertilizer samples according to 2.073–2.075 (2), modified to use 25 g sample.

(*2*) Prepare 2 standard curves, one with biuret Standard A and another with biuret Standard B.

(*3*) Determine the melting points for the 2 biuret standards.

(*4*) Report the results obtained by using both standard curves.

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Results and Discussion

Six collaborators reported 176 individual results; 4 of them reported 5 values for each sample and each curve, and 2 reported single values for each sample and curve. The single values and the averages are shown in Tables 1 and 2, respectively. The equipment used by the collaborators included: Klett-Summerson colorimeter (4 cm cell and No. 54 filter); Unicam-SP Model 8003 ultraviolet spectrophotometer (3 cm cell); Senior spectrophotometer; and Prolabo, Jean et Constant spectrophotometer (4 cm cell).

From the values reported in Table 2, it is evident that the values obtained by using biuret Standard B are significantly different from those obtained by using biuret Standard A. Four collaborators reported melting points for the biurets (Table 3) and it was confirmed that biuret Standard B contains more impurities.

The values obtained for the 4 samples by Collaborators 1–4, using the 2 standard curves, were averaged, and the differences were calculated (Table 4). The error introduced by using a low melting biuret standard may be as high as 50%. The values for Samples 10 and 13 obtained by using biuret Standard A agreed with the theoretical values. The apparent deviation for Sample 12 could be attributed to sample preparation. The deviation for Sample 11 is a result of the low single values from Collaborator 4.

The standard deviations obtained in the previous study (1) for samples with biuret contents lower than 1% were compared with those obtained for biuret contents between 1.5 and 3.0%. The S_r, S_d, and S_b values were calculated for the results from Collaborators 1, 2, 3, and 4. The

Table 1. Individual collaborative results reported for the determination of per cent biuret in urea and mixed fertilizers

	Biuret std A						Biuret std B					
Detn	1	2	3	4	5[a]	6[a]	1	2	3	4	5[a]	6[a]
						Sample 10 (46–0–0)						
1	3.04	2.67	3.05	1.33	2.88	2.94	4.32	3.85	3.92	1.80	4.08	4.15
2	3.04	2.64	3.00	1.57[b]	—	—	4.32	3.82	3.87	2.05[b]	—	—
3	3.04	2.64	3.05	1.33	—	—	4.32	3.82	3.92	1.80	—	—
4	3.06[b]	2.67	3.00	1.33	—	—	4.33[b]	3.85	3.87	1.80	—	—
5	3.04	2.64	3.05	1.33	—	—	4.32	3.82	3.92	1.80	—	—
						Sample 11 (23–0–24)						
1	1.38	1.72	1.40[b]	0.62	1.44	1.54	1.91	2.48	1.74[b]	0.82	2.00	2.18
2	1.40	1.60	1.56	0.62	—	—	1.95	2.32	1.98	0.82	—	—
3	1.38	1.60	1.60	0.62	—	—	1.91	2.32	1.96	0.82	—	—
4	1.43	1.60	1.56	0.62	—	—	1.98	2.32	1.98	0.82	—	—
5	1.38	1.72	1.56	0.62	—	—	1.91	2.48	1.98	0.82	—	—
						Sample 12 (38–14–0)						
1	1.92	1.84	1.80	0.74	2.08	1.96	2.69	2.64	2.46	1.02	2.92	2.75
2	1.92	1.76	1.80	0.74	—	—	2.69	2.56	2.44	1.02	—	—
3	1.92	1.96	1.88	0.74	—	—	2.69	2.80	2.52	1.02	—	—
4	1.91[b]	1.76	1.84	0.74	—	—	2.68[b]	2.56	2.50	1.02	—	—
5	1.92	1.96	1.84	0.74	—	—	2.69	2.80	2.50	1.02	—	—
						Sample 13 (31–9–10)						
1	1.74	1.76	1.81	0.56	1.72	1.70	2.44	2.56	2.50	0.73	2.48	2.42
2	1.73	1.76	1.80	0.56	—	—	2.42	2.56	2.44	0.73	—	—
3	1.73	1.72	1.84	0.56	—	—	2.42	2.48	2.46	0.73	—	—
4	1.74	1.76	1.78	0.56	—	—	2.44	2.56	2.42	0.73	—	—
5	1.74	1.96[b]	1.76	0.56	—	—	2.44	2.80[b]	2.38	0.73	—	—

[a] These collaborators reported only a single value.
[b] These values were rejected according to the criteria for testing for extreme values, $\alpha = 0.05$.

expressions used in the calculations were as follows (5):

(*a*) Criteria for testing for extreme values—

$$r = (x_d - x_c)/(x_k - x_m)$$

With a critical value of $\alpha = 0.05$ and n determinations, in Table A-8e (6), we found r'. Rejection condition: $r > r'$, where x_d = doubtful value; x_c = next value to x_d; x_k = largest value of the series; and x_m = smallest value of the series.

(*b*) Standard deviation for collaborator—

$$S = \sqrt{\frac{\sum(X_i - \bar{X})^2}{n - 1}}$$

where X_i = individual values; $\bar{X}$ = mean for each collaborator; n = number of determinations for each collaborator; and S = standard deviation for each collaborator.

(*c*) Precision standard deviation—

$$S_r = \sqrt{\frac{\sum S^2}{N}}$$

where N = number of collaborators considered; and S_r = precision standard deviation.

(*d*) Standard deviation referring to the distribution of the actual data—

$$S_d = \sqrt{\frac{\sum(\bar{X} - \bar{\bar{X}})^2}{N - 1}}$$

where $\bar{\bar{X}}$ = overall data average; and S_d = standard deviation among laboratories.

(*e*) Standard deviation of the systematic error—

$$S_b = \sqrt{S_d^2 - \frac{S_r^2}{n}}$$

where S_b = standard deviation of the systematic error. The values for S_r, S_d, and S_b for each sample are shown in Table 5. The S_b values obtained are higher than S_b values reported in the first part of the study (1), and we can conclude that the S_b values increase with the increase in biuret content, even when a biuret of low melting point is used as the standard.

Table 2. Collaborative results for the determination of per cent biuret in urea and mixed fertilizers

Coll.	Biuret std A		Biuret std B	
	$\bar{X}$[a]	Std dev.	$\bar{X}$[a]	Std dev.
	Sample 10 (46–0–0)			
1	3.04	0.0000	4.32	0.0000
2	2.65	0.0164	3.83	0.0164
3	3.03	0.0273	3.90	0.0273
4	1.33[b]	0.0000[b]	1.80[b]	0.0000[b]
	Sample 11 (23–0–24)			
1	1.39	0.0219	1.93	0.0319
2	1.65	0.0657	2.38	0.0876
3	1.57	0.0200	1.97	0.0100
4	0.62	0.0000	0.82	0.0000
	Sample 12 (38–14–0)			
1	1.92	0.0045	2.69	0.0000
2	1.86	0.1003	2.67	0.1213
3	1.83	0.334	2.48	0.0328
4	0.74[b]	0.0000[b]	1.02[b]	0.0000[b]
	Sample 13 (31–9–10)			
1	1.74	0.0054	2.43	0.0109
2	1.75	0.0200	2.48	0.0400
3	1.80	0.0303	2.44	0.0447
4	0.56[b]	0.0000[b]	0.73[b]	0.0000[b]

[a] $\bar{X}$ = per cent biuret, average of 5 determinations.
[b] These values were rejected according to the criteria for testing for extreme values, $\alpha = 0.05$.

Recommendations

It is recommended—

(1) That the melting point of the standard biuret should be checked, and if it is <192°C, the standard should be recrystallized.

(2) The study should be continued with samples containing >1% biuret.

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The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee A and were accepted by the Association. *See* (1975) *JAOAC* **58**, 307–308.

Table 3. Melting points for biuret standards

Coll.	Melting point range, °C	
	Biuret std A	Biuret std B
1	190–195	165–173
2	198–205	170–185
3	183–185	155–158
4	198–202	170–180
Av.	194	169

Table 4. Error in biuret determination resulting from the use of an impure standard

Sample	Av. % biuret from std A	Av. % biuret from std B	Diff. % biuret	Theor. % biuret[a]
10	2.91	4.02	1.11	3.0
11	1.31	1.77	0.46	1.5
12	1.87	2.61	0.74	2.1
13	1.76	2.45	0.69	1.8

[a] Values expected from the urea content in the samples based on 3.0% biuret in urea = 100%.

Table 5. Statistical evaluation[a] of the collaborative results for the determination of biuret

Sample	Av. % biuret	S_r	S_d	S_b
	Biuret Std A			
10	2.91	0.0183	0.2223	0.2221
11	1.31	0.0360	0.4710	0.4707
12	1.87	0.0610	0.0458	0.0368
13	1.76	0.0211	0.0321	0.0307
	Biuret Std B			
10	4.02	0.0183	0.2650	0.2649
11	1.77	0.0468	0.6683	0.6680
12	2.61	0.0725	0.1159	0.1113
13	2.45	0.0351	0.0264	0.0212

[a] S_r = precision standard deviation; S_d = standard deviation among laboratories; and S_b = standard deviation of the systematic error.

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This report of the Associate Referee was presented at the 88th Annual Meeting of the AOAC, Oct. 14–17, 1974, at Washington, DC.

New Statistical Manual

The *Statistical Manual of the AOAC,* a 96-page booklet, is a composite, with revisions, of the popular AOAC monograph, *Statistical Techniques for Collaborative Tests,* by the late W. J. Youden, and a companion volume, *Planning and Analysis of Results of Collaborative Tests,* written by E. H. Steiner and originally published in 1974 by the British Food Manufacturing Industries Research Association, who have assigned their copyright to the AOAC. This book is intended for use in the statistical analysis of the results of interlaboratory collaborative tests, such as those required by the AOAC before its adoption as official of analytical methods for agricultural products, foods, beverages, drugs, cosmetics, color additives, and other commodities important in public health.

The Youden monograph was specifically written as a "do-it-yourself" manual for those who are at home in the analytical laboratory but who have little or no experience with formal statistics. It presents simple and flexible statistical techniques, using examples related to familiar questions concerning the significance of apparent differences among results. The Steiner manual, intended for those who are already familiar with the fundamentals covered by Youden, presents guidelines for the planning of collaborative tests and introduces the analysis of variance technique with examples of how it may be used in the analysis of interlaboratory tests.

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FORENSIC SCIENCES

Study of Speaker Identification by Aural and Visual Examination of Non-Contemporary Speech Samples

LONNIE SMRKOVSKI
Voice Identification Unit, Scientific Crime Laboratories Section, Michigan Department of State Police, 714 S. Harrison Rd, East Lansing, MI 48823

In this study, 10 pairs of unfamiliar voices were aurally and visually (with spectrograms) compared by 12 examiners to determine whether they belonged to the same speaker. Five decisions could be made: positive identification, positive elimination, probable identification, probable elimination, and unable to make a decision with the samples submitted. Each speaker recorded 9 cue words twice, spoken as a sentence, within a 15 month interval. The pair of speakers in each task was of the same sex. Match and no match constituted 50% each of the total, presented in a random fashion. For each group of examiners there were 40 possible positive correct decisions (20 identifications and 20 eliminations). Group I (professional examiners with >2 years experience) reported 90% correct positive identifications; the remaining 10% corresponded to probable identifications and no decision. Correct positive eliminations were 80%; the remaining 20% corresponded to probable eliminations and no decision. Group II (trainees with <2 years experience) reported 80% correct positive identifications; the remaining 20% corresponded to probable identifications. Correct positive eliminations were 75%; the remaining 25% corresponded to probable eliminations and no decision. No errors were produced by either Group I or II. Group III (novices) reported 40% correct positive identifications; probable identifications and no decision constituted 35%. Correct positive eliminations were 75%; probable eliminations were 20%. The percentages of error for false identifications and false eliminations were 5 and 25, respectively. The collaborative results suggest that professional examiners perform better than trainess and the performance of both groups is far superior to that of novices. Examiners with academic training in speech and hearing sciences and with over 2 years of experience perform better than those lacking academic and/or practical experience. The 15-month interval between the non-contemporary speech samples used in this study did not yield any errors for Groups I and II and no significant difference was found in examiners' ability to identify or eliminate male or female voices. Group III had more difficulty with female speakers than with male speakers. The results also suggest that trained professional examiners can make very reliable decisions using no more than 9 cue words, regardless of speaker sex, and non-contemporary speech samples up to 15-month intervals. On the basis of this study, the official first action voiceprint method, 45.A01–45.A03, has been adopted as official final action.

The method of speaker identification used in this study consisted of aural and visual comparison of speech spectrograms of pairs of voice samples to decide whether they belonged to the same person. Previous research dealing with speaker identification indicates that speakers can be identified with high reliability within certain conditions (1–3).

The present study was conducted to determine what effect (if any) time lapse has on the voice and the examiners' ability to identify and/or eliminate male and female speakers. It was also of interest to study and compare the results of 3 examiner experience levels (novice, trainee, and professional).

Collaborative Study

Subjects

Fourteen speakers, 7 male and 7 female, were employed for this study. All spoke general American English dialect, possessed normal hearing, and had no known speech defects. Their ages ranged from 25–55 years. Education ranged from a high school diploma to a Ph.D. degree.

Twelve (12) examiners (from the United States, Canada, and South Africa) were used to produce discrimination trials, based on aural and visual examination of speech spectrograms from

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samples obtained from the speakers. Four of the examiners were professional voice identification examiners and members of the International Association of Voice Identification (IAVI) with >2 years of forensic experience in the field. Four of the examiners were trainees (<2 years of practical experience) of the IAVI, and 4 were novices (≤1 month of training).

Phonetic Materials

Fourteen speakers, 7 male and 7 female, recorded twice the words "it is on you and the I to me" (9 cue words) spoken as a sentence, i.e., ongoing speech. These recordings, spaced 15 months apart, were recorded on a Sony 110 cassette magnetic tape recorder in a normal environment, i.e., the working place of each individual.

Experimental Procedure

Each examiner received a cassette tape containing 10 recordings numbered 1 to 10. Each recording consisted of 2 samples of the same text, either spoken by the same person (matched task) within a 15-month interval, or by 2 different persons (no match). The pairs of speakers in each sample were of the same sex. Fifty per cent were male and 50% were female. Match and no-match tasks constituted 50% of the total presented in a random fashion. The examiners were advised that, for each task, a match may or may not exist.

METHOD

The method of examination was that adopted as official first action in 1975(4), with the following change:

Change **45.A02(a)**, lines 4–5, to read as follows: "(available from Audio Intelligence Devices, PO Box 23130, Ft. Lauderdale, FL 33307), or equiv."

Results and Recommendation

The percentage of correct positive identifications obtained by the professional examiners (Group I) was 90; the remaining 10% corresponded to probable identifications and no decision. The percentage of correct positive eliminations was 80; the remaining 20% corresponded to probable eliminations and no decision one way or the other. No errors were made by the professional examiners.

The percentage of correct positive identifications obtained by the trainees (Group II) was 80; the remaining 20% corresponded to probable identification. The percentage of correct positive eliminations was 75; the remaining 25% corresponded to probable eliminations and no decision. No errors were made by the trainee group.

The percentage of correct positive identifications obtained by the novice examiners (Group III) was 40; probable identifications and no decision constituted 35%. The percentage of correct positive eliminations was 75; probable eliminations were 20%. The percentage of error for false identifications was 5 and for false eliminations 25. Complete results are shown in Tables 1–3. Table 4 shows results of individual examiners.

This study suggests that professional examiners in voice identification with >2 years of experience perform better than trainees with <2 years of experience. Novice performance was far inferior in performing discrimination tasks than the other 2 groups.

No error of false identification was produced by examiners in Groups I and II for the 15-month-interval, non-contemporary speech samples. However, Group III produced 5% false identifications for these samples. Concerning false eliminations, the 15-month-interval gap yielded 0% error for Groups I and II, and 25% error for Group III.

No significant difference was found in examiners' ability to identify or eliminate male or female speakers for Groups I and II. However, Group III had more difficulty with female than with male speakers.

The results also suggest that reliable decisions in voice identification and elimination can be rendered by using 9 cue words or less. Professional voice identification examiners presently use a minimum of 10 similar pairs of speech sounds in rendering positive decisions in forensic cases. This study tends to support that criterion for rendering positive decisions.

No errors were produced by examiners in Groups I and II, but those with academic de-

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**, 399–400.

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

Table 1. Number of responses made by Group I (trained professional examiners)

Task	Correct response	Correct pos. ident. ($N = 4$, $N_T = 20$)[a]	Correct probable ident.	Correct pos. elim. ($N = 4$, $N_T = 20$)	Correct probable elim.	No decision	Error: False ident.	Error: False elim.
1	match	4	0	–	–	0	–	0
2	match	3	0	–	–	1	–	0
3	no match	–	–	3	1	0	0	–
4	match	4	0	–	–	0	–	0
5	no match	–	–	2	0	2	0	–
6	no match	–	–	4	0	0	0	–
7	match	4	0	–	–	0	–	0
8	no match	–	–	4	0	0	0	–
9	no match	–	–	3	1	0	0	–
10	match	3	1	–	–	0	–	0
Decisions (D)								
No.		18	1	16	2	3	0	0
%(= D/N_T)		90	–	80	–	7.5		

[a] N = maximum correct answers for each task; N_T = maximum correct answers for 10 tasks.

Table 2. Number of responses made by Group II (trainees)

Task	Correct response	Correct pos. ident. ($N = 4$, $N_T = 20$)[a]	Correct probable ident.	Correct pos. elim. ($N = 4$, $N_T = 20$)	Correct probable elim.	No. decision	Error: False ident.	Error: False elim.
1	match	3	1	–	–	0	–	0
2	match	4	0	–	–	0	–	0
3	no match	–	–	2	2	0	0	–
4	match	4	0	–	–	0	–	0
5	no match	–	–	2	1	1	0	–
6	no match	–	–	4	0	0	0	–
7	match	4	0	–	–	0	–	0
8	no match	–	–	4	0	0	0	–
9	no match	–	–	3	1	0	0	–
10	match	1	3	–	–	0	–	0
Decisions (D)								
No.		16	4	15	4	1	0	0
%(= D/N_T)		80	–	75	–	2.5		

[a] See footnote, Table 1.

Table 3. Number of responses made by Group III (novices)

Task	Correct response	Correct pos. ident. ($N = 4$, $N_T = 20$)[a]	Correct probable ident.	Correct pos. elim. ($N = 4$, $N_T = 20$)	Correct probable elim.	No decision	Error: False ident.	Error: False elim.
1	match	2	1	–	–	0	–	1
2	match	1	2	–	–	1	–	0
3	no match	–	–	3	0	0	1	–
4	match	3	1	–	–	0	–	0
5	no match	–	–	2	2	0	0	–
6	no match	–	–	3	1	0	0	–
7	match	2	2	–	–	0	–	0
8	no match	–	–	4	0	0	0	–
9	no match	–	–	3	1	0	0	–
10	match	0	0	–	–	0	–	4
Decisions (D)								
No.		8	6	15	4	1	1	5
% = (D/N_T)		40	–	75	–	2.5	5	25

[a] *See* footnote, Table 1.

Table 4. Number and percentage of responses made by collaborators

Coll.	Correct pos. ident. ($N = 5$, $N_T = 20$)[a] No.	(%)	Correct probable ident. No.	Correct pos. elim. ($N = 5$, $N_T = 20$) No.	(%)	Correct probable elim. No.	No decision No.	Error: False ident. No. (%)	Error: False elim. No. (%)
Trained Professional Examiners									
1	5	(100)	–	5	(100)	–	0	0	0
2	5	(100)	–	4	(80)	–	1	0	0
3	5	(100)	–	5	(100)	–	0	0	0
4	3	(60)	1	2	(40)	2	2[b]	0	0
	18	(90)	1	16	(80)	2	3	0	0
Trainees									
5	5	(100)	–	5	(100)	–	0	0	0
6	3	(60)	2	5	(100)	–	0	0	0
7	4	(80)	1	3	(60)	1	1	0	0
8	4	(80)	1	2	(40)	3	0	0	0
	16	(80)	4	15	(75)	4	0	0	0
Novices									
9	2	(40)	2	4	(80)	1	0	0	1 (20)
10	0	(0)	2	4	(80)	–	1	1 (20)	2 (40)
11	2	(40)	2	4	(80)	1	0	0	1 (20)
12	4	(80)	–	3	(60)	2	0	0	1 (20)
	8	(40)	6	15	(75)	4	1	1 (5)	5 (25)

[a] See footnote, Table 1.
[b] One no decision corresponded to a matching task. The other no decision corresponded to a no-match task.

grees in the speech and hearing sciences performed better than those with little or no academic training. In Group III, an academic degree in speech and hearing sciences possessed by one examiner did not improve performance over peers of the group with no such degree. The results suggest that examiners with academic training in speech and hearing science and over 2 years of experience perform at a higher level of confidence than those lacking academic training and/or practical experience.

The Associate Referee recommends that the official first action voiceprint identification method, **45.A01–45.A03**, be adopted as official final action.

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REMINDER . . .

FULL REGISTRATION FEE—$10.00

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In 1969, the AOAC established a registration fee for attendance at the Annual Meeting of the Association. The fee has been instituted to help defray the meeting costs. For the convenience of attendees, special advance prepaid registration forms are printed on the insert at the back of this issue of *JAOAC*.

MEAT AND MEAT PRODUCTS

An Improved Protein Parameter for Analytical Control of Cured Pork Production

CARROL H. PERRIN and PATRICK C. KELLY
Canada Packers Ltd, Research Centre, 2211 St. Clair Ave W, Toronto, Ontario, Canada M6N 1K4

Protein content expressed on a fat-free basis, PR_F, has been proposed as a control of weight gain or meat content of cured pork. However, PR_F is complicated to use, since it is a direct measure of meat content of the fat-free part of the sample. It is doubtful that this was the intention of the control. We propose the use of protein, fat-corrected basis, PR_C, which is directly related to meat content of the sample as well as to weight gain. This would mean that conclusions about product quality could be made with greater certainty. $PR_C = PR + kF$, where PR = % meat protein, F = % fat, and k is a constant.

In May 1975 it was announced that the U.S. Department of Agriculture (USDA) "is readying a new standard for hams and pork shoulders which will limit added substances by using a standard chemical determination of protein on a fat-free basis . . ." (1). In July 1974 it was reported that the USDA "is considering a new method for determining the weight gain of ham" (2). This proposed new method is currently recommended by the FAO/WHO Codex Committee on Processed Meat Products as a control on international trade. The formula used is protein, fat-free basis, $PR_F = (100 \times \%\ \text{protein})/(100 - \%\ \text{fat})$. For compliance a product would be required to have a minimum value for PR_F. In order to select an appropriate minimum value for such a regulation, it is highly desirable to know the relationship between PR_F and percentage weight gain or meat content of the product. However, as will be shown below, the relationship is indirect and unnecessarily complicated. This paper introduces a more simple and exact method for correcting for differences in the fat content of the meat.

In 1919, Stubbs and More (3) proposed a method for the estimation of the meat content of sausages and meat pastes which is based upon the well known observation that fresh, fat-free meat has a fairly constant percentage protein. For example, the protein content of fresh, fat-free ham meat is 22% (4, 5), so that the fat-free meat content of a sample is its meat protein percentage divided by 0.22. The symbols used in this paper are defined in Table 1. For ham, the total meat content, MT, is (PR/0.22 + F) and its "non-meat" content, NM, is 100 − (PR/0.22 + F). From this we obtain the formula PR = 22 − 0.22F − 0.22NM, which is the basic equation in Table 1.

The Stubbs and More method (3) is used in Great Britain for control of the meat content of meat mixtures. Although the Stubbs and More calculation of meat content uses a standard value of PR_F for the raw meat involved, it is not based upon the PR_F of the *product* as is commonly believed.

Actually the Stubbs and More method is based upon a protein parameter which we call PR_C (i.e., per cent protein, fat-corrected basis). It is the purpose of this paper to prove that under many practical production conditions PR_C offers substantial advantages over PR_F. This is because the calculation of PR_F implies removal of all solvent-extractable fat from the sample and the performance of a protein analysis on the residue. No allowance is made in the calculation for the weight of fat hypothetically removed. Therefore, there is an implied concentration of all the curing pickle in the remaining lean meat. This causes PR_F to be a direct measure of NM_F rather than of NM (*see* formulas in Table 1). In this respect the use of PR_F would not be consistent with either the current USDA method for "added substance" or the Stubbs and More method for "meat content."

Proposed Method of Calculating Protein on a Fat-Corrected Basis

From Table 1 we have the formula for meat protein in cured ham, PR = 22 − 0.22F − 0.22NM. Since the *presence* of each per cent unit of fat in the same reduces the original 22% units of

Table 1. Definitions and relationships of ham parameters

Name	Symbol and formula	$(\partial/\partial F)_{NM}$	$(\partial/\partial NM)_F$
Meat protein, %	$PR = 22 - 0.22F - 0.22NM$[a]	−0.22	−0.22
Meat (% in sample prepared for analysis) is % lean meat + % fat	$MT = PR/0.22 + F = PR_C/0.22$ $= PR_F \times (100 - F)/22 + F$		
Non-meat (% in sample prepared for analysis) (This usually is curing pickle.)	$NM = 100 - MT = 100 - PR_C/0.22$		
Non-meat, % (fat-free basis)	$NM_F = 100NM/(100 - F) = 100 - PR_F/0.22$		
Protein, % (fat-free basis)	$PR_F = 100PR/(100 - F)$ $= 100 \times (22 - 0.22F - 0.22NM)/(100 - F)$	$-22NM/(100 - F)^2$	$-22/(100 - F)$
Protein, % (fat-corrected basis)	$PR_C = PR + 0.22F$ $= 22 - 0.22NM = 0.22MT$	$(-0.22) + (0.22)$ $= 0$	−0.22
Meat fat (% extractable by official solvent)	$F = (PR_C - PR)/0.22$		

[a] For ham and side bacon use factors 22 and 0.22 as in formulas above. For back bacon use 23 and 0.23. For shoulder pork meats use 21 and 0.21 (*see* Table 3).

protein in fresh, fat-free ham meat by 0.22, we may exactly correct for the dilution of the protein (by fat) by adding back 0.22F. Then any residual dilution of the protein is caused by the presence of non-meat substance in the sample. Thus, the corrected protein analysis, $PR_C = PR + 0.22F$. The partial derivatives of PR_C show that it is completely insensitive to variation in fat content and is depressed by a constant 0.22 for the presence of each percentage unit of non-meat in the sample. However, the corresponding partial derivatives of PR_F show a much more complex behavior since they are functions of the fat content.

Figure 1 shows that the interpretation of a given value of PR_F in terms of meat content becomes unsatisfactory as per cent fat and/or non-meat increase. This difficulty cannot be ignored when PR_F is applied to shoulder meats and side bacon. The top line of the graph expresses the equation $PR_C = 22 - 0.22NM$. Clearly PR_C is not

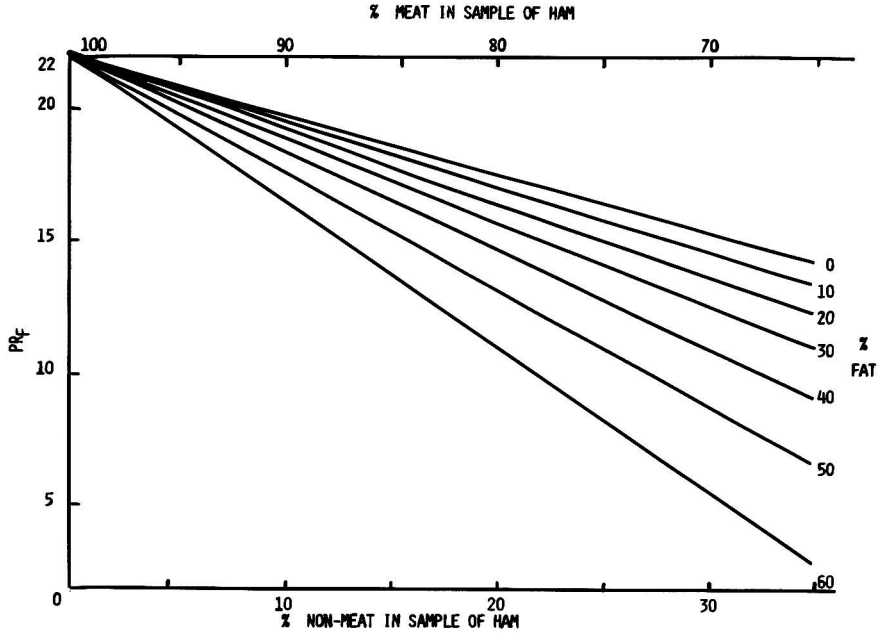


FIG. 1—Effect of fat and non-meat on protein (fat-free basis). Top line (% fat = 0) also represents PR_C. Note that PR_C needs only this 1 line for all levels of fat.

Table 2. Composition of cured hams

Calculated analyses and other values	C_1	C_2
Original moisture, %	55.728	43.344
Protein, %	15.840	12.320
Residual, %	0.432	0.336
Fat, %	8.000	24.000
Non-meat (curing matter)	20.000	20.000
Total	100.000	100.000
Weight increase, % (raw meat basis)	25	25
Weight increase, % (cured product basis)	20	20
Meat content, %	80	80
PR_C	17.60	17.60
PR_F	17.22	16.21
Weight increase required to produce a standard $PR_F = 17.00$	26.47% of raw meat	20.59% of raw meat

affected by fat content. However, when we plot PR_F against F we obtain an hyperbola for each level of NM with curvature increasing as *NM increases.*

Examples of Calculations

The calculations of PR_C, PR_F, and NM for 2 artificial samples of cured ham are illustrated in Table 2. It will be assumed that the starting material is the edible portion of pure raw ham. The first example (C1) starts with ham containing exactly 10% fat. According to average values in ref. 5, this starting material will contain 69.66% moisture and 19.80% protein. The second example (C2) starts with ham containing exactly 30% fat, 54.18% moisture, and 15.40% protein.

To illustrate the principles we shall assume that 80 lb of either starting material combines with and retains 20 lb curing pickle. For the resulting compositions, consult Table 2. We also assume that the curing process involves no removal of skin, bone, or fat and no shrink due to loss of moisture and protein.

Practical Advantages of PR_C over PR_F

Table 3 shows that for pure, fat-free ham meat $PR = PR_C = PR_F = 22$ (with somewhat different values for shoulders and backs). In Table 2 note that $PR_C = 17.60 = 80\%$ of 22 for both examples. Therefore, both C1 and C2 contain 80% meat. If we had wanted to make a product with a standard value of $PR_C = 17.6$, we would merely arrange conditions so that $17.6/0.22 = 80$ lb of *both* of the pure, raw ham samples would combine with and retain 20 lb curing pickle. Of course no prior knowledge of the fat content of the raw meat is required. Clearly, PR_C is directly related to weight gain in these examples.

By contrast, suppose we wanted both samples to have a standard value of $PR_F = 17.00$. Table 2 indicates that at a starting value of 10% fat, 100 lb of the raw meat would have to combine with and retain 26.47 lb curing pickle. The corresponding value for 30% fat would be 20.59 lb. The reader should satisfy himself that these calculations for reaching a standard value for PR_F are much more difficult than those for control of PR_C. Furthermore, the calculated values can never be exact, since we never know the exact fat content of each ham before curing. However, control over individual hams must be exercised, since the use of a zonal sampling system has been proposed (6). Thus, the chance selection of a single ham or shoulder cut which was unusually fat or lean could result in a false conclusion concerning the average compliance of the entire lot if PR_F is used instead of PR_C. Indeed, a system based upon PR_F could conceivably lead to abuse through visual selection of samples.

Of course, in practice, control of PR_C or PR_F would require allowance for many other variables such as trim, shrink, and meat loss. A major concern is the effect of error in sample preparation and analysis. Since a company's production would be subject to evaluation by a number of laboratories, it would be necessary to be prepared for standard deviations as high as those obtained in meat check sample programs. These may exceed 0.5 for per cent protein and 1 for per cent fat

Table 3. PR_F (or PR_C) for pure, raw pork (edible portion)

Cut	No. of entire pieces analyzed	Av. PR_F (or PR_C)	Std dev. of PR_F (or PR_C)
Ham	25	22.0[a]	0.5
Back	10	23.3[b]	0.8
Shoulder	24	21.4[b]	0.6

[a] From ref. 5.

[b] From later analysis performed in our laboratory with equal care.

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(7). In the calculation of PR_F an error in protein analysis is multiplied by $100/(100 - F)$, whereas in the calculation of PR_C an error in protein analysis is not magnified by the fat content of the sample analyzed. Although a complete error analysis is complex, and not given here, it can be shown that PR_F is more vulnerable than PR_C to analytical inaccuracy. Since PR_C contains a constant which has some natural uncertainty, one might conclude that PR_F has less error than PR_C. However, exactly the same constant must be applied to PR_F if it is to have any meaning in terms of "meat content" or weight gain. (*See* second formula for PR_F, Table 1.)

Criteria for Selecting an Analytical Control Method

(*1*) The method should measure that property of the sample which is most directly and simply related to the characteristic of the product (or process) for which a control is intended. For example, the USDA Meat Inspection Regulations (8) anticipate a control on the gain in weight in going from the fresh to the final state. Therefore, if a method is intended for this control, the property measured should, as nearly as possible, correlate with the change in weight of the meat rather than with that of the fat-free part of the meat.

(*2*) The method should not inadvertently control a characteristic, such as fat content, for which no control is authorized.

(*3*) The accuracy and precision of the method should be limited only by the representativeness of the sample, the reliability of the analytical work, and the correctness of the factors used to interpret the analysis.

(*4*) The method should be as absolute as possible and have no empirical factors or corrections. It need not, and should not, include allowance for processing variables such as average protein loss, yield, skinning and boning yield, and average composition of curing pickle used. If desired, such allowances could be incorporated in limits or tolerances that are subject to revision without affecting the basic method.

(*5*) If possible, the method should be directly and simply related to a parameter of the meat (such as weight) that is capable of control during production. (This would be particularly important in the production of canned meats where reprocessing is expensive.)

(*6*) If possible, the method should be inexpensive and rapid. The calculations and interpretation of the analyses should be simple.

Received September 2, 1975.

Discussion

It has been shown that a control based upon the calculation of PR_C or upon "meat content" would satisfy all the listed criteria for selection. The reporting of "meat content," however, would raise arguments about terminology because a cooked product does not contain raw meat. "Cooked meat" content could not be reported because cooked meat does not have a standard average composition independent of the process used and, therefore, the correctness of the estimate could be challenged reasonably. For the same reason it would be unsatisfactory to report "added substance" or "yield."

A control of meat content or weight gain based upon PR_F meets only criterion 4. The argument that PR_F would be desirable because it indirectly controls the fat content of the product is not convincing (*see* criterion 2). All percentages of fat, including the ideal level for eating pleasure, would be penalized. A control on fat content should not be vicarious or difficult to understand and comply with. It should begin to penalize only above a deliberately chosen maximum.

Protein analysis by itself is superior to PR_C and PR_F as a measure of the weight of protein purchased, but it is not directly related to weight gain or to NM. Since PR is depressed equally by NM and fat content, an accurate prior knowledge of the latter would be required to estimate and control NM.

The proposal to use PR_C represents no change in the aim of the control but, rather, a more exact realization of this aim. It is analogous to the recommendation of a more accurate and more generally applicable method of analysis. Superior methods of measurement reduce the cost of both production and official evaluation since fewer repetitions and less tolerance need be allowed for error inherent in the measuring process.

Conclusion

If a protein parameter is used to estimate "meat content" of a sample or weight gain, protein (fat-corrected) is more accurate than protein (fat-free). When the total of per cent fat and per cent non-meat in the sample exceeds approximately 20 (as is the case with side bacon and many shoulder meats) the error in using PR_F would be unacceptable.

Numerically PR_C is equal to the percentage protein of fat-free cured meat having exactly the same concentration of non-meat substance as the product under examination.

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TECHNICAL COMMUNICATIONS

Comparison of Wet Ash and Dry Ash Methods for the Digestion of Mineral-Mix Feeds

WILLIAM L. HOOVER
Department of Agricultural Analytical Services, Texas Agricultural Experiment Station, College Station, TX 77843

When some mineral-mix feed samples are digested by AOAC dry ash methods, the mineral components are not dissolved. A 50% error in phosphorus values is observed when these digestion methods are used. It is suggested that the wording in AOAC dry ash methods for minerals in feeds be changed to exclude mineral-mix feeds.

As the Associate Referee for minerals in feeds, I have been alerted to problems in phosphorus in mineral-mix feeds. There are 2 options for digesting feeds for mineral analyses in the 12th Edition of *Official Methods of Analysis* (1). One method uses nitric and perchloric acids to digest the organic materials in the sample prior to the determination of the mineral content. An optional technique consists of ashing the sample at 550°C and dissolving the ash in hydrochloric and nitric acids. A majority of laboratories use the 550°C ashing method since it is the simplest. Unfortunately, some mineral-mix feeds contain components that form refractory compounds at 550°C. These compounds will not completely dissolve in the acid solutions recommended in the AOAC methods. This problem was not recognized in the collaborative study (2) reported in 1967, because mineral-mix feeds were not included in the test. However, one can observe that the wet digestion values were higher than the dry ashing values in every case for the data presented for calcium with lanthanum added.

An examination of the difference between the 2 options for digesting the mineral-mix feeds has been made. Twelve mineral-mix feeds were selected at random from samples taken by Texas feed and fertilizer inspectors. A portion of each sample was digested according to **7.080(a)** (1) and another portion was digested according to **7.080(b)** (1). Phosphorus was determined on the digests by the quimociac method (**2.028(b)** (1)). Other elements (Ca, Mg, Fe, Mn, Zn, Cu, and Co) were determined by the atomic absorption method (**7.078** (1)).

Table 1. Phosphorus analysis (%) in mineral-mix feeds[a]

Sample No.	Dry ash	Wet ash
1	5.3±0.2	9.9±0.5
2	4.3±0.5	8.8±0.5
3	8.5±0.7	12.9±1.8
4	9.9±1.5	13.3±1.0
5	5.8±0.3	6.6±0.4
6	6.4±0.7	6.5±0.4
7	16.2±0.9	16.3±1.4
8	9.0±0.5	9.0±0.5
9	9.2±0.6	9.2±0.5
10	5.6±0.3	5.5±0.3
11	16.2±0.9	15.9±1.0
12	12.1±1.0	12.3±0.7

[a] Mean and standard deviation of 6 analyses.

Results

The values in Table 1 indicate that there are differences in analytical results when using the 2 optional ashing methods with some samples of mineral-mix feeds. Ashing at 550°C definitely caused the phosphorus results for Samples 1, 2, 3, and 4 to be low when compared with the nitric acid-perchloric acid digests. It should also be observed that two-thirds of the sample values in Table 2 are essentially the same by either digesting method. Further studies of feed samples other than mineral-mix feeds failed to show any differences between results obtained by wet and dry ash techniques.

Analyses for other minerals in the first 4 samples indicate that some elements, in addition to phosphorus, were affected by heating to 550°C. The values for P, Ca, Fe, Mn, Zn, and Cu are lower for dry ash for some of the samples in Table 2. Only Mg seems to be unaffected by heating to 550°C.

Conclusion

Since it is apparent that dry ashing methods

W. L. Hoover is Associate Referee on Minerals.

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

Table 2. Effect of wet and dry ashing on mineral-mix feeds[a]

Sample No.		P, %	Ca, %	Mg, %	Fe, %	Mn, ppm	Zn, ppm	Cu, ppm	Co, ppm
1	dry ash	5.3±0.2	7.5±0.3	2.2±0.1	0.5±0.1	74±4	13±1	—[b]	—
	wet ash	9.9±0.3	9.4±0.7	2.4±0.2	1.0±0.1	215±11	39±2	—	—
2	dry ash	4.3±0.5	5.8±0.6	2.8±0.3	0.6±0.1	87±5	14±1	—	—
	wet ash	8.8±0.6	9.3±0.7	3.0±0.2	1.2±0.1	200±10	37±1	—	—
3	dry ash	8.5±0.7	9.0±0.6	0.5±0.1	1.2±0.1	233±12	73±5	10±1	—
	wet ash	12.9±0.9	12.1±0.9	0.5±0.1	1.2±0.1	260±15	74±4	22±1	—
4	dry ash	9.9±1.5	7.7±0.8	2.7±0.2	0.8±0.1	900±50	40±2	128±6	90±6
	wet ash	13.3±1.0	17.3±0.3	2.6±0.2	0.9±0.1	910±45	46±1	126±8	105±5

[a] Mean and standard deviation of 6 analyses.
[b] Not detectable.

do not yield correct answers for some mineral-mix feeds, I suggest that the statement "not applicable for mineral-mix feeds" be added to the dry ashing methods for minerals in feeds.

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Gas-Liquid Chromatographic Analysis of *N*-(4-Chlorophenyl-*N'*-2,6-difluorobenzoyl)urea Insecticide After Chemical Derivatization

JAMES F. LAWRENCE and KANTH M. S. SUNDARAM*
Food Research Division, Health Protection Branch, Health and Welfare Canada, Tunney's Pasture, Ottawa, Ontario, Canada K1A 0L2
** Chemical Control Research Institute, Environment Canada—Forestry Service, 25 Pickering Place, Ottawa, Ontario, Canada K1A 0L2*

The gas-liquid chromatographic analysis of TH 6040 (*N*-(4-chlorophenyl)-*N'*-(2,6-difluorobenzoyl)urea) was carried out after the insecticide was converted to its *N,N'*-dimethyl analog (*N*-methyl-*N*-(4-chlorophenyl)-*N'*-methyl-*N'*-(2,6-difluorobenzoyl)urea). The methylation was accomplished in DMSO with sodium hydride and methyl iodide. The derivative was thermally stable and chromatographed as a single peak on 3% OV-1 at 235°C. Support for the structure of the product was given by mass spectrometry. The sensitivity of the product to electrolytic conductivity detection was approximately 50 ng for 50% full-scale deflection in the nitrogen mode and 70 ng for the same response in the chloride (reductive) mode. About 0.25 ng was required for 50% full-scale deflection by electron capture (at 1×10^{-9} amp standing current). Foliage samples were analyzed for TH 6040, using the proposed method.

Thompson-Hayward TH 6040 (*N*-(4-chlorophenyl)-*N'*-(2,6-difluorobenzoyl)urea) is a promising new insecticide of low mammalian toxicity. The compound is thought to interfere in insect cuticle formation (1).

Attempts at the gas-liquid chromatographic (GLC) analysis of this compound at normal GLC temperatures and on columns commonly used by pesticide residue analysts proved unsuccessful (2).

However, a high-pressure liquid chromatographic (HPLC) technique has been developed for the determination of TH 6040 in milk (2). This approach is capable of detecting 10 ng insecticide. Unfortunately, most government regulatory laboratories in Canada are not equipped with liquid chromatographs at present. Thus a GLC method for TH 6040 was desired. The present paper reports on a new GLC method for TH 6040 after the compound is converted to a derivative amenable to GLC. The technique increases the sensitivity of TH 6040 analysis and also may be used for confirmation of the HPLC method if desired. The reaction consists of forming the dimethyl analog by treatment with sodium hydride-methyl iodide in dimethylsulfoxide (DMSO). This method has been used for the analysis and/or confirmation of a number of pesticides (3–5), including urea herbicides similar in structure to TH 6040 (3, 5).

METHOD

Reagents and Apparatus

(a) *Analytical standard.*—Technical TH 6040 (99.4%), obtained from Thompson-Hayward Chemical Co., PO Box 2383, Kansas City, KN 66110.

(b) *Sodium hydride.*—50% oil dispersion (Fisher). Wash with hexane before use and store in 20 ml vial with Teflon-lined screw cap. *Caution:* Reagent is flammable and reacts rapidly with water, evolving hydrogen.

(c) *Gas chromatograph.*—Micro-Tek MT 220 equipped with Coulson electrolytic conductivity detector set for nitrogen determination. Detector sensitivity was improved 2–3-fold by insertion of 0.003″ stainless steel wire into capillary water entrance of detector cell mixing chamber (6).

Operating conditions: temperatures (°C)—column 235, furnace 800, transfer unit 220; gas flows (ml/min)—helium carrier 100, helium sweep 100, hydrogen 50; dc bridge potential 30 v. Column consisted of 3% OV-1 coated on 80–100 mesh Chromosorb W (HP) packed in 1.3 m × 6 mm od borosilicate glass column. Column effluent was vented for 1 min after injection.

Electron capture analysis was carried out with Hewlett-Packard 5713A gas chromatograph on 3% OV-210 coated on 80–100 mesh Chromosorb W (HP), packed in 2 m × 6 mm od glass column. Temperatures (°C)—injector 250, column 235, detector 250; argon-methane (95+5) carrier 60 ml/min.

Extraction of Foliage

Homogenize samples (20 g) of chopped foliage in Sorvall blender for 5 min with two 100 ml portions of acetonitrile. Filter homogenates under suction through Whatman No. 1 paper. After washing residue with 20 ml acetonitrile, pool extracts and pass through column of 50 g Na_2SO_4. Rinse column with 50 ml solvent and evaporate filtrate on rotary evaporator to ca 100 ml. Equilibrate 3 times with 30 ml hexane. Discard nonpolar phase after partitioning with 30 ml CH_3CN. Pool polar phases and evaporate to near dryness. Dissolve residue in four 1 ml portions of acetone-hexane (1+4) mixture and pass through column (35 cm × 14 mm) containing 10 g partly deactivated Florisil (5.5% water) sandwiched between 5 g Na_2SO_4 and prewashed with 50 ml petroleum ether. Elute column in succession with 45 ml hexane, 30 ml acetone-hexane (1+9), and 10 ml acetone-hexane (1+4) and discard all eluates; then elute with 50 ml acetone-hexane (1+4). Evaporate final eluate containing TH 6040 to dryness for derivatization.

Alkylation

To pesticide residue in test tube add 0.5 ml DMSO (British Drug Houses) followed by 0.3 ml methyl iodide (Fisher) and ca 20 mg NaH. Stopper tube and shake gently. Let reaction proceed for >15 min. Add 3 ml hexane and shake. Carefully add, dropwise, 1 ml distilled water to destroy excess NaH (until effervescence ceases); then add another 9 ml and shake vigorously. Let layers separate (centrifuge if necessary) and inject 3 μl upper (hexane) phase into gas chromatograph for analysis.

For electron capture or Coulson detection in chloride mode, remove hexane layer by Pasteur pipet to clean test tube and wash twice with distilled water. Remove water layer with Pasteur pipet each time and discard. Add small quantity (ca 0.5 g) anhydrous Na_2SO_4 to remove remaining water and decant hexane into 5 ml centrifuge tube. Rinse test tube with 1 ml hexane and add rinse to centrifuge tube. Evaporate hexane to dryness under stream of nitrogen at room temperature. Dissolve residue in 1 ml hexane for GLC analysis.

Results and Discussion

Alkylation

A reaction scheme for the alkylation was described earlier (5). After destruction of excess sodium hydride by conversion to sodium hydroxide with distilled water, the methylated product, excess methyl iodide, and traces of DMSO remained in the hexane layer. The DMSO was removed by 2 further washings with water while the methyl iodide was removed when the hexane was evaporated to dryness. Both DMSO and methyl iodide interfered in the detection of the derivative by electron capture and in the chloride

mode of the Coulson detector. The complete removal of DMSO and methyl iodide was not required for detection in the nitrogen mode of the Coulson detector if the venting time was long enough (about 1 min). Only the strontium hydroxide scrubber needed to be replaced about every 100 injections.

The reaction conditions reported above are considered optimum for the authors' purposes. However, the parameters were not critical, since quantities of DMSO, methyl iodide, and sodium hydride could be varied. The only restriction was that enough of the reagents be present to quantitatively react with the pesticides. Since water destroys sodium hydride, wet solvents were dried before use. A minimum time of 15 min at room temperature was required for highest yield of product. Times as long as 2 hr had no further effect on the product yield. The reaction appeared to be quantitative based upon the nitrogen response of the product compared to that for a similar urea.

The dimethylated derivative was found to be the main product as determined by mass spectrometry. A strong molecular ion corresponding to m/e 338 indicated that 2 methyl groups were added. The derivative was found to be completely stable for 2 months (the longest time studied) in hexane solution at concentrations from 0.3 to 100 μg/ml.

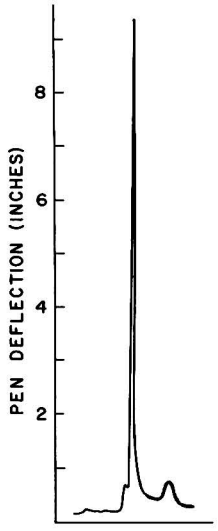


FIG. 1—Chromatogram of TH 6040 with electrolytic conductivity detection in nitrogen mode. 40 ng TH 6040 injected. 1 × attenuation. GLC conditions as described in *Method*. (Retention time = 2:40.)

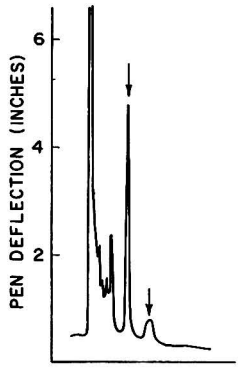


FIG. 2—Chromatogram of 7.5 ng (16 × attenuation, 1 × 10^{-9} amp) TH 6040 with electron capture detection. GLC conditions as described in *Method*. Arrows indicate derivative peaks. (Retention time = 2:50, first peak.)

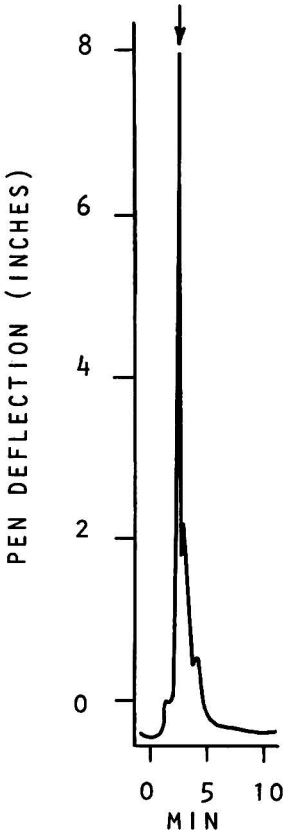


FIG. 3—Chromatogram of spruce foliage extract containing 10.0 ppm TH 6040. 16 mg sample injected. 4 × attentuation.

Gas Chromatography

Figure 1 shows a chromatogram of the alkylated derivative of TH 6040 obtained with the Coulson detector in the nitrogen mode. The smaller peak at longer retention time (7 min) was the only other product observed. The identification of this peak was not attempted but it could possibly be the monomethylated product.

Electron capture detection was found to be at least 200-fold more sensitive than the Coulson detector. As little as 0.25 ng gave 50% full-scale deflection by electron capture compared to 50 and 75 ng with the Coulson detector in the nitrogen and chlorine modes, respectively. However, the electron capture detector was unsuitable for routine operation at maximum sensitivity. Figure 2 shows an electron capture chromatogram of the same reaction mixture as shown in Fig. 1 but after the cleaning step mentioned in the method. Direct electron capture analysis of the hexane extract after the reaction was impossible due to the presence of DMSO and methyl iodide.

Figure 3 shows the Coulson detector (nitrogen mode) results obtained for the analysis of TH 6040 spiked in spruce foliage at 10.0 ppm. With the extraction method described, as little as 0.2–0.5 ppm could be detected. Recoveries were generally >80% above 1.0 ppm. Difficulty was encountered in the removal of foliage coextractives and a more efficient cleanup would be needed in order to determine lower concentrations.

Acknowledgment

The authors are indebted to David Lewis for technical assistance.

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Distribution of Aflatoxins in Some Samples of Peanuts

VINCENT P. DiPROSSIMO
Food and Drug Administration, 850 Third Ave, Brooklyn, NY 11232

Naturally occurring G aflatoxins were found at approximately 20 times the level of B aflatoxins in one lot of roasted, blanched peanuts. Official methods, as well as high-pressure liquid chromatography, were used to confirm this finding. Additional routine sample analysis data of raw and roasted peanuts revealed that this finding is not so unusual as formerly thought. It was found that 9.8% of the raw peanut samples contained higher levels of G than B aflatoxin; 4.9% containing 2–8 times more G than B aflatoxins. In a smaller sampling of roasted peanuts, 28% of the contaminated peanuts contained more G than B aflatoxins.

Received September 23, 1975.

A sample consisting primarily of Virginia peanuts was recently analyzed in our laboratory and was found to contain 21 μg total aflatoxins/kg with almost all of the aflatoxin content due to aflatoxins G_1 and G_2. At present, samples containing aflatoxins at levels >20 μg/kg are violative of Food and Drug Administration (FDA) guidelines, but the aflatoxin contamination must be confirmed by 2 methods: one a chemical derivatization (1) which can detect only aflatoxins B_1 and G_1, the second, a bioassay (2, 3) which is much more sensitive to aflatoxin B_1 than any of the other aflatoxins. Since this violative sample could not be confirmed by bioassay, we attempted to use another confirmatory procedure, high-pressure liquid chromatography (HPLC), that is

more sensitive for aflatoxins G_1 and G_2. We also became interested in obtaining information on the incidence of cases where quantities of G aflatoxins exceed those of B aflatoxins.

Experimental

Apparatus

DuPont Model 841 high-pressure liquid chromatograph, equipped with Model 836 fluorescence detector and 25 cm × 2.1 mm id Zorbax-Sil column, was used. Mobile phase was CH_2Cl_2-$CHCl_3$-methanol (60+40+0.3) with flow rate of 0.72 ml/min (2000 psi). Corning CS-7-60 optical excitation and Corning CS-3-72 emission filters were used. Under these conditions aflatoxins G_1 and G_2 had retention times of 5.6 and 8.1 min, respectively.

Procedure

Aflatoxins were determined by using official AOAC Method I (4, 5). Confirmation of aflatoxins B_1 and G_1 was obtained by chemical derivatization with trifluoroacetic acid (1). HPLC confirmation of aflatoxins G_1 and G_2 was made on the same peanut extract used for thin layer chromatography (TLC) after it had been further cleaned up with $(NH_4)_2SO_4$ (6, 7).

Results and Discussion

Table 1 summarizes the results obtained by 4 analysts. Figure 1 shows liquid chromatograms of a 25 μl injection of the sample and a 50 μl injection of a mixed aflatoxin standard in chloroform containing approximately 0.2 ng aflatoxins B_1, B_2, G_1, and G_2/μl, using the fluorescence detector. The retention time of the G_1 sample peak matches that of the standard G_1 peak. However, the sample G_2 peak appears as a shoulder on an interfering peak. The B aflatoxins are not detectable by fluorescence at this level because of quenching by the eluting solvent mixture. Figure 2 is the chromatogram of a 50 μl sample injection after the ammonium sulfate cleanup. The G_2 area of the sample is now clear of interferences and the retention times of the 2 sample peaks match the G_1 and G_2 aflatoxin standard peaks, respectively. Official AOAC Method II (8, 9) was used to confirm the level of G_1 and the absence of significant amounts of B_1 and B_2 in the sample (interference in the G_2 area prevented quantitation of G_2). Since Method II uses no column, loss of aflatoxins B_1 and B_2 in the column chromatographic step of Method I can be ruled out.

The effects of water content (10), relative humidity (11), temperature (10–13), mold species or strain (12, 14–16), plant variety (16), and trace metals (17) on the formation of aflatoxins in foods have been discussed in the literature. A review by Jarvis (18) discusses factors affecting the formation of aflatoxins and other mycotoxins. Schindler *et al.* (12) found that the ratio of production of aflatoxin B_1 to G_1 varied with temperature when *Aspergillus flavus* was grown on wort media. One *A. flavus* strain (M 93) isolated from wheat produced more G_1 than B_1 aflatoxin after 5 days while another strain (M 122) isolated from peanuts produced more B_1 than G_1. Rabie and Smalley (13) found the optimum temperatures for B_1 and G_1 formation by *A. flavus* were 24 and 30°C, respectively. Of 128 fungi isolated by van Walbeek *et al.* (14) from 74 food samples, 14 were found to produce aflatoxins and 3 were found to produce ochratoxin A (one produced both aflatoxins and ochratoxin A). Of the 14 aflatoxin-producing isolates, 3, identified as *A. flavus, A. ochraceus,* and *Rhizopus* sp., produced more G than B aflatoxins. One known toxin-producing strain of *A. flavus* (C. cir-2), used as a control, produced approximately 10 times more G_1 than B_1 aflatoxin. The same strain produced only 3.2 times as much G_1 as B_1 in a different growth medium. Six strains identified as *A. flavus var. columnaris* produced aflatoxin B_2 only. While this literature search is by no means complete, it appears that with experiments involving laboratory media, at least, the ratio of B to G aflatoxins produced depends on fungal

Table 1. Quantitation and identification of aflatoxins in a sample of roasted, shelled peanuts (μg aflatoxin/kg sample)

	Analyst				Confirm. of ident.	
Aflatoxin	1[a]	2[b]	3[a]	4[a]	Deriv. (1)	HPLC
B_1	tr(<3)	tr	1	2	+	
B_2	ND[c]	ND	tr	tr		
G_1	16	19	22	17	+	+
G_2	5	4	3	4		+
Total	21	23	26	23		

[a] 50 g sample (4).
[b] 1 kg sample (4).
[c] ND = none detected.

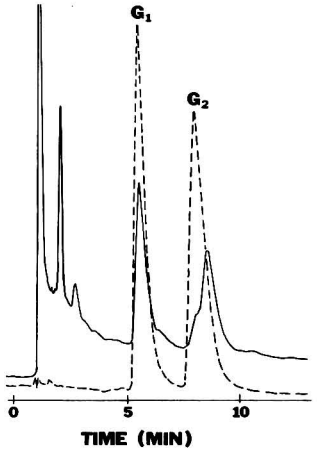


FIG. 1—Liquid chromatogram of aflatoxin standards (- - -) and peanut sample extract (—) before cleanup with $(NH_4)_2SO_4$.

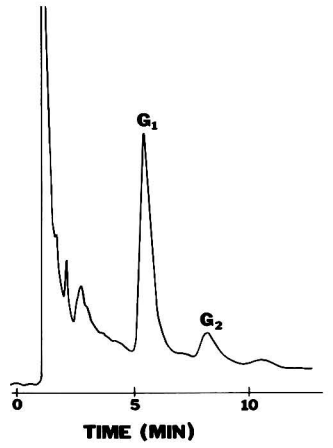


FIG. 2—Liquid chromatogram of peanut sample extract after $(NH_4)_2SO_4$ cleanup.

species and strain, temperature, and growth medium.

A literature search for naturally occurring ratios of B and G aflatoxins in peanuts proved fruitless. A survey of several experts revealed the general belief that the natural occurrence in peanuts of G aflatoxins in greater quantity than B aflatoxins either did not occur or was extremely rare. Analytical data from large numbers of samples could indicate relationships between commodities and distribution of naturally occurring aflatoxins. Shotwell *et al.* (19) reported that most of the contaminated white corn they analyzed in a 1971 study of Missouri corn contained only B_1 and B_2 aflatoxins, with the quantity of B_2 approximately 0.18 that of B_1. Very few of the aflatoxin-containing samples contained aflatoxin G_1. Pistachio nuts analyzed in our laboratory and found to be positive for aflatoxin have exhibited definite patterns of contamination. During the period May 1971 to May 1975, 189 pistachio nut samples contained aflatoxins (unpublished data). Of 132 contaminated Iranian pistachio nut samples, 128 contained B aflatoxins but no G aflatoxins. In contrast, contaminated pistachio nut samples from Turkey and Afghanistan usually contained both B and G aflatoxins (72.5% of Turkish samples, 94.1% of those from Afghanistan). Of 57 contaminated pistachio nut samples from Turkey and Afghanistan, only 1 had more total G aflatoxins than total B aflatoxins.

Examination of data provided by Joyner (20) for 368 contaminated raw peanut samples revealed that 36 (9.8%) of the samples contained higher levels of G than B aflatoxins. Eighteen (4.9%) of these samples contained from 2 to 8 times more G than B aflatoxins. The most outstanding results were (μg/kg) 1.5 B_1, 0.5 B_2, 12 G_1, and 4 G_2 (18 total), which is similar to the case reported here.

The data on raw peanuts demonstrate that higher levels of G than B aflatoxins occur naturally in a small percentage of samples, and suggest that roasting of the lot of peanuts described here probably did not selectively destroy B aflatoxins. The results from a few peanut samples analyzed in our laboratory tend to confirm the fact that some samples contain more G than B aflatoxins. Of 18 samples examined, 5 contained more G than B aflatoxins. The findings reported in this paper illustrate the need to develop additional confirmatory methods since we now know that in a significant number of cases it is possible to find samples containing total aflatoxins violative of FDA guidelines but difficult to confirm by bioassay because of low levels of B_1. Recent work by Ashoor and Chu (21) shows promise as an additional confirmatory method for aflatoxins.

Acknowledgments

The author wishes to express his appreciation to Joseph D. Rosen, Science Advisor, FDA, New York District, and Research Professor of Food Science, Cook College, Rutgers University, New Brunswick, NJ, for his invaluable assistance in the preparation of this paper; A. D. Campbell, S. Nesheim, and L. Stoloff, FDA, Washington, DC, O. L. Shotwell, U.S. Department of Agriculture (USDA), Peoria, IL, for their advice; B. L.

Joyner, USDA, Dothan, AL, for supplying the data for raw peanuts; and L. Allen, FDA, Brooklyn, NY, and N. L. Brown and C. W. Thorpe, FDA, Washington, DC, for their analytical work confirming aflatoxin levels in the peanut sample.

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CORRECTIONS

JAOAC **59**, 62–66 (1976), "Interlaboratory Study of Acid Orange 12 Dye Binding for Measuring Protein in Meat," by S. N. Heller and J. W. Sherbon, p. 62 and p. ii (Table of Contents)

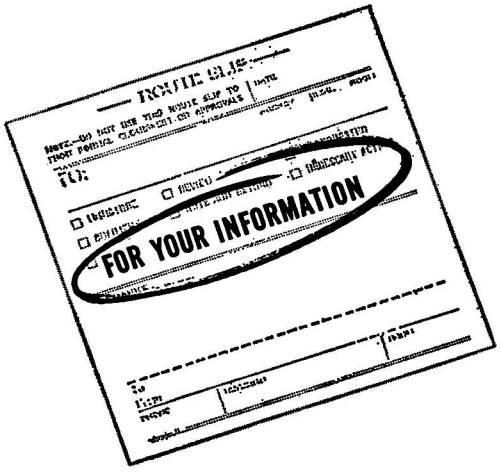
Change "Dairy Products" to "Meat and Meat Produets"

JAOAC **59**, 384–387 (1976), "Report of Subcommittee C on Recommendations for Official Methods," by D. E. Coffin, A. D. Campbell, D. J. Mitchell, A. R. Johnson, and J. A. Springer, p. 386, left column, after line 4

Add the following:

(15) *Nitrates and Nitrites:* Continued study.
(16) *Nitrosamines:* Continued study.
(17) *Polysorbates:* Continued study.
(18) *Polypropylene Chlorohydrin:* Continued study.
(19) *Sodium Lauryl Sulfate:* Continued study.

Corrected
V.P.
20 Dec '76



AOAC Commemorates the 100th Anniversary of ACS

The AOAC offers heartiest congratulations to the American Chemical Society on the occasion of the Society's 100th anniversary. The AOAC is an organization devoted to the development and standardization of improved analytical methods; therefore the information and services provided by the Society in the areas of chemical nomenclature, specifications for reagents, advanced instrumentation, and new analytical techniques, have been of special benefit and usefulness. The AOAC has also profited by the high standards set by the American Chemical Society in its scientific publications and has noted the innovations advanced by the Society in publishing and information retrieval. The AOAC recognizes the world-wide prominence of the Society in many aspects of chemistry and salutes its efforts to foster international cooperation in dealing with problems common to the scientific community. The AOAC hopes that the Society will continue to be a leader in the field of chemistry and that its second century will be as fruitful as its first 100 years.

Ernest A. Epps, Jr.
President

Meetings and Symposia

The International Union of Pure and Applied Chemistry (IUPAC) has scheduled the following meetings for 1976 and 1977:

IUPAC-IUFOST Symposium on Advances in Smoking of Foods—Warsaw, Poland—September 8–10, 1976

III International Symposium on Mycotoxins in Foodstuffs — Paris, France — September 16–18, 1976

Symposium on Clinical Chemistry and Chemical Toxicology of Toxic Metals—Monte Carlo, Monaco—March 3–5, 1977

International Symposium on Microchemical Techniques—Davos, Switzerland—May 22–27, 1977

For further information on the meetings, write: IUPAC, Bank Court Chambers, 2–3 Pound Way, Cowley Centre, Oxford OX4 3YF, UK.

* * *

The International Organization for Standardization (ISO) has scheduled the following meetings in 1976:

Spices and Condiments, TC 34/SC 7—Geneva—September 6–8

Tea, TC 34/SC 8—Geneva—September 9–10

Milk and Milk Products, TC 34/SC 5—Berlin—4th Quarter (Date to be announced)

Dr. Hoffman Receives Fisher Award

Dr. I. (Al) Hoffman, past president of AOAC and executive secretary of the Canadian National Research Council's Associate Committee on Scientific Criteria for Environmental Quality, Ottawa, has been named winner of the Fisher Scientific Lecture Award for 1976 by The Chemical Institute of Canada (CIC). The Award for distinguished contributions in analytical chemistry, consisting of a framed scroll and a $500 honorarium by Fisher Scientific Co. Ltd., was presented to Dr. Hoffman during the 59th Canadian Chemical Conference and Exhibition in London, Ontario, June 6–9, 1976.

Dr. Hoffman has played a major role in advancing analytical chemistry in agriculture in Canada and throughout the world. The author of more than 80 research papers on analytical methods relating to agricultural problems, he has been a leader in the field of thermogravimetry and its applications to problems of minerals in soils. His works on arsenic and selenium residues in agricultural products are classics and many of his methods are used internationally.

Born in Ottawa in 1919, Dr. Hoffman joined Agriculture Canada in 1938 and then served with the RCAF from 1940 to 1945. He received his B.S. from Queens University and his M.A. and Ph.D. in Analytical Chemistry from the University of Toronto. In 1970 he took his present position with the Canadian National Research Council (NRC) and also became Head of the Environmental Secretariat of NRC. Dr. Hoffman has served as president and chairman of the executive committee, AOAC (1974–75), as well as chairman of the CIC Analytical Chemistry Division.



New Managing Editor

Nancy Palmer has joined the AOAC staff as Managing Editor of the Journal of the AOAC. A native of Ithaca, NY, Mrs. Palmer attended Cornell University and received a B.S. degree in biochemistry in 1964. She worked analyzing blood samples in the Diagnostic Chemistry Laboratory at the Johns Hopkins Hospital in Baltimore, MD, for one year before she joined the Food and Drug Administration as a research chemist in 1965. Her first assignment was in pesticide methodology development in the Bureau of Foods; she then transferred to the Editorial Office where she was Associate Editor for the Journal of the AOAC and Assistant Editor for *Bureau By-Lines,* an FDA scientific publication, for 2 years. Mrs. Palmer resigned from fulltime employment in 1970 but continued editing for *JAOAC* and preparing methods for inclusion in "Changes in Methods" on a parttime basis. She also edited symposium papers for the Advances in Chemistry Series for the American Chemical Society. In 1975, Mrs. Palmer joined Tracor Jitco Inc. as Technical Editor of the Report Series on Bioassay of Potential Carcinogenic Chemicals, prepared and published under contract to the National Cancer Institute. Mrs. Palmer resides in Gaithersburg, MD.

AOAC to Hold Annual October Meeting

The Association of Official Analytical Chemists (AOAC) will hold its 90th Annual Meeting Oct. 18–21, 1976 at the Marriott Hotel, Twin Bridges, Washington, DC. About 1300 regulatory scientists and researchers from government, industry, and academia in North America and Europe are expected to attend. They will present approximately 240 papers on new techniques, methods, and instrumentation for analysis of foods, drugs, pesticides, cosmetics, forensic materials, hazardous substances, feeds, fertilizers, and other materials related to agriculture and public health and welfare.

Three symposia and 2 workshops are scheduled to take place during the 4-day convention:

A symposium on the Computerized Automated Nutrient Analysis Laboratory will be held Wednesday, October 20, 1976. Mike J. Deutsch, U.S. Food and Drug Administration, Washington, DC, is chairman. The focus will be on food applications. Major topics of discussion will include analytical laboratory techniques such as gas-liquid chromatography and high-pressure liquid chromatography; use of computerization for determining dietary intake; computer predictions of nutrient content in food processing; cost effectiveness; and quality control and quality assurance (complete schedule printed below).

A Symposium on Forensic Sciences will be held Tuesday, Oct. 19, 1976. Richard L. Brunelle, Bureau of Alcohol, Tobacco and Firearms, Department of the Treasury, Washington, DC, is chairman.

A Joint Symposium of the Society of Cosmetic Chemists and the AOAC will take place on Monday, Oct. 18, 1976. Justin McCarthy, Malmstrom Chemical Co., Linden, NJ, is chairman.

A Thin Layer Chromatography Workshop conducted by Camag, Inc. of New Berlin, WI, will be held Tuesday evening, Oct. 19. An Automated Analysis Workshop run by Technicon Industrial Systems, Tarrytown, NY, will be held Wednesday evening, Oct. 20.

Immediately before the AOAC meeting, on Oct. 16 and 17, the American Chemical Society (ACS) will hold a course on "Solving Problems with Modern Liquid Chromatography" conducted by J. J. Kirkland, E. I. Dupont de Nemours & Co., and Lloyd R. Snyder, Technicon Instruments Corp. This new problem-oriented course is designed for chemists with experience in high-pressure liquid chromatography. For registration or information, write or call Department of Educational Activities, ACS, 1155 16th St NW, Washington, DC 20036 (202/872-4508). The fee is $195 for non-members, $160 for ACS members, and includes a textbook.

A reception will be held Monday evening, Oct. 18, at 6:30 p.m. to be followed by the Harvey W. Wiley banquet at 7:30 p.m. Entertainment will be provided by The Young Columbians in a musical tribute to the USA. The highlight of the banquet will be the presentation of the Wiley Award to Walter A. Pons, Jr., Southern Regional Research Laboratories, U.S. Department of Agriculture, for his original research on gossypol and aflatoxin.

Nearly 40 firms will exhibit the latest laboratory equipment and supplies. Registration will take place from 1 p.m. Sunday, Oct. 17 through Thursday morning, Oct. 21. Registration fee is $5 for one day or $10 for 2 or more days. All interested persons are invited to attend. For further information contact Luther G. Ensminger, AOAC, Box 540, Benjamin Franklin Station, Washington, DC 20044.

Computerized Automated Nutrient Analysis Laboratory

This symposium will be held Oct. 20, 1976, during the 90th Annual Meeting of the AOAC, Oct. 18–21, 1976, at the Marriot Hotel, Twin Bridges, Washington, DC. The symposium schedule is as follows:

8:00 am: Use of Computerization for Determining Dietary Intake. Prentiss E. Schilling, Louisiana State University, Baton Rouge, LA 70803.

Discussion Leader—Edith Christensen, USDA, Washington, DC 20250.

8:30 am: Automated Vitamin Analysis in Foods. I. B_1, B_2, C, and Niacin. David C. Egberg, General Mills, Minneapolis, MN 55427.

Discussion Leader—Larry Hambleton, Purdue University, West Lafayette, IN 47907.

9:00 am: Automated Vitamin Analysis in Foods. II. A, B_1, B_2, and C. James Kirk, Michigan State University, East Lansing, MI 48824.

Discussion Leader—Elwyn D. Schall, Purdue University, West Lafayette, IN 47907.

9:30 am: Use of the AutoTurb System in Food Analysis. David Rodriguez, Nabisco Inc., Fairlawn, NJ 07410.

Discussion Leader—Robert A. Moffitt, Carnation Co., Van Nuys, CA 91412.

10:00 am: The Devil's Advocate—AOAC Manual Assays. Wayne Thornburg, Del Monte Corp., Walnut Creek, CA 94598.

Discussion Leader—C. W. Gehrke, University of Missouri, Columbia, MO 65201.

10:30 am: Automated Protein and Amino Acid Assays. Larry Wall, University of Missouri, Columbia, MO 65201.

Discussion Leader—Elmer George, New York State Food Laboratory, Albany, NY 12203.

11:00 am: Automated Protein Assay. Robert Revesz, Leco Corp., 3000 Lakeview Ave, St. Joseph, MI 49085 and N. Aker, Kellogg Co., Battle Creek, MI 49016.

Discussion Leader—Donald J. Mitchell, State Chemical Laboratory, University of South Dakota, Vermillion, SD 57069.

11:30 am: Automated Analysis of Fatty Acid Composition. Arthur Foltz, General Foods, White Plains, NY 10625.

Discussion Leader—H. P. Moore, Ohio Department of Agriculture, Reynoldsburg, OH 43068.

1:30 pm: HPLC Quantitative Differentiation of Folates in Food. Andrew Clifford, University of California at Davis, Davis, CA 95616.

Discussion Leader—Fred J. Baur, Procter and Gamble Co., Cincinnati, OH 45224.

2:00 pm: Computerized Prediction of Nutrients in Canned Foods. Kan-ichi Hayakawa, Rutgers University, New Brunswick, NJ 08903.

Discussion Leader—Ed Elkins, National Canners Association, Washington, DC 20036.

2:30 pm: Sonics as a Quantitative Tool in Food Production. W. C. Winder, University of Wisconsin, Madison, WI 53076.

Discussion Leader—Robert Weik, FDA, Washington, DC 20204.

3:00 pm: A Computerized Food Laboratory. Arvid W. Munson, Ralston Purina, St. Louis, MO 63188.

Discussion Leader—Charles Austin, Kellogg Co., Battle Creek, MI 49016.

3:30 pm: Computerization for Nutrient Labeling. Harry G. Lento, Campbell Soup Co., Camden, NJ 08102.

Discussion Leader—Phil Derse, WARF Institute Inc., Madison, WI 53704.

4:00 pm: Quality Control and Quality Assurance in a Computerized Automated Nutrient Analysis Laboratory. Foster McClure, FDA, Washington, DC 20204.

Discussion Leader—James DeVoe, National Bureau of Standards, Washington, DC 20234.

4:30 pm: Cost Effectiveness vs. Automation. David J. Aulik, WARF Institute Inc., Madison, WI 53704.

Discussion Leader—Gus Kuhn, Quaker Oats Research Laboratories, Barrington, IL 60010.

5:00 pm: Summary. Alan J. Senzel, AOAC, Washington, DC 20044.

AOAC Publishes Bacteriological Analytical Manual (BAM)

AOAC has published the fourth edition of the Bacteriological Analytical Manual for Foods (BAM), of the Division of Microbiology, Food and Drug Administration. These analytical methods provide Federal and State regulatory and industry quality control laboratories with effective means for qualitative and quantitative detection of microorganisms and certain of their metabolic products. Applicable primarily to foods, the manual will be updated by supplements issued to users at no additional charge.

The first 4 chapters of the manual cover sampling and sample handling, preparation of food homogenates, microscopic examination of foods, and aerobic plate count. Bacteria included are coliform organisms, enterococci, *Salmonella* (both cultural and fluorescent antibody methods), *Shigella, Vibrio parahaemolyticus, Vibrio cholerae, Staphylococcus aureus* and its enterotoxins, *Clostridium botulinum, Clostridium perfringens,* and *Bacillus cereus.*

The manual also discusses enumeration of yeasts and molds in foods, examination of sterilized or microbiologically stable milk products, direct microscopic examination of eggs, and detection of penicillin and other microbial inhibitors in milk and milk products. Appendices cover culture media, reagents and diluents, stains and staining procedures, and most probable number tables.

The manual is available from the AOAC at a cost of $10 per copy including postage and handling. Supplements will be forwarded automatically to users at no additional charge. Orders may be sent to: AOAC, Box 540, Benjamin Franklin Station, Washington, DC 20044.

AOAC Spring Meeting in Denver

AOAC's first Annual Spring Workshop was held May 10–12, 1976 in Denver, Colorado. About 330 scientists attended.

Topics discussed at the meeting included Mycotoxin Analysis; Analysis of Drug Residues in Animal Tissues and in Animal Feeds; Microscopy; Statistical Techniques, General Theory and Application to the Magruder Fertilizer Check Sample Program; Automated Analysis with Computer-Based Data Acquisition and Report Generation; Particulate Matter Related to Filth in Foods. The sessions on Mycotoxins, Drug Residues in Animal Tissues and in Animal Feeds, and the HPLC and Electrochemical Methods were especially well attended.

Special events included a panel discussion entitled "Are Colleges Preparing the Student for the Real Laboratory Work Life?" Warren Bontoyan of EPA gave a special HPLC course—"Participation Analysis of Pesticide Formulations using HPLC." AAFCO and AOAC held a round table discussion on "What industry can do to improve communications between itself and state government."

The meeting was planned and coordinated by Luther Ensminger, Executive Secretary of the AOAC; Clyde Jones and Lon Ullrich, Colorado Department of Agriculture; Dr. Theodore O. Meiggs, EPA/NFIC Denver Federal Center; Dave Root and Chester Ball, U.S. Food and Drug Administration, Denver District; Floyd J. Gregarek, Denver Health Department, and other support staff in these agencies.

Plans are now underway for the Second Spring Meeting to be held May 4–6, 1977 at Stouffer's Inn, Cincinnati, Ohio. For further information contact: Howard P. Moore, Chief, Consumer Analytical Laboratories, Ohio Dept. of Agriculture, Reynoldsburg, Ohio 43068 (614/866-6361); or John Feldman, Laboratory Director, Food and Drug Administration, 1141 Central Parkway, Cincinnati, Ohio 45202 (513/684-3511).

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.

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 methods have been adopted as

interim official methods, in addition to those reported in the May 1976 issue of *JAOAC* (p. 724), since the 1975 Annual Meeting of the AOAC: mineral oil extraction of light filth from ground paprika, submitted by Joel J. Thrasher (Food and Drug Administration, Washington, DC) and Rose Gentry; determination of moisture in corn sirups, submitted by C. Earl Engel (Clinton Corn Processing Co., Clinton, IA); extractable color of capsicums and oleoresin paprika, submitted by James E. Woodbury (Cal-Compack Foods, Inc., Santa Ana, CA); semiautomated method for the analysis of prednisolone and prednisone tablets, submitted by James F. Brower (Food and Drug Administration, St. Louis, MO); an automated method for the determination of crude protein in meat and meat products submitted by Rodney J. Noel (Purdue University, West Lafayette, IN) (the basic method was published in (1976) *JAOAC* 59, 141–147; additional data were submitted to support adoption of the method for meat and meat products); a modified method for erythromycin thiocyanate in pelleted feeds, submitted by Victor W. Winkler (Abbott Laboratories, N. Chicago, IL). These reports are scheduled for publication in the January 1977 issue of *JAOAC*. 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.

OBITUARY

Dr. Morris Fishbein

Dr. Morris Fishbein, a microbiologist with the Food and Drug Administration for 28 years, died of cancer at his home in Silver Spring, MD on March 26, 1976. He was 59 years old.

Dr. Fishbein was born in New York City and graduated from CCNY in 1938 with a B.S. in Zoology. He received his Master's degree in 1940 from the University of Kentucky and was first employed by the Florida State Board of Health in Pensacola. In 1942, he joined the Army Air Force, serving as a radio operator and aerial gunnery officer with the 15th Air Force Division. He flew more than 30 heavy bombardment missions over Europe during World War II. After the war, he was employed by Schwartz Laboratories in New York in brewing technology. He left to become a Research Fellow with the U.S. Fish and Wildlife Service at the University of Maryland, and to study for his Ph.D.

In 1948, he joined the Food and Drug Administration's Division of Microbiology, and in 1951 completed his dissertation. He received his Ph.D. degree from the University of Maryland in the same year. Dr. Fishbein's years at FDA were spent studying the physiology of the coliform group of organisms and its importance as an index of food sanitation. More recently, he studied the marine microbe *Vibrio parahaemolyticus*, a causative agent of food poisoning. He had also been involved with studies of *E. coli* and *Shigella* and their role as food poisoning organisms. His counsel and collaboration were sought by university, industry, and public health personnel both here and abroad.

Dr. Fishbein was a member of the Editorial Board of *Applied Microbiology*, and an Associate Referee on *Shigella* in the AOAC. He was also a member of the AOAC, the New York Academy of Science, the American Society for Microbiology, the American Association for the Advancement of Science, and Sigma Xi.

He is survived by his wife, Eleanor, a daughter, Susan, and son, Sanford, at home, and a son, David, of Euclid, OH.

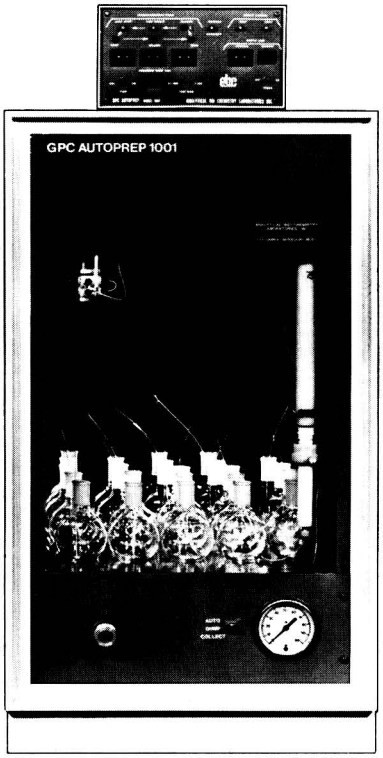
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